Ferrous Iron Oxidation by Intestinal Mucosa: Possible Role in Mucosal Iron Metabolism[1] (37734)

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(Introduced by I. L. Schwartz)

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A two-step mechanism for iron absorption has been described in man as well as in experimental animals (1–6). The first, relatively rapid, step is uptake of luminal iron by the mucosal cell. Subsequent transfer out of the cell into the circulation or underlying tissues is slower and only partially complete, thereby providing an intestinal mechanism for the regulation of iron absorption. Unabsorbed iron is postulated to remain sequestered in the mucosal cell until it is lost in the feces when the cell is desquamated (4, 5).

An earlier study (7) with rat duodenal gut sacs demonstrated that the iron in the mucosa reacted as two chemically distinct forms, Fe^{2+} and Fe^{3+}. In addition, the Fe^{2+} fraction was the precursor of the Fe^{3+} fraction, and the properties of the latter were consistent with the aforementioned sequestered portion of mucosal cell iron. Variable, small portions of the trivalent iron have been identified as ferritin (5, 7, 8), but the major fraction of this pool remains unidentified.

These findings suggested the presence of an intracellular mechanism for the oxidation of Fe^{2+} to Fe^{3+}, and the present report describes the discovery of such a ferrous-oxidizing activity in the intestine. The physical and chemical properties of this activity are consistent with those of an enzyme and suggest a possible role in mucosal cell iron metabolism for this activity.

Methods. Analytical methods. Total iron was determined by the α,α'-dipyridyl method of Ramsay (9), modified as previously described (3). Aliquots of unknown were mixed with 0.5 ml of 0.5% α,α'-dipyridyl in 1.0 *M* sodium acetate buffer (pH 4.0) and 0.25 ml of 0.2 *M* sodium sulfite, diluted to a total volume of 3.25 ml, heated 10 min at 100°, then centrifuged in a clinical centrifuge till clear. The optical density of the supernatant was measured at 520 nm in a Beckman DU Spectrophotometer. Under these conditions, 100% of added $FeCl_3$ and $FeSO_4$ reacted to form the red dipyridyl complex.

Ferrous iron was similarly measured, but the sodium sulfite and heat were omitted, and the mixture was centrifuged for 10 min immediately after the addition of the α,α'-dipyridyl reagent. Under these conditions, 97% of added $FeSO_4$ reacted to give the colored complex, whereas only 2.9% of added $FeCl_3$ reacted with the α,α'-dipyridyl. The amount of Fe^{2+} oxidized was calculated from the difference between the initial and the final Fe^{2+} concentration of the incubation medium.

Protein was determined by the method of Lowry *et al.* (10) using bovine albumin as a standard.

Activity assay system and procedure. Activity was assayed in a test system that contained 5 μmoles of Tris (pH 6.0) and 400 nmoles of $FeSO_4$ plus 1–2.5 mg of protein in a total volume of 2.5 ml of 0.125 *M* sucrose. The test mixture was incubated for 60 min at 37°, and the reaction stopped by the addition of the α,α'-dipyridyl reagent used for the iron determination.

Results. Assay system stability. Iron in the assay system was stable in the divalent form for 60 min at 37°, as illustrated by the dark circles in Fig. 1. Ninety-five percent of the

[1] This work was supported by Public Health Service Grant AM 13221. Portions of this work were presented at the Joint Meeting of The American Federation For Clinical Research and The American Society For Clinical Investigation, Atlantic City, NJ, May 4, 1969.

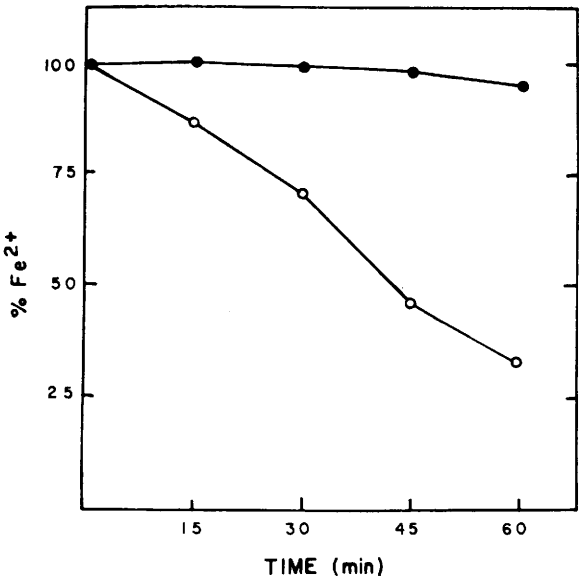

FIG. 1. Ferrous oxidation at 37° in the presence (—O—) and absence (—●—) of crude rat intestinal homogenate. Protein (2.5 mg) was incubated as described in *Methods*.

$FeSO_4$ remained unoxidized after 60 min under these conditions. The buffer composition and pH were critical for the stability of the Fe^{2+}. Sodium phosphate and acetate buffers, pH 6.0, and Tris, pH 7.4, rapidly oxidized Fe^{2+} when assayed as described in *Methods*. Ferrous iron was stable in sodium acetate buffer, pH 4.8 and 5.3, but optimal mucosal oxidizing activity was found using Tris, pH 6.0. The latter assay system was used in all the following experiments. Figure 2 shows the linear relationship which exists between the concentration of extracted protein and the velocity of the reaction under the conditions defined.

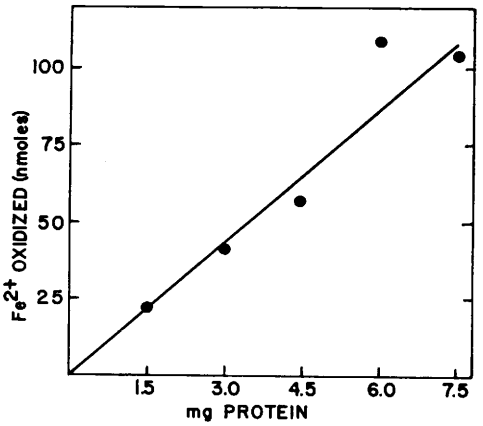

FIG. 2. Ferrous oxidation activity related to the concentration of intestinal extract. Varying amounts of the partially purified 30–40% ammonium sulfate fraction obtained from bovine intestine acetone powder (See text) were assayed with 176 nmoles of Fe^{2+} as described in *Methods*.

Preparation and assay of crude homogenates. Male CFE rats (Carworth Farms, 80–200 g) were fasted overnight and killed by exsanguination. The small intestine was removed, opened longitudinally, rinsed in 0.146 *M* NaCl/0.004 *M* KCl (0–5°), and the mucosa scraped from the underlying layers with a spatula and homogenized in 10 vol of 0.25 *M* sucrose for one minute in a Potter–Elvehjem homogenizer. The crude homogenate was dialyzed overnight against 0.25 *M* sucrose, then assayed for ferrous oxidation. The results are illustrated by the open circles in Fig. 1. Sixty-six percent of the initial Fe^{2+} was oxidized during the 60-min incubation. Total iron determination on aliquots of the final reaction mixture recovered 100% of the iron added to the system initially, confirming that the Fe^{2+} had been converted to Fe^{3+}.

Table I shows the oxidizing activity of crude rat mucosal homogenate under varying conditions. Ninety-seven percent of the activ-

TABLE I. Ferrous Oxidation by Crude Rat Intestinal Homogenate Before Dialysis, After Dialysis, and After Boiling.[a]

Sample	Fe^{2+} Oxidized (nmoles/mg protein/30 min)	% Activity remaining
Initial homogenate	76	—
Boiled homogenate	2	3
Dialyzed homogenate	74	97

[a] Identical samples of homogenate, containing 2.3 mg of protein were treated as follows: Initial homogenate = No treatment. Boiled homogenate = Heated 10 min in boiling water bath. Dialyzed homogenate = Dialyzed 17 hr in 0.25 *M* sucrose at 0–5°. Samples were then incubated 30 min at 37° with 240 nmoles of Fe^{2+} in 3.0 ml of incubation medium.

ity was nondialyzable, and heating for 10 min at 100° destroyed 97% of the initial activity. These data suggested that the activity was associated with a protein, perhaps an enzyme, and the following experiments were planned to explore this possibility.

Isolation, partial purification, and chemical properties of the activity. Acetone powder of the mucosa of rat small intestine was prepared by homogenizing mucosal scrapings in a Waring Blender for 2 min in 10 vol of acetone at −10°, then filtering under suction, and air-drying the powder. The acetone powder was extracted for 30 min in 10 vol of 0.25 *M* sucrose at 0–5°, then centrifuged at 10,000*g* in a refrigerated centrifuge. Table II summarizes the subsequent fractionation of the soluble proteins with ammonium sulfate. The resultant fractions were dissolved in minimal quantities of 0.25 *M* sucrose and dialyzed 17 hr against 0.25 *M* sucrose (0–5°) prior to assay. The ferrous-oxidizing activity of rat intestine was extractable as a soluble protein from the acetone powder, and the 0–40% ammonium sulfate fraction demonstrated a twofold increase in specific activity (Table II). Furthermore, the activity in the partially purified fraction was heat labile, as shown in Table II.

These results were confirmed when acetone powder was similarly prepared from mucosa scraped from bovine small intestine. Ferrous oxidation was present in the soluble protein, and a 50% increase in specific activity was obtained by separation of the protein precipitated by 30–40% ammonium sulfate saturation. The bovine protein was approximately half as active as that of the rat, and a lesser degree of purification was obtained by ammonium sulfate fractionation.

The reaction characteristics, when increasing amounts of substrate (Fe^{2+}) were incubated with constant amounts of the partially purified rat extract (ammonium sulfate 0–40%, Table II), were consistent with Michaelis–Menten kinetics, as illustrated in Fig. 3. V_{max} was 70 nmoles Fe^{2+} oxidized/mg protein/60 min, and apparent K_m calculated from these data was 7.6×10^{-5} *M*. Oxygen was the electron acceptor in the ferrous oxidation described. Partially purified extract

TABLE II. Ferrous Oxidation by Acetone Powder Extract of Rat Intestinal Mucosa Following Ammonium Sulfate Fractionation.[a]

Fraction	Specific activity (Fe^{2+} oxidized nmoles/mg protein)	Total protein (mg)	Total activity	% Activity recovered
Crude extract	68	1,929	131,000	—
0–40% AS	147	253	37,200	28
40–60% AS	23	771	17,400	14
60–90% AS	8	591	5,280	4
0–40% AS (heated 15 min at 100°)	4			

[a] The acetone powder was extracted as described in the text. The fractions were assayed as described in the *Methods*. AS = Ammonium sulfate saturation.

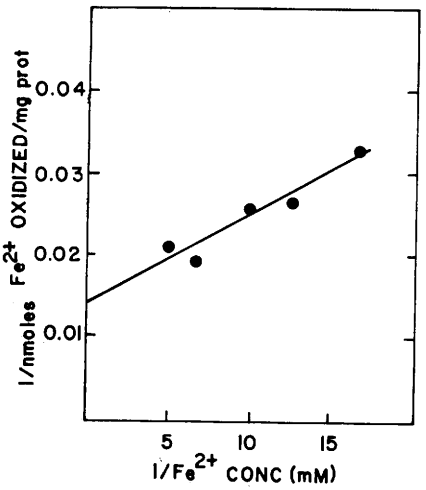

FIG. 3. Lineweaver–Burk plot of rat intestinal ferrous oxidizing activity. One milligram of the partially purified rat extract (0–40% AS, Table II) was incubated as described in *Methods*. The line drawn represents the straight line of regression calculated by the method of least squares. The standard error of the estimate is ±0.001.

(Table II) oxidized 146 nmoles of Fe^{2+}/mg protein/60 min when incubated aerobically, compared to 10 nmoles Fe^{2+} oxidized when incubated anerobically (N_2).

Earlier reports described the serum protein, ceruloplasmin, as a ferroxidase (11–15) with the physiologically important role of oxidizing extracellular Fe^{2+} to Fe^{3+} prior to its incorporation into transferrin (16). The ferrous-oxidizing activity of human ceruloplasmin (Sigma Chemical Co., St. Louis, MO) in this test system was 18% that of the partially purified rat extract, 27 to 147 nmoles Fe^{2+} oxidized/mg protein, respectively, suggesting that the oxidative activity in intestinal extracts was not due to contamination with serum ceruloplasmin.

Distribution. Table III illustrates the relative activity of homogenates prepared from tissues of various species. The activity was present in the intestine, liver, and kidney cortex of all the species tested, whereas splenic extracts varied in activity. Extracts of rat skeletal and cardiac muscle were active contrasted to mouse preparations which had negligible activity.

Discussion. The present report describes the discovery of a heat-labile, nondialyzable ferrous-oxidizing activity in intestinal mucosa. Furthermore, the activity is present in the soluble protein extracted from acetone powder of rat and bovine intestinal mucosa, and ammonium sulfate fractionation of this protein results in partial purification of the activity. The activity also demonstrates Michaelis–Menten kinetics. These physical and chemical properties suggest that the activity described may be enzymic in nature.

Crosby and coworkers (4, 17) have proposed that total iron absorption is regulated at the mucosal level by variable sequestration of iron in the intestinal epithelium. Similarly, Charlton *et al.* (5) and Bothwell (18) have proposed that ferritin formation is a mechanism for preventing excessive absorption of iron from the gut. If ferritin or sequestered iron, in a trivalent form, provide a protective mechanism in the intestine for preventing absorption of excess iron, then the activity described here may have a significant metabolic role in this phase of the regulation of iron absorption. The proposed mucosal pathways for iron may briefly be summarized as follows: bivalent iron is preferen-

TABLE III. Ferrous Oxidation by Tissue Homogenates from Various Species.[a]

Tissue	Fe^{2+} Oxidized (nmoles/mg protein)				
	Rat	Mouse	Hamster	Rabbit	Guinea pig
Intestinal mucosa	192	198	94	110	45
Liver	94	83	68	115	34
Kidney cortex	92	106	99	80	44
Spleen	8	2	57	82	48
Skeletal muscle	55	5	NT	NT	NT
Cardiac muscle	98	5	NT	NT	NT

[a] Protein (1.0 mg) was assayed as described in *Methods*. NT = Not tested.

tially taken up by the mucosal cell into a portion of cellular iron that reacts chemically as a ferrous pool; this pool is a precursor of both the iron transported to the serosa and the sequestered trivalent pool (3–5). The ferrous-oxidizing activity, described here, is postulated to be an intracellular enzymic mechanism for the formation of trivalent iron prior to its incorporation into the latter pool.

The data reported characterize the activity in association with a soluble mucosal protein; however, definitive proof of its enzymic nature remains to be obtained. The ferrous oxidizing activity does not appear to be a factitious oxidation of Fe^{2+}, since some species, organ, and protein specificity has been demonstrated. Furthermore, this intestinal activity has a greater oxidative capability than the previously described circulating ferroxidase, ceruloplasmin. The exact intracellular role of ferrous oxidation remains unknown, but the isolation and identification of the ferric-iron-containing compounds of the mucosal cell should aid greatly in defining this role.

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Received June 21, 1973. P.S.E.B.M., 1973, Vol. 144.