UTILIZATION OF IRON FROM ENRICHED WHEAT BREAD BY NORMAL AND ANEMIC RATS

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ABSTRACT

Hemoglobin and hematocrit levels were normal in rats fed for 6 weeks from weaning on diets of bread supplemented with a minimum amount of lysine to produce significant growth. Supplementation of the diet with soybean meal or with methionine and additional lysine improved growth rate and feed efficiency. The effects of the two kinds of supplement were additive. Iron storage in the liver was increased by supplementation with the two amino acids, but not with soybean meal. Anemic rats were provided with graded levels of iron by addition of either ferrous sulfate or bread to their diets. The relative biological value of bread iron, compared to ferrous sulfate iron, differed for the several parameters of iron metabolism that were evaluated. Bread as a source of dietary iron for anemic rats appeared to stimulate production of red blood cells to a greater extent than could be accounted for by the iron available for hemoglobin synthesis or for restoration of iron content of solid tissues.

As a result of recent nutrition surveys which indicated a significant incidence of iron deficiency anemia, an increase in the level of iron fortification of wheat flour has been recommended. This has spurred renewed interest in the biological availability of iron in wheat products. Absorption of iron from bread is inefficient in comparison to its absorption from some other foods (1,2) and the form of iron used to enrich the flour from which bread is made has a significant effect on its availability (3,4). There is also considerable interest in improving the quality of protein in cereal products by supplementing them with amino acids or with concentrates of high-quality proteins. For example, Stillings *et al.* (5) have shown that addition of fish protein concentrate to flour or bread would raise the protein efficiency ratio (PER) of these products to match that of the casein standard. The PERs of the products were also improved by addition of lysine but not to a level equal to the casein diets. Pomeranz *et al.* (6) developed a process by which as much as 16% of soy flour or other protein-rich products can be added to wheat flour to produce a bread having acceptable loaf characteristics. Although there is evidence in the literature that the protein quality of a food has some effect on iron absorption (7), effects of amino acid composition of bread on utilization of its iron have received little attention.

Previous studies indicated that absorption of iron by rats was increased when diets containing wheat gluten or flour were supplemented with lysine or with lysine and methionine (8). The studies reported here were conducted to investigate effects of amino acid supplementation on absorption of iron from bread. In the first study, rats were raised from weaning on diets of bread supplemented with amino acids, soybean meal, or both. The second experiment was designed to measure the effectiveness of iron contained in bread in regeneration of hemoglobin in rats that were iron deficient.

MATERIALS AND METHODS

Bread, made by a standard white bread recipe, was obtained from a commercial bakery. It was reportedly fortified with iron in the form of ferrous sulfate. Solvent-extracted soybean meal (SBM) was purchased from a commercial feed manufacturer. The bread was dried to about 5% moisture and both the bread and SBM were ground to pass a 20-mesh sieve. Both dietary ingredients were analyzed for amino acid composition by ion-exchange chromatography on a Durrum 500 amino acid analyzer after acid hydrolysis, and for mineral content by emission spectrography on a Jarrell-Ash Direct Reading Emission Spectrograph. In addition, iron and copper contents of the bread and the diets in the first experiment were determined by colorimetric methods indicated below.

The basal diet in the first experiment (Table I) contained bread supplemented with 2.2% of a complete vitamin mixture and 1.8% of a salt mixture formulated to increase the content of various minerals of the diet to the level required for growth of young rats. Since this diet contained only 30% of the lysine and 45% of the methionine recommended for these animals, it was anticipated that the weanling rats would grow very poorly unless the protein quality was improved. Therefore, sufficient lysine (0.35% of l-lysine) was added to raise the dietary content to approximately two-thirds of that recommended. A second diet, similar to the basal one, was supplemented with 0.7% of l-lysine and 0.2% of l-methionine which increased the lysine content to that recommended and the methionine to about 75% of the suggested level. The third and fourth diets contained 13% of SBM added to the basal diet at the expense of bread. The third diet, fed without amino acid supplementation, contained approximately the same amounts of lysine and methionine as the basal diet. The fourth diet was supplemented with these two amino acids to increase their levels to that of the second diet. Weanling rats were divided into four groups of 15 animals each on the basis of weight. Each group was fed, ad libitum, one of the diets described above for 6 weeks.

The basal diet in the second experiment (Table II) was nutritionally adequate

TABLE I
Composition per Kilogram of Diets Used in Experiment 1

Diet	Bread: + Lysine	Bread: + Lysine + methionine	Bread + Soybean Meal[a]	Bread + Soybean Meal[a]: + Lysine + methione
Bread, g	956	951	830	825
Soybean meal, g	...	...	130	130
Lysine, g	3.5	7	...	3.0
Methionine, g	...	2	...	1.5
Vitamin mix[b], g	22	22	22	22
Salt mix[c], g	18	18	18	18
Total in diet				
Lysine, g	6.4	9.9	7.2	10.2
Methionine, g	2.9	4.9	3.3	4.5
Iron, mg.	53	53	75	75

[a]The bread and soybean meal contained 55 and 230 mg Fe/kg, respectively.
[b]Vitamin diet fortification mixture, Nutritional Biochemical Corp., Cleveland, Ohio.
[c]Provided per kg of diet (mg): Ca, 5070; K, 1780; Mg, 406; Mn, 45; Cu, 4; I, 0.17.

except for iron content. The salt mix used was formulated as described by Williams *et al.* (9) except that ferric citrate was omitted. Chemical analysis indicated that the diet contained less than 1 mg of iron per kg of diet. This diet was fed to weanling rats for 25 days at which time hemoglobin levels of the animals averaged 6%. The animals were divided into 11 groups of seven rats each on the basis of their hemoglobin content and one group was sacrificed immediately. A second group of rats was continued on the iron-free diet and the other nine groups were fed the diets described below for 15 days. To establish standards for comparison, three groups of rats were fed the basal diet supplemented with either 6, 12, or 24 mg/kg of iron, as ferrous sulfate tritiated in confectioners sugar. Diets of the remaining groups of animals contained enough bread to supply either 10 or 20 mg/kg of iron. Bread was added to the diets at the expense of casein and dextrin in proportion to the protein and nonprotein content of the bread. At each level of bread, one diet was supplemented with lysine and one with lysine and methionine as indicated in Table II.

The various indices of iron metabolism obtained from the animals fed the several levels of ferrous sulfate were plotted on graph paper and the points joined by straight lines. Relative biological value of the iron supplied by bread was calculated by locating the appropriate point on the standard response curve and dividing iron content of the experimental diet into the figure indicated on the abscissa to be the amount of iron in the standard diets that would give the same response (10).

In both experiments, diets and deionized water were provided ad libitum to rats housed individually in stainless-steel cages. At the end of the feeding period, the animals were exsanguinated under ether anesthesia and tissues were taken for analyses as described previously (8). Total hemoglobin was determined by the method of Evelyn and Malloy (11), hematocrits were measured in heparinized

TABLE II
Composition per Kilogram of Diets Used in Experiment 2

	Basal	Bread[a] 10 mg Fe/kg			Bread[a] 20 mg Fe/kg		
Bread, g	...	180	180	180	360	360	360
Casein, g	127.5	104.5	104.5	104.5	81.5	81.5	81.5
Dextrin, g	400	243	243	243	86	86	86
Lysine, g	...	...	1.5	1.5	...	2.5	2.5
Methionine, g	3	...	...	1.5	...	...	2.5
Corn oil, g	50	50	50	50	50	50	50
Vitamin mix[b], g	22	22	22	22	22	22	22
Salt mix[c], g	37.5	37.5	37.5	37.5	37.5	37.5	37.5
Cellulose, g	15	15	15	15	15	15	15
Sucrose, g	345	348	346.5	345	348	345.5	343
Total in diet							
Lysine, g	9.8	8.5	10	10	7.3	9.8	9.8
Methionine, g	6.0	3.3	3.3	4.8	3.0	3.0	5.5

[a]The bread contained 55 mg Fe/kg.
[b]Vitamin diet fortification mixture, Nutritional Biochemical Corp., Cleveland, Ohio.
[c]Williams and Briggs salt mix, modified (9), with ferric citrate omitted.

capillary tubes, and red blood cells were counted in a hemacytometer. Ceruloplasmin was measured by its para-phenylenediamine oxidase activity (12). Transferrin was saturated with iron as described by Goodwin *et al.* (13). Samples for mineral analyses were wet-ashed in sulfuric, nitric, and perchloric acids. Copper was measured by the method of Carter (14); iron in serum was quantitated with Ferrozine (15) and in other samples with ortho-phenanthroline (16).

Data were subjected to analysis of variance and significant differences evaluated by Duncan's multiple range test (17) where appropriate.

RESULTS AND DISCUSSION

The animals grew fairly well on the diets containing 95% bread (Table III), though feed efficiency was less than is normally obtained with diets of high-quality protein. In addition to deficiencies of lysine and methionine, the bread diets contained only 70 to 80% of the rats' requirement for histidine, isoleucine, and valine, and about 60% of the recommended amount of arginine and threonine. Supplementation of the 95% bread diet with methionine and an additional

TABLE III
Iron and Copper Utilization by Rats Fed from Weaning on Diets of Enriched Bread Supplemented with Amino Acids and Soybean Meal. Experiment 1

	Bread		Bread + Soybean Meal		Standard Error of Mean[a]	Probability Level[b] %
Lysine added, g/kg	3.5	7.0	...	3.0		
Methionine added g/kg	...	2.0	...	1.5		
Weight gain, g	200b	214b	279a	297a	7.36	0.01
Feed efficiency[c]	0.247d	0.262c	0.303b	0.321a	0.004	0.01
Hemoglobin, %	16.0	15.9	15.5	15.9	0.198	n.s.
Packed cell volume, %	52.9	53.0	51.3	52.2	0.519	n.s.
Red blood cells, millions	9.42	9.07	8.81	9.01	0.161	n.s.
Mean cell volume, μ^3	56.2	58.5	58.4	58.4	0.934	n.s.
Mean cell hemoglobin, pg	17.0	17.6	17.7	17.7	0.301	n.s.
Serum						
Iron, γ/100 ml	269	267	288	329	27.1	n.s.
Total iron-binding capacity	580	570	636	624	37.2	n.s.
Copper, γ/100 ml	136	148	150	163	6.75	n.s.
Ceruloplasmin, mg/100 ml	45.9	51.1	52.1	56.1	2.80	n.s.
Iron, γ/g						
Spleen	149	113	108	101	15.0	n.s.
Liver	53.7b	63.1a	46.3b	63.2a	3.00	0.01
Carcass	15.8a	16.1a	14.2b	14.8b	0.50	0.05
Copper, γ/g						
Liver	4.30	4.32	4.48	4.71	0.16	n.s.
Carcass	1.40a	1.40a	1.25b	1.15b	0.04	0.01

[a]Standard error of the mean calculated from polled error variance, 15 rats per diet.
[b]Values in a row not followed by the same letter are significantly different at the value of P indicated according to Duncan's multiple range test. n.s. indicates that the value of P is greater than 5%.
[c]Feed efficiency, weight gained per gram food consumed.

quantity of lysine improved the feed efficiency. Substitution of SBM for a portion of the bread increased both growth rate and feed efficiency. The diet containing 83% bread and 13% SBM supplied the animals' requirements for all amino acids except for lysine and methionine. When supplements of these amino acids were added, feed efficiency was increased still further.

The hematological parameters measured in this study were not altered by the dietary treatments. The animals were obtained from a commercial breeder and were presumed to have normal hematological parameters and iron stores at the beginning of the feeding trial. Furthermore, the concentration of iron in all of the diets was greater than the 38 mg/kg considered necessary for rats. Under these conditions, the animals were able to absorb sufficient iron to maintain blood values within normal levels even though the protein quality of some of the diets was poor. The values obtained for hemoglobin and hematocrit were somewhat higher than we have observed before in animals fed diets containing adequate protein and similar levels of iron (18). The sodium content of these diets was unusually high (5.8 g/kg bread) and the animals consumed large quantities of water and excreted more urine than is normal. The blood may have been somewhat concentrated by the necessity for eliminating so much sodium through the kidneys. The blood appeared to clot more rapidly than usual during exsanguination.

Amino acid supplementation of both the bread and the bread-plus-soybean-meal diets significantly increased stores of iron in the liver but not in the spleen or carcass of the animals. Since the increase in liver iron content was accomplished without additional feed consumption, absorption of iron must have been increased by amino acid improvement of the diet. Copper content of the liver and carcass was not altered significantly by amino acid supplementation. The

TABLE IV
Response of Anemic Rats to Several Levels of Dietary Iron Supplied as Ferrous Sulfate or in Enriched Bread. Experiment 2

Iron Source	None	$FeSO_4$			Bread		Standard Error of Mean
mg/kg	0	6	12	24	10	20	
Final weight, g	197	211	222	222	217	228	6.84
Hemoglobin, %	5.4	9.0	11.7	15.3	9.6	13.2	0.26
Packed cell volume, %	26.6	37.3	45.0	52.6	40.3	49.5	0.77
Red blood cells, millions	6.26	8.46	9.84	9.60	9.23	9.68	0.31
Mean cell volume, μ^3	42.5	44.1	45.7	54.8	43.7	51.1	1.20
Mean cell hemoglobin, pg	8.7	10.6	11.9	15.9	10.4	13.7	0.30
Serum							
Fe, γ/100 ml	107	129	233	241	153	185	23
TIBC, γ/100 ml	945	862	873	680	905	799	39
Cu, γ/100 ml	122	129	133	122	124	122	8.33
Ceruloplasmin, mg/100 ml	46.4	54.5	50.2	50.0	50.0	51.0	3.33
Iron, γ/g							
Spleen	53	71	72	108	71	78	4.23
Liver	20	23	26	45	22	30	1.61
Carcass	5.0	5.7	7.7	10.7	5.5	8.0	0.30
Copper, γ/g							
Liver	8.75	6.50	5.01	5.06	6.61	6.16	0.66
Carcass	1.37	1.38	1.38	1.43	1.32	1.37	0.03

soybean meal had no effect on iron content of spleen and liver or on copper content of liver but concentration of both minerals was decreased in carcasses of animals given the legume. Though the diets with soybean meal contained more iron than those without, the animals given this supplement grew so much faster that their rate of growth must have exceeded their capacity to store iron in the carcass.

In the second experiment, the animals weighed about 162 g and had an average hemoglobin content of 6% after 25 days on the iron depletion diet. In animals sacrificed at this time, the average total iron content, including that in blood, was 1.76 mg. Though animals were alloted to the regeneration diets on the basis of hemoglobin concentration, statistical analysis indicated that there was no difference in the average weight of rats allocated to the several diets.

In this study, the amino acid supplements added to the diets containing bread had no significant effects on the parameters measured. In the first experiment, dietary iron was abundant and lysine and methionine were the most limiting nutrients. Under these conditions, amino acid supplements in the diet increased iron absorption. In the second experiment, the animals had been depleted of iron stores and iron itself was the most limiting nutrient in the diet. The animal's ability to adapt to such treatment by increasing the proportion of dietary iron absorbed probably was operating at maximum capacity when the regeneration diets were first offered. Since these diets were fed for only 15 days before the animals were sacrificed, the adaptive effect may have masked any additional benefit that would be derived by improving the quality of dietary protein. Data for the three diets containing each level of bread were averaged for presentation in the tables.

Regeneration of hemoglobin in rats of the dietary groups fed graded increments of ferrous sulfate followed the course anticipated (Table IV). Packed cell volume (PCV, hematocrit) also increased as iron content of the diet was raised. The number of red cells per unit volume of blood increased with dietary additions of iron up to 12 mg/kg but was not further increased by adding 24 mg of iron per kg. The mean cell volume (MCV) of the RBCs, as indicated by the ratio of packed cell volume to number of cells, was small in animals fed diets with 0, 6, or 12 mg of iron as ferrous sulfate and was not significantly altered by these increments of dietary iron. Only when the mineral content of the diets was increased to 24 mg/kg did the size of the cells increase, thus accounting for the increase in hemoglobin and packed cell volume without an increase in numbers of cells.

Serum iron was depressed and transferrin, as measured by serum iron binding capacity, was elevated by the low iron diets. Transferrin saturation was less than 12% in animals fed the diet with no iron added and was only slightly improved by supplements of 6 mg/kg of ferrous sulfate or 10 mg/kg of iron from bread.

Iron contents of spleen, liver, and carcasses were significantly reduced by the low iron diets. Only in animals fed the diet containing 24 mg/kg of ferrous sulfate iron did these values approach concentrations obtained for animals in the first experiment. Concentration of copper in the carcass was not affected by dietary iron levels in the second experiment and was approximately the same as for animals in the first study. Liver copper was increased in the animals fed the low iron diets and tended to be inversely related to iron content of the diet as was reported by Sourkes *et al.* (19).

Relative biological values for several of the parameters measured are shown in Table V, and indicate some interesting aspects of iron metabolism by rats fed the diets containing bread. PCV was restored more effectively than was hemoglobin concentration of the blood by iron in bread. Though the relative biological value as indicated by numbers of RBCs is more difficult to evaluate because of the nonlinear response of rats fed the control diets, it can be seen from the data in Table IV that the RBC count of animals fed 10 mg/kg of iron from bread was approximately the same as would have been expected from rats fed an equal quantity of ferrous sulfate iron. The relative biological value of bread iron for replenishing the mineral in the solid tissues, spleen, liver and carcass was considerably less than that for restoration of the blood parameters.

For most of the measures of iron metabolism shown in Table V, the relative biological value of 20 mg/kg of iron supplied by bread was greater than that of 10 mg/kg of bread iron. A notable exception to this general response was the serum iron concentration of the animals fed the two levels of bread, in which case the lower supply of bread iron had a higher relative biological value.

One possible interpretation of the results is that bread contains some component that specifically stimulates the production of erythrocytes from erythroblasts in anemic rats even though the supply of iron is suboptimal. Thus at comparable hemoglobin levels, the blood of animals given bread contains more RBCs which are smaller and have less hemoglobin per cell than that of rats fed the standard iron diet. This increased rate of erythron maturation might stimulate mobilization of iron from storage sites or divert newly absorbed dietary iron toward the areas of erythropoiesis and thus account for the decreased content of the mineral in solid tissues.

When the content of bread, and therefore of iron, in the diet was doubled, the characteristics of the RBCs approached those of animals fed a comparable quantity of iron from ferrous sulfate while iron content of other tissues was still significantly below that of controls. This again indicates selective stimulation of erythrocyte production by bread. In this case, the quantity of iron available from the diet was sufficient for synthesis of a nearly complete complement of

TABLE V
Relative Biological Value[a] of Iron in Enriched Bread Compared to that in Ferrous Sulfate

Bread in diet, %	**18**	**36**
Fe in diet, mg/kg	**10**	**20**
Hemoglobin, %	75	86
Packed cell volume, %	83	96
Mean cell volume, μ^3	45	96
Mean cell hemoglobin, pg	54	87
Serum, γ/100 ml		
Iron	74	46
Total iron-binding capacity	29	83
Fe, γ/g		
Spleen	59	74
Liver	44	72
Carcass	45	71

[a]Calculated as described in paragraph 4 of Materials and Methods.

hemoglobin for the RBCs produced, but not for restoration of the tissue stores to levels achieved with a comparable amount of ferrous sulfate in the diet.

The unique reversal of the relative biological value of the two levels of bread with respect to serum iron content may result from the increased mobilization of iron from storage sites or from the initial point of absorption in the intestinal mucosa through serum to the area of hematopoiesis.

The first experiment indicates that enriched bread fed as the major dietary constituent is an adequate source of iron for weanling rats when it is supplemented with sufficient lysine to promote nearly normal growth. Use of SBM rather than lysine to improve protein quality of the basal diet increased growth and feed efficiency but decreased concentration of iron and copper in the carcass, while having no effect on hematological status of the animals. Further supplementation of the diets with methionine or additional lysine increased iron absorption without altering the blood parameters.

Data obtained in the second experiment suggest that bread selectively enhances proliferation of red blood cells even though its iron is less available than that of ferrous sulfate for synthesis of hemoglobin or for storage in solid tissues.

Supplementation of bread and other wheat products with lysine and methionine, the most limiting amino acids, might enhance iron absorption enough to be beneficial to persons whose dietary intake of the mineral is suboptimal. Improvement of the protein quality would be especially effective in cases where bread provides the major portion of the protein in a meal.

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