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Enhancement of Intestinal Iron Absorption by a Humoral Effect of Hypoxia in Parabiotic Rats (32973)

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In recent years much has been learned about the kinetics of the mechanism for the intestinal absorption of iron and the diverse conditions associated with increased iron absorption (1–4); yet the link between these conditions and the absorptive process is unknown. The possibility that a humoral factor might be involved has led us to use parabiotic rats in an attempt to determine if iron absorption can be promoted by an indirect effect (5,6). The results indicate that hypoxia can increase iron absorption when the possibility of a direct effect on the bowel is excluded and that this action must be mediated by a transferable humoral factor. Our findings, with those of other workers, suggest that this factor probably is not erythropoietin.

Materials and Methods. Highly inbred female rats of strain LEW/Mai, weighing 60–75 gm each, were parabiosed by a modification of the Bunster-Meyer technique. These inbred animals were selected because parabionts could be prepared without the occurrence of the intoxication reaction observed with outbred strains. Twelve of 19 pairs were successfully used in the study. Of the remaining seven pairs, three died spontaneously, two were killed accidentally, one did not gain weight normally, and one was excluded because of imperfect dosing. They were given Purina rat chow *ad libitum*, and 4–5 weeks later, when the combined weight of a pair was approximately 250 gm, hematocrits were measured in blood taken from the tail vein of each animal. Patency of the vascular anastamosis between parabionts was established by injecting a small quantity of red cells labeled with ^{51}chromium into the tail of Rat A, the member that would subsequently be subjected to hypoxia, and 3–6 hours later measuring the radioactivity in 40 μl of tail vein blood from Rat B. Equilibration of injected red cells occurred within 3 hours. The parabiotic rats continued to grow normally: they did not develop anemia, parabiotic shift of red cell mass or intoxication. Occasional testing of vascular anastamoses with ^{51}chromium labeled red cells during the course of the experiment showed them to be functional in all cases.

Intestinal iron absorption was measured as follows: both members of a pair were starved but given tap water *ad libitum* for 24 hours. Rat B was then given 0.6 ml of fluid containing 0.2 μC ^{59}ferrous citrate and 500 μg of carrier iron as freshly prepared ferrous sulfate via a polyethylene tube passed down the esophagus into the stomach. Each pair was then housed individually in a stainless steel cage which was designed to permit collection on aluminum foil of all feces passed by both rats. After 6 days the feces were put into a polyethylene container (250-ml capacity) and the aluminum foil was washed extensive-

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ly with gauze sponges wet with Radiacwash.[2] Radioactivity of feces plus washings was then measured by counting them in a small animal whole body counter. Sufficient counts were obtained to give a counting error of less than 1%. The amount of radioactivity originally administered was determined by pipetting an aliquot of the dose into a polyethylene container filled with guinea pig food and mixing thoroughly. Comparison of this standard with standards made up in nonradioactive rat feces showed that the self absorption and geometry of standards and collected feces and washings were comparable. Intestinal iron absorption was calculated by subtracting radioactivity excreted in the feces in 6 days from the amount administered to Rat B.

It has been found that when one rat of a parabiotic pair was exposed to hypoxia, the oxygen saturation of the blood of the other member remained normal (5). In this experiment only Rat A of each pair was subjected to 24 hours of continuous hypoxia; Rat B of each pair breathed room air. This was accomplished by placing the parabiotic pairs in specially constructed chambers, as described by Rosse and Waldmann (6). The gas mixtures were humidified by bubbling through water, and the oxygen content of both sides of the chamber was monitored frequently with a Fryrite[3] oxygen analyzer. The oxygen content was maintained at 9–11% for Rat A. Flow rate was approximately 7 liters/min. Hematocrit and reticulocytes were determined on tail vein blood immediately preceding and following the period of hypoxia. While the rats were in the chambers they were given water *ad libitum* but no food. At the end of the hypoxic period, the pairs were removed from the chambers, so that both rats breathed room air for the balance of the experiment. Rat B was given radioactive iron by stomach tube as described above, immediately at the end of the period of hypoxia. Four hours later both rats were given access to food. Rat A of each pair was subjected to hypoxia and to normal oxygenation on alternate weeks, and iron absorption was measured only in Rat B of each pair.

In our experiments with rats the dose of radioactive iron was administered via a polyethylene tube passed down the esophagus under light ether anesthesia. An occasional animal regurgitated a portion of the dose, and such animals were removed at once from the experiment. In working on many hundreds of rats we found that even though the dosing appeared to be technically perfect there was faulty administration of the dose or occult regurgitation and aspiration of a portion of the dose in as many as 5% of the rats. This fact was discovered by measuring the radioactivity in the lungs of animals which had unexpectedly high apparent iron "absorption," i.e., more than 60% of the administered dose in Osborne-Mendel rats that are not iron deficient. We found that the radioactivity in the lungs of properly dosed rats was less than 2% of the administered radioactivity and that most (but not all) animals with apparent iron "absorption" of more than 60% had significant isotope in the lungs. We therefore excluded from our results all values for iron absorption greater than 60%, because of the possibility that such results represent faulty dosing and/or occult regurgitation and aspiration of a portion of the dose. Our studies indicate that imperfect dosing of animals with absorption values less than 60% is rare.

Rats used to measure the effects of hypoxia and erythropoietin upon iron absorption were Osborne-Mendel females weighing 130–160 gm. This strain was used for experiments with individual animals, because of its ready availability to us and because of our prior experience with it. These experiments with individual animals were performed before it was discovered that the Osborne-Mendel rats were not sufficiently inbred to permit successful parabiosis; therefore, LEW/Mai rats were used for the experiments with parabiotic animals. The individual rats were randomized and divided into three groups. The hypoxic group were exposed to an atmosphere of 10% oxygen at a flow rate of approximately 14 liters/min for 24 hours.

[2] Atomic Products Corp., Center Moriches, L. I., New York.

[3] Bacharach Industrial Instrument Company, Pittsburgh, Pennsylvania.

The second group were given erythropoietin prepared from the urine of a patient with hypoplastic anemia by the method of Stohlman (7), 5 units subcutaneously every 4 hours for 24 hours. The third group served as controls. All animals were starved but given water *ad libitum* for the 24-hour period, after which they were given radioactive iron as described for parabiotic rats. Administered radioactivity was measured by counting each rat in a small animal whole body counter. After 6 days the animals were killed by cervical fracture and the entire gastrointestinal tract was removed intact. Blood loss was negligible. Absorbed radioactivity was measured by counting the carcass in the whole body counter, and the amount absorbed was expressed as percentage of administered dose. In a previous experiment we found that 45 hours after dosing, the washed gastrointestinal tracts of control rats and rats exposed to hypoxia or erythropoietin contain only approximately 2% of the administered dose. There was no significant difference in the radioactivity of the washed gastrointestinal tracts of the 3 groups. It was therefore considered that measurement of iron absorption by whole body counting should give results very close to those obtained by fecal excretion.

Rats used in studies of the effects of bilateral nephrectomy and hypoxia were also Osborne-Mendel females weighing 130–160 gm. Bilateral nephrectomy was performed under ether anesthesia through a midline abdominal incision. The kidneys were first decapsulated and the adrenal glands carefully preserved. Blood loss was minimal. The kidneys of sham-operated rats were decapsulated but not removed. Nephrectomized rats were then exposed either to hypoxia or to room air for 20 hours as described above. Measurement of pCO_2 of the atmosphere surrounding the rats showed that there was no accumulation of carbon dioxide. At the end of the 20-hour period the rats were given radioactive iron by stomach tube. Nine hours later the animals were killed by cervical fracture and the gastrointestinal tract was removed intact. This 9-hour period was selected for 2 reasons: (i) nephrectomized rats were still in relatively good health at this time; and (ii) it has been shown that most of the absorption of a single intragastric dose of iron has occurred by this time (8). Reticulocytes and hematocrits were obtained on tail vein blood from all animals on the day before operation and again at dosing. Reticulocytes were enumerated by the method of Brecher and Schneiderman (9).

In order to assess the degree of uremia produced by bilateral nephrectomy and hypoxia, two groups of 4 Osborne-Mendel rats, each weighing approximately 260 gm, were subjected to bilateral nephrectomy. One group was then exposed to hypoxia for 20 hours while the other group breathed room air. Two rats with intact kidneys were placed in the hypoxic chamber along with the nephrectomized animals. At the end of the period of hypoxia the rats were bled from the heart and measurements were made of blood pH and blood urea nitrogen.

Results. Forty-one measurements of intestinal iron absorption were made in the left partner (Rat B) of the 12 parabiotic pairs. Nineteen of these determinations followed 24 hours of hypoxia of the right partner (Rat A) while 22 followed normal oxygenation. The distribution of values in both cases was bimodal: values were either between 0–38% or greater than 79%, and there were no intermediate values. Four values were greater than 79% following hypoxia of Rat A, whereas there was one such value following normal oxygenation. The frequency of these high values in the parabiotic rats was much greater than their frequency in single animals (where we have encountered them in about 5% of animals); and although the administration of radioactive iron appeared technically perfect in every case, the group of high values was excluded from consideration, because of the possibility that they represented intrapulmonary dosing (see "Methods").

The values for iron absorption between 0–38% appeared normally distributed. The mean absorption of Rat B following hypoxia of Rat A was 18.4% (SE 2.6%) and the mean value following normal oxygenation of Rat A was 12.0% (SE 1.6%) (Table I). A two-tailed Student's test indicated that these

TABLE I. Iron Absorption of Rat B of Parabiotic Rat Pairs Following Hypoxia and Normal Oxygenation of Rat A.

Air of Rat A	No. of measurements	Iron absorption in 6 days (% ± SE)
Following hypoxia	15	18.4 ± 2.6
Following normal oxygenation	21	12.0 ± 1.6

values differed significantly ($0.025<p<0.05$). Comparison of the absorption values following the first and second exposures to hypoxia or normal oxygenation did not indicate a possible bias reflecting the younger age of the animals during the first exposure.

Hematocrit and reticulocyte values of both members of a pair were averaged, since we have found that there are apparently random fluctuations of red cell mass or plasma volume between the two animals. While the rats were in the chambers, they drank sparingly, and during the 24-hour period of hypoxia of Rat A the hematocrit increased 5.2 ± 0.7%. This increase in hematocrit was interpreted as relative polycythemia secondary to dehydration. Reticulocytes were 2.65 ± 0.27% just prior to the institution of hypoxia and 2.89 ± 0.26% at the end of the period of hypoxia. This difference is not statistically significant ($0.50<p<0.60$).

The effects on iron absorption of exposing individual rats to hypoxia for 24 hours and of giving intermittent injections of erythropoietin are shown in Table II. Both hypoxia and erythropoietin increased iron absorption significantly ($p<0.005$). The distribution of absorptive values in the 3 groups appeared Gaussian. This experiment was also performed using somewhat larger rats (mean weight 163 gm) and longer exposure to hypoxia or erythropoietin (45 hours) with similar results ($p<0.01$), (Table III).

Results of the experiments designed to determine the effect of hypoxia on iron absorption in the absence of significant elaboration of erythropoietin are shown in Table IV, Exp. 1. Nephrectomy itself appeared to influence iron absorption, since nephrectomized normally oxygenated rats had significantly lower absorption than sham-operated controls ($0.025<p<0.05$). In the normally oxygenated nephrectomized animals there was a small and statistically insignificant difference between the reticulocytes measured before and after the 22-hour period (7.25% ± 0.47 before, and 6.46% ± 0.60 after; $0.30<p<0.40$). However, there was found to be a significant correlation between the iron absorption values and the changes in reticulocytes in these animals during the experiment ($r = -0.67$; $0.25<p<0.05$). This correlation indicates that the low iron absorption of this group was associated with a decrease in erythropoiesis, presumably caused by the removal of the capacity to elaborate erythropoietin. Hypoxic nephrectomized animals had significantly greater iron absorption than normally oxygenated nephrectomized rats ($0.005<p<0.01$). In the hypoxic group there was a significant decrease in reticulocytes during the experiment ($p<0.01$), and there was poor correlation between iron absorption and this change in reticulocytes. These findings suggest that hypoxia increased iron absorption in the absence of enhanced erythropoiesis. An earlier experiment (Expt. 2, Table IV) without

TABLE II. Iron Absorption of Rats Exposed to Hypoxia or Given Erythropoietin (ESF) for 24 Hours.

	Before hypoxia or ESF		At completion of hypoxia or ESF		22 Hours after completion of hypoxia or ESF; HCT (%)	Iron absorption (%)
	Wt. (gm)	HCT (%)	Wt. (gm)	HCT (%)		
Hypoxia (16)[a]	148.1	46.7 ± 0.66[b]	124.5	52.5 ± 0.38	48.8 ± 0.58	14.7 ± 1.29
ESF every 4 hours (13)	146.8	46.7 ± 0.71	124.2	51.1 ± 0.68	50.4 ± 0.77	13.31 ± 1.18
Control (13)	146.4	—	125.6	—	—	8.40 ± 0.88

[a] No. of animals in parentheses.

[b] SE of the mean.

TABLE III. Iron Absorption of Rats Exposed to Hypoxia or Given Erythropoietin (ESF) for 45 Hours.

	Before hypoxia or ESF		At completion of hypoxia or ESF; HCT (%)	Iron absorption (%)	Iron in washed bowel (%)
	Wt. (gm)	HCT (%)			
Hypoxia (8)	156.7	48.9 ± 0.55	55.8 ± 0.67	15.2 ± 3.0	2.57 ± 0.50
ESF every 6 hours (11)	170.2	49.1 ± 0.44	55.1 ± 0.39	11.9 ± 1.2	2.06 ± 0.26
Control (12)	163.1	49.6 ± 0.73	49.6 ± 0.76	7.4 ± 0.64	2.45 ± 0.29

sham operated controls gave similar differences between hypoxic and normally oxygenated nephrectomized rats ($p<0.001$).

The effects of bilateral nephrectomy and hypoxia on blood pH and blood urea nitrogen of rats are shown in Table V. At the end of 20 hours the nephrectomized animals had considerable nitrogen retention; those subjected to hypoxia in addition were moderately acidotic.

Discussion. A number of conditions capable of influencing iron absorption have been clearly delineated: anemia per se, the rate of erythropoiesis, a variety of dietary factors, and hypoxia. Each of these variables can influence iron absorption while the others are kept constant (except that severe anemia produces tissue hypoxia). In particular, it has been shown in mice that hypoxia can increase iron absorption when changes in erythropoiesis are prevented by radiation induced marrow aplasia (10). Similarly, iron absorption varies inversely with the hemoglobin level in patients with aplastic anemia, i.e., without concomitant changes in erythropoiesis (11). It appeared unlikely that each of these factors could influence the intestinal absorptive mechanism by a separate pathway. Previous suggestions (12,13) that the level of unsaturated transferrin might serve as a common regulator, have not been substantiated (14–16). However, a humoral factor has not been excluded as a possible mediator. An attempt to demonstrate such a factor in iron deficient rats has been reported (17), but the results are inconclusive. It was thought that just as with erythropoietin the technique of parabiosis might afford a better chance to demonstrate the factor than does simple transfer of plasma (5).

Our data (Tables II and III) confirms the findings of others that hypoxia and erythro-

TABLE IV. Iron Absorption of Rats Subjected to Bilateral Nephrectomy and Hypoxia for 20 Hours.

	Wt. (gm)	Before operation		At dosing		Iron absorption (%) (9 hours)
		HCT (%)	Retics. (%)	HCT (%)	Retics. (%)	
Expt. 1						
Nephrectomy + hypoxia (11)[a]	140.0 ± 2.1[b]	43.4 ± 0.8	7.34 ± 0.38	43.4 ± 1.5	5.70 ± 0.26	8.31 ± 1.10
Nephrectomy + room air (11)	143.8 ± 2.8	45.0 ± 2.3	7.25 ± 0.47	40.4 ± 1.1	6.64 ± 0.60	4.51 ± 0.57
Sham operated + room air (9)	139.3 ± 1.6	44.7 ± 2.0	6.76 ± 0.45	42.7 ± 1.2	6.16 ± 0.28	8.94 ± 1.80
Expt. 2						
Nephrectomy + hypoxia (7)	169.3 ± 4.6					11.4 ± 1.2
Nephrectomy + room air (11)	172.6 ± 2.0					5.1 ± 0.7

[a] No. of animals in group is given in parentheses.
[b] SE of the mean.

TABLE V. Blood pH and Blood Urea Nitrogen of Rats Subjected to Bilateral Nephrectomy and Hypoxia for 20 Hours.

	Blood pH	BUN (mg/100 ml)
Nephrectomy + hypoxia (4)[a]	7.29 ± 0.03[b]	158 ± 6.4
Nephrectomy + room air (4)	7.38 ± 0.03	138 ± 4.0
Intact kidneys + hypoxia (2)	7.38	25

[a] No. of rats in group is given in parentheses.
[b] Standard deviation.

poietin increase iron absorption in intact animals (18,19). This action of hypoxia is known to be rapid (18), occurring within 8–12 hours, and can occur independently of accelerated erythropoiesis (10). Erythropoietin, which is elaborated rapidly (2–4 hours) in response to hypoxia (20), has been shown to increase iron absorption only indirectly and not independently of accelerated erythropoiesis (10). The mechanism of action by which hypoxia enhances iron absorption has not been elucidated. Studies in the rat using a loop of gut with an artificial circulation indicate that hypoxia was also found to inhibit active transport of iron in isolated duodenal segments, but did increase permeability to iron in segments of distal small bowel (1). The possibility had therefore to be considered that hypoxia exerts its effect directly on the bowel.

The results of our experiment with parabiotic rats indicate that hypoxia can increase iron absorption in the intact animal independently of any direct action on the gut. In the parabiotic system the bowel of the animal in which absorption was measured (Rat B) was not directly exposed to hypoxia, and could only have been influenced by some humoral factor transferred from his hypoxic partner (Rat A) (5).

It is of interest to note that iron absorption in individual control rats in 3 separate experiments was 8.45, 7.4 and 8.9% (Tables II, III, and IV), whereas iron absorption in Rat B of the parabiotic animals following normal oxygenation of Rat A was 12.0%. Although fecal excretion was used to measure iron absorption in the parabiotic rats rather than whole body counting, it is likely that differences of strain, weight, age, and season are more responsible for the difference in iron absorption of control animals in the experiments with individual and parabiotic rats. Jacobs *et al.* (21) and Yeh *et al.* (22) have called attention to the importance of these variables.

The experiments employing hypoxia in bilaterally nephrectomized animals confirm the findings of Yeh and Chow (23) and provide suggestive evidence that the humoral factor implied by the parabiotic experiment is not erythropoietin and is not elaborated from the kidney. It has been reported that bilateral nephrectomy in rats decreased iron absorption, and that this decrease is closely related to the degree of uremia as measured by increased blood urea nitrogen (24). Consistent with this report is our finding that normally oxygenated nephrectomized rats developed nitrogen retention and had decreased iron absorption compared to sham-operated controls (Tables IV and V). By contrast, bilaterally nephrectomized rats subjected to hypoxia became even more uremic than normally oxygenated animals, yet their absorption of iron was significantly increased over that of normally oxygenated nephrectomized animals.

Yeh and Chow (23) postulated that the increased iron absorption in bilaterally nephrectomized rats exposed to hypoxia was secondary to increased erythropoiesis. Although it is possible that erythropoietin may be produced in sites other than the kidney (6), bilateral nephrectomy is followed by a marked decrease in the ability of animals to elaborate this hormone (25). The decrease in reticulocytes during exposure to hypoxia and the poor correlation between the change in reticulocytes and iron absorption in the hypoxic animals of our experiment weigh against the interpretation of Yeh and Chow. Our findings and those of Mendel (10) suggest that hypoxia may enhance iron absorption without stimulation of erythropoiesis.

In view of the multiple variables known to influence iron absorption, the postulate of a

humoral factor as a common pathway for regulation of iron absorption must remain speculative, since we have assumed that what applies in the single animal will also hold for the parabiotic pair and vice versa. It is still conceivable that hypoxia may stimulate iron absorption in the single animal by direct action on its intestine, while the increased iron absorption in the parabiotic partner of an hypoxic animal could theoretically be caused by the erythropoietic effects of transferred erythropoietin.

Summary. Parabiotic rats have been employed in an attempt to determine if hypoxia can enhance intestinal iron absorption by a humoral effect. The mean absorption of iron by rats whose partners were exposed to hypoxia was significantly greater than that of rats whose partners were normally oxygenated ($0.025 < p < 0.05$). Additional experiments using bilaterally nephrectomized rats exposed to hypoxia indicated that (i) bilaterally nephrectomized rats absorbed less iron than sham-operated controls; (ii) hypoxia increased the absorption of iron in bilaterally nephrectomized animals over that of nephrectomized rats that were normally oxygenated; and (iii) this enhanced absorption of iron by the bilaterally nephrectomized rats was not associated with increased erythropoiesis. If one assumes that what applies in the single animal also holds for the parabiotic pair and vice versa, the results of these experiments suggests that intestinal iron absorption may be mediated by a transferable humoral factor that is not erythropoietin.

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