THE EFFECT OF PYRIDOXINE DEFICIENCY ON THE ABSORPTION OF IRON BY THE RAT

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The factors which govern the absorption of iron are not well understood. The current theory of iron absorption assigns to bodily need for iron the chief rôle in the regulation of iron absorption (1, 2). Conclusive evidence is needed, however, to demonstrate the correctness of such a view.

In an earlier study from this laboratory (3), it was demonstrated that the high serum iron values and hemosiderosis of the liver and spleen, which are characteristic of pyridoxine deficiency in swine, do not appear when the experimental animals are fed a diet very low in iron content, even though all other manifestations of pyridoxine deficiency develop. This observation indicates that, at least in pyridoxine-deficient swine, iron continues to be absorbed even though it cannot be utilized and in spite of the fact that the body stores are replete with iron.

Although it is evident that iron absorption in pyridoxine deficiency may represent unusual circumstances which are of no significance in the normal animal or in other types of deficiency, it was thought important to study the problem further. One point needing elucidation is whether or not iron absorption is normal, reduced, or even possibly increased in the face of pyridoxine deficiency as compared with normal. Since the answer to this question could only be obtained by measuring the total iron content of experimental animals, and since this could not be done easily in swine, the rat was chosen for the experimental studies to be described. The rat differs from swine and dogs in that little or no anemia develops when pyridoxine deficiency is produced, even though various other signs of this deficiency do (4, 5).

Materials and Methods

Experiment I—Weanling Sprague-Dawley rats were used for this study. From the outset they were divided into three groups: Group A, six rats; Group B, ten rats; and Group C, ten rats. All rats were placed on a basal diet consisting of crude casein (Sheffield "new process") 27 per cent, sugar

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58.0 per cent, Snowdrift, a hydrogenated vegetable oil, 5.0 per cent, lard 5.0 per cent, salt mixture1 4.0 per cent, and cod liver oil 1.0 per cent. Vitamin E in the form of mixed tocopherols2 was incorporated into the cod liver oil and mixed with the diet in the amount of 1.5 mg. per kilo of diet.

Group A (controls fed ad libitum) and Group C (pyridoxine-deficient rats fed ad libitum) received the following vitamin supplements (mg. per kilo of diet): thiamine 10, riboflavin 10, calcium pantothenate 40, choline chloride 500, nicotinic acid 100, p-aminobenzoic acid 600, and inositol 1000. These were mixed with the diet. In addition, Group A received 50 γ per rat per day of pyridoxine hydrochloride dissolved in the iron citrate solution.

Group B (restricted controls) received their vitamin B supplements in the form of powdered yeast, making up 4 per cent of the diet. Vitamins A, D, and E were given as in the other groups.

All three groups received the equivalent of 1 mg. of iron per day in the form of 11.0 mg. of ferric citrate in aqueous solution administered orally by pipette every other day. Iron administration was not started until the first signs of pyridoxine deficiency appeared, i.e. untidy fur, red whiskers, and xanthurenic acid excretion in the urine (12). This was done in order to rule out any absorption during the period before the pyridoxine deficiency began exerting its effect.

Group B was included in order to obviate any results due to differences in weight of Groups A and C.

Hemoglobin determinations were made about every 2 weeks with blood obtained by clipping the tail. The hemoglobin was determined as oxyhemoglobin by means of the Evelyn photoelectric colorimeter.

The rats were kept on this regime for 12 weeks, at which time they were all sacrificed by ether anesthesia and stored in the refrigerator until ashed. Iron feeding was discontinued 4 days before the rats were to be sacrificed, in order to permit elimination of any unabsorbed iron from the gut.

Experiment II—A second experiment was carried out in which thirteen Sprague-Dawley rats were divided into two groups: Group A, controls, six rats; Group B, pyridoxine-deficient, seven rats. The dietary intake of rats of Group A was restricted in order to keep their weights approximately the same as those of Group B. Group II-B rats were about a week older than those in Group I-C when they were started on the experimental diet. The care, feeding, etc., was exactly the same as in Experiment I, except that Experiment II was carried on for 8 weeks instead of

1 The salt mixture had the following percentage composition: NaHCO₃ 27.4, CaHPO₄ 25.0, MgSO₄ 14.0, K₂CO₃ 4.65, KCl 31.2, CuSO₄ 0.24, MnSO₄ 0.15, KI 0.15, NaF 0.03.

2 Parke, Davis and Company.
12. All rats in Group I-C manifested symptoms of severe pyridoxine deficiency, while those in Group II-B never exhibited extreme outward symptoms of the deficiency, although they showed a considerable excretion of xanthurenic acid.

The determination of the total iron in such biological material as a whole rat presents considerable difficulties due to the large amount of material and to the large quantities of salts present in the animal carcass, notably phosphates and calcium. Since wet ashing is entirely impractical for such a large animal, dry ashing was the only recourse open. Hence the carcasses were dry ashed in large porcelain trays, the heating being started at 100-200° until danger of excessive foaming was passed; then the temperature was raised to 600° for 12 to 18 hours. The ashing proceeded smoothly, and the ash was easily dissolved in 25 ml. of concentrated HCl with gentle warming.

It has been suggested that dry ashing leads to loss of iron through volatilization as FeCl₃ (6). Experience in this laboratory, as well as that of Fabian et al. (7), would indicate that there is very little, if any, loss by this means. It is quite likely that the losses suggested were due to lack of full color development as a result of phosphate interference.

The dissolved ash was transferred to a 100 ml. volumetric flask and made to volume with double distilled water. 2 to 5 ml. aliquots of this were transferred to 50 ml. volumetric flasks and made to volume with double distilled water. Aliquots (1 to 2 ml.) were then taken for the colorimetric determination of iron, with use of the Evelyn photoelectric colorimeter and α,α-dipyridyl as the color reagent. The method finally used was a combination of the methods of Kitzes, et al. (8) and Woiwod (9) modified to suit the ashed material.

The high concentration of salts, especially calcium and phosphates, in the ash from a whole rat interferes in the determination of iron by the usual colorimetric methods. A number of procedures have been suggested to overcome this interference, such as by boiling the ash with NaOH (10), with HCl or HNO₃ (11, 12), by precipitation of the iron as the sulfide followed by filtration and resolution in acid (6, 9), or by fusing the ash with calcium or sodium carbonate (13). These are all designed to hydrolyze the pyrophosphates, presumably formed during ashing, which produce non-ionized complexes with iron and thus retard or prevent its combination with the color reagent. However, these methods all involve considerable additional manipulation, which increases the chances for loss or contamination with iron and also the addition of reagents which are high in iron, notably NaOH. In view of the disadvantages cited, these methods are generally quite unsatisfactory. α,α-Dipyridyl has been found more satis-

* Unpublished.
factory for use with this type of material. It forms a more stable complex than thiocyanate and is less sensitive to interference from phosphates than is either thiocyanate or o-phenanthroline. As a result the color develops more rapidly and more completely. If 24 to 48 hours are allowed for color development with \( \alpha, \alpha \)-dipyridyl, reproducible results are obtained which compare well with those obtained with the titrimetric dichromate method of Bernhardt and Skeggs (14) as modified by Sill \(^4\) to prevent air oxidation.

The method employed in this work was as follows: Suitable aliquots of the diluted rat digest solution were pipetted into iron-free test-tubes. To this was added 1.0 ml. of freshly prepared (10 per cent) sodium sulfite solution followed by thorough mixing. Then 0.5 ml. of a 1.0 per cent solution of \( \alpha, \alpha \)-dipyridyl in 0.1 n HCl was added and mixed well. A sufficient volume of saturated sodium acetate was then added to make the pH basic (red) to Congo red paper. Usually 2 ml. were required. Double distilled water was then added volumetrically to make the total volume to 10 ml. The tubes were shaken, covered, and set aside for at least 24 hours before reading. The blank was prepared in the same manner as the samples, except that water was used in place of the aliquot of sample.

The readings were made with use of the Evelyn photoelectric colorimeter and Filter 520 after adjusting the blank to 100.

In the second experiment total body copper determinations were also made on the rat digest solutions by a modification of McFarlane's (15) method as suggested by Tompsett (16) for urine, feces, and other materials high in iron and calcium. 0.5 ml. of the concentrated rat digest solution was pipetted into acid-washed test-tubes. To this, 4.5 ml. of saturated sodium citrate solution were added to bind the calcium in a soluble complex. To this were added, in the following order, 1 ml. of saturated \( \text{Na}_4\text{P}_2\text{O}_7 \) (sodium pyrophosphate) solution, 2 ml. of concentrated \( \text{NH}_4\text{OH} \), and 1.0 ml. of 0.1 per cent sodium diethyl dithiocarbamate solution, with mixing after each addition. Water was then added to give a total volume of 15 ml. The color was read immediately with the Evelyn photoelectric colorimeter with Filter 440.

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\frac{\text{Concentration}}{D} = K
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Concentration (mg. per rat) = \( D \times K \times \frac{100}{\text{ml. sample}} \)

Results

The data for both experiments are presented in Table I. The hemoglobin, total body iron, and iron per 100 gm. of body weight are expressed

\(^4\) Sill, C., unpublished personal communication.
as the mean for the group along with the respective standard deviations. The data were analyzed statistically and the significance of the difference between the means was tested by Fisher's t test. The increase in iron per 100 gm. of body weight in the pyridoxine-deficient Group I-C is significant when compared with either Group I-A (ad libitum controls, \( t = 3.94 \)) or Group I-B (restricted controls, \( t = 4.31 \)). The same is true in comparing the pyridoxine-deficient Group II-B with Group II-A (restricted controls, \( t = 4.48 \)). The differences in total body iron between Groups I-A and I-C and Groups II-A and II-B are not significant, but this is due to the larger body weight of the controls (\( i.e., \) 325 gm. and 226 gm., respectively for Groups I-A and II-A as compared to 213 gm. and 192 gm., respectively for Groups I-C and II-B). The increase in total iron in Group I-C as compared to Group I-B, however, is significant at the 1 per cent level (\( t = 3.62 \)).

One rat in Group I-C was omitted from the statistical analysis because both its total iron (51.5 mg.) and iron per 100 gm. (26.1 mg.) were found to be 8 standard deviations from the mean. Since this rat exhibited more severe symptoms of deficiency than the others, it seemed justifiable to consider the animal separately.

The pyridoxine-deficient Group II-B also shows a significant increase in both total copper and copper per 100 gm. when compared to control Group II-A (\( t = 4.47 \) and 6.25, respectively).

The hemoglobin values show no significant reduction in the pyridoxine-deficient groups as compared with the controls.
The results of these experiments indicate that the absorption of iron is increased during pyridoxine deficiency. This is an interesting finding in view of the fact that Granick (2) and Whipple and coworkers (1, 17) have suggested that the intestinal mucosa has the property of accepting or rejecting iron, depending on the state of the iron stores in the body. According to this hypothesis, since in pyridoxine deficiency the synthesis of hemoglobin is retarded and the serum iron is elevated, it would be expected that the need of the body for iron would be reduced. Yet, as has been demonstrated in these experiments, iron continues to be absorbed even to the extent that the total body iron is increased. This condition would seem to present an exception to the "mucosal block" theory. Dubach, Callender, and Moore (18), with use of the isotope technique for studying iron absorption, found a similar situation to exist in patients with untreated pernicious anemia, refractory anemia, and hemolytic anemia, even though the tissues were replete with iron in these conditions. These authors suggest that the mucosal block theory must be thought of only in relative terms.

Many factors which play a role in the regulation of iron absorption are certainly still unknown. It is possible that the theory of Granick, Whipple, and their associates describes the mechanism operating under normal conditions but that this mechanism breaks down and does not function in such conditions as pernicious anemia and pyridoxine deficiency. For example, apoferritin, a protein, has been postulated to play a central role in iron absorption (2). Pyridoxine has been shown to be involved in protein metabolism as a component of transaminase systems (19) and also of amino acid decarboxylase systems (20). It is also essential to normal tryptophan metabolism (21, 22). It is possible that pyridoxine deficiency leads to some defect in apoferritin formation or breakdown which may, in turn, affect iron absorption. On the other hand, further study may yield data for the elaboration of a new theory of iron absorption.

The increased retention of copper in pyridoxine deficiency is extremely interesting, though also difficult to explain with the few facts available at present. Whether the copper retention is the result of an effect of pyridoxine deficiency per se on copper absorption or excretion or whether it is a secondary effect due to changes in iron metabolism cannot be stated. Serum copper has been shown to be slightly lowered in pyridoxine-deficient swine (23), and whole blood copper in pyridoxine-deficient dogs (24). Since copper is apparently excreted much as other heavy metals through the urine and bile (25-27), there is no evidence that copper absorption is regulated by body need to the extent that iron is regulated. Hence, the
low serum copper might not be expected to exert an influence on copper absorption.

Bing et al. (28) demonstrated a considerable increase in total copper in young rats given 0.5 mg of iron per day intraperitoneally when compared to similar rats given the same amount orally. The iron (total) of these two groups was 6.78 mg and 2.12 mg, respectively. The copper in the group given iron intraperitoneally was as high as in a group receiving 0.025 mg of copper intraperitoneally per day along with 0.5 mg of iron orally. This latter group had a total iron content of 3.63 mg. This would suggest that copper absorption may be influenced by the iron levels in the body. The observations reported in the present paper and unpublished data from our laboratory seem to suggest the same relationship. The converse, i.e. that copper affects iron retention, has been shown not to be true (29, 30).

It has been suggested that copper enhances the utilization and mobilization of iron for hemoglobin synthesis (29, 31, 32). This function, however, does not explain the increase of copper in a condition in which there is diminished utilization of iron and hence a decreased need for iron mobilization from the stores, unless it represents an overcompensation in an attempt to supply iron to a bone marrow which is unable to use it. This might be one explanation for the very high serum iron values, i.e. an increased mobilization coupled with an increased absorption.

It is evident from the questions raised by these findings that more work needs to be done on the interrelationships of copper and iron metabolism and their influence on erythropoiesis.

SUMMARY

The total body iron and copper have been determined in pyridoxine-deficient and in control rats.

Both iron and copper were significantly increased in the pyridoxine-deficient groups.

It is shown that the "mucosal block" theory of iron absorption is not consistent with the findings in pyridoxine deficiency.

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