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Research Article

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In vitro studies suggest that heme complexes with a substance present in IF-containing materials. Whether a gastric glycoprotein similar to IF serves as an intestinal transport factor for heme, similar to transport of vitamin B_{12}, or whether normal gastric juice acts by another mechanism cannot be determined at this time.

Malabsorption of Hemoglobin Iron in Pernicious Anemia: Correction with Intrinsic Factor–Containing Substances

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ABSTRACT Hemoglobin iron absorption in patients with treated pernicious anemia (PA) and concomitant iron deficiency was low compared to absorption in patients with iron deficiency alone. Crude and purified hog intrinsic factor (IF) concentrates doubled the absorption of hemoglobin iron in these patients as did normal (neutralized depepsinized) human gastric juice. Hemoglobin iron absorption was not significantly enhanced by PA gastric juice. Absorption of *heme* iron, like that of *hemoglobin* iron, was enhanced by normal neutralized depepsinized gastric juice. No enhancement of hemoglobin iron absorption by these substances was obtained in the normal or iron-deficient non-PA control subjects. Preincubation of the hog IF concentrate with antisera to IF significantly reduced the enhancement of hemoglobin iron absorption due to the concentrate.

In vitro studies suggest that heme complexes with a substance present in IF-containing materials. Whether a gastric glycoprotein similar to IF serves as an intestinal transport factor for heme, similar to transport of vitamin B_{12}, or whether normal gastric juice acts by another mechanism cannot be determined at this time.

INTRODUCTION

Hemoglobin is a significant source of dietary iron (3, 4) and is poorly absorbed in the iron deficiency anemia of gastrectomized patients (5, 6), a factor which perhaps contributes to the anemia. Heme iron appears to have a different mode of intestinal absorption from that of inorganic iron salts (7–9). The association of iron deficiency, achlorhydria, and pernicious anemia (PA) has been extensively documented (10–13). Gastric parietal cell antibody has been found in nearly all cases of PA and achlorhydric iron deficiency anemia (14–16). This evidence suggests that a normal stomach is required for absorption of both iron and vitamin B_{12}. Moreover, the ring structure (but not necessarily the spatial configuration) of porphine (the basic porphyrin structure) and the corrin nucleus of B_{12} are almost identical, differing mainly by lack in corrin of an alpha methene bridge between positions 1 and 19 and also by lack of some double bonds in corrin. A number of workers (17–19) postulated that a gastric intrinsic factor (IF), similar to that required for B_{12} absorption, was required for *inorganic* iron absorption. The current studies were performed to determine whether IF-containing substances were needed for absorption of hemoglobin iron, which is a form of *organic* iron.

METHODS

Iron absorption was studied in patients with treated PA with iron deficiency as well as in normal and iron deficient subjects. Subjects were selected who had no recent blood loss or iron therapy. The diagnosis of PA was established by absence of IF in gastric juice (20) and by normalization of absorption of B_{12}-^{60}Co by IF (21). Iron deficiency was established by transferrin saturation of less than 16% (22), as determined by the iron method of Ramsey (23) and the iron-binding capacity method of

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Herbert, Gottlieb, Lau, Fisher, Gevirtz, and Wasserman (24).

Methods. Hemoglobin labeled with ^{59}Fe was prepared by injecting 1.2–2.0 mc of ^{59}Fe into anemic Dorset sheep; 7–10 days later the animals were bled, and the radioactive red cells were separated from plasma, hemolyzed, and fed to recipients.

Radioactive hemin was prepared from labeled hemoglobin by the method of Labbe and Nishida (25). Since hemin is a heme, for simplicity we will subsequently refer to our prepared product as heme. Hog IF concentrates were generously provided by Dr. Leon Ellenbogen of Lederle Laboratories, Pearl River, N. Y. The *crude* IF concentrate was active in man in a daily oral dose of 50 mg. The *purified* IF concentrate was 100 times more concentrated (active in a daily oral dose of 500 μg. Gastric juices were depepsinized by bringing them to pH 11 with 1 N NaOH for 20 min; they were then neutralized and stored at −20°C. Antisera to human and hog IF concentrates were utilized, and antibody activity was measured by blocking B_{12} binding to IF (20).

Administration. The hemoglobin-^{59}Fe was taken orally in 40 ml of tomato juice after an overnight fast. The radioactivity administered ranged from 1 to 2 μc; the amount of hemoglobin iron was 4 mg.

The percentage of iron absorption was determined by whole body counting[1] at 7 and 14 days after isotope administration, and the 4 hr value was 100% (26). The counting period was 10 min, and the counts under both ^{59}Fe full energy peaks (1.098 and 1.297 Mev) were summated.

The entire 2 wk absorption study was then repeated, and either crude IF (50 mg), purified IF (1 mg), or neutralized depepsinized gastric juice (20 ml) was given to the same patients immediately after the same amount of hemoglobin iron had been previously ingested. In certain studies, autoantibody to human IF (20 ml of serum from appropriate patients with PA) was incubated 30 min with the IF concentrate and then administered before hemoglobin was added. Taylor had shown that such serum blocked the action of hog IF (27).

RESULTS

In vivo experiments. Hemoglobin iron absorption was studied in four control (i.e., non-PA) subjects (Table I). These controls included two normal subjects and two subjects with iron deficiency. The iron-deficient patients absorbed almost twice as much iron as did the normal subjects. Crude IF concentrate and gastric juice did not significantly enhance hemoglobin iron absorption in either group. Also noted in Table I are the similar results of previous investigators.

[1] Whole Body Counting Facility at New York University was used with the kind courtesy of Dr. B. Pasternack and with the aid of Miss Linda Rose.

TABLE I

Hemoglobin Iron Absorption in Controls (Normal and Iron-Deficient Non-PA)

Controls	Hb	Fe/TIBC	Absorption of hemoglobin-^{59}Fe: Hb alone, 4 mg iron	+ Crude IF	+ Normal GJ
	g%		%	%	%
Normal					
E. F.	15.4	107/209	11.9	11.7	11.2
E. F. (duplicate)			10.9	—	—
S. W.	14.6	96/242	8.5	7.6	10.3
Iron-deficient non-PA					
C. E.	9.2	34/360	18.1	16.6	22.4
J. R.	9.8	24/298	21.0	18.3	18.2

Results of Previous Investigators

Investigators	Dose of Hb iron	Absorption of hemoglobin-^{59}Fe: Normal	Iron-deficient non-PA
	mg	%	%
Callender (7)	5	11.0	22
Turnbull (8)	5	12.7	15.6
Conrad (9)	1	22.1	35.1

PA, pernicious anemics; TIBC, total iron-binding capacity; IF, intrinsic factor; GJ, gastric juice.

In sharp contrast to control subjects, the absorption of iron after the administration of 4 mg of hemoglobin iron to five patients *with PA in remission and concomitant iron deficiency* averaged 10.4% with a range from 2.7 to 21.4% (Table II). This is approximately half the absorption expected in the uncomplicated iron-deficient patient. Crude IF concentrate and normal gastric juice each *enhanced* hemoglobin iron absorption *more than twofold.* However, there was no significant increase in hemoglobin iron absorption with the administration of PA gastric juice. The enhancement by purified was less than by crude IF, perhaps suggesting that purification removed the potentiator of hemoglobin iron absorption. An additional patient with treated PA with iron deficiency absorbed 2.6% of the ^{59}Fe in 112 mg of heme and 27% when 20 ml of normal gastric juice was added.

Antibody to IF was given with the crude hog IF to three patients with PA and iron deficiency and reduced the enhancing effect of IF on absorp-

TABLE II

Hemoglobin Iron Absorption in Patients with Treated for Pernicious Anemia and Concomitant Iron Deficiency: Effect of Intrinsic Factor-Containing Substances

Subject	Hb	Fe/TIBC	Absorption of hemoglobin-^{59}Fe				
			Hb alone	+ Crude IF	+ Purified IF	+ Normal GJ	+ PAGJ
			%	%	%	%	%
S. T.	11	40/344	9.7	22.4	17.7	21.1	12.8
A. H.	9.6	27/317	10.7	30.8	24.3	23.0	—
I. S.	12.2	41/277	2.7	15.6	3.6	19.8	4.1
C. D.	12.1	53/317	21.4	41.7	38.5	36.5	20.6
C. H.	11.8	38/324	7.7	24.2	12.8	16.3	10.2
Average			10.4	26.9	19.4	23.3	11.9

PA, pernicious anemia; TIBC, total iron-binding capacity; PAGJ, gastric juice from patient with pernicious anemia.

tion of hemoglobin iron (Table III). There was only minimal reduction in hemoglobin iron absorption when normal serum was substituted for IF antiserum, a phenomenon probably due to haptoglobin binding of hemoglobin-^{59}Fe. The finding with serum containing IF antibody suggests that a specific substance mediating hemoglobin iron absorption has been blocked.

In vitro experiments. Various experiments were designed to provide an in vitro model of the IF concentrate–hemoglobin interaction.

Demonstration using coated charcoal of heme binding by IF. Heme-^{59}Fe was dissolved in 0.1 N NaOH and diluted with 0.1 M Tris, pH 8.0, to a concentration of 400 μg/ml (as determined by spectrophotometry at wavelength 610 mμ). Increasing amounts of crude IF (1–14 mg) and purified IF (0.1–1.4 mg) were incubated with heme-^{59}Fe (40 μg) in 0.1 M Tris buffer, pH 8.0, at 37°C for 30 min. Dextran-coated charcoal (DCC) (1 : 10 ratio of Dextran 10 [Pharmacia Fine Chemicals Inc., Piscataway, N. J.] to charcoal; 0.25 g/100 ml of dextran) was used to separate the unbound heme (adsorbed by DCC) from bound heme (remaining in supernatant). The binding curve (Fig. 1) demonstrates that a given weight of hog IF concentrate has a maximal fixed capacity to result in binding of heme. In the linear portion of the curve, 1 mg of crude IF binds approximately 7 μg of heme when the crude IF : heme weight ratio was less than 100 : 1. Purified IF (100 times more concentrated) similarly studied bound 14 μg/mg of purified IF when the heme : purified IF weight ratio was less than 15 : 1. On a weight ratio, purified IF did not appear to bind more heme than the crude IF, a finding which suggests that the heme-binding substance is not the same as the binding substance for B_{12}. Preincubation of the crude IF

TABLE III

Effect of IF Antibody on the Absorption of Hemoglobin Iron in Treated PA with Iron Deficiency

Subject	Absorption of hemoglobin ^{59}Fe			
	Alone	With crude IF	With crude IF + AB IF serum	With crude IF + normal serum
	%	%	%	%
A. H.	10.7	30.8	14.5	26.4
I. S.	2.7	15.6	8.0	12.8
C. D.	21.4	41.7	11.3	—

PA, pernicious anemia; IF, intrinsic factor; AB, antibody.

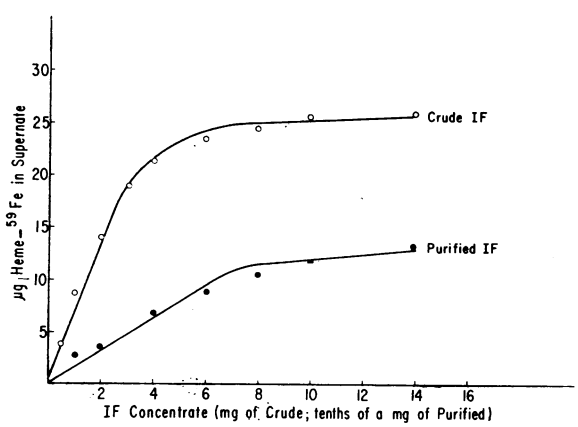

FIGURE 1 Apparent binding curve of heme by intrinsic factor (IF)-containing substances determined by dextran-coated charcoal separation.

concentrate with heme did not reduce the ability of IF to subsequently bind B_{12}-^{57}Co (as determined by coated charcoal (20). Conversely, preincubation of IF concentrate with B_{12} did not reduce subsequent binding by crude IF of heme-^{59}Fe.

Sucrose density gradient ultracentrifugation. Crude IF concentrate (10 mg/ml) labeled with B_{12}-^{57}Co was incubated for 30 min at 37°C in 0.1 M acetate buffer, pH 4.5, alone or with hemoglobin-^{59}Fe (10 mg/ml). Sucrose density gradient (10–40%) ultracentrifugation was performed on each solution in a Beckman preparative ultracentrifuge at 40,000 rpm for 16 hr similar to the method of Martin and Ames (28). The sucrose fractions were then counted for both ^{59}Fe and ^{57}Co activity in a Picker Autowell II. (Picker Instrument Corp., New York). Hemoglobin-^{59}Fe and IF-^{57}Co centrifuged separately are both found in the low molecular weight fraction (Fig. 2). When incubated together, 40% of the hemoglobin-^{59}Fe was found in a higher molecular weight range, whereas the ^{57}Co-labeled IF remained in the low molecular weight fraction. When the solutions were incubated at alkaline pH (8.5) hemoglobin did not migrate to a larger molecular weight range when mixed with IF-^{57}Co.

Sucrose density gradient patterns were done on solutions of heme-^{59}Fe (1 mg), crude IF concentrate (10 mg), labeled with B_{12}-^{57}Co, and a mixture of the two prepared in 0.1 M Tris buffer pH 8.0. In the mixture of heme and crude IF, 60% of the heme-^{59}Fe is found in a higher molecular weight fraction (Fig. 3). Therefore, hog IF concentrates appear to increase the molecular size of heme or hemoglobin, but, in the case of hemoglobin, not by combination with that fraction of IF concentrate which binds to B_{12}.

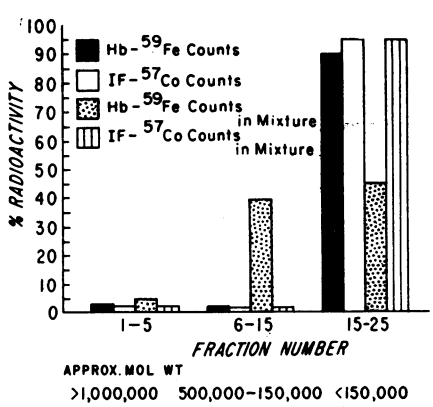

FIGURE 2 Sucrose density gradient ultracentrifugation patterns of hemoglobin-^{59}Fe and IF-^{57}Co interaction.

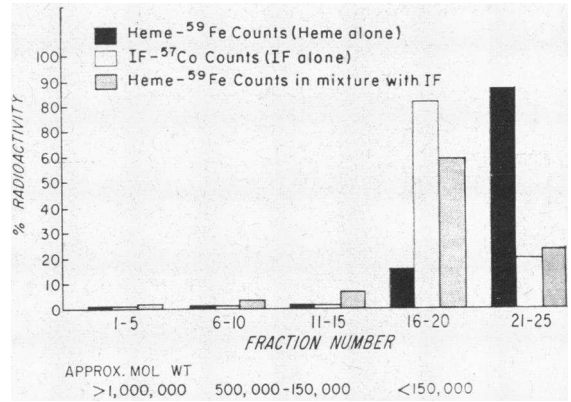

FIGURE 3 Sucrose density gradient ultracentrifugation patterns of heme-^{59}Fe and IF-^{57}Co interaction.

Sephadex gel filtration studies. G-15 Sephadex gel was expanded and washed overnight in 0.10 M phosphate buffer, pH 8.0, and then packed in a Pharmacia column K 9/15. Thereafter, heme-^{59}Fe (0.1 mg) alone or after incubation for 30 min at 37°C with either crude IF (1.0 mg), purified IF (0.1 mg), normal neutralized depepsinized gastric juice (concentrated 20 times), or PA gastric juice (concentrated 20 times) were passed through the column in 0.1 M phosphate buffer, pH 8.0. Several void volumes were collected and counted for ^{59}Fe activity. Heme-^{59}Fe alone (Ta-

TABLE IV

G-15 Sephadex Gel Filtration Studies of Heme-^{59}Fe-IF Interaction

	Heme-^{59}Fe alone	Heme-^{59}Fe + crude IF	Heme-^{59}Fe + pure IF	Heme-^{59}Fe + normal GJ	Heme-^{59}Fe + PAGJ
	%	%	%	%	%
Void volume					
1–2	17	90	75	85	72
3–4	3	2	4	3	2
5–6	0	0	0	0	2
^{59}Fe activity recovered in eluates	20	92	79	88	76
^{59}Fe activity remaining on column	80	8	21	12	24

IF, intrinsic factor; GJ, gastric juice; PAGJ, gastric juice from patient with pernicious anemia.

ble IV) was in the main retarded in the gel (80%), while in the presence of IF-containing substances and both normal GJ and PAGJ it was mainly recovered in the first void volume. This finding may be interpreted as meaning that heme alone is retarded because the molecular size is less than 1500 or is insoluble in this medium and adheres to the gel. In the presence of IF-containing substances, normal GJ, and PAGJ, a larger molecular weight or soluble heme complex is formed, and the heme complex rapidly passes through the column.

DISCUSSION

Heme iron is absorbed via a different mechanism from that for inorganic iron (7–9). Recent studies by Conrad, Weintraub, Sears, and Crosby (9). demonstrating that hemoglobin is split by duodenal enzymes suggested that the iron was absorbed into the intestinal epithelial cell as a metalloporphyrin. This phenomenon would explain why agents such as phytates (which complex free iron) and ascorbic acid (which promotes absorption of free iron) have no significant effect on hemoglobin iron absorption. There is a decrease in percentage absorption but an increase in total absorption as larger amounts of both inorganic and hemoglobin iron are ingested, and there is enhanced absorption of both inorganic and hemoglobin iron in iron-deficient patients. Intestinal factors such as pH and coordinating substances (i.e., ligands) play a role in the regulation of hemoglobin iron absorption by their effect on heme polymerization (29), monomeric heme compounds being better absorbed than heme polymers of large molecular size.

Before the present study, there was evidence to suggest that *gastric factors* play a role in hemoglobin iron absorption, particularly in iron deficiency anemia. There is a 10–40% occurrence of iron deficiency anemia in gastrectomized patients. Studies of iron absorption (5, 6) in such patients reveal malabsorption of hemoglobin iron in the iron-deficient gastrectomized patient, because the expected increase in hemoglobin iron absorption in iron deficiency does not occur. Iron-deficient gastrectomized animals also malabsorb inorganic iron; this malabsorption is not corrected by ascorbic acid (30). The occurrence of iron deficiency (not associated with blood loss) in 30% of patients with treated PA (10) further suggests the importance of the normal gastric mucosa in iron absorption. Interestingly, gastric parietal cell antibody is found in about ⅕–⅓ of patients with iron deficiency (14–16). Biggs, Bannerman, and Callender (31) found normal absorption of ^{59}Fe-labeled hemoglobin in achlorhydric subjects. Jacobs, Bothwell, and Charlton (32) studied patients with PA in remission and found that HCl enhanced the absorption of ferric chloride but not ferrous ascorbate or hemoglobin iron. Cook, Brown, and Valberg (33) found that achlorhydric patients absorbed ½ the expected amount of inorganic iron given with bread; the malabsorption was corrected by the addition of gastric juice but not as well by neutralized gastric juice. The presence of a gastric protein with ability to affect inorganic iron absorption has been suggested (17–34). Koepke and Stewart (18) described a factor in gastric juice from anemic dogs which significantly increased the absorption of such iron. This factor was stated to be a glycoprotein with a molecular weight of 4500.

In the current study, we found diminished hemoglobin iron absorption in patients with treated PA and coincident iron deficiency; this malabsorption was corrected by IF concentrates and normal neutralized depepsinized gastric juice, but not by PA gastric juice. The iron deficiency in these patients with PA may be related to the malabsorption of hemoglobin iron, since other causes of iron deficiency, such as blood loss, were absent. In addition, antiserum to IF markedly reduced the enhancing effect of IF on hemoglobin iron absorption. This finding suggests that a gastric factor other than acid, present in the normal stomach, potentiates the absorption of hemoglobin iron. Our in vitro studies suggest that heme complexes with a substance present in IF-containing materials. Sucrose density gradient ultracentrifugation revealed that hemoglobin migrated to a higher molecular weight position than did hemoglobin alone or B_{12}-^{57}Co attached to IF when ^{59}Fe-labeled hemoglobin and B_{12}-^{57}Co IF were simultaneously present.

Conrad, Benjamin, Williams, and Foy (35) showed that agents which retard the passage of heme through Sephadex enhanced heme absorption. The in vitro evidence in this study suggests that the molecular size of the heme iron complex

increases in the presence of IF-containing substances. Thus, the active agent or agents in IF-containing substances are probably *not* one of the depolymerizing agents studied by their group, since IF concentrates *speed* the passage of heme through Sephadex (Table IV). PA gastric juice similarly appeared to increase the molecular size of the heme iron complex, a finding which suggests that other factors in gastric juice may interact with heme iron and not enhance intestinal absorption. This factor may be similar to the non-IF B_{12} binders in normal and PA gastric juice which do not enhance the intestinal absorption of B_{12}. Whether a specific gastric glycoprotein similar to IF serves as an intestinal transport factor for heme iron, in a manner similar to IF-facilitated B_{12} absorption, or whether normal gastric juice contains nonspecific solubilizing proteins which aid in hemoglobin iron transport remains to be determined.

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