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SYNTHETIC CHELATING AGENTS IN IRON METABOLISM *

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Iron in man is held within the cells incorporated in organic complex molecules, either as functional iron, i.e., hemoglobin and tissue enzymes, or as storage iron, i.e., ferritin and hemosiderin. Owing to the requirements of the erythroid marrow in particular, large amounts of iron must constantly pass from one cell to another through the plasma. This plasma transport is brought about by a β -globulin transferrin, which handles iron in a highly selective fashion, removing it from donor tissues such as the reticuloendothelial cells and releasing it to those tissues which have a need for iron. It is likely that this direction of motion is accomplished by the relative affinity of individual cellular receptors for iron as compared to that of transferrin. Thus weaker receptors yield iron to transferrin and stronger receptors assimilate it.

Recently, powerful chelating agents have been developed which may participate in tissue iron exchange (1-10). Such chelates have been shown to compete *in vitro* with transferrin for iron (5, 6). They have also been shown to mobilize iron from body tissues when injected parenterally (3, 4). It is the aim of the present study to investigate the effects of this competitive binding of chelates in reactions of iron transport. The observations made further define the mode of action of these compounds on intermediate iron metabolism and indicate their usefulness in the treatment of acute or chronic iron overload.

MATERIALS AND METHODS

The three chelating agents employed were ethylenediaminedi-(*o*-hydroxyphenylacetic acid) (EDDHA), di-

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ethylenetriaminepentaacetic acid (DTPA), and ethylenediaminetetraacetic acid (EDTA).¹ The formulas of the first two are indicated in Figure 1. These three chelating agents bind ferric iron strongly over a pH range from 4 to 9, whereas the ferrous chelates are less stable and readily oxidized to the ferric form. The stability constants for the ferric chelates have been estimated as 10^{22} for DTPA and 10^{25} for EDTA (3, 10). EDDHA and DTPA were available as the free acids in powder form. Trisodium calcium DTPA was available as a powder and as a sterile 25% solution. Commercial preparations of disodium EDTA in powder form (Eastern Organic Chemicals) were used. DTPA labeled with C¹⁴ in the alpha positions of the acetate groups with a specific activity of 1.74 mc per g was also employed. Fresh solutions of these compounds were made in iron-free saline. Two moles of NaOH were added for each mole of the chelating compound and the pH adjusted to 7.8 with iron-free HCl (0.5 N). Unsterile solutions were used in the *in vitro* studies and the animal experiments. Solutions of EDDHA for clinical use were sterilized by autoclaving. This procedure did not alter the iron-binding properties of the compound. Solutions of chelating agents had less than 30 μ g Fe per gram of chelate.

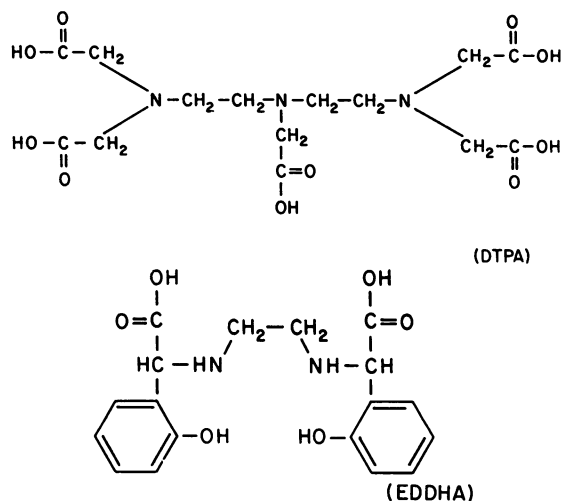


FIG. 1. DIETHYLENETRIAMINEPENTAACETIC ACID (DTPA) AND ETHYLENEDIAMINEDI-(*O*-HYDROXYPHENYLACETIC ACID) (EDDHA).

¹ Supplied through the courtesy of Dr. M. Weiner of Geigy Research Laboratories, Yonkers, New York.

Experimental procedures

In vitro studies. The competitive binding of iron between plasma and synthetic chelating agents was measured in mixtures that were prepared by adding solutions of varying concentrations of chelating agents to equal volumes of plasma. The mixtures were buffered with 0.1 volume of 0.1 M Tris buffer (pH 7.4). In observations of transfer of iron from plasma to synthetic chelating agents, plasma was first incubated with tracer amounts of $\text{Fe}^{59}\text{Cl}_3$ for 15 minutes at room temperature before addition of synthetic chelating agents. In studies of iron transfer from chelating agents to plasma, a similar incubation of synthetic chelating agents was carried out with radioiron before addition of plasma. The mixtures were incubated for 1 to 4 hours at 37° C, and then chelate was separated from protein through dialysis in cellophane bags against iron-free saline buffered with Tris at 4° C for 24 hours. Transfer of iron between transferrin and synthetic chelate was thus possible over this whole period. The amount of transfer was estimated by comparing the radioactivity in the cellophane bags with that in the dialysis fluid. A similar procedure was used to study the distribution of iron added to a mixture of synthetic chelate and plasma, which was allowed to stand for 10 minutes at room temperature before dialysis.

The capacity of chelates to bind nontransferrin iron in plasma was likewise determined by iron analysis after dialysis against saline and EDDHA (50 μg per ml) solutions. The plasma containing excess nontransferrin iron in these experiments was obtained by the administration of 250 mg of iron per kg by gastric intubation in rabbits and the collection of the iron-loaded plasma 3 hours later.

Rabbit reticulocytes were used in studies relating to the transfer of iron from chelating agents to young red cells. Reticulocyte-rich blood was obtained from donor rabbits four days after the intraperitoneal injection of 20 mg of phenylhydrazine HCl per kg body weight. Red cells were separated from plasma by centrifugation and washed thrice in twenty times their volume of iron-free saline at 4° C. The red cell suspensions contained from 3 to 5 million red cells per mm^3 and 10 to 30% reticulocytes. Glucose was added to all incubation mixtures to achieve a final concentration of 150 mg per 100 ml. Solutions of plasma and EDDHA were so prepared as to contain the same total iron-binding capacity and per cent saturation with iron. One-milliliter samples of reticulocyte-rich cell suspensions were incubated at 39° C for 4 hours in a shaking incubator with 4 ml of EDDHA solution in saline. Incubation was also carried out in various mixtures of either of the two solutions. Fe^{59} was used to label either the chelating agent or the plasma. Samples were taken hourly during incubation, and uptake of radioiron by red cells was determined. Samples of plasma-chelate mixtures were dialyzed before and after incubation to determine the amount of iron transfer between the two compounds. The cells were washed three times in ten times their volume of saline at 0° C, and

radioactivity was determined. In certain studies radioactivity in heme was also measured.

In vivo studies in animals. The distribution and fate of synthetic chelates and of iron bound to chelates after intravenous administration were studied in New Zealand white rabbits weighing from 2 to 4 kg with hematocrits above 35%. Solutions of chelating agents and preparations of iron-chelate were injected into the marginal ear vein. Two animals were pretreated by the intravenous injection of 4 g of iron in the form of iron dextran² and studied six months later to determine the effect of iron overload. In some studies of the internal distribution of iron-bound chelate, nephrectomy was performed to eliminate renal excretion. The iron-chelate was injected within 30 minutes after the operation, when the animals had largely recovered from anesthesia.

To determine the distribution of chelate, C^{14} -DTPA was injected in a total dose of 50 mg of chelating agent. In studies of the distribution of chelate iron, EDDHA was employed in amounts from 9 to 200 mg with 10 to 20 μc of Fe^{59} as ferric chloride (1 to 4 μg of iron). The solutions injected had an initial pH of 8 and were well tolerated. Blood samples were obtained by cardiac puncture at regular intervals after injection. Urine was collected through a soft rubber catheter (12 F) from the bladder over a 4-hour period following injection of chelating agents. Depending on the nature of the experiment, determinations were made either of the isotopically labeled chelating agent or of the radioiron in the urine. Tissue distribution of radioiron bound to EDDHA was measured at 5 hours and at 7 days after the intravenous injection. In such instances the animals were killed by exsanguination and the visceral organs isolated. The entire gut was dissected free and washed of its contents. Muscle, skin, and skeleton were separated after autoclaving of the carcasses.

The influence on iron absorption of an increase of plasma iron-binding capacity due to the injection of synthetic chelates was also studied. The iron-binding capacity of the plasma was increased by the injection of EDDHA in a dose of 100 mg, which produced a level of EDDHA in the plasma estimated to be in the range of 100 to 200 μg per ml of plasma; this is equivalent to an iron-binding capacity of 16 to 32 μg per ml. In two rabbits, fasted overnight, iron was given by gastric tube in a dose of 0.50 mg per kg body weight as ferrous sulfate, with a total of 5 μc of Fe^{59} added. The chelate was injected immediately after intubation and a dose of 50 mg was given every 30 minutes by intraperitoneal injection for 4 hours thereafter. Two days later the experiment was repeated with the same dose of iron (including 50 μc of Fe^{59}) without the administration of EDDHA. An identical experiment was carried out on two other animals with the sequence of the tests reversed. Urine was collected for 4 hours for measurement of any chelate-bound radioiron excreted. The rabbits were killed on

² Imferon, kindly supplied by Lakeside Laboratories, Milwaukee, Wisconsin.

day 10, and the radioiron in the blood and the tissues was determined.

To determine the *in vivo* effect of a chelating agent on massive iron influx from the gut, animals were given 250 mg per kg body weight of iron as ferrous sulfate by gastric tube. Some animals served as controls, and the others were given the same treatment with EDDHA as in the absorption experiments. Iron excretion in the urine was measured during a 3-hour period following ingestion of iron. At 3 hours a blood sample was taken for determination of iron in the plasma. Samples of this plasma were dialyzed against saline to separate chelate-bound iron from protein-bound iron. The plasma was subsequently dialyzed against a solution of EDDHA (50 μg per ml) to separate transferrin-bound iron and iron bound in a nonspecific manner to other proteins.

Clinical studies with EDDHA and DTPA. Experiments were designed to determine the role of synthetic chelating agents in mobilization, transport, and excretion of iron in man. Subjects for these studies included normal volunteers, patients with various forms of iron overload, and patients with varying rates of erythropoiesis. In each subject $\text{Fe}^{59}\text{Cl}_3$ (40 μc) bound to 15 to 20 ml of normal plasma was injected intravenously, and samples of blood were drawn for the determination of plasma iron turnover. Two hours later an intravenous infusion was started of 2 to 4 g of EDDHA or DTPA with a tracer amount of $\text{Fe}^{59}\text{Cl}_3$ (5 μc) in 100 ml of saline; this was administered over a period of 5 to 6 hours. The urine was collected in 4-hour samples, usually during a 24-hour period, but in several instances for 3 days. Excretion of isotopic and nonradioactive iron in the urine was measured, and the utilization of iron by circulating red cells was determined after 14 days. Plasma volume was calculated either on the basis of an assumed blood volume of 60 ml per kg body weight or from the initial dilution of plasma-bound radioiron injected intravenously. In man the mean body hematocrit was assumed to be 0.92 times the venous hematocrit.

Chemical, hematologic, and isotopic techniques

All glassware employed in studies involving chelating agents was rendered iron-free by acid washing. Determination of iron in blood, urine, and tissues was done after acid digestion by the sulfosalicylic acid method of Lorber (11). Heme was isolated from blood or hemolysates by repeated crystallization according to the method of Labbe and Nishida (12). Known quantities of rabbit hemoglobin labeled with Fe^{59} were added so that loss of heme in the crystallization procedure could be determined. Plasma iron and iron-binding capacity were measured by the methods of Bothwell and Mallet (13) and Ressler and Zak (14). Plasma iron turnover was carried out as described by Bothwell, Hurtado, Donohue, and Finch (15). Hemoglobin was estimated as oxyhemoglobin or cyanmethemoglobin in an Evelyn colorimeter at 540 $m\mu$. Hematocrits were spun in Wintrobe tubes at 2,400 g for 30 minutes. Red cell counts were carried out by enumerating at least 1,000 cells in duplicate; sepa-

rate pipettes and counting chambers were used. Reticulocyte counts were performed on dried smears stained with new methylene blue, and 2,000 red cells were counted. The radioisotopes of iron employed, Fe^{55} and Fe^{59} , had a specific activity of 4 to 5 and 5 to 15 μc per μg , respectively. Samples containing both isotopes were digested in acid and the iron was electroplated onto copper disks for differential counting (16). Analysis of C^{14} in plasma and in urine was performed in a Tricarb liquid scintillation counter. In this procedure 0.1 ml of unknown solution was added to 2 ml of hyamine and 5 ml of toluene scintillator according to standard techniques. Correction for quenching was made by the addition of an internal standard and recounting of the samples.

RESULTS

1. Competitive binding of iron between synthetic chelating agents and transferrin in vitro. Three types of studies were performed: one in which the ability of chelating agents to remove iron from transferrin was tested, a second in which the removal of iron from chelating agents by transferrin was studied, and a third in which the iron was added to a mixture of the two substances and the total initial uptake and subsequent exchange by each was determined. When radioiron was bound to transferrin first and chelating agents were added, there was a transfer of radioiron of less than 1% in 24 hours, even when the amount of chelating agents was 100 times that of transferrin on a molar basis.³ On the other hand, transferrin easily removed iron which was initially bound to chelating agents in the mixture. When the molar ratio of the two binding substances was 1:1, removal from EDDHA, DTPA, and EDTA was 41, 21, and 78%, respectively. This transfer was reduced to less than 10% when the molar ratio between transferrin and the chelating agents was 1:10. In Figure 2 the distribution of radioiron added to mixtures of transferrin and chelating agents is shown. This distribution, determined after 24-hour dialysis, was again dependent on the concentration of chelating agents relative to unsaturated transferrin. With a 1:1 molar ratio of chelating agents to transferrin, EDDHA,

³In the calculation of the molar ratio between transferrin and the chelating agents, it should be remembered that each molecule of transferrin can bind two atoms of iron, and the synthetic chelating agents used bind only one atom per molecule. Normal human plasma was used, about one-third saturated with iron. Only the unsaturated fraction of transferrin was taken into account.

DTPA, and EDTA assimilated 18, 3, and 2%, respectively, of the radioiron added. These results clearly indicate that under the conditions of the experiment, transferrin was considerably more effective in binding iron than the various chelating agents employed, and that the chelating agents showed a binding strength bearing a general relationship to the stability constants of their ferric chelates.

Further studies were carried out in which the transferrin was saturated and excess iron was bound to other plasma protein. Plasma obtained from two animals with experimental iron poisoning contained 2,140 μg and 635 μg of iron per 100 ml before and 2,122 μg and 516 μg per ml after dialysis against saline. When EDDHA was added to the dialysis fluid, only 217 μg and 190 μg of iron were retained in the plasma samples. This residual fraction of iron corresponded to the transferrin-bound iron of the plasma. These results indicate that any iron in excess of transferrin is quickly taken up by the chelate.

2. *Transfer of iron from synthetic chelating agents to reticulocytes in vitro.* Immature red cells can absorb iron from transferrin *in vitro*, but adult cells do not take up iron under these circumstances (17). It was found that iron was taken up from iron-EDDHA solutions in saline only by immature red cells, the uptake being small

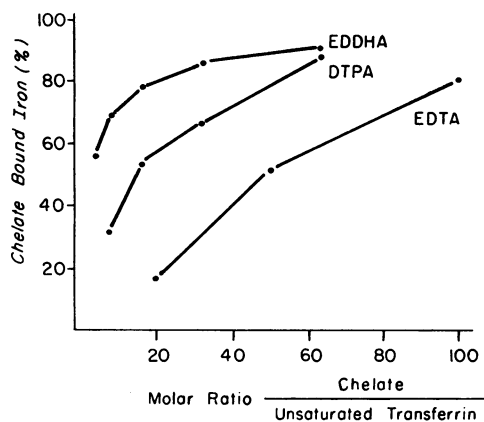


FIG. 2. COMPETITIVE BINDING OF IRON OF TRANSFERRIN AND SYNTHETIC CHELATING AGENTS. Tracer quantities of Fe^{59} were added to mixtures of plasma and synthetic chelating agents, and chelate-bound iron was separated from protein through dialysis. Each point in the curves represents an average of the results from three different plasma samples.

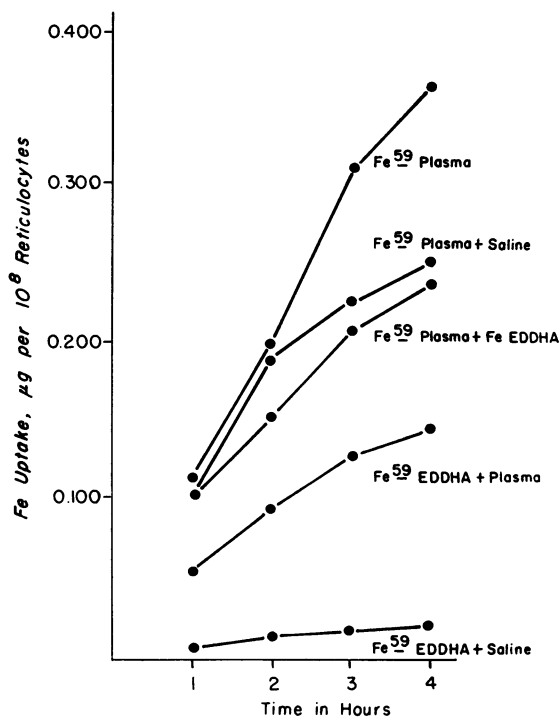


FIG. 3. UPTAKE OF IRON FROM PLASMA AND EDDHA SOLUTIONS IN SALINE BY RABBIT RETICULOCYTES. Fe^{59} was added in tracer quantities. Details of the conditions of incubation are given in the text under Experimental Procedures.

compared to that from iron-transferrin (Figure 3). The presence of nonlabeled plasma in the incubation mixtures increased the iron uptake to varying degrees. This increase could be accounted for by transfer of iron from EDDHA to transferrin during the 4-hour incubation, as would be anticipated from studies reported under Section 1. A large excess of EDDHA (exceeding by 20 times the iron-binding capacity of the plasma) with or without carrier iron did not inhibit the uptake of transferrin-bound iron by immature red cells. To determine the subsequent handling of iron assimilated by the red cells, heme was isolated from the incubated cells. The heme fraction was found to contain 50 to 90% of the total red cell iron (Fe^{59}), regardless of whether the iron had been originally bound to plasma or to EDDHA.

3. *Distribution and excretion of injected DTPA in rabbits.* Disappearance from plasma and urinary excretion of radioactivity after intravenous administration of 50 mg of C^{14} -DTPA was measured

TABLE I
Initial distribution and plasma disappearance rate of C^{54} -labeled DTPA*
(50 mg/kg) in normal (1-4) and in iron-heavy (5-6) rabbits

Rabbits	1	2	3	4	5	6
Plasma iron, $\mu\text{g}/100\text{ ml}$	183	191	270	243	165	153
Total plasma iron binding capacity, $\mu\text{g}/100\text{ ml}$	229	246	392	342	186	181
DTPA in plasma, % of injected radioactivity	6	11	10	9	12	10
Clearance of DTPA from plasma, t_4 in min.	50	45	50	70	41	46

* DTPA = diethylenetriaminepentaacetic acid.

in four normal rabbits and also in two "iron-heavy" animals to determine the possible influence of a large excess of iron in the body on the distribution of DTPA. The results are presented in Table I. Radioactively labeled DTPA was so rapidly lost from plasma after intravenous injection that after 10 minutes only 10 to 15% of the injected dose could be detected in the plasma. Subsequent disappearance from the plasma occurred in an exponential fashion with a half-time of about 50 minutes (Figure 4). DTPA was found solely in the plasma phase of whole blood. These data indicate that DTPA is initially distributed in a theoretical volume approximately 8 to 10 times greater than the plasma volume. There was no apparent difference in distribution of DTPA in the "iron-heavy" animals as compared to the normal. Urinary excretion varied from 41 to 85% over a period of 4 hours. Because of technical difficulties, it is possible that the urine collections may have been incomplete and the amount of DTPA excreted was underestimated.

4. *Distribution and excretion of injected Fe^{59} bound to EDDHA in the rabbit.* The results of distribution studies of radioiron bound to EDDHA are presented in Table II. As observed with the labeled chelating agent itself, a large portion of the radioiron disappears from the plasma in the first 10 to 15 minutes after injection. Subsequent disappearance of Fe^{59} from plasma occurs with a half-time of about 100 minutes. This time interval was significantly longer than anticipated from the results obtained with the labeled DTPA. The possibility existed that this represented not only iron bound to EDDHA but also iron now exchanged to transferrin. Such a transfer could indeed be demonstrated by separating the EDDHA-bound iron and the transferrin-bound iron by dialysis in a series of samples taken after injection

of Fe^{59} bound to EDDHA. An average of 50% of the injected EDDHA-bound iron was taken up by transferrin in the course of two hours in three rabbits which had been nephrectomized to prevent urinary excretion of the chelate.

Urinary excretion of Fe^{59} reached a maximum in the first hour after injection of chelate, with more than one-third of the amount injected excreted by that time. Approximately two-thirds of the total amount of radioiron excreted had been collected in the urine by 3 hours. In two animals killed 5 hours after injection of Fe^{59} -EDDHA, the radioiron was found widely distributed throughout all body tissues (Table III). In the nephrectomized animal the tissues contained greater amounts of radioactivity, but there was again little indication of specific localization. In the normal animal after 7 days, the major fraction was found in the circulating blood with a smaller amount present in the liver (Table IV).

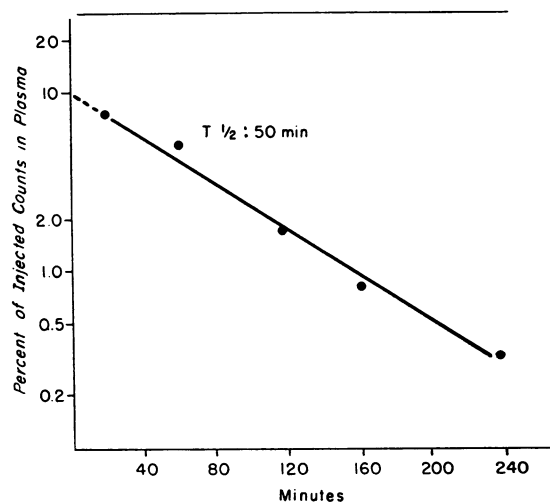


FIG. 4. C^{14} -ACTIVITY IN PLASMA AFTER INTRAVENOUS INJECTION OF C^{14} -LABELED DTPA. Carrier DTPA, 25 mg per kg body weight in a rabbit.

TABLE II
Distribution and excretion of Fe⁵⁹ injected as Fe⁵⁹-EDDHA in rabbits *

Rabbits	7	8	9
Plasma iron, $\mu\text{g}/100\text{ ml}$	181	167	266
Total plasma iron binding capacity, $\mu\text{g}/100\text{ ml}$	293	321	321
Fe ⁵⁹ in plasma, % of injected dose	8.6	12.5	9.1
Plasma Fe ⁵⁹ clearance, $t_1/2$ in min.	81	135	100
Renal excretion of Fe ⁵⁹ in 3 hours, % of injected dose	58	62	65

* EDDHA = ethylenediaminedi-(*o*-hydroxyphenylacetic acid).

Virtually no radioactivity was found in the remainder of the carcasses.

5. *Effect of chelates on iron absorption.* It has been postulated that absorption of iron is controlled by the amount of unsaturated transferrin in the plasma (18). Studies were therefore made of the effect on absorption of the repeated parenteral administration of sufficient chelate to cause a marked increase in the iron-binding capacity of the plasma. Enough chelating agent was injected to raise the binding capacity of the body fluids to 100 times that of circulating transferrin. Four rabbits were each given the same dose of labeled ferrous sulfate by gastric tube with and without parenteral administration of chelate (Table V). Different isotopes of iron were used for the two studies so that a comparison could be made. Absorption was unchanged in three animals and perhaps slightly better with EDDHA in the fourth. There was no significant difference in the amount of the activity found in the body tissues; over the 3-hour period of study about 10% of the iron was

excreted in the urine when the chelating agent was given.

The situation was entirely different when toxic doses of iron were administered by intubation. After saturation of the circulating transferrin, additional iron entering the plasma becomes loosely bound in a nondialyzable state by other protein, but can easily be complexed by EDDHA. In two animals given 250 mg iron per kg body weight by intubation and a total of 700 mg EDDHA by injection, the plasma iron in excess of that bound to transferrin was found to be dialyzable against saline (Table VI). In control studies it was shown that the excess iron was only dialyzable after addition of chelate (50 μg EDDHA per ml) to the dialysis fluid. The chelation of the excess iron in the treated animals was further indicated by the passage of dark urine, the color being due to the excretion of a large amount of the iron-chelate complex.

6. *Clinical studies with EDDHA and DTPA.* Table VII summarizes relevant hematologic data in patients given chelating agents. There were no serious side effects of the infusions of 2 to 4 g of chelating agents. Several patients complained of anorexia and nausea on the day following the

TABLE III
Tissue distribution of radioiron 5 hours after intravenous injection of Fe⁵⁹ as Fe⁵⁹-EDDHA (9 mg) in rabbits *

Rabbits	10	11
Urine	47.60	
Blood	5.12	8.24
Liver	11.19	29.21
Kidneys	2.78	
Spleen	0.03	0.04
Gut	5.64	8.69
Skeleton	17.47	23.66
Muscle	13.26	17.65
Skin and brain	8.89	16.50
Total activity	112%	104%

* Results are expressed as percentage of injected dose. Blood activity refers to that removed by exsanguination. Rabbit 10 was a normal animal and rabbit 11 was nephrectomized before injection of the iron-chelate. EDDHA = ethylenediaminedi-(*o*-hydroxyphenylacetic acid).

TABLE IV
Disposition of chelated iron in the rabbit*

Rabbits	12	13	14	15	16	17
Blood	26.0	28.3	55.6	54.9	57.7	29.9
Liver	11.7	12.0	14.5	12.3	5.9	5.7
Spleen	0.05	0.1	0.1	0.1		
Kidneys	0.9	0.9	0.3	1.3	1.0	1.0
Total urine of 7 days	†	†	†	†	37.7	64.6
Total recovery	39.1	30.5	70.5	68.6	101.4	100.3

* Tissue distribution of radioiron 7 days after iv injection of Fe⁵⁹ as Fe⁵⁹-EDDHA in rabbits. Results are expressed as percentage recovered of amount injected. Urinary losses were not measured in animals 12 to 15. Blood activity was calculated on the basis of an assumed blood volume of 60 ml/kg body weight.

† Not collected.

TABLE V
Effect of intravenous EDDHA on the absorption of iron in rabbits*

Rabbits	18		19		20		21	
Hemoglobin, g/100 ml	10.8		9.0		10.1		10.0	
Plasma iron, $\mu\text{g}/100\text{ ml}$	208		165		193		147	
Total plasma iron binding capacity, $\mu\text{g}/100\text{ ml}$	293		298		297		288	
Study	EDDHA	Control	EDDHA	Control	EDDHA	Control	EDDHA	Control
Blood	3.87	2.84	0.67	0.59	2.60	2.90	2.34	2.70
Liver	0.71	0.50	0.21	0.10	0.35	0.71	0.23	0.35
Kidneys	0.09	0.06	0.07	0.02	0.06	0.11	0.04	0.06
Urine	0.40		0.14		0.27		0.37	
Total	5.07	3.40	1.09	0.71	3.28	3.72	2.98	3.11

* Results are expressed as percentage of orally administered dose of radioiron. Blood values were determined before the first absorption study. Iron was given in a dose of 0.050 mg/kg body weight as ferrous sulfate. Fe^{59} was used as a tracer in the absorption studies during EDDHA treatment, and Fe^{55} was used in the control studies. EDDHA = ethylenediaminedi-(*o*-hydroxyphenylacetic acid).

administration of the chelates. Some patients experienced polyuria which did not correspond to the amount of iron excreted. There was no change in blood urea nitrogen levels or creatinine levels in the blood and no proteinuria after the injection of DTPA and EDDHA. One patient with aplastic anemia had a temperature rise to 40°C and also muscular pains for 3 days after the infusion of 3 g of DTPA.

To determine the transferrin-chelate interrelationship, studies were carried out with one isotope of radioiron bound to transferrin and the second bound to a chelate. These observations indicated that the infusion of chelating agents had

no effect on the kinetics of transferrin iron in the body as measured by plasma iron levels and by the rate of plasma iron turnover. A typical example of this is shown in Figure 5. After the intravenous injection of radioiron-labeled chelate, a urinary loss of 26 and 28% of Fe^{59} was found in

TABLE VI
Effect of chelate on plasma iron overload*

Rabbits	22	23
Plasma iron, $\mu\text{g}/100\text{ ml}$, before oral iron	275	112
Plasma iron, $\mu\text{g}/100\text{ ml}$, 3 hours after oral iron	1,223	2,140
Plasma iron, $\mu\text{g}/100\text{ ml}$, after dialysis against saline	384	2,122
Plasma iron, $\mu\text{g}/100\text{ ml}$, after dialysis against EDDHA	235	217
Iron, μg , excreted in the urine 3 hours after oral iron.	8,800	<200

* Plasma iron levels and urinary excretion of iron after administration of 250 mg of iron as ferrous sulfate per kg body weight by gastric tube. One rabbit (22) was treated with EDDHA iv in a total dose of 400 mg. The other rabbit (23) served as a control. EDDHA = ethylenediaminedi-(*o*-hydroxyphenylacetic acid).

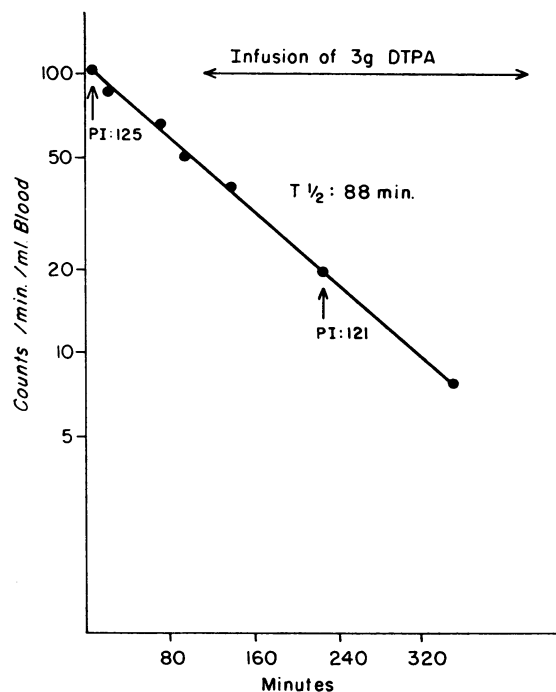


FIG. 5. PLASMA RADIOACTIVITY IN A NORMAL SUBJECT AFTER INTRAVENOUS INJECTION OF Fe^{55} BOUND TO HIS OWN PLASMA. Trisodium calcium DTPA, 3 g, was given in 500 ml of saline as an intravenous drip over a 5-hour period. PI = plasma iron.

TABLE VII
Subjects infused with chelate*

Patient	Diagnosis	Age	Sex	Hgb.†	Hct.†	Plasma iron	Total iron binding capacity	Bone marrow iron	Plasma iron turnover	Transfused iron
				g %	%	μg %	μg %			g
R.B.	Normal subject	23	M	14.1	44	88	209		0.58	
L.N.	Normal subject	22	M	14.2	48	125	283		0.77	
C.R.	Hereditary spherocytosis	18	F	11.8	33	81	221		2.02	
C.A.	Idiopathic hemochromatosis	71	M	13.8	41	65	359		1.04	
W.W.	Idiopathic hemochromatosis	51	M	14.5	39	273	349		0.98	
S.A.	Refractory anemia with hyperplastic bone marrow	68	M	5.6	19	190	260	increased	3.14	12
M.B.	Refractory anemia with hypoplastic bone marrow	33	F	9.1	34	261	261	normal	0.36	7
K.B.	Refractory anemia with hypoplastic bone marrow	28	F	5.8	18	130	210	increased	0.44	25

* Relevant clinical and laboratory data on subjects used in the study of synthetic chelating agents. Patients W.W. and C.A. had typical skin pigmentation, diabetes, and disturbed liver function. The diagnosis was verified by liver biopsy. Both patients had undergone treatment with repeated phlebotomy. Examination of the spleen and a liver biopsy in patient C.R. at the time of splenectomy showed moderate siderosis. Plasma iron turnover is expressed as mg per 100 ml of whole blood per day.

† Hgb. = hemoglobin; Hct. = hematocrit.

two normal subjects, whereas in a group of patients the urinary losses varied from 22 to 65%. Of the chelate-iron retained in the body, the average amount taken up by the bone marrow for heme synthesis was in most cases only slightly below that found with transferrin-iron (Table VIII).

The amount of nonradioactive iron excreted in the urine was also measured. Although this was less than 1 mg in the two normal subjects, in patients with iron overload, the excretion was increased to as much as 102 mg after a single chelate injection. Two patients with idiopathic hemochromatosis excreted a lesser amount, but the body stores of these patients had been depleted by repeated phlebotomy over the years prior to the study. The amounts of iron excreted by the three patients with transfusion hemosiderosis seemed related to the quantity of iron acquired by transfusion.

DISCUSSION

To provide a guide for the more complicated *in vivo* relationships, *in vitro* experiments have been reported concerning iron exchange between plasma transferrin and chelates. Although these studies are limited in scope, they indicate the amount of exchange which might be expected over the short

time of contact involved in the *in vivo* studies. Under the conditions employed, the natural protein, when present in equimolar amounts, i.e., twice the binding capacity for transferrin as compared with chelates, took up or held over 80% of radioiron added. Equal distribution of iron between chelates and transferrin was obtained at 24 hours only when the molar ratio of EDTA to transferrin was 5:1, of DTPA, 10:1, and of EDTA, 50:1. These results, in general agreement with the findings of Rubin, Houlihan, and Princiotto (8), indicate a clear-cut superiority in binding of iron by transferrin over any of the other chelates tested in a plasma environment.

In translating such data to *in vivo* conditions, one must allow both for the distribution of chelates within a large volume of body water and their rapid excretion in the urine. Our observations and those of Foreman and associates using EDTA (19, 20) indicate that virtually all of the injected chelate is excreted within 24 hours. In rabbits about two-thirds of the complexed radioiron accompanies the chelate in the urine, whereas in normal human subjects, our results (Table VIII) and those of Korman (21), would indicate that about one-third is excreted. Radioiron bound to chelate and injected intravenously was found initially widely distributed through the body tissues

TABLE VIII
Disposition of chelated iron in man*

Patients and normal subjects	R.B.	L.N.	C.R.	C.A.	W.W.	S.A.	M.B.	K.B.
Red cell utilization, %	83	87	65	95	72	17	14	2
Transferrin-iron (Fe ⁵⁹)								
Red cell utilization, %	68	72	66	48	67	10	13	2
Retained chelate-iron (Fe ⁵⁹)								
Excretion of chelate-Fe ⁵⁹ in the urine, %	26	28	28	30	44	55	65	58
Excretion of iron in the urine after 3 g DTPA, mg	0.8	0.7	8	0.4	13	70	7	102
Excretion of iron in the urine after 3 g EDDHA, mg					25		18	22

* Distribution of intravenously injected Fe⁵⁹ chelated to EDDHA and DTPA in man. Data of radioactivity are expressed in terms of percentage of dose injected. Relevant clinical and laboratory data on these subjects are presented in Table VII. Red cell utilization was measured 14 days after injection of radioiron. DTPA = diethylenetriaminepentaacetic acid; EDDHA = ethylenediaminedi-(*o*-hydroxyphenylacetic acid).

of rabbits, whereas at 7 days after injection, the retained radioiron was distributed in a fashion similar to that of transferrin-bound iron (Table IV). Evidence of direct transfer of iron from EDDHA to transferrin *in vivo* was found in nephrectomized rabbits, whereas transfer from transferrin to the chelate could not be demonstrated. These observations are then consistent with the *in vitro* observations indicating the greater binding affinity of transferrin as compared with chelates, and indicate that a considerable portion of chelate-bound iron may be taken up by transferrin.

An attempt was made to demonstrate the transfer of iron from the gastrointestinal tract to chelate in plasma and extracellular fluids. In animals given EDDHA with a binding capacity 100 times that of circulating transferrin, up to 10% of the radioiron absorbed from the gastrointestinal tract was excreted in the urine bound to chelate. The actual amount of iron taken up from the intestinal mucosa by the chelate must have been somewhat greater, since the iron-EDDHA complex would be partly dissociated in the body. Total absorption of iron, however, did not appear to be altered by parenteral administration of EDDHA (Table V).

The ability of chelates to participate in the normal return of catabolized red cell iron from reticuloendothelial cells to transferrin was examined in normal individuals and in a group of patients with varying rates of plasma iron turnover. In two

normal subjects, plasma iron turnover, as measured by plasma iron levels and the disappearance rate of injected transferrin-bound radioiron, appeared unchanged during an infusion of 3 g of DTPA over a period of 5 hours (Figure 5). No relationship was discernible between rate of plasma iron turnover, i.e., the rate at which transferrin exchanged iron with the tissues, and the amount of iron in the urine bound to chelate (Table IX). However, the extreme variations in iron excretion due to variable iron stores may have obscured other relationships.

To observe the release of chelate-bound iron to tissue receptors, studies were carried out *in vitro* with reticulocytes. In the absence of transferrin, only a small fraction of chelate-bound iron was absorbed onto the surface of the immature red cell,

TABLE IX
Relation of plasma iron turnover to urinary excretion of iron after infusion of 3 g of a synthetic chelating agent (EDDHA or DTPA) *

Plasma iron turnover	Excretion of iron
mg/hr	mg
0.54	7.0
0.71	102.0
1.04	0.3
1.37	0.7
1.54	0.8
1.87	13.0
3.12	6.0
5.58	7.0

* EDDHA = ethylenediaminedi-(*o*-hydroxyphenylacetic acid); DTPA = diethylenetriaminepentaacetic acid.

and this amount might well have been explained by the residual transferrin on such cell surfaces (22). The red cell uptake of radioiron was increased when plasma was added to the incubation mixture, in keeping with the ability of transferrin to assimilate iron from the chelate and to transfer it to the reticulocyte. The synthetic chelates, on the other hand, in no way inhibited transfer of iron from transferrin to the reticulocyte. Once iron gained access to the red cell, either directly from transferrin or indirectly from the iron-chelate complex in the presence of transferrin, its incorporation into heme appeared to be the same. These results strongly suggest that these chelates are unable to transfer iron directly to tissue receptors, but do yield their iron to transferrin. These various observations of the inability of ferric iron-binding chelates to participate in normal iron exchange give further support to the concept that iron must be reduced before exchange can occur between tissues and plasma.

Although chelates are relatively ineffective in participating in normal iron transport, it is evident that they can bind iron in pathologic states of iron overload. In acute iron toxicity following the ingestion of large amounts of iron by mouth, circulating transferrin is saturated and trivalent iron may be found in loose complexes with various plasma proteins. Such iron is rapidly and completely taken up by the chelates and subsequently eliminated through urinary excretion.

Although the toxicity in iron poisoning may not be related to this protein-bound fraction of iron, since it has been shown to be relatively nontoxic when injected intravenously (23), it is nevertheless reasonable to assume that such free iron on gaining initial access to the circulation may exert toxic effects before it becomes bound to the plasma proteins, and that the presence of chelates might provide protection in iron poisoning. There have been reports on the use of EDTA in this connection, both in animal experiments and also in accidental iron poisoning in children (24, 25).

Considerable attention has been directed to the use of these chelates in causing iron excretion from the body in states of iron excess. Thus Fahey and associates (5) have reported increased amounts of iron up to 100 mg a day in the urine, and Figueroa and Tuttle (7) have reported more

detailed studies in idiopathic hemochromatosis. Our own observations further demonstrate that these chelates effect excretion of iron in patients with iron overload. It is not clear what source of body iron is available to the chelates. Our results and those of Figueroa and Tuttle (7) indicate that tissue iron becomes available to chelates only when an excess is present. The rate of plasma iron turnover did not correlate with the amount of iron excreted after administration of chelate. Korman has suggested that the chelates may remove iron from ferritin (21). Our own attempts to demonstrate uptake by chelate of radioiron from broken cells containing ferritin and hemosiderin injected subcutaneously in rats have failed. In this connection it is important to remember that there is no evidence that chelates can pass cell membranes and come into contact with intracellular iron. One might therefore speculate that the available iron is located on the cell membrane.

SUMMARY

Three synthetic chelating agents, ethylenediaminedi-(*o*-hydroxyphenylacetic acid), diethylenetriaminepentaacetic acid, and ethylenediaminetetraacetic acid, have been shown to be less effective than transferrin in binding iron *in vitro*.

When injected intravenously, they are distributed in the extracellular space and rapidly excreted. The iron-chelate complex is partly dissociated *in vivo*, and iron is yielded to transferrin. Synthetic chelates do not participate significantly in normal iron exchange between donor tissues, such as intestinal mucosa and reticuloendothelial cells, and the receptor cells of the erythroid marrow and do not remove iron from transferrin.

Chelates do bind iron when present in plasma associated with proteins other than transferrin and bind noncirculating iron in patients with iron overload. In these two conditions the chelates may be useful in effecting excretion of iron.

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