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Research Article

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With incubation times of 3-10 min, ^{59}Fe appeared promptly in the main hemoglobin. The entry of ^{59}Fe into ferritin paralleled that of hemoglobin but was smaller in amount. When the marrow cells were incubated with ^{59}Fe for 15-20 min and reincubated without radioactive iron, movement of ^{59}Fe into main hemoglobin was observed, and essentially all this iron came from the particulate fraction (stroma, mitochondria, and microsomes). In these chase experiments there was no change in the total quantity of ^{59}Fe in ferritin. There was no evidence of a significant hemoglobin precursor other than low molecular weight iron.

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Studies on the Partition of Iron in Bone Marrow Cells

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ABSTRACT Canine marrow cells were incubated with transferrin-bound ^{59}Fe , and the partition of cellular iron was studied by chromatographic and gel filtration methods. Splitting-off of iron from the stromal fraction was avoided by lysing the cells in Tris HCl buffer at pH 8.6. Cellular iron was divided into four major compartments: stroma, microsomes, main hemoglobin, and fraction I. The iron in fraction I was found in ferritin, heme proteins, and low molecular weight iron.

With incubation times of 3–10 min, ^{59}Fe appeared promptly in the main hemoglobin. The entry of ^{59}Fe into ferritin paralleled that of hemoglobin but was smaller in amount. When the marrow cells were incubated with ^{59}Fe for 15–20 min and reincubated without radioactive iron, movement of ^{59}Fe into main hemoglobin was observed, and essentially all this iron came from the particulate fraction (stroma, mitochondria, and microsomes). In these chase experiments there was no change in the total quantity of ^{59}Fe in ferritin. There was no evidence of a significant hemoglobin precursor other than low molecular weight iron.

Depending upon concentration, lead was observed to inhibit cellular iron metabolism at several points: uptake of iron by the cell, movement of iron from stroma to the soluble intracellular compartment, and synthesis of hemoglobin. The most pronounced inhibitory effect of lead was always on hemoglobin synthesis with an increase in ferritin:hemoglobin ratio. Bipyridine appeared to trap intracellular ferrous iron and to inhibit synthesis of both hemoglobin and ferritin.

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It was concluded that iron moves from the stroma into the soluble intracellular compartment as low molecular weight iron, probably as a complex of ferrous iron with low molecular weight components of the cytoplasm, that serves as the source of iron for both hemoglobin and ferritin synthesis.

INTRODUCTION

Iron moves through the plasma bound to a plasma protein, transferrin. At the surface of the red cell precursor it is released from transferrin onto the cell membrane and is incorporated rapidly into hemoglobin (1–4). The mechanism of the movement of iron from the cell membrane to the site of hemoglobin synthesis is not understood. Several studies have indicated that iron enters a non-heme precursor before being incorporated into hemoglobin (3–7). Some reports have suggested that ferritin is an intracellular precursor of hemoglobin (8, 9). Zail, Charlton, Torrance, and Bothwell observed rapid incorporation of ^{59}Fe into the ferritin of bone marrow in vitro without a decrease in radioactivity of ferritin on continued incubation without ^{59}Fe (7). They inferred that ferritin was not an immediate precursor of hemoglobin. This report describes a study of cellular iron partition by chromatographic and gel filtration methods, together with observations of cellular iron kinetics and the effects of inhibitors of hemoglobin synthesis.

METHODS

Isotopes and counting. $^{59}\text{FeCl}_3$, specific activity 8–25 $\mu\text{C}/\mu\text{g}$, was obtained from Abbott Laboratories, North Chicago, Ill. ^{59}Fe was bound to transferrin by incubation of 100 μC of $^{59}\text{FeCl}_3$ for 20 min at 37°C with 25–50 ml of

heparinized plasma. The quantity of iron added was always less than the binding capacity of the serum. Radioactive iron was counted in a well scintillation counter.¹

Marrow procurement. The canine marrow donor was sacrificed with pentobarbital. Marrow was obtained from the long bones and suspended in Ringer's solution. The marrow was sieved through a stainless steel tea strainer and then passed through a stainless steel screen with openings 200 μ square (4). Fat was removed by centrifugation at 240 *g*. The marrow was processed and used within 1 hr after removal from the body.

Incubation of marrow cells. Incubations were carried out at 37°C in air with gentle shaking. The incubation medium was approximately 50% autologous plasma (with or without transferrin-bound ⁵⁹Fe) in Hanks' solution at pH 7.6 with 10,000 U of penicillin, 60 μ g of streptomycin, and 5 mg of glucose per ml. The quantity of marrow incubated ranged from 5 to 30 $\times 10^9$ nucleated cells, and the volume of incubation medium ranged from 35 to 100 ml. After incubation in ⁵⁹Fe-containing medium, the reaction was stopped by rapid cooling to 0°C, and the cells were washed five times with a 10–20-fold volume of Ringer's solution. In chase experiments, an aliquot of washed cells was reincubated for 75 min at 37°C in plasma-Hanks' solution without added ⁵⁹Fe.

Incubation with lead. In some experiments lead acetate was added to the incubation medium. In other experiments, lead-containing cells were obtained by allowing an aliquot of marrow to stand at room temperature for 5 min in 50 ml of saline containing 2×10^{-4} M lead acetate. The cells were then centrifuged at 240 *g* for 5 min, resuspended, and incubated under conditions identical for the aliquot of marrow cells not exposed to lead. Exposure to lead in this fashion produced a moderate inhibition of hemoglobin synthesis without disruption of all metabolic processes (3).

Lysis of cells and preparation of supernatant. In some experiments washed, packed cells were lysed by overnight exposure to 4 volumes of distilled water at 4°C. In other experiments lysis was accomplished by a similar exposure to 0.01 M Tris HCl buffer at pH 8.6. The lysate was centrifuged at 34,800 *g* for 20 min to remove the large particle fraction composed of stroma and "mitochondria," here referred to as the stromal fraction. The stromal fraction was washed three times in 10 volumes of 0.01 M Tris HCl buffer at pH 8.6, and the supernatants were combined. The supernatant was centrifuged at 100,000 *g* for 70 min to remove the "microsome" fraction. The clear red supernatant was referred to as "hemoglobin solution."

Chromatography on IRC-50. The hemoglobin solution was chromatographed on the cation exchange resin, Amberlite IRC-50, (Rohm and Haas, Philadelphia, Pa.) in two steps as previously described (4). The first fraction composed of heterogeneous compounds not adsorbed to IRC-50 was referred to as "fraction I." The hemoglobin eluted from IRC-50 was referred to as "main he-

moglobin." This fraction contained about 98% of the heme of the hemoglobin solution.

Chromatography on DEAE cellulose. Commercial DEAE cellulose² was sieved through U. S. standard sieves, Nos. 20 and 100. The material remaining in the No. 100 sieve was suspended in distilled water and freed from the fines by repeated decantation. The DEAE cellulose was then treated alternately with 0.1 N NaOH and 0.1 N HCl with intermediate washing with distilled water through three or four cycles ending on the NaOH phase. It was then resuspended in 0.01 M acetate buffer at pH 5.6 with repeated adjustments of the pH with HCl and transferred to a 1 \times 40 cm column. The column was then washed with starting buffer until the effluent had the desired pH. The sample of fraction I in a volume of 10–30 ml of 0.0067 M monosodium phosphate with 0.0033 M KCN was applied to the column. A linear gradient of increasing ionic strength and decreasing pH was used with a starting buffer of 0.01 M acetate, pH 5.6, and a limiting buffer of 0.01 M acetate, pH 4.0, containing 0.1 M NaCl. After collection of the first two peaks, the column was washed with 8 ml of distilled water, a second gradient was applied with a starting buffer of 0.005 M phosphate, pH 7.7, and a limiting buffer of 0.05 M phosphate, pH 7.7. When the third peak had been collected, a fourth peak was obtained by clearing the column with 0.5 M KCl in 0.05 M phosphate at pH 7.7. Total recovery of radioactivity in the peaks from the DEAE columns was about 60%.

Separation with Sephadex. Sephadex G-25, G-100, or G-200³ was allowed to swell in 0.05 M Tris HCl buffer, pH 7.7, for 24 hr and freed of fines by repeated decantation. Columns of 2 \times 35 or 2 \times 55 cm were filled with Sephadex and covered with a 2 cm disc of filter paper. Samples were applied in a volume not exceeding 8 ml for the 35 cm column or 11 ml for the 55 cm column. Gel filtration was carried out with an eluting buffer of 0.05 M Tris HCl at pH 7.7. In some experiments ethylenediaminetetraacetate (EDTA) was added to a final concentration of 0.01 or 0.02 mole/liter to prevent low molecular weight iron from adhering to the column. Recovery of radioactivity from Sephadex columns was 60–80% without EDTA and 85–95% with EDTA.

Preparation of ferritin and rabbit antiferritin. Recrystallized ferritin was prepared from canine liver by the method of Granick (10). 8.8 mg of ferritin in 2 ml of Freund's adjuvant was injected intramuscularly into two rabbits, on two occasions, 41 days apart. Sera were collected 23 and 30 days later. Using a modified Ouchterlony double diffusion technique (11), the lowest detectable concentration of ferritin was 138 μ g/ml. Total ferritin was determined by precipitation with antibody as described by Zail, Charlton, Torrance, and Bothwell (7).

Starch-gel electrophoresis. Starch-gel electrophoresis was carried out at pH 8.6 by a discontinuous buffer system as described by Poulik (12).

¹ Packard Auto-gamma Spectrometer, Series 410A, Packard Instrument Company, Inc., La Grange, Ill.

² Carl Schleicher and Schuell Co., Keene, N. H.

³ Pharmacia Fine Chemicals Inc., Piscataway, N. J.

RESULTS

Separation with DEAE. Bone marrow cells incubated with transferrin-bound ^{59}Fe took up iron rapidly. Study of the soluble portion of the lysate of these cells indicated that a part of the ^{59}Fe was not in heme and that the nonheme fraction could be separated by DEAE chromatography (4). Accordingly, a series of additional experiments was carried out, and a representative experiment is shown in Fig. 1. Canine marrow cells were incubated with transferrin-bound ^{59}Fe for 25 min. The cells were washed with Ringer's solution and lysed with distilled water. The ultracentrifuged lysate was chromatographed on Amberlite IRC-50 to remove the main hemoglobin component (4). The unadsorbed protein mixture (fraction I) was separated by means of a DEAE column into four peaks (Fig. 1). Table I shows the relative activity of these peaks in four representative experiments where distilled water was used for lysis.

The first peak, emerging with the column volume, can be shown by starch-gel electrophoresis to contain at least nine different proteins, two of which contain heme. The ^{59}Fe in this peak is in the heme proteins as indicated by close agreement between the activity obtained by chromatography compared with the activity obtained by Myhre's method (13), which permits the differentiation of

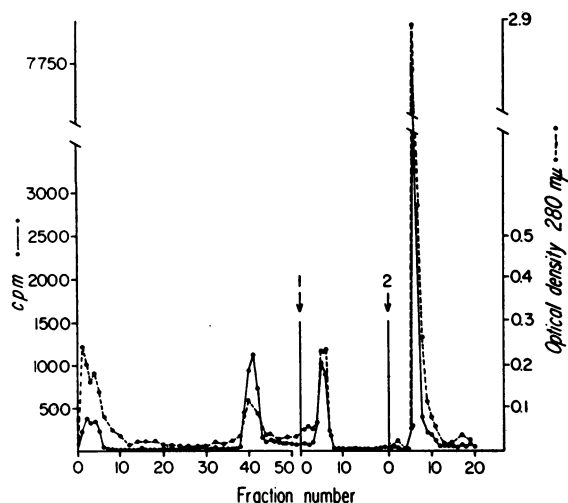


FIGURE 1 Separation of fraction I on DEAE. 30 ml of fraction I containing 35,670 cpm of ^{59}Fe and corresponding to 2.1×10^8 marrow cells was used. The first arrow indicates the change to the phosphate gradient. The second arrow indicates the change to 0.5 M KCl. Column, 1×40

TABLE I
Separation of Fraction I by DEAE Chromatography

Experiment	^{59}Fe			
	Peak 1	Peak 2	Peak 3	Peak 4
13	6.0	19.6	10.4	18.0
15	7.2	12.8	3.3	44.9
16	5.8	18.5	5.9	25.2
18	6.7	11.0	8.0	36.5

transferrin and ferritin from heme-containing proteins. This peak showed only a slight decrease in radioactivity in chase experiments. Microsomes appeared in peak 1 when they were not separated by ultracentrifugation. If transferrin were present, it would appear in this peak, since separate experiments showed that transferrin was not bound to DEAE under these conditions.

The second peak emerged with a steep ascending and a less steep descending side, with a tendency to tail, indicating incomplete elution. Starch-gel electrophoresis showed 10 or 11 proteins in this peak. The radioactivity was associated with Prussian blue positive zones in locations similar to crystalline dog liver ferritin. Agar gel diffusion showed reactions of identity between the radioactive compound in peak 2 and crystalline dog liver ferritin when reacted with rabbit antidog ferritin antiserum (Fig. 2).

The third peak contained at least eight electrophoretically different benzidine-negative proteins. The small amount of ^{59}Fe present in peak 3 did not decrease during chase experiments, and, therefore, the compounds in this peak were not characterized further.

The fourth peak showed the greatest decrease in activity in chase experiments. Separation of peak 4 on Sephadex G-200 showed it to contain some residual ferritin, a small fraction of iron-containing proteins not completely excluded by the gel and a large fraction of lower molecular weight. Gel filtration on Sephadex G-25 indicated the latter fraction had a molecular weight of less than 5000.

Change in the lysis procedure. The finding of a large low molecular weight fraction in the eluate from the DEAE column suggested that iron might

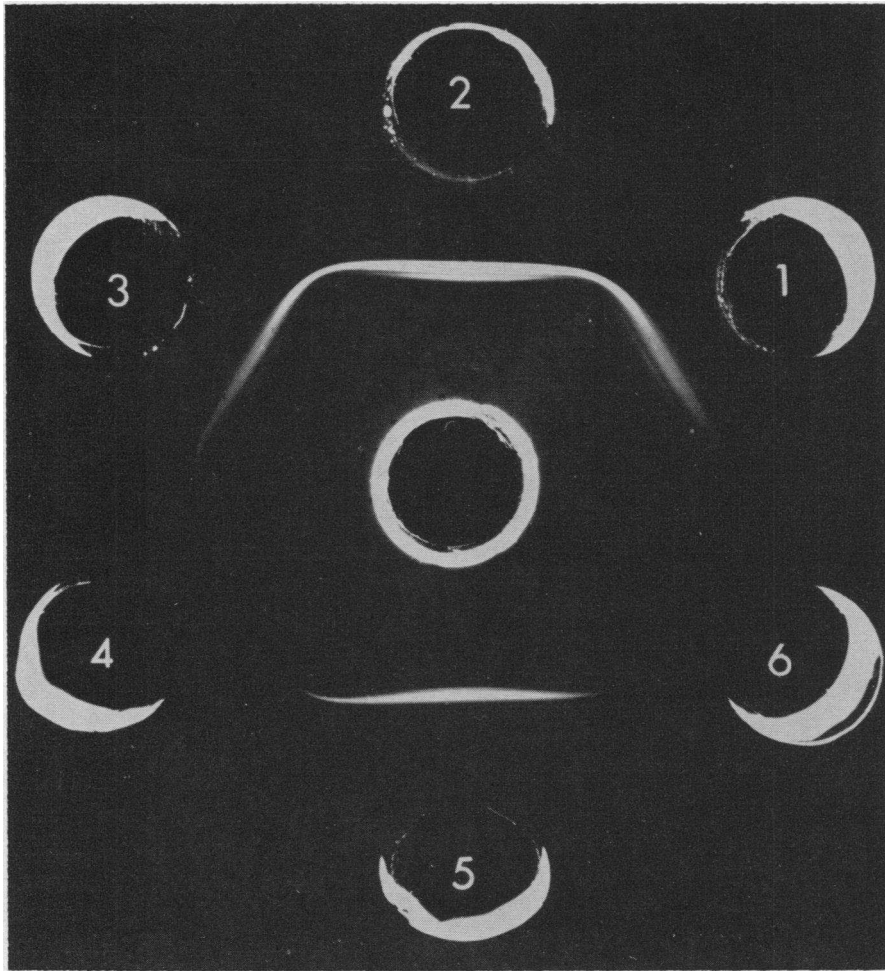


FIGURE 2 Identification of ferritin by agar gel diffusion. Center well, 1:8 rabbit anti-serum against recrystallized dog liver ferritin. Wells 1, 3, and 5, peak 2 as eluted from DEAE and concentrated 10-fold. Wells 2, 4, and 6, dog liver ferritin, 550, 275, and 138 $\mu\text{g/ml}$, respectively.

be split off from other compounds by exposure to relatively low pH. During lysis of cells with distilled water the pH was found to be in the range of from 5.6 to 6.0. Since Cawley, Goodwin, Schneider, and Eberhardt (14) identified transferrin in the lysate of human erythrocytes, and since Laurell (15) observed that transferrin-bound iron dissociates below pH 7.2, it appeared desirable to avoid a similar iron-protein dissociation by lysis at a higher pH. Accordingly, lysis was carried out with 0.01 M Tris HCl buffer at pH 8.6, with a final pH of 8.2, and was compared to lysis with distilled water (Table II). Lysis with buffer gave much less iron in fraction I. Fractionations of the ultracentrifuged lysate in Sephadex G-100 showed

a two- to tenfold increase in the low molecular weight iron fraction in distilled water lysates, as compared with the buffer lysates. There was also an increase in the amount of iron in the main hemoglobin fraction with buffer lysis, due to a better elution of hemoglobin from the microsomes pellet. Lysis with distilled water for 15 min or for 16 hr gave similar results.

Separation with Sephadex. Sephadex columns were utilized to obtain separation of the lysates and of fraction I with the very mild condition of gel filtration at a pH of 7.7. Fig. 3 illustrates the separation of the ultracentrifuged lysate of equal aliquots of marrow after 20 min incubation with transferrin-bound ^{59}Fe and after 75 min additional

TABLE II
Comparison of the Per Cent of ^{59}Fe in Different Fractions after Lysis of Cells with Distilled Water or with 0.01 M Tris-HCl Buffer at pH 8.6

	^{59}Fe			Fraction I
	Stroma	Microsomes	Main hemoglobin	
			%	
Lysis with H ₂ O	19	33	29	19
Lysis with buffer	27	21	41	10

incubation without ^{59}Fe . EDTA, 0.01 mole/liter final concentration, was added to the lysate to prevent the nonprotein iron from sticking to the column. From these aliquots the total radioactivity for each peak was calculated.

Immunoprecipitation showed the ^{59}Fe in peak I to be composed principally of ferritin. About 80% of the radioactivity could be precipitated by anti-ferritin antiserum. This peak contained a total of 13,900 cpm of ^{59}Fe before the chase and 14,500 cpm after the chase. The total radioactivity in the

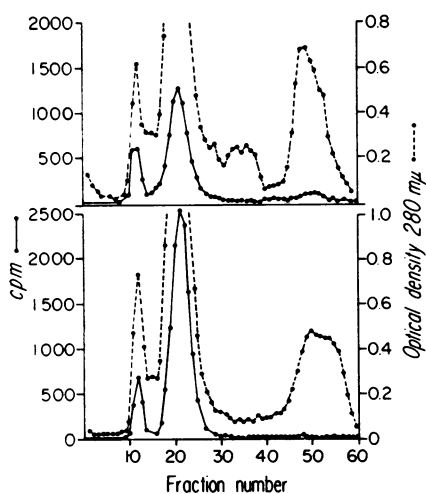


FIGURE 3 Separation of the ultracentrifuged supernatant of a marrow lysate on Sephadex G-100. 17.7×10^9 nucleated marrow cells were incubated for 20 min at 37°C in 25 ml of autologous plasma and 20 ml of Hanks' solution containing 100 μc of transferrin-bound ^{59}Fe . The upper portion of the figure shows the iron partition of an aliquot obtained at the end of the 20 min incubation. The lower portion of the figure shows the iron partition of an equal aliquot obtained after an additional incubation of 75 min without ^{59}Fe . Lysates were prepared in Tris HCl buffer. Column, 2×55 cm; flow rate, 18 ml/hr; fraction volume, 3.6 ml.

ferritin of the lysate before ultracentrifugation was also determined by immunoprecipitation. Total ferritin radioactivity was 31,700 cpm before the chase and 32,700 cpm after the chase. The difference in the total ferritin activity and in the ferritin activity from peak I represents the loss of ferritin into the microsome fraction during ultracentrifugation. Peak I, therefore, does not represent a quantitative determination of ferritin. In this and several other experiments the activity of peak I was found to represent about 40–45% of the activity of the total ferritin.

Peak II consisted mainly of hemoglobin with a minor fraction composed of ^{59}Fe in proteins that appear in peaks 1 and 3 of the DEAE separation. This peak contained a total of 60,300 cpm before and 106,300 cpm after the chase.

Peak III contained low molecular weight iron with a total of 9300 cpm before and 2010 cpm after the chase. In separate experiments with added inorganic ^{59}Fe it was found that recovery of peak III from the column averaged about 80% even with excess EDTA.

Fig. 4 illustrates the separation of fraction I on Sephadex G-200 before and after a chase. Peak I, the ferritin fraction, showed no change in radioactivity during the chase. Since the main hemoglobin had been removed, the second peak contained relatively little radioactivity. It also showed no significant change in radioactivity during the chase. Peak III, low molecular weight iron, decreased from 4020 to 1040.

Characterization of the low molecular weight iron fraction. As indicated above peak 4 from the DEAE columns and peak III obtained by gel filtration contained ^{59}Fe with a molecular weight of less than 5000, as shown by its retention in Sephadex G-25. Comparison of this low molecular weight iron with inorganic ferric iron showed identical behavior on the Sephadex columns. Like inorganic iron, it could be separated from the hemoglobin solution at pH 7.0 by pressure ultrafiltration through cellophane, it was not precipitated by saturated ammonium sulphate at pH 5.0, and it was precipitated by 8-hydroxy-quinoline at pH 7.7 (with ferric ammonium sulfate as carrier). That this "inorganic iron" fraction was not adsorbed to protein is indicated by the fact that it did not move with proteins on gel filtration in the absence of EDTA, and by the fact that it could

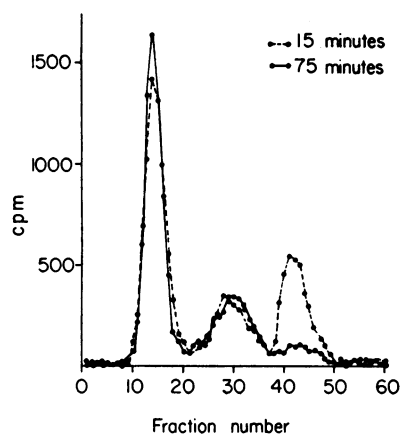


FIGURE 4 Separation of fraction I on Sephadex G-200. 6.9×10^9 nucleated marrow cells were incubated for 15 min at 37°C in 17 ml of autologous plasma and 20 ml of Hanks' solution containing $100 \mu\text{c}$ of transferrin-bound ^{59}Fe . An aliquot of this marrow was reincubated for an additional 75 min in the absence of ^{59}Fe . Lysates were prepared in Tris HCl buffer and ultracentrifuged, and fraction I was obtained from IRC-50 columns. Fraction I from aliquots before and after the chase was then separated on Sephadex G-200. Column, 2×55 cm; flow rate, 8.6 ml/hr; fraction volume, 4.3 ml.

be separated from proteins by ultrafiltration. These observations do not exclude complex formation by the intracellular ^{59}Fe with low molecular weight compounds, such as amino acids (16).

Fractionation of cellular iron. The technique described permitted the separation of cellular iron into four major compartments: Stroma, microsomes, main hemoglobin, and fraction I. Fraction I could be further divided into three parts: ferritin, heme proteins, and low molecular weight iron. Table III shows the uptake of transferrin bound ^{59}Fe into each of these compartments after 15 min incubation and the transfer of ^{59}Fe between compartments upon a further incubation of 75 min without ^{59}Fe . Movement of ^{59}Fe into main hemoglobin during the chase is evident. Essentially all this iron came from the particulate compartment (stroma, mitochondria, and microsomes). The ^{59}Fe in fraction I decreased only slightly. Radioactivity of the low molecular weight iron in fraction I decreased moderately, but there was no change in the ferritin fraction (peak I) nor in the heme-protein fraction (peak II).

In order to look for an early precursor of hemoglobin, fractionation of intracellular iron after periods of incubation of from 3 to 10 min was

studied in several experiments. Table IV shows the partition of iron in marrow from a normal dog, a dog made iron deficient by bleeding and a dog given Imferon (1 g, 3 wk before sacrifice) to increase iron stores. The iron-deficient marrow showed a greater ^{59}Fe uptake with some increase in fraction I, but there was otherwise no significant difference. In the three experiments shown in Table IV the lysate was ultracentrifuged in order to separate microsomes. Since ultracentrifugation results in the loss of ferritin, two additional experiments were performed in which total ferritin was determined by immunoprecipitation. The results, Table V, show that hemoglobin and ferritin take up ^{59}Fe in parallel. Neither a delay in the appearance of the label in hemoglobin nor a buildup of ^{59}Fe in ferritin preceding hemoglobin could be detected. Hemoglobin always showed a greater uptake of ^{59}Fe than ferritin.

Influence of lead on intracellular iron partition. Table VI shows the results of a series of experiments designed to investigate the effects of lead on intracellular iron partition. In the first experiment aliquots of bone marrow were incubated for 45 min in the presence of transferrin-bound iron without lead and with 7×10^{-4} M lead. This relatively high concentration of lead caused a marked

TABLE III
Partition of ^{59}Fe into Various Compartments

	Normal dog			
	15 min incubation		Additional 75 min incubation with "cold" iron	
	cpm	%	cpm	%
Total cpm in washed cells	784,000		710,810	
Stroma	368,325	47	215,050	30
Microsomes	128,950	16	117,070	16
Main hemoglobin	226,600	29	329,275	46
Fraction I	60,130	8	49,410	7
Subfractions of fraction I from Sephadex G-100				
Peak I (ferritin)	34,390	4.3	34,875	4.9
Peak II	5,125	0.6	5,405	0.7
Peak III (low molecular weight)	11,720	1.4	7,060	0.9

TABLE IV
Partition of ⁵⁹Fe into Various Compartments after Short Incubation Time

Time of incubation	Normal dog*				Iron-deficient dog*				Dog given iron*			
	5 min		10 min		3 min		8 min		3 min		8 min	
	cpm	%	cpm	%	cpm	%	cpm	%	cpm	%	cpm	%
Total cpm in washed cells	33,816		61,615		87,167		183,955		29,669		59,360	
Stroma	14,809	43.8	21,319	34.6	47,365	52.0	86,680	47.1	18,688	63.0	31,312	52.7
Microsomes	4,182	12.4	10,472	16.9	6,203	7.2	10,800	5.8	1,506	5.0	5,330	8.9
Main hemoglobin	9,251	27.4	18,702	30.3	21,499	24.6	55,692	30.2	6,709	22.6	18,311	30.8
Fraction I	5,574	16.5	11,122	18.1	12,093	15.0	30,786	16.7	2,766	9.3	4,407	7.4
Subfractions of fraction I on Sephadex G-100												
Peak I	3,735	11.1	8,201	13.3	4,434	5.1	7,004	3.8	1,648	5.5	2,430	4.0
Peak III	963	2.8	1,461	2.4	3,392	3.8	10,550	5.7	890	3.0	1,113	1.8

* Corrected to correspond to 1×10^9 marrow cells.

TABLE V
Distribution of ⁵⁹Fe in the Supernatants of Marrow Lysates

Time of incubation	Experiment 1*				Experiment 2*			
	3 min		8 min		3 min		8 min	
	cpm	%	cpm	%	cpm	%	cpm	%
Supernatant‡	28,019	100	40,999	100	13,577	100	18,447	100
Main hemoglobin	8,160	29.1	12,560	30.6	6,550	48.1	11,866	64.1
Total ferritin§	1,344	4.8	3,279	8.0	2,253	16.6	2,840	15.4

* Corrected to correspond to 1×10^9 marrow cells.

‡ Obtained without ultracentrifugation.

§ Determined by immunoprecipitation.

TABLE VI
The Effect of Lead on Cellular ⁵⁹Fe Partition

Experiment	Time	Total uptake		Stroma		Microsomes		Main hemoglobin		Ferritin		Low molecular weight iron	
		cpm	%	cpm	%	cpm	%	cpm	%	cpm	%	cpm	%
I Control	45	1,163,000	100	429,000	36.8	212,000	18.2	381,000	32.7	91,300	7.8	—	—
	7×10^{-4} Pb	234,000	100	119,000	50.8	48,000	20.5	27,400	7.4	28,000	12.0	—	—
II Control	45	2,011,000	100	601,700	29.9	428,000	21.3	755,000	37.5	76,500	3.8	27,500	1.4
	2×10^{-4} Pb	1,802,000	100	799,400	44.3	741,700	41.2	269,700	14.9	165,200	9.2	76,400	4.2
III Control	45	497,400	100	194,600	39.1	73,300	14.7	192,600	38.7	26,400	5.3	10,300	2.1
	2×10^{-4} Pb	509,200	100	245,900	48.3	138,500	27.2	74,500	14.6	37,100	7.3	14,400	2.8
IV Control	45	1,000,000*	100	657,300	65.7	112,000	11.2	148,700	14.8	104,800	10.4	11,000	1.1
	2×10^{-4} Pb	1,000,000*	100	728,000	72.8	124,400	12.4	73,500	7.4	78,700	7.9	15,100	1.5
IV Control	90	1,000,000*	100	621,200	62.1	120,900	12.1	161,300	16.1	134,900	13.5	10,900	1.1
	2×10^{-4} Pb	1,000,000*	100	699,800	69.9	147,300	14.7	95,600	9.6	108,100	10.8	11,600	1.2

* Corrected for minor differences in the aliquots of the cell suspension.

reduction in total uptake of radioactive iron by all the iron-containing fractions including stroma. Lead caused a relative accumulation of iron in stroma and ferritin with marked inhibition of the entrance of iron into hemoglobin.

The second and third experiments (Table VI) were similar to the first experiment, except that the marrow cells were exposed to 2×10^{-4} M lead for 5 min at room temperature and then removed from the lead-containing medium. Exposure to lead in this fashion produced a moderate inhibition of hemoglobin synthesis without disruption of metabolic processes. The total uptake of iron by the control and the lead-exposed cells was virtually identical, but in the presence of lead there was an increased proportion of iron in the stroma, microsomes, and ferritin with a corresponding decrease of iron in the main hemoglobin fraction. The low molecular weight iron fraction was also slightly increased in the presence of lead. Fig. 5 shows the separation of the lysate on Sephadex G-200 for the second experiment.

In the fourth experiment bone marrow cells were incubated for 20 min in the presence of transferrin-bound iron. At the end of this period the cells were washed and divided into four equal aliquots; two aliquots were exposed for 5 min to 2×10^{-4} M lead acetate and two aliquots served as control. The control and the lead-exposed aliquots were then incubated for an additional 45 or 90 min. In this chase experiment, the initial total uptake was, of course, identical. As shown in Table VI, movement of iron from the stromal fraction was inhibited by lead with a corresponding inhibition of hemoglobin synthesis and ferritin synthesis. However, the inhibition of hemoglobin

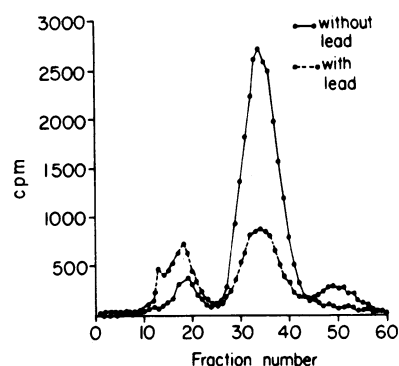


FIGURE 5 Separation on Sephadex G-200 of the supernatant of marrow lysates obtained with and without exposure to lead. Two aliquots of marrow were prepared, each containing 11×10^9 nucleated cells. 1 aliquot was exposed to 2×10^{-4} M lead for 5 min. Each aliquot was then incubated for 45 min in 25 ml of autologous plasma and 12 ml of Hank's solution containing $50 \mu\text{c}$ of transferrin-bound ^{59}Fe . Ultracentrifuged lysates were prepared with Tris HCl buffer. Equal aliquots of the lysates were separated on Sephadex G-200. The aliquot without lead contained 35,200 cpm, and the aliquot with lead contained 20,800 cpm. Column, 2×55 cm; flow rate, 7.8 ml/hr; fraction volume, 3.9 ml.

synthesis was much greater than the inhibition of ferritin synthesis.

Influence of bipyridine on intracellular iron partition. Table VII shows the results of incubation of equal aliquots of marrow cells with transferrin-bound iron in the absence of and in the presence of 5×10^{-4} M 2,2'-bipyridine. Bipyridine "trapped" intracellular ferrous iron with a small increase in the low molecular weight fraction. There was marked inhibition of synthesis of hemoglobin and ferritin by bipyridine. The total uptake of iron in the presence of bipyridine was 41% of that of the control.

TABLE VII
The Effect of Bipyridine on Cellular ^{59}Fe Partition

	Time	Total uptake*		Stroma		Microsomes		Main hemoglobin		Total ferritin†		Low molecular weight iron	
		min	cpm	%	cpm	%	cpm	%	cpm	%	cpm	%	cpm
Control	60	225,991	100	85,974	37.9	46,776	20.6	69,379	30.7	37,244	16.4	1,468	0.65
5×10^{-4} bipyridine	60	93,522	100	57,046	60.9	25,388	27.1	2,244	2.4	7,185	7.7	1,589	1.7

* Calculated for 1×10^9 nucleated marrow cells.

† Determined by immunoprecipitation without prior ultracentrifugation.

DISCUSSION

This report describes the partition of intracellular iron after incubation of canine marrow cells with transferrin-bound ^{59}Fe . Four major fractions were obtained: stroma, microsomes, hemoglobin, and nonhemoglobin iron (fraction I). Fraction I was further fractionated into three iron-containing components: a small amount of hemoglobin not bound to cation or anion exchange columns, ferritin and low molecular weight iron. Lysis of the marrow cells with buffer at pH 8.6 resulted in less fraction I than perviously reported (4). After short periods of incubation (3–8 min) the majority of the ^{59}Fe was found in the particulate fractions (stroma and microsomes). There was also a considerable amount in the main hemoglobin fraction. Chase experiments performed by incubation for an additional 75 min without ^{59}Fe showed a large increase in the amount of ^{59}Fe in the main hemoglobin fraction. Essentially all of this radioactive iron came from the particulate fraction. The low molecular weight iron in fraction I showed a decrease in activity during the chase, but there was no significant decrease in the activity of the other components of fraction I. With incubation times of only 3 min, 48% of the ^{59}Fe in the not ultracentrifuged supernatant was found to be in the hemoglobin (average of four experiments). Therefore, these experiments do not indicate the existence of a soluble, intracellular iron protein precursor of hemoglobin.

Greenough, Peters and Thomas reported evidence for the existence of an intracellular iron protein precursor for hemoglobin synthesis (4). At all incubation times they found a larger proportion of ^{59}Fe in fraction I than was observed in the present experiments. In their experiments ferrous citrate- ^{59}Fe was added directly to the marrow incubation flask. Adding iron in this fashion causes more iron to be bound to the stroma than when ^{59}Fe bound to transferrin is used as in the present experiments (2). For cell lysis they used distilled water as did other investigators who have reported evidence for the existence of a hemoglobin precursor in the lysate (3, 7). As shown in the present experiments, fraction I obtained by water lysis contained a much greater amount of the cellular iron than that obtained by buffer lysis. The findings by Greenough, Peters,

and Thomas of larger amounts of fraction I and a higher fraction I to hemoglobin ratio appears explained by the greater nonspecific stromal binding of the ferrous citrate followed by an increased splitting-off of low molecular iron by water lysis. The present experiments do show a decrease in the low molecular weight iron components of fraction I in chase experiments. The total amount of ^{59}Fe in this low molecular weight fraction is very small at any given time. However, this fraction may be turning over rapidly and thus serve as a principal pathway of movement of iron from stroma to the site of synthesis of hemoglobin.

In the previous study, ferritin was not identified in the soluble fraction of the marrow cell lysate (4). This fraction was prepared by ultracentrifugation which results in loss of ferritin as pointed out by Zail, Charlton, Torrance, and Bothwell (7) who did find ferritin in fraction I. An iron-containing fraction was found that appeared to differ from liver ferritin by electrophoresis, but Alfrey, Lynch, and Whitley (17) and Gabuzda and Gardner (18) have demonstrated a difference in the electrophoretic mobility of marrow ferritin and ferritin obtained from liver or spleen. The present immunoprecipitation studies show that ultracentrifugation of the lysate resulted in the loss of about 55 to 60% of the ferritin from the soluble fraction. The ferritin remaining in the soluble fraction was readily separated by Sephadex chromatography. In chase experiments we found, as did Zail et al. (7) that the amount of ^{59}Fe in ferritin did not change. However, in the presence of a large stromal iron pool, the finding of a persistence of the label in the ferritin in short-term chase experiments does not exclude a turnover of the iron in this compound. Mazur and Carleton (9) found that in the reticulocyte, after reincubation for 1 hr in unlabeled medium, there was a release of iron from the ferritin with a concomitant degradation of the protein moiety. Since we did not determine the specific activity of the iron in the ferritin before and after the chase, we cannot estimate the contribution of ^{59}Fe for heme synthesis by turnover of ferritin. Our experiments in the first 8 min of incubation show no buildup of ^{59}Fe in ferritin before the label appears in hemoglobin. The two compounds are labeled in parallel with hemoglobin showing the faster rate. It appears that iron leav-

ing the stromal pool need not pass through ferritin before it can be utilized for heme synthesis.

The effect of lead on the partition of intracellular iron appeared to depend on the concentration of lead employed. High concentrations of lead reduced strikingly the total uptake of ^{59}Fe by marrow cells with a corresponding decrease of ^{59}Fe in all fractions, but particularly the hemoglobin fraction. Brief exposure to a lower concentration of lead did not inhibit ^{59}Fe uptake by the cell. It did, however, produce a moderate inhibition of hemoglobin synthesis with an increase of ^{59}Fe uptake into ferritin and the "inorganic" iron fraction. Chase experiments showed that low concentration of lead inhibited the movement of ^{59}Fe from the stromal fraction into the soluble intracellular compartment. Hemoglobin synthesis was reduced by 50%, whereas ferritin synthesis showed minimal inhibition. Evidently lead can interfere with the metabolism of iron by marrow cells at a number of sites, but the greatest inhibition is always in hemoglobin synthesis.

Marrow cells were incubated with bipyridine in order to "trap" the intracellular ferrous iron. There was marked inhibition of hemoglobin synthesis as had been observed by others (9, 19). Bipyridine also caused inhibition of ferritin synthesis and an accumulation of iron in the low molecular weight fraction. The fact that bipyridine blocked both hemoglobin and ferritin synthesis suggests that ferrous iron within the cell is the source of iron for both hemoglobin and ferritin synthesis.

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