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CONTROL OF IRON DEFICIENCY
DSAN-C-0045

University of Kansas Medical Center

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Total Current Year Costs: 100,375
Projected Third Year Costs: 220,581

Narrative Summary

The prime objective of this AID contract is to develop intervention strategies to combat nutritional iron deficiency. The following five areas of activity were proposed in the original contract: 1) factors affecting iron bioavailability of food, 2) iron availability from LDC diets, 3) development of iron fortification systems, 4) food technology, and 5) pilot fortification trials. With few exceptions, these areas involve measurements of radioiron absorption in man. A total of 656 iron absorption measurements have been performed during the past year in 164 volunteer subjects. The salient features are as follows.

Most of the effort during the past year has focused on the development of iron fortification systems. Several studies were performed to further define the effect of soy protein on iron absorption. The inhibitory effect of soy is of major importance in iron nutrition because infant protein supplements contain large quantities of the protein. We have shown that several soy products (whole soybean, fat free soy flour, textured soy protein, and isolated soy protein) markedly inhibit the absorption of nonheme iron and this inhibition is enhanced by the process of protein purification. Baking does not destroy the inhibiting effect but the latter can be partly reversed by ascorbic acid or meat products.

The other major focus has been on sodium iron EDTA (NaFeEDTA) because of interest in using it for fortification of sugar and other food vehicles. We have demonstrated conclusively that NaFeEDTA exchanges completely with dietary nonheme iron. Of more practical importance is that EDTA blocks the enhancing effect of animal foods or ascorbic acid on nonheme iron absorption. We have found that NaFeEDTA improves iron absorption from a meal with low iron availability but impairs absorption from a meal of high availability.

In studies of iron availability from LDC diets, two diets typical for low and moderate income families in the Philippines were studied. Iron absorption from six separate meals was remarkably similar and uniformly low.

A. General Background

Iron deficiency anemia is widely recognized as the most prevalent nutritional deficiency in the world. While the impact of the anemia which accompanies iron lack has not been fully defined, severe iron deficiency is known to impair work capacity, increase the risk of maternal and infant morbidity and mortality, reduce resistance to infection, and impair the physical and mental growth of school-age children.

Two major approaches have been discussed as intervention strategies to combat iron deficiency anemia. The most direct approach is iron supplementation or the supply of medicinal iron to children or pregnant women. However, iron supplementation programs are costly to administer and usually fail due to lack of compliance. An alternative to iron supplementation is some form of dietary manipulation; either adding more iron to the diet (iron fortification) or adding a substance that increases food iron availability (enhancement fortification). A critical question at present concerns the efficacy of either or both of these procedures because their effectiveness has never been clearly established. Furthermore, because no single method of iron delivery will be suitable in all LDCs, several fortification systems should be made available which could be applied in specific situations with minimal modification.

The purpose of the present project is to develop methods of iron delivery that would be suitable for field testing and eventual implementation on a national level. The optimal approach will depend on the level of dietary iron and the degree of its availability. At one extreme, diets with low-iron content but high availability would be best suited to iron fortification. On the other hand, a diet with high content but low assimilation would be more suitable for enhancement fortification. The best strategy may be to employ both of these approaches in tandem.

B. Statement of Project Objectives

The development of an effective food intervention strategy to combat iron deficiency requires consideration of the absorption of a particular form of iron, its stability when added to a particular food vehicle, and the effect that it may have on the vehicle itself. The selected food vehicle must be consumed in sufficient quantities by the deficient population, be compatible with the form of added iron, and be centrally processed to permit adequate regulation of the fortification process.

Before selecting a particular fortification strategy, a number of studies must be conducted in human subjects with radioisotopes to determine basal iron availability of the diet, to measure absorption of fortification iron, and to estimate the gain in iron balance that would be achieved with implementation of the strategy. Field trials will eventually be required to establish the effectiveness of a given strategy but such trials are costly and should be limited to those approaches that appear most promising.

The present project as originally submitted was to conduct research in 5 closely related areas:

(1) Evaluation of factors affecting food iron availability.
(2) Measurement of iron availability from LDC diets.
(3) Development of effective systems of iron or enhancement fortification.
(4) Food technology studies to establish iron/vehicle compatability.
(5) Pilot fortification trials to measure the efficacy of the intervention strategy.

There has been no major change in these basic objectives as defined in the original contract. Efforts in developing iron fortification systems depend largely on isotopic studies of food iron absorption. These studies are still the core of the present contract. As compared with the initial year, a much greater investment was placed during the second year of this contract in the development of delivery systems for iron fortification although factors affecting food iron availability continue to be of interest. Studies were initiated during the past year to measure iron availability from LDC diets; two Filipino diets were recently evaluated and studies will be undertaken shortly to measure iron availability from a typical Egyptian diet. Other areas of potential collaboration of this type include Guyana, the Caribbean, and Cameroon. Limited food technology studies have been conducted in relation to rice fortification although these studies are not supported under this contract. We plan to conduct some pilot iron fortification trials (iron absorption studies) in the coming year to examine the efficacy of iron fortification of rice.

C. Continued Relevance of Objectives

The stated objectives of this contract remain relevant and there will be no major modification of these in the coming year. The program is gradually expanding with respect to collaborations in LDCs.

D. Accomplishments to Date

The body of this progress report is contained in the following appendices:

Appendix A: Iron absorption studies
Appendix B: Publications
Appendix C: LDC collaborations
Appendix D: Iron bioavailability protocol

A detailed description of 16 separate iron absorption studies conducted during this second year are contained in Appendix A. Each of these studies is presented under headings of summary, rationale, design, results, and comment. The following is a list of these studies:

No.	Page	Subjects	Title
11	7	12	Effect of Cooked and Uncooked Egg Albumin
12	14	10	Effect of Soy Isolates on Iron Absorption
13	23	9	Effect of Coffee on Iron Absorption
14	32	11	Radioiron Exchange with NaFeEDTA
15	40	9	Iron Absorption from Filipino Low Cost Meals
16	48	8	Iron Absorption from Filipino Moderate Cost Meals
17	57	9	Effect of Cooking Soy Protein Isolates
18	66	8	Interaction of NaFeEDTA and Ascorbic Acid
19	74	10	Interaction of NaFeEDTA and Coffee
20	81	14	NaFeEDTA Absorption at Two Iron Levels
21	89	11	Effect of Soy Protein on a Meal Containing Meat
22	96	8	Interaction of NaFeEDTA and Ascorbic Acid
23	102	10	Interaction of NaFeEDTA and Meat
24	112	7	Effect of Coffee Before, During, and After a Meal
25	119	11	Interaction of Soy Protein and Ascorbic Acid
26	128	7	Interaction of Soy Protein and Meat

Of the 16 studies performed this past year, only three were directed solely to the identification of dietary factors affecting food iron bioavailability. In the first year tentative evidence was obtained that conalbumin, a constituent of egg albumin, inhibits nonheme iron absorption. Additional evidence that ovalbumin impairs absorption is based on recent studies showing that when the protein content of the SS meal is reduced, a dramatic increase in iron absorption occurs. Study 11 was performed to determine whether prior heating of egg albumin to destroy the iron binding activity of conalbumin abolishes the inhibitory effect of ovalbumin. A slightly higher absorption was observed from a high iron meal when the protein was cooked prior to administration. The results are consistent with a modest inhibitory effect of conalbumin. However, the magnitude of this effect was considered too small to be of importance in a normal diet.

Studies during the first year demonstrated a pronounced inhibitory effect of coffee on iron absorption. Two studies were performed during the past year to further define this effect. In study 13 the SS meal was consumed without coffee, with drip coffee, and with one and two cups of instant coffee; a striking 72, 83, and 91% inhibition respectively was observed in the absorption of nonheme iron. Because much of this beverage may be consumed apart from meals, study 24 was performed to determine whether coffee ingested apart from a meal might influence absorption. The study was performed with a high availability meal (hamburger). Basal absorption (no coffee) of 8.1% absorption fell to 5.6% when coffee was ingested either during the meal or one hour following. A

lesser decrease, to 6.3%, was observed when coffee was taken one hour before the meal. These two studies demonstrate conclusively that coffee, as well as tea, severely inhibits absorption of dietary iron. From the standpoint of iron nutrition, sugar may be less desirable as a vehicle for iron fortification in countries with a high consumption of coffee.

Most of the remaining iron absorption studies were directed to the development of iron fortification systems. During the first year of this contract, soy protein was identified as a potent inhibitor of nonheme iron absorption and was believed to account for the consistently low assimilation of iron contained in various infant protein supplements. A major effort was made during the past year to define more clearly the inhibiting effect of soy. Study 12 was performed to determine whether all soy products impair iron absorption. When full fat soy flour, textured soy protein, and isolated soy protein were substituted for ovalbumin in the SS meal, iron absorption fell from 5.5% to 1.0, 1.9, and 0.4% respectively. Soy inhibition therefore appears to be enhanced with the process of protein purification.

Our observations with soy contradict earlier studies in the literature indicating a relatively high bioavailability of iron contained in whole soybeans. Study 17 was performed to determine whether this discrepancy was related to the method of preparing soybean; in earlier studies showing high availability, soybeans were boiled at low temperature before serving. A slight but significant increase in absorption was observed when isolated soy protein or whole soybean was baked prior to administration, but the degree of change was too small to account for the inhibition of soy observed in our

recent studies. Two other studies with soy were performed to determine the extent to which the inhibitory effect of soybean is counteracted by substances in the diet that enhance iron availability. In study 25, 100 mg ascorbic acid added to the SS meal containing isolated soy protein produced a striking increase in iron absorption, from 0.56 to 3.2%. The same quantity of ascorbic acid increased absorption with egg albumin in the SS meal from 5.1 to 10.2%. In study 26 a similar but less dramatic effect was seen when meat was added to a soy-containing meal. Iron absorption from a soy meal increased from 0.36 to 1.44% when ingested with meat as compared with an increase from 5.9 to 7.5%. with an ovalbumin meal. In conclusion, the inhibitory effect of soy can be partly neutralized by enhancers such as ascorbic acid or animal foods.

A major effort was made in the past year to define more precisely the advantages and/or disadvantages of NaFeEDTA for iron fortification. This iron form is of key importance at present because of its use for fortification of sugar in a field study in Guatemala. Interest in NaFeEDTA for iron fortification is partly based on the reduction in unwanted reactions with food such as discoloration and rancidity which may occur with other forms of iron. There is mounting interest in several countries for fortifying other food products such as salt, wheat, rice, etc. Our studies of EDTA during the past year were designed to determine more completely its effect on iron availability and its interaction with other dietary substances known to influence iron absorption.

The effect of EDTA on nonheme iron absorption is still controversial. An earlier study showed progressive inhibition in iron absorption with increasing amounts of EDTA. These results were in sharp contrast to the findings of Layrisse (Venezuela) and

Viteri (Guatemala) who reported that NaFeEDTA is absorbed at least as well as the common pool of dietary nonheme iron, and in some instances is more available. One possible explanation for these discrepancies is that Na_2EDTA was used in the study demonstrating an inhibitory effect of the chelate, whereas NaFeEDTA was employed in later studies that demonstrated favorable absorption. With this background, studies were performed during the past year to compare absorption of NaFeEDTA with absorption of a mixture of $FeCl_3$ and Na_2EDTA. In study 14 absorption of NaFeEDTA (synthesized prior to administration) was identical to absorption from a mixture of Na_2EDTA and $FeCl_3$ when added to the same meal, and both forms of iron exchanged completely with dietary nonheme iron.

The same question was examined in study 20 where the degree of exchange between NaFeEDTA and dietary nonheme iron was examined at two levels of iron intake. Absorption of ($FeCl_3$ + Na_2EDTA) and NaFeEDTA was again the same although when the iron and EDTA content of the meal was increased ten-fold, percentage absorption was actually higher than absorption with the low iron meal. The higher quantity of NaFeEDTA may have facilitated absorption of nonheme iron as compared with the low iron meal. In study 22 the extent to which NaFeEDTA exchanges with dietary nonheme iron in the presence of ascorbic acid was evaluated. Absorption of NaFeEDTA and iron ascorbate added to the same meal was again identical although somewhat higher absorption of both radioiron forms was observed when ascorbic acid was added before EDTA.

A second study was performed with NaFeEDTA to determine its interaction with ascorbic acid. In study 18 the addition of 200 mg ascorbic acid to the SS meal (egg albumin) produced the expected sharp increase in absorption, from 7.3 to 23.2%. However, when radioiron was added to the SS meal in the form of NaFeEDTA, there was only a slight increase in iron absorption, from 6.8 to 7.3%. Therefore, there is a strong neutralizing effect of NaFeEDTA on ascorbic acid, the most potent enhancer of nonheme iron absorption that has been identified to date.

The interaction between NaFeEDTA and coffee was examined in study 19 because of the interest in using this form of iron for sugar fortification. When taken with water, NaFeEDTA was absorbed slightly better than $FeCl_3$ (5.0 and 3.8% respectively). When these forms of radioiron were taken with coffee rather than water, absorption fell sharply to 1.57 and 1.14% respectively. EDTA was therefore unable to counteract the inhibiting effect of coffee on iron absorption.

The most important of the EDTA studies was study 23 which was designed to determine the interaction between NaFeEDTA and meat on nonheme iron absorption. When the SS meal was administered without meat, mean absorption was 1.1% with $FeCl_3$ and 2.1% with NaFeEDTA; this twofold difference was statistically significant. When meat was taken with these meals, absorption of NaFeEDTA and $FeCl_3$ rose to 6.24 and 5.4% respectively, consistent with the well known enhancing effect of meat. This study demonstrated that meat enhances the absorption of NaFeEDTA but does so less dramatically than nonheme iron in the absence of EDTA.

More importantly, study 23 provides evidence that the availability of NaFeEDTA in relation to nonheme iron absorption depends on basal availability of the meal. With a low availability meal, absorption was twofold higher with NaFeEDTA than with $FeCl_3$ (1.1 and 2.1% respectively), whereas NaFeEDTA was less well absorbed than $FeCl_3$ from a high availability meal containing meat (5.4 and 6.2% respectively). This observation may explain the disparity in results with EDTA. In earlier studies by Cook and Monsen, progressive inhibition by EDTA was demonstrated in studies employing a high availability meal (STD) while Latin American workers demonstrated an enhancing

effect of EDTA by using meals with relatively low iron bioavailability. The choice of NaFeEDTA for iron fortification may depend, therefore, on basal availability of a diet in a given geographic region.

The first studies to assess iron bioavailability of LDC diets were conducted during the second year and the results are presented in studies 15 and 16. Two diets were chosen as typical for low income and moderate income families in the Philippines. With each of these diets, absorption of nonheme iron was measured from breakfast, lunch, and dinner meals. Foods for this study were purchased locally (Kansas City), but were served as they would be eaten in the Philippines. The main objective of this study was to test the validity of measuring absorption from typical LDC diets using food items and volunteer subjects from the U.S. rather than the LDC. The counterpart of this study is currently being conducted in the Philippines - that is, administration of the same meals to volunteer subjects in the Philippines with food purchased there.

The results of the measurements in Kansas City were instructive from the standpoint of iron nutrition. First, absorption of nonheme iron was the same with low and moderate income diets. The main difference was the content of heme iron which was significantly higher with the moderate income diet. However, the middle income diet offered no advantages in either availability of nonheme iron or in total iron content. Presumably, iron balance would be more favorable with the middle income diet because of its higher content of heme iron. However, it should be noted that absorption of heme iron was assumed to be 40% and was not measured directly. Moreover, the content of heme iron

is no adequate method for determining dietary nonheme iron. The content of heme iron in the two Philippine diets is critically important because our iron absorption values suggest that little would be accomplished by fortification with nonheme iron.

Several steps were taken during the second year to facilitate collaboration in developing countries. Studies will be undertaken within the next few weeks of iron bioavailability from a typical Egyptian diet and preliminary efforts have been made to study diets in other LDCs.

E. Dissemination and Utilization of Research Results

The findings that have been developed under this contract have been communicated through formal presentations at national and international meetings, through exchange on an informal basis between investigators visiting the iron nutrition unit in Kansas City, and by reciprocal visits by co-investigators in LDCs. The following is a summary of these various avenues of communication.

1. Presentations

a. Dr. Cook presented recent findings on food iron nutrition at the Workshop on Nutrition and Health; Cairo, Egypt, October 20-22, 1979.

b. Dr. Cook presented an overview of the findings obtained during the first year of the contract at the annual meeting of the International Nutritional Anemia Consultative Group (INACG); Cairo, Egypt, October 24-26, 1979.

c. Dr. Cook presented a paper entitled "Nutritional Iron Deficiency" at Ohio State University; Columbus, Ohio, November 14, 1979.

d. Dr. Cook was the chairman of the Nutritional Anemia Symposium held at the American Society of Hematology annual meetings; Phoenix, Arizona, December 1-4, 1979. Drs. Finch, Crosby, Hallberg, and Viteri participated in this symposium.

e. Dr. Lynch presented a paper entitled "Interaction of Vitamin C and Iron" at the New York Academy of Sciences Annual Meeting; New York, February 3-5, 1980.

f. Dr. Lynch presented a paper entitled "Food Iron Absorption" and Dr. Cook presented a paper on "Newer Prospectives in Iron Nutrition" to the Philippine Association of Nutrition and the Philippine Society

of Dietitians and Nutritionists, Inc.; Manila, Philippines, March 14, 1980.

g. Dr. Cook was a visiting professor at Cornell University, Division of Nutritional Sciences, and presented a paper entitled "Food Iron Absorption in Man"; Ithaca, New York, March 23-24, 1980.

h. Dr. Morck presented a paper entitled "Enhancement of Food Iron Absorption by Ascorbyl Palmitate" at the annual FASEB meetings; Anaheim, California, April 13-16, 1980.

i. Dr. Cook presented a paper entitled "Animal Products and Iron Absorption" at the International Symposium on Animal Products in Human Nutrition; Iowa State University, Ames, Iowa, June 2-3, 1980.

j. Dr. Cook presented a paper entitled "Evaluation of Iron Status" at the International Society of Hematology meetings; Montreal, Canada, August 15-20, 1980.

Scientific Publications

Pre-reprints of the following publications are included in Appendix B.

a. Simpson, K.M., Morris, E.R., and Cook, J.D. The inhibitory effect of bran on iron absorption in man. American Journal of Clinical Nutrition (in press).

b. Lynch, S.L. and Cook, J.D. Interaction of vitamin C and iron. Annals of the New York Academy of Science (in press).

c. Cook, J.D., Morck, T.A., Skikne, B.S., and Lynch, S.R. The importance of animal products in human iron nutrition. The Nutrition Foundation (in press).

d. Morck, T.A., Lynch, S.R., and Cook, J.D. Iron availability from infant food supplements. (in preparation).

3. Scientific Exchange

The following individuals visited our research laboratory at the University of Kansas Medical Center during the second year of the contract.

a. Pensri Pootrakul, M.D., Division of Hematology, Siriraj Hospital, Mahidol University, Bangkok, Thailand, September-October 1979.

b. Anita Marzan, Nuclear Research Associate IV, Philippines Atomic Energy Commission, Quezon City, Philippines, September-October 1979.

c. William K. Simmons, Ph.D., Caribbean Food and Nutrition Institute, Jamaica, West Indies, September 3-8, 1979.

d. Benjamin de la Cruz, M.D., Biomedical Research Division, Philippine Atomic Energy Commission, Quezon City, Philippines, September 17-29, 1979.

e. Vernon Young, Ph.D. and Morteza Janghorbani, Ph.D., Department of Nutrition, Massachusetts Institute of Technology, Boston, December 12, 1979.

f. Dan Hopkins Ph.D. and Fred Steinke, Ph.D., Nutritional Biochemistry Laboratory, Ralston Purina Co., St. Louis, Missouri, December 12, 1979.

g. Juanita Madriaga, Food and Nutrition Institute, Manila, Philippines, April-July 1980.

h. Ernest Beutler, M.D., Department of Clinical Research, Scripps Clinic and Research Foundation, La Jolla, California, May 20-21, 1980.

i. Allan Forbes, Associate Director for Nutrition and Consumer Sciences, Food and Drug Administration, May 28-29, 1980.

j. Malee Sritongkul, Siriraj Hospital, Mahidol University, Bangkok, Thailand, July 1980-June 1981.

k. Sally Mulhern, Ph.D., Food and Drug Administration, September 10-11, 1980.

F. Statement of Expenditures and Obligations

Category	Amt. Awarded*	Amt. Used**	% Used
Personnel	$ 90,482	$ 63,520	70
Travel	7,800	8,736	100
Shipping	8,000	--	--
Equipment	5,000	3,300	66
Supplies	16,750	10,242	61
Volunteers	22,500	11,015	49
Contracts	4,305	2,623	61
Publication	1,000	939	94
	$155,837	$100,375	64
Indirect Costs	54,289	38,112	70
Total	$210,126	$138,487	66

* Total three-year award (1 October 77-30 September 81).

** Second year (1 September 79-31 August 80).

About 15% of the total effort was invested in studying factors influencing iron availability, 70% of the effort in developing iron fortification systems and the remaining 15% in evaluating iron bioavailability of LDC diets. These proportions can be applied directly to the total figure of $100,375 in direct costs to determine the amount actually expended in these three categories of work activity. Thus, $15,056 was for factors affecting food iron availability, $70,263 for studies to develop a fortification system, and $15,056 for studying iron bioavailability in LDC diets.

The total expenditures during the second 12 months of this contract were $100,375 as compared with the budgeted amount of $155,837. The major reason for the disparity in budgeted and expended amounts relates to the difficulty in volunteer recruitment. Whereas we can still obtain volunteers, it has been difficult at times and several studies had to be delayed in the past year because of this problem. In addition, the number of subjects who entered each study was often much below that planned. Finally, recruitment of volunteer subjects is not feasible during the summer months or during school breaks. Another factor contributing to low expenditure relates to the difficulty in developing effective collaborations in LDCs. For example, it has taken Ms. Marzan nearly a year to initiate the counterpart of the iron availability study in the Philippines because of delays in funding and difficulty in obtaining supplies.

G. Work Plan and Budget Forecast for the Coming Year

The work on this contract will continue to follow the guidelines outlined in the original proposal. Much of the effort will still be devoted to studies of food iron absorption in man, but there will be a greater investment in developing overseas collaboration. The major areas of proposed study are:

1. Factors affecting food iron availability.

   It is planned to make a limited but continued investment in identifying biochemical factors that influence availability of food iron. Studies in this area during the first two years have included comparisons of purified proteins, and the effects of soy products, coffee, and bran.

   It is estimated that 3-4 separate studies will be performed in this area in the coming year. We hope to

develop further information on the importance of fiber. One study will involve a comparison of purified sources of fiber (e.g. pectin, cellulose) to determine whether any one form of fiber is particularly inhibitory. We plan to perform a study in which two meals are designed that have comparable energy, protein, carbohydrate, and fat content but which differ maximally in the content of naturally-occurring fiber. One additional study may be performed with coffee to determine whether, like tea, tannate is responsible for its inhibiting effect.

One of the striking features in our studies during the first 2 years is that virtually all nonanimal sources of proteins inhibit iron absorption. This has been observed not only with soy but with other semipurified proteins such as casein or ovalbumin. For example, by reducing the protein content of the SS meal, mean absorption of nonheme iron is increased approximately three-fold. One approach to studying the protein effect may be to predigest these purified proteins *in vitro* prior to administration but such tests meals are usually highly unpalatable. Another approach would be to administer the same quantity of amino acids as would be contained in the whole protein and then attempt to identify those amino acid(s) that facilitate or impair iron absorption. Finally, the relative importance of different forms of carbohydrate and fat have been neglected in our studies to date in that we have only studied a single source of purified carbohydrate (dextrimaltose) and fat (corn oil). It would be of interest to determine the extent to which these semipurified ingredients are representative of other foods in their respective categories.

2. Development of iron fortification systems.

The major effort during the coming year will still be the development of effective methods of iron fortification. During the first year of the contract, iron absorption from infant protein supplements was extremely low due mainly to isolated soy protein as well as a high content of calcium and phosphate. Since iron deficiency during infancy occurs regularly without iron fortification, iron deficiency might be reduced by a more effective method of fortification. Simply adding additional amounts of iron to infant protein supplements (currently ferrous fumarate is used) will not improve iron balance significantly. We plan to examine systems that would enhance the availability of iron contained in these infant supplements. Recent studies suggest that NaFe-EDTA may be better absorbed than the common pool of nonheme iron when added to foods of very low availability. We therefore plan to compare absorption of ferrous fumarate with NaFeEDTA when used to fortify CSM. A three to four-fold increase in iron absorption of the iron may have major impact on iron nutrition in infancy. If EDTA does not enhance iron availability, we may perform feasibility studies of adding ascorbic acid to CSM to enhance iron availability.

The pros and cons of various food vehicles has been discussed at length in the application for this contract and will not be reviewed here. However, because rice is the staple food for a large portion of the world's population and has a fairly even distribution of intake in the population, it has advantages as a food vehicle.

During the past year, we have been conducting feasibility studies with Hoffmann-LaRoche to develop a system for coating rice. Whole grain rice is coated by rinse-resistent technic and diluted 1:199 with unenriched rice. We have been able to coat rice with ferrous sulfate in this way at level that would increase iron intake in a rice-eating population by approximately 8 mg/day. There is only a minimal difference in color between premix and uncoated rice grains, and less than 99% of the iron is eluted by conventional rinse tests. In the last 3 months, we have prepared a large batch of radiolabeled premix (radioactive ferrous sulfate) in sufficient amounts to conduct aproximately 80 iron absorption tests. We will perform pilot fortification studies with this material in Kansas City within the next 2-3 months. It is anticipated that the radioiron used to coat rice (7 gm ferrous sulfate/lb. rice) is absorbed to the same extent as the common pool of nonheme iron, but there are other technical reasons that could result in reduced availability of this iron. If pilot studies in Kansas are successful, we will continue these in the Philippines.

One of the vexing problems in iron fortification programs has been the difficulty in determining the bioavailability of various iron forms that might be used for iron fortification without resorting to radioactive measurements. There are a variety of <u>in vitro</u> methods (such as solubility, particle size, etc.) and some animal models such as the hemoglobin repletion assay in the rat that are presently used to assess bioavailability but the correlation with iron absorption in man is inconsistent. In collaboration with Dr. Allen Forbes who heads a task

force at INACG to develop protocols for iron availability, we plan to perform certain studies in the coming year in human subjects; the protocol for this project is included in Appendix D. Briefly, it will involve studying three types of fortification iron that differ widely in their availability. These iron compounds will be radiolabeled and a battery of *in vitro* and animal measurements will be performed to evaluate bioavailability. In addition to *in vitro* measurements, two different animal assays will be used (hemoglobin repletion method and radioiron absorption). Finally, these labeled compounds will be administered to human volunteers to measure iron availability in man. By correlating these results with studies in animals and *in vitro* methods, it is hoped to develop a simplified and more practical approach for monitoring iron bioavailability. As many as six separate iron absorption studies may be required as part of this project which could account for 40-50% of our efforts in the coming year.

3. Bioavailability of LDC Diets

A major objective in the initial contract was to measure availability in various LDC diets. These studies have progressed slowly because of the difficulty in identifying sources of support for studies in LDCs. One of the more effective collaborations to date has been in the Philippines and further collaboration is planned. This be particularly important if there are any major disparities in the data currently being obtained in Kansas and in the Philippines. We also plan to conduct pilot studies of radiolabeled fortified rice as described previously. Finally, we hope to perform some preliminary studies with bagoong, a fish paste, that is a close second

candidate to rice as a vehicle for iron fortification (see Appendix C).

Dr. Shobaki, a nutritional biochemist from Cairo, Egypt will spend 2 months in our laboratory beginning in October of the present year. During this time we will plan to measure iron availability from one or more typical Egyptian diets using foods purchased locally. It is hoped that the counterpart of these studies can be performed in Cairo on his return as is being done with the Philippine collaboration.

There are three other LDCs with the potential for collaboration in studies of food iron availabiltiy during the coming year. Dr. Lynch recently completed a three-week study of anemia in Cameroon and his report is included in Appendix C. While iron deficiency was not the sole cause of anemia, it was thought to be an important component. We hope to have an opportunity to study iron availability from that diet which is predominantly vegetable and believed to have low availability of iron. Another possible country is Guyana, and a report of a site visit to that country in 1978 is also included in the Appendix. A study is currently being conducted in Guyana to identify causes of anemia in that country. If iron deficiency again surfaces as the major cause, there is a potential for collaborative studies of iron availability. Finally, a meeting is planned for the Caribbean in January, 1981 by Dr. Gurney and Dr. Simmons. Data on the prevalence of iron deficiency anemia in the Caribbean will be reviewed and it is hoped that some collaborative efforts can be developed to measure iron availability of diets from those regions.

GENERAL METHODOLOGY

The following is an interim report of investigations of food iron availability performed under USAID Contract No. DSAN-C-0045 from 1 September 1979 to 30 August 1980. The results are presented as a series of 16 consecutively-numbered absorption studies performed in groups of 7-15 subjects each. The following aspects of methodology and data analysis are common to all of these studies.

Experimental Subjects. Each study was performed in normal male volunteer subjects between the ages of 18 and 65 years. Fully informed consent was obtained from the subjects, all of whom were remunerated for their participation in the studies. The majority of subjects were enrolled as medical or dental students at the University of Missouri, Kansas City. None of the subjects gave a history of hematological abnormalities or diseases known to influence the gastrointestinal absorption of iron. The majority of volunteers had normal iron status as gauged as serum ferritin levels; an occasional subject had mild iron deficiency due to either participation in other experimental studies or frequent blood donations.

Test Meals. Except for absorption from the reference dose or inorganic iron which is described below, the test meals contained either a mixture of food items or a combination of semi-purified dietary ingredients. The methodology used to measure the absorption of nonheme dietary iron was based on the principle of extrinsic radioiron tagging. With this technic, it is possible to measure absorption from the common pool of nonheme iron that is formed when several food items are ingested in the same meal. Extrinsic tagging is performed by adding 0.1 mg elemental iron as labelled $FeCl_3$ to the meal. This radioiron label is either mixed with the entire meal when studying liquid diets or is added to one of the liquid items in a conventional meal.

Composition of the test meals differed in each study depending on the nature of the experiment; specific meals are described under each study. Many studies were performed with one of two standardized meals that have been used extensively in published studies from this laboratory. The first meal was designed to represent a typical American meal. Originally this meal consisted of beef, corn, potatoes, bread, margarine, peaches and ice milk; it contained a total of 696 kcal; 29.4 gm protein, 67.6 gm carbohydrate, 34.9 gm fat, 202 gm calcium, 414 mg phosphorus and 4.1 mg iron. To facilitate the preparation of large number of test meals for the AID studies, a similar type of meal was purchased the day before each study from a local McDonald's Restaurant. The meal consisted of a quarter-pound hamburger, regular size order of french fries, and one-half of a vanilla milkshake. The french fries and hamburger were reheated in a microwave oven immediately prior to administration. The milkshake served as a convenient vehicle for introduction of the extrinsic tag or other test materials. The composition of this standard (STD) meal as determined by analysis at WARF Institute is as follows: 791 kcal, 33.7 gm protein, 85 gm carbohydrate, 35.3 gm fat, 662 mg calcium, 361 mg phosphorus, and 5.7 mg iron. In a preliminary study performed in 10 male volunteer subjects, absorption from the original standard meal and the McDonald's meal averaged 4.0 and 3.6% respectively, a difference that was not statistically significant.

A second test meal that has been used extensively in published studies of food iron absorption from this laboratory was designed to match identically the biochemical composition of the original standard meal. The meal consists of semi-purified ingredients, the amount of which can be altered in each experiment so as to standardize total content of the major dietary ingredients in each test meal. This semisynthetic (SS) meal contains 37.4 gm egg albumin as protein, 66.8 gm dextrimaltose

as carbohydrate, 34.8 gm corn oil as fat, and sufficient dibasic calcium phosphate, dibasic potassium phosphate, and ferric chloride to bring the calcium, phosphorus, and iron content to 262, 361, and 4.1 mg, respectively. The composition of the SS meal was not revised to match the biochemical composition of the STD meal obtained from McDonald's.

A final test dose that was used in many studies consisted of a solution of inorganic iron and is referred to as reference absorption (REF). This test dose contained 3 mg iron as ferrous sulfate and 2 moles freshly prepared ascorbic acid/mole of iron in 50 ml iron-free water. The purpose of the REF test is to provide a measure of the intestinal avidity of iron in each subject and thereby permit standardization of absorption measurements performed in groups of subjects that differ in iron status. By expressing absorption from a given test meal as a ratio of REF absorption, the effect of differences in iron status between subjects or groups of subjects is eliminated.

Iron Absorption Measurements. In all studies four iron absorption tests were performed in each subject as two sets of parallel double isotope measurements. The day before the first meal the subjects reported to the laboratory, were told the nature of the study, and gave their informed consent. Age, height, and weight of each subject was recorded, and 30 ml whole blood was obtained for measurements of hematocrit, serum iron, total iron-binding capacity, serum ferritin, and background blood radioactivity. On the following day, the first test meal tagged with either ^{59}Fe or ^{55}Fe was administered to fasting subjects between 8 and 10 a.m.; nothing further was allowed by mouth for 4 hours. A second meal tagged with the alternate form of radioiron was fed under identical conditions the following day. Fourteen days later the subjects returned to the laboratory and 10 ml whole blood was obtained for duplicate measurements of ^{55}Fe and ^{59}Fe. A second pair of absorption tests

were again performed with ^{59}Fe and ^{55}Fe on two successive days, and a final blood sample was obtained 14 days later to measure the rise in ^{55}Fe and ^{59}Fe blood radioactivity. The total dose of radioactivity administered to each subject was 4 uCi ^{59}Fe and 10 uCi ^{55}Fe.

Simultaneous measurement of ^{55}Fe and ^{59}Fe activity in 10 ml blood (day 0 x 1, day 14 x 2, day 28 x 2) was performed by liquid scintillation counting using a modification of the Eakins and Brown method. Standards representative of each test dose were processed along with the blood samples. Percentage absorption was calculated from whole blood radioactivity and total blood volume, the latter estimated on the basis of height and weight. In these iron replete volunteers it was assumed that 80% of the absorbed iron was incorporated into circulating red cells 14 days after administering each test meal.

Statistical Analysis and Data Presentation. Iron absorption measurements expressed as a percent of the administered dose are highly skewed and the degree of skewing is particularly marked at low levels of absorption which are frequently encountered in studies of food iron availability. Previous studies of this problem have established that log transformation of percentage absorption will produce a Gaussian distribution. Statistical analysis was performed on logarithms of percentage absorption and the results transformed as antilogs to recover the original units of percent absorption.

When testing the significance of differences in mean absorption between any two test meals in a given study, analysis was performed on logarithms. Student's t was calculated to determine whether the mean difference in log absorption between any pair of meals was significantly different than zero; this is equivalent to testing in original units for a ratio different than unity.

In the main table prepared for each study, percent absorption from each meal is listed individually in each subject and the most important ratios are similarly tabulated. Also included for each study is a table which lists mean absorption ±1 SE for all possible meal combinations. The reciprocal mean ratio (1/ratio) is also listed for each meal but not the associated +1 SE which can be calculated as reciprocals of ±1 SE for the original ratios. Values for Student's t and the associated P values are also listed for each meal. In addition, correlation coefficients for absorption from each pair of meals (performed on logarithms) are listed together with the associated P value. These correlation coefficients reflect the precision of each paired comparison. Lower correlation coefficients are usually obtained when comparing absorption from test meals administered 14 days apart rather than 24 hours apart--that is, when comparing absorption from either meals A or B with either meals C or D. Note that the meal designations (A,B,C,D) do not necessarily indicate the order in which the meals were actually administered; this order was sometimes altered in the tables to emphasize the most important comparisons in each study. Graphs are also included for each study showing percentage absorption and the important absorption ratios, both plotted on a logarithmic scale.

APPENDIX A

IRON ABSORPTION STUDIES

STUDY 11

EFFECT OF COOKED AND UNCOOKED EGG ALBUMIN

SUMMARY

To determine whether conalbumin, an iron binding protein contained in egg albumin, impairs iron absorption, cooked and uncooked albumin were administered as the protein source in the SS meal. No effect was seen at the usual iron content of the SS meal (4.1 mg) but when the study was repeated at double the iron concentration, a small but significant 29% increase in absorption was observed with the precooked protein. A borderline increase in iron absorption was also observed when the iron content of the meal was increased to exceed the iron binding capacity of conalbumin. It is concluded that the iron binding protein, conalbumin has a minimal affect on iron assimilation.

RATIONALE

The SS meal, used extensively in this laboratory during the past 5 years, contains ovalbumin as the protein source which is simply spray-dried egg white. Conalbumin constitutes about 12% of this protein which is similar, if not identical, to transferrin, the iron binding protein in serum. The SS meal contains 4.4 gm conalbumin, an amount capable of binding approximately 4.4 mg iron.

Conalbumin could impair iron absorption by binding iron and rendering it unavailable to the intestinal mucosa. However, iron binding by this protein is strongly pH dependent; the affinity for iron is lost at a pH below 6. Thus, minimal binding may occur in the proximal duodenum where iron uptake by the intestinal mucosa is maximum. It is of interest that recent animal studies indicate that a transferrin-like protein may actually be excreted by the intestinal mucosal and complex iron to facilitate uptake by the mucosal cell.

In a previous study (study 4), egg albumin was administered with uncooked ovalbumin and with albumin that had been cooked at 70°C for 15 minutes, a temperature that is known to destroy the iron binding capacity of transferrin. If ovalbumin affects iron absorption, it was reasoned that cooking the protein would ablate this effect. Absorption with uncooked ovalbumin averaged 2.49% and with prior heating, 6.21%; the increase in absorption was highly significant ($p < 0.001$).

In the present study, the effect of precooking ovalbumin was again examined at the usual iron level of the SS meal (4.1 mg). In a second pair of test meals, the content of the SS meal was doubled to 8.2 mg, a level that far exceeds the iron binding capacity of conalbumin. If conalbumin impairs iron absorption, an increase would be anticipated at a higher iron content.

DESIGN

Four separate test meals were administered to 12 male volunteer subjects. All four meals consisted of the SS meal with no added calcium or phosphate. The protein source in meals A and C was uncooked ovalbumin whereas in meals B and D, the ovalbumin was precooked at 70°C for 15 minutes. Meals A and B contained the usual amount of iron--0.4 mg as ovalbumin and 3.7 mg as ferric chloride added in 1 ml 0.01 N HCl. To meals D and C, 7.8 mg iron as $FeCl_3$ was added to bring the total iron content to 8.2 mg.

Meal	Ovalbumin	Iron
A	Uncooked	4.1
B	Cooked	4.1
C	Uncooked	8.2
D	Cooked	8.2

RESULTS

The results are shown in Table 11-1. Absorption from meal A averaged 3.0% which is similar to the means of 2.5% and 4.7% in studies 4 and 5 respectively with this same test meal. Mean absorption from meal B was virtually identical, 3.03%.

Surprisingly, absorption increased when the iron content was doubled; absorption from meals C and meal D averaged 3.7 and 4.8% respectively. The increase was not significant with uncooked albumin (mean ratio C/A=1.24, p=0.16) but was sig-

nificant with cooked albumin (mean ratio D/B=1.58, $p=0.02$). In addition, significantly higher absorption was observed when the protein was heated prior to administration (mean ratio D/C=1.29, $p< 0.01$).

COMMENT

In contrast to a previous study, iron absorption was not higher when ovalbumin was cooked and added to a meal containing 4.1 mg iron. There is no obvious explanation for the disparity in the two results. However, when the quantity of iron in the test meal was doubled, absorption increased rather than decreased as one would predict; absorption is usually inversely proportional to the size of the test meal. Moreover, there was a significant 58% increase in absorption when cooked ovalbumin was administered at this higher iron dose. No definite conclusion in regard to the effect of conalbumin on iron absorption can be drawn from this study. While the results do not exclude the possibility that conalbumin impairs iron assimilation, the inhibition, if it exists, is minimal.

TABLE 11-1

STUDY 11. The Effect of Uncooked and Cooked Egg Albumin on Iron Absorption.

Subject	Age	Packed Cell Volume (%)	Serum Iron (µg/dl)	Tsf Sat (%)	Serum Ferritin (ng/ml)	IRON ABSORPTION (% of dose) Low Iron Uncooked(A)	Low Iron Cooked(B)	High Iron Uncooked(C)	High Iron Cooked(D)	Absorption Ratio B/A	D/C	C/A	D/B
1	25	43	88	31	59	0.81	0.83	2.91	5.12	1.02	1.75	3.59	6.16
2	34	43	150	56	132	1.27	1.95	1.77	2.68	1.53	1.51	1.39	1.37
3	27	43	92	33	79	1.31	1.30	2.73	3.33	0.99	1.21	2.08	2.56
4	21	44	52	13	115	2.07	1.42	2.21	2.82	0.68	1.27	1.06	1.98
5	21	48	102	30	26	2.15	1.91	3.66	3.00	0.88	0.81	1.78	1.57
6	25	46	128	38	62	3.10	2.47	4.51	8.30	0.79	1.84	1.45	3.36
7	23	46	78	26	22	3.50	4.17	2.93	4.10	1.19	1.39	0.83	0.98
8	28	41	106	40	94	4.30	5.41	2.05	3.08	1.25	1.50	0.47	0.56
9	21	47	96	28	25	5.25	5.48	8.48	11.35	1.04	1.33	1.61	2.07
10	23	44	102	24	105	5.41	1.25	2.07	1.71	0.23	0.82	0.38	1.36
11	22	46	103	28	94	5.50	6.68	3.05	4.02	1.21	1.31	0.55	0.60
12	41	36	27	6	5	12.38	42.60	44.47	47.32	3.44	1.06	3.59	1.11
MEAN	26	44	114	29	51*	3.00*	3.03*	3.73*	4.79*	1.01*	1.29*	1.24*	1.58*
					-1 SE	2.40	2.23	2.89	3.71	0.85	1.19	1.01	1.30
					+1 SE	3.75	4.13	4.82	6.19	1.21	1.38	1.53	1.92

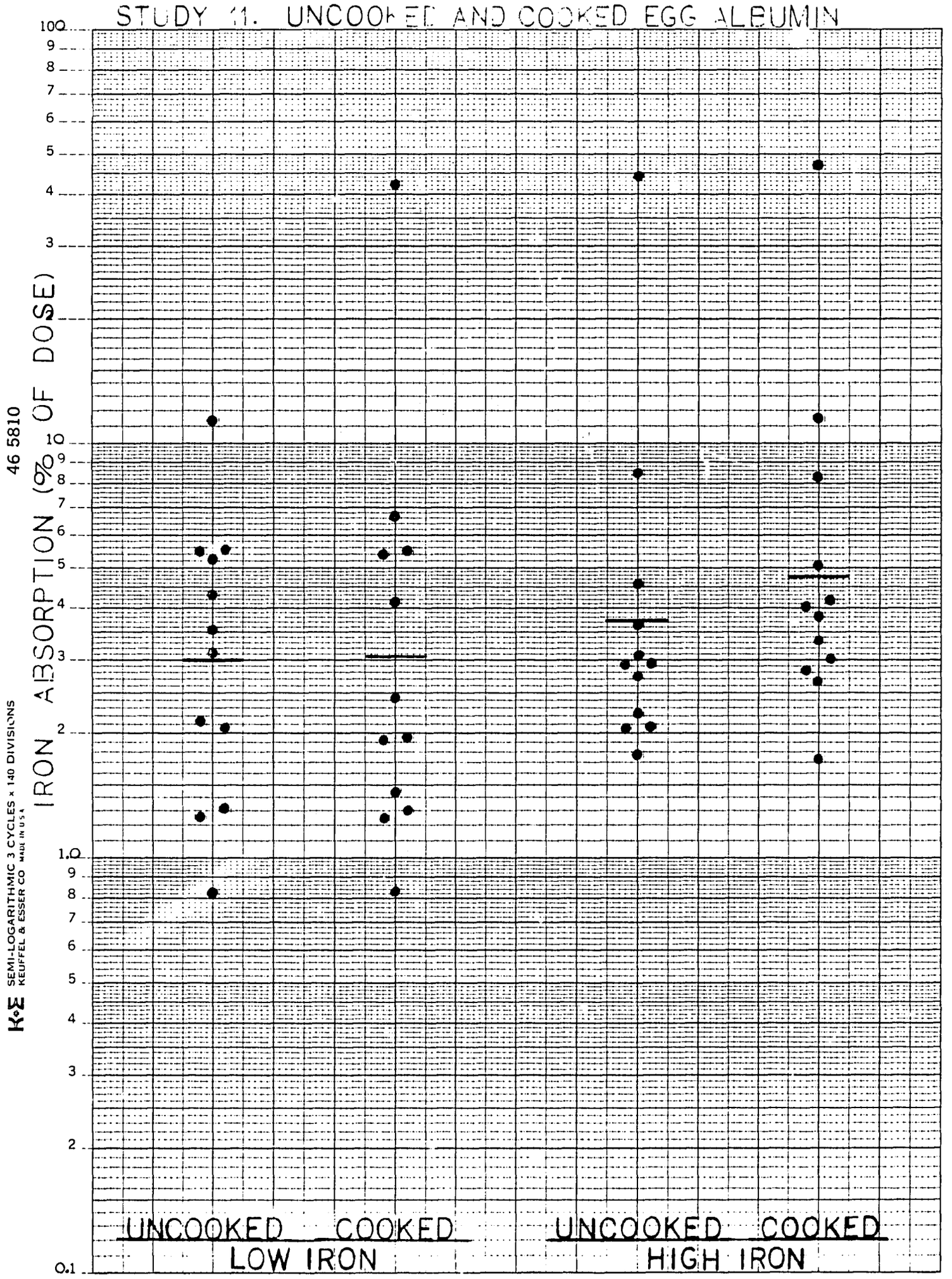
* geometric mean

TABLE 11-2.

STUDY 11. The Effect of Uncooked and Cooked Egg Albumin on Iron Absorption.

	ABSORPTION RATIOS					
	A/B	A/C	A/D	B/C	B/D	C/D
Mean Ratio	0.99	0.80	0.63	0.81	0.63	0.78
-1 SE	0.83	0.65	0.49	0.67	0.52	0.72
+1 SE	1.18	0.99	0.79	0.98	0.77	0.84
1/ Ratio	1.01	1.24	1.60	1.23	1.58	1.29
Student's *t*	0.064	1.031	1.989	1.087	2.315	3.392
P value	0.475	0.162	0.036	0.150	0.020	0.003
Correlation* (r)	0.823	0.620	0.521	0.791	0.771	0.957
P value	< 0.001	0.016	0.041	0.001	0.002	< 0.001

* Log scale


STUDY 11. UNCOOKED AND COOKED EGG ALBUMIN
IRON ABSORPTION (% OF DOSE)
100
9
8
7
6
5
4
3
2
10
9
8
7
6
5
4
3
2
1.0
9
8
7
6
5
4
3
2
0.1
UNCOOKED
COOKED
LOW IRON
UNCOOKED
COOKED
HIGH IRON
46 5810
K+E SEMI-LOGARITHMIC 3 CYCLES x 140 DIVISIONS
KEUFFEL & ESSER CO. MADE IN U.S.A.

STUDY 12

EFFECT OF SOY ISOLATES ON IRON ABSORPTION

SUMMARY

The effect on iron absorption of the following soy isolates was tested by making protein equivalent substitutions for ovalbumin in the SS meal: full fat soy flour (FFSF), textured soy protein (TSP) and isolated soy protein (ISP). Mean absorption from the SS meal of 5.5% fell significantly with all three soy isolates; mean absorption averaged 0.4%, 1.9% and 1.0% with ISP, TSP, and FFSF respectively. It is concluded that soy products markedly inhibit iron absorption and this inhibition is enhanced in the purification of soy protein.

RATIONALE

In a previous study (study 4), isolated soy protein (ISP) markedly inhibited iron absorption from the SS meal as compared with egg albumin; absorption averaged 0.46 and 2.49% respectively. However, published studies with intrinsically tagged soybeans have shown a relatively high availability of iron as have studies of extrinsically labeled soy. Moreover, ISP is about 60% as available as ferrous sulfate based on the rat hemoglobin repletion assay. The present study was undertaken to determine whether inhibition by ISP was related to the purification process; various soy isolates were substituted for ovalbumin in the SS meal to determine their effect on iron assimilation.

There are several steps involved in processing soybeans to obtain ISP (Figure 12-1). After soybeans are cracked, dehulled, and flaked, the food can be ground to obtain full fat soy flour (FFSF). Alternatively, the flakes can be extracted with solvent to remove soybean oil; the higher protein flakes are then either ground to obtain defatted soy flour (DFSF) or extruded to obtain granules with a meat-like consistency upon dehydration. This latter isolate is marketed as textured soy protein (TSP). Water extraction of the defatted flakes precipitates about one-third of the total protein. The soluble fraction is then acidified to pH 4.5 (isoelectric point for soy protein) which causes about one-half of the remaining soy protein to precipitate. The soluble fraction (termed 'whey') which contains about one-third of total soy protein, is discarded and the precipitate is then marketed as ISP. As the protein content of these soy fractions increases (due to solvent extraction and isoelectric precipitation), the iron content per unit weight increases in parallel.

DESIGN

Four separate meals were fed to 10 male volunteer subjects. The ovalbumin in the SS meal was used as a basis for comparing iron absorption from different soy isolates. Meal A was the SS meal with one-half quantities of ovalbumin and no added calcium or phosphate; the protein content was lowered to maintain a reasonable total meal volume when substituting soy products having a lower protein concentration. In meal B, ISP (Supro 710, Ralston Purina Co.) was substituted for egg albumin in protein equivalent amounts. In meal C, the protein source was TSP, (Supro 50A, Ralston Purina Co.), while meal D contained FFSF (El Molino Mills, City of Industry, CA). The composition of the four test meals is shown in Table 12-1. The amount of dextrimaltose and corn oil in each of the meals was varied to maintain the same total content of these constituents. $FeCl_3$ was added to bring the total iron content of each meal to 4.1 mg.

Meals A, B, and D were thoroughly mixed and served as a drink. For meal C containing TSP, dry protein granules were hydrated in a styrofoam bowl with twice their weight of water. This mixture was then heated in a microwave oven and a portion of the liquid (containing fat and carbohydrate) was poured over it. TSP was eaten with a spoon while the other components of the SS meal were taken as a drink. Meal C was the most difficult for the subjects to ingest and many complained of nausea for several hours following the test meal. Side effects of the other meals were minimal.

RESULTS

The results are listed in Table 12-2. Iron absorption from meal A averaged 5.5%, nearly double the absorption observed with the

SS meal in previous studies; the difference is explained by the lower content of ovalbumin used in the present study.

Compared to ovalbumin, all meals containing soy isolates sharply inhibited iron absorption. Absorption with ISP (meal B), averaged 0.41% which represented an inhibiton of 92% relative to ovalbumin. Absorption with TSP (meal C) averaged 1.9%, an inhibition of 65% relative to meal A. Absorption with FFSF (meal D) averaged 0.97%, an 82% reduction relative to ovalbumin.

Absorption from all meals containing any one of the soy isolates also differs significantly from each other (see Table 12-3). Even when absorption was relatively similar, as with meals C and D (TSP and FFSF), the difference was still highly significant (mean ratio C/D=1.95, $p < .01$).

COMMENT

All soy products examined in this study showed a marked inhibition in iron absorption as compared with ovalbumin. Apparently there are substances in this protein that bind iron and render it unavailable to the intestinal mucosa. The extremely low value of 0.4% for ISP is virtually identical to the mean of 0.46% observed in study 4. Because the same degree of inhibition was observed in the present study with half quantities of protein, the iron binding capacity of ISP is high and is still not saturated at an iron protein ratio in this study of 4 mg/15 g.

The inhibiting effect of soy products is closely related to the protein content. For example, FFSF contains 42% as much protein as ISP on an equal weight basis and the absorption ratio for ISP:FFSF (A/D) averaged 0.42. The result with TSP is not

entirely consistent with this hypothesis because the protein content of FFSF is 37% as compared with 51% for TSP and yet mean absorption with TSP was significantly higher than FFSP (mean ratio C/D=1.95, $p < 0.01$). The difference may relate to the extrusion process that is applied to defat soy flour and obtain a texture similar to that of meat. The high temperature and pressure conditions in the extrusion process may in part neutralize the inhibiting factor. The inhibiting effect of soy might also relate to its content of phytic acid which is concentrated in parallel with protein during purification. ISP contains 1.8% phytic acid but the phytate content of the other soy products is unknown. Recent studies with wheat bran, however, indicate that phytate content is much less inhibitory to iron absorption than previously believed.

All three soy products studied in this report inhibited iron absorption and the greatest inhibition was seen with ISP. The nature of this inhibiting effect is not known but the present results suggest that it may be protein or nitrogenous in nature. If the biochemical nature of the inhibition can be identified and perhaps eliminated, the nutritive value of soy products as food supplements will be enhanced.

FIGURE 12-1.

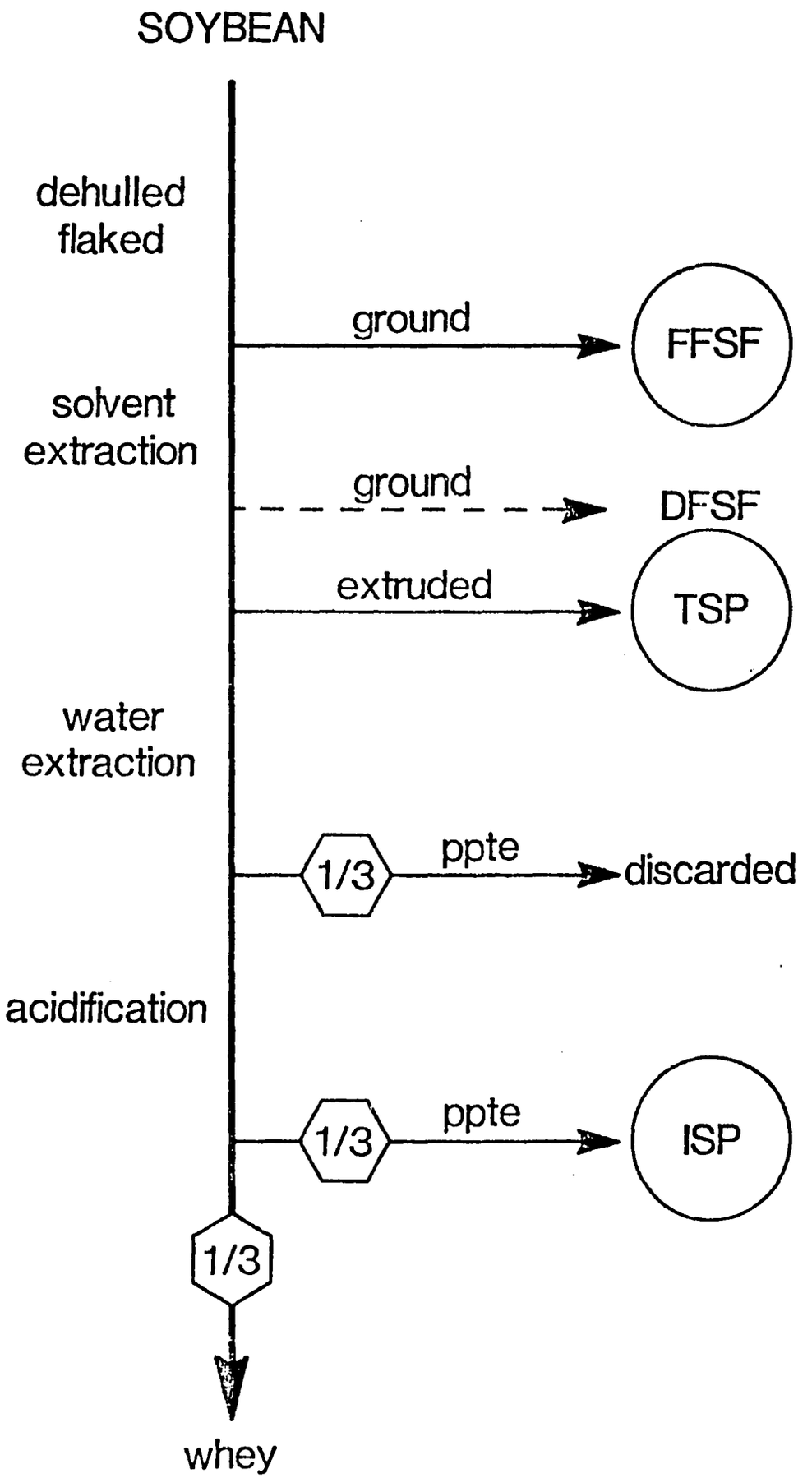

SOYBEAN
dehulled
flaked
ground
FFSF
solvent
extraction
ground
DFSF
extruded
TSP
water
extraction
1/3
ppte
discarded
acidification
1/3
ppte
ISP
1/3
whey

TABLE 12-1. Composition of Test Meals.

	Amount (gm)	Energy (kcal)	Protein (gm)	Iron (mg)
MEAL 1				
Egg albumin	18	68	15	0.1
Dextrimaltose	67	254	-	-
Corn oil	35	308	-	-
$FeCl_3 \cdot 6H_2O$	19*	-	-	3.9
Water	220	-	-	-
Total	340	630	15	4.0
MEAL 2				
ISP	17	53	15	2.0
Dextrimaltose	67	257	-	-
Corn oil	35	380	-	-
$FeCl_3 \cdot 6H_2O$	10*	-	-	2.0
Water	220	-	-	-
Total	340	618	15	4.0
MEAL 3				
TSP	29	93	15	3.3
Dextrimaltose	57	219	-	-
Corn oil	35	305	-	-
$FeCl_3 \cdot 6H_2O$	3*	-	-	0.7
Water	220	-	-	-
Total	340	617	15	4.0
MEAL 4				
FFSF	40	169	15	3.4
Dextrimaltose	55	210	-	-
Corn oil	27	236	-	-
$FeCl_3 \cdot 6H_2O$	3*	-	-	0.6
Water	228	-	-	-
Total	350	615	15	4.0

* mg

TABLE 12-2

STUDY 12. The Effect of Soy Products on Iron Absorption.

Subject	Age	Packed Cell Volume (%)	Serum Iron (µg/dl)	Tsf Sat (%)	Serum Ferritin (ng/ml)	Iron Absorption (% of dose) ALB (A)	ISP (B)	TSP (C)	FSF (D)	Absorption Ratio C/B	D/B	C/D
1	24	44	163	40	83	2.01	0.16	1.00	0.81	6.25	5.06	1.23
2	21	46	106	31	105	3.28	0.52	0.90	0.91	1.73	1.75	0.98
3	35	40	99	28	149	3.46	0.28	1.71	0.40	6.11	1.43	4.27
4	24	46	117	39	59	3.75	0.61	3.30	1.52	5.41	2.49	2.17
5	42	42	106	29	94	4.11	0.13	1.47	0.46	11.31	2.49	3.19
6	25	47	144	40	30	5.53	0.17	2.40	0.57	14.12	3.35	4.21
7	24	44	78	19	112	6.08	0.33	2.82	1.21	8.55	3.67	2.33
8	24	46	107	27	46	8.37	1.27	2.73	4.67	2.15	3.68	0.58
9	34	46	96	26	86	14.03	1.55	1.06	0.66	0.68	0.43	1.60
10	25	42	81	22	26	18.36	0.72	4.43	1.85	6.15	2.57	2.39
MEAN	28	44	110	30	69*	5.50*	0.41*	1.91*	0.97*	4.62*	2.37*	1.95*
					-1 SE	4.42	0.31	1.60	0.77	3.43	1.89	1.59
					+1 SE	6.83	0.54	2.27	1.23	6.21	2.96	2.39

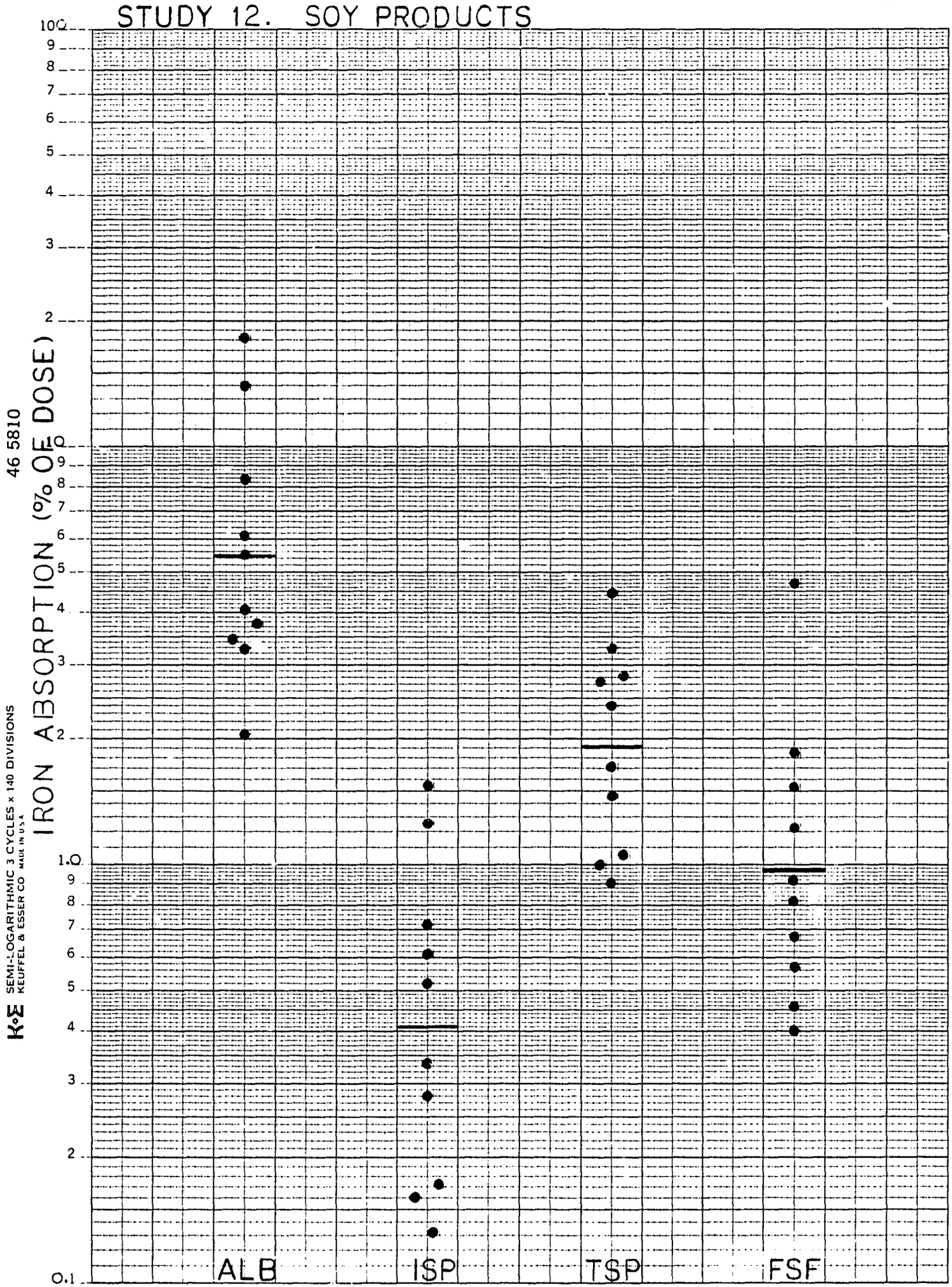
* geometric mean

TABLE 12-3

STUDY 12. The Effect of Soy Products on Iron Absorption.

	ABSORPTION RATIOS					
	A/B	A/C	A/D	B/C	B/D	C/D
Mean Ratio	13.30	2.88	5.62	0.22	0.42	1.95
SE	10.81	2.35	4.37	0.16	0.34	1.59
+1 SE	16.37	3.53	7.22	0.29	0.53	2.39
1/ Ratio	0.08	0.35	0.18	4.62	2.37	0.51
Student's *t*	12.462	5.169	6.888	5.163	3.834	3.298
P value	0.001	0.001	0.001	0.001	0.002	0.004
Correlation* (r)	0.664	0.471	0.385	0.185	0.618	0.542
P value	0.018	0.084	0.135	0.304	0.028	0.053

* Log scale


STUDY 12. SOY PRODUCTS
IRON ABSORPTION (% OF DOSE)
100
10
1.0
0.1
ALB
ISP
TSP
FSF
K+E SEMI-LOGARITHMIC 3 CYCLES x 140 DIVISIONS
KEUFFEL & ESSER CO. MADE IN U.S.A.
46 5810

STUDY 13

EFFECT OF COFFEE ON IRON ABSORPTION

SUMMARY

The effect of coffee on nonheme iron absorption from the SS meal was evaluated in 9 volunteer subjects. Iron absorption from the SS meal taken without coffee averaged 5.9%. When consumed with drip and instant coffee, absorption fell significantly to 1.64 and 0.97%, respectively. When taken with two cups of instant coffee, absorption was further reduced to 0.53%. This study demonstrates a striking inhibition of iron absorption by coffee similar to that previously observed with tea.

RATIONALE

In a previous study (study 9), the effect of coffee and tea on iron absorption from the STD meal was examined. Baseline absorption of 3.7% decreased to 2.3 and 1.3% when the meal was taken with coffee and tea respectively. The present study was performed to extend this observation - a) to evaluate the effect of coffee on a meatless meal, b) to determine whether the type of coffee affected the inhibition, and c) to determine if further inhibition occurs when the amount of coffee is doubled.

DESIGN

Eight men and one post-menopausal woman participated in this study. All four test meals consisted of the SS meal without added calcium and phosphate salts and one-half protein (ovalbumin) content (same as meal A in study 12). The amount of water added to the SS meal was decreased (220 ml) so that liquid intake would be the same as meals consumed with coffee. Meal A was taken with 200 ml water, meal B with drip coffee prepared by mixing 250 ml water with 7 gm coffee, and meal C with instant coffee prepared by mixing 1.5 gm coffee (Taster's Choice) with 200 ml water. In meal D, twice the amount of instant coffee (3.0 gm) was taken in the same volume of water (200 ml). Sufficient $FeCl_3$ was added to all meals to bring the total iron content to 4.1 mg.

One additional methodologic question was addressed in this study. In all prior studies, the interval between administering a test meal and drawing blood to measure incorporated red cell activity has been 14 days. We have

consistently observed a much higher correlation in absorption between test meals given on two successive days than when given 14 days apart. One explanation is that absorbed radioiron is not fully incorporated into circulating red cells by day 14. If true, increasing the interval between administration of the test meal and blood sampling, would alter absorption values. This question was examined in this study by obtaining blood samples on all subjects on both day 14 and day 21 following administration of the labeled test meal. In other words, instead of feeding the second pair of meals on day 14, blood was drawn on day 14 and day 21; meals C and D were then administered on the following day. Similarly, blood was drawn on day 35 and again on day 42 of the study, 14 and 21 days following administration of the second pair of test meals.

RESULTS

The results are outlined in table 13-1. Mean absorption from the SS meal averaged 5.88%, a value almost identical to that obtained from this meal in study 12 (5.55%). Administration of coffee with the SS meal produced a striking inhibition. Mean absorption with drip coffee was 1.64% while absorption with single and double strength instant coffee averaged .97 and .53% respectively. Absorption from all meals taken with coffee was significantly lower than when the SS meal was taken with water. Mean ratios in (with/without) coffee averaged 0.28, 0.17, and 0.09 with drip, instant, and double strength instant respectively; thus, inhibition averaged 72, 83, and 91% respectively.

Differences in absorption between all three meals taken with coffee were also statistically significant. Absorption with instant coffee was significantly lower than with percolated beverage (ratio C/B=0.59, p=.045) and the effect of doubling the amount of instant coffee was also significant (mean ratio D/C=0.55, p=.002).

A comparison of iron absorption values based on samples obtained 14 and 21 days following test meal administration are listed in Table 13-3. The differences are small but not negligible; for meal C, 7% higher absorption was obtained when the sampling interval was 21 rather than 14 days although in no instance did this change the level of statistical significance between absorption studies. In some instances, absorption ratios changed by as much as 5%.

COMMENT

This study provides convincing evidence that coffee inhibits iron absorption and that the degree of inhibition can be influenced by both the type and strength of coffee used. It is of interest that coffee inhibition with the STD meal (containing meat) was less pronounced (about 40%) than that observed with the SS meal in the present study--72 and 83% with drip and instant coffee respectively. In a previous study by Derman et al (Br. J. Nutr. 38:261, 1977), one cup of coffee produced an inhibition of 59% with a maize meal containing 100 mg ascorbic acid.

The results of the present study have an important bearing on iron nutrition in view of the high con-

sumption of coffee throughout the world. Indeed, when the equivalent of two cups of coffee are ingested with a meal, absorption is reduced by a factor of 10. This may also have an important bearing on the vehicle used for iron fortification. The use of sugar as a vehicle has the disadvantage that a significant amount of this fortification iron in adults would be ingested with coffee.

TABLE 13-1

STUDY 13. Effect of Coffee on Iron Absorption From a Semisynthetic Meal.

Subject	Age	Packed Cell Volume (%)	Serum Iron (μg/dl)	Tsf Sat (%)	Serum Ferritin (ng/ml)	IRON ABSORPTION (% of dose) SS Meal A	Coffee Drip B	Coffee Instant C	Coffee Instant x2 D	Absorption Ratio B/A	C/A	D/A	C/B	D/B	D/C
1	44	50	166	41	55	1.35	1.06	0.36	0.23	0.78	0.26	0.17	0.33	0.21	0.63
2	30	49	197	50	76	3.22	2.45	2.57	1.65	0.76	0.79	0.51	1.04	0.67	0.64
3	24	39	183	50	114	4.48	1.88	0.43	0.33	0.41	0.09	0.07	0.22	0.17	0.76
4	23	43	126	35	114	5.11	1.10	0.27	0.23	0.21	0.05	0.04	0.24	0.20	0.85
5	24	44	145	38	132	6.22	0.86	0.40	0.17	0.13	0.06	0.02	0.46	0.19	0.42
6	50	37	123	28	56	9.03	3.37	2.45	2.21	0.37	0.27	0.24	0.72	0.65	0.90
7	28	45	118	28	46	9.51	0.83	2.62	1.43	0.08	0.27	0.15	3.15	1.72	0.54
8	27	45	157	39	86	10.00	1.91	1.11	0.28	0.19	0.11	0.02	0.58	0.14	0.25
9	31	43	106	20	181	15.98	3.56	2.65	0.90	0.22	0.16	0.05	0.74	0.25	0.33
MEAN	31	44	147	38	87*	5.88*	1.64*	0.97*	0.53*	0.28*	0.17*	0.09*	0.59*	0.33*	0.55*
					-1 SE	4.60	1.36	0.70	0.38	0.22	0.12	0.07	0.45	0.25	0.48
					+1 SE	7.52	1.98	1.36	0.75	0.36	0.22	0.13	0.78	0.43	0.64

* geometric mean

TABLE 13-2

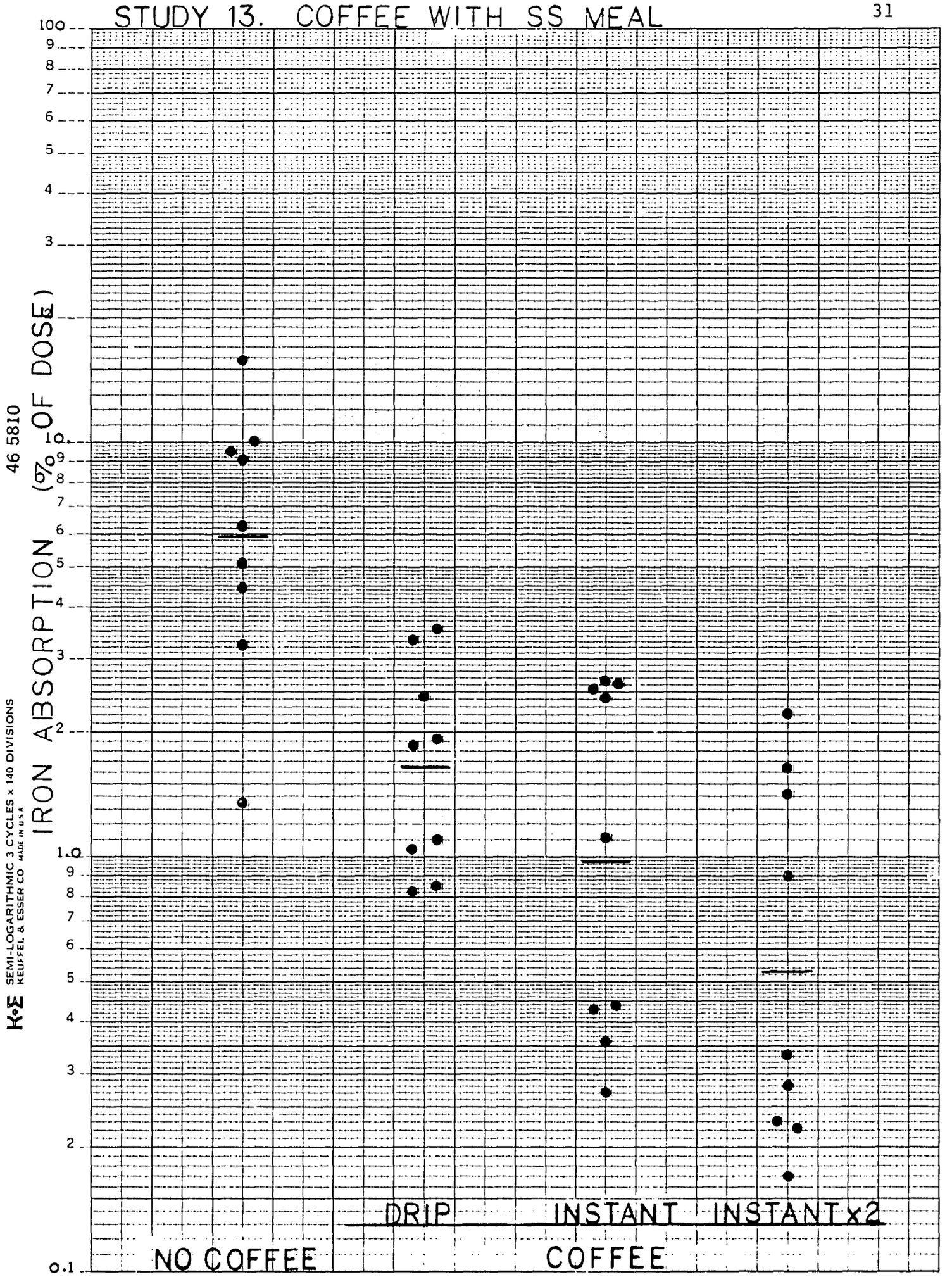
STUDY 13. Effect of Coffee on Iron Absorption From a Semisynthetic Meal.

	ABSORPTION RATIOS					
	A/B	A/C	A/D	B/C	B/D	C/D
Mean Ratio	3.58	6.03	10.90	1.68	3.05	1.81
-1 SE	2.79	4.53	7.78	1.28	2.31	1.57
+1 SE	4.59	8.01	15.29	2.21	4.01	2.09
1/ Ratio	0.28	0.17	0.09	0.59	0.33	0.55
Student's *t*	5.14	6.30	7.07	1.92	4.03	4.11
P value	0.0004	0.0001	0.0001	0.0453	0.0018	0.0017
Correlation* (r)	0.367	0.543	0.343	0.576	0.553	0.905
P value	0.165	0.065	0.183	0.052	0.061	0.0004

* Log scale

TABLE 13-3. Effect of Interval Between Meal Administration and Blood Sampling.

MEAL	Interval		Mean Iron Absorption				Iron Absorption Ratio					
	A/B	C/D	A	B	C	D	B/A	C/A	D/A	C/B	D/B	D/C
	(weeks)											
	2	2	5.51	1.53	0.91	0.55	.278	.165	.099	.594	.357	.601
	2	3	5.92	1.65	0.91	0.55	.279	.154	.092	.552	.332	.601
	3	2	5.47	1.53	0.97	0.53	.279	.178	.098	.637	.352	.552
	3	3	5.88	1.64	0.97	0.53	.279	.165	.091	.594	.328	.552


STUDY 13. COFFEE WITH SS MEAL
IRON ABSORPTION (% OF DOSE)
100
9
8
7
6
5
4
3
2
10
9
8
7
6
5
4
3
2
1.0
9
8
7
6
5
4
3
2
0.1
NO COFFEE
DRIP
INSTANT
INSTANT x2
COFFEE
46 5810
K+E SEMI-LOGARITHMIC 3 CYCLES x 140 DIVISIONS
KEUFFEL & ESSER CO. MADE IN U.S.A.

STUDY 14

STUDY OF RADIOIRON EXCHANGE WITH NaFeEDTA

SUMMARY

The present study was the first of a series to better define the value of sodium iron EDTA for iron fortification. In the present study complete isotopic exchange was observed between nonheme dietary iron of the SS meal (labeled extrinsically) and either preformed NaFeEDTA or a mixture of disodium EDTA and ferric chloride ($Na_2EDTA + FeCl_3$). Furthermore, no difference was observed in absorption of the preformed complex (NaFeEDTA) and (Na_2EDTA and $FeCl_3$) added separately at the time of administration.

RATIONALE

There has been much interest in recent years in the use of NaFeEDTA as a compound for food iron fortification. Studies more than a decade ago by Garby demonstrated an increase in hemoglobin levels when used for iron fortification of fish sauce in Thailand. More recently, a large fortification trial has been undertaken in Guatemala to examine the efficacy of sugar fortified with NaFeEDTA. Preliminary studies that led to this trial indicated that this form of iron avoids certain unfavorable reactions with foods such as discoloration or rancidity. For example, when ferrous sulfate is added to tea, a heavy black precipitate forms immediately whereas the same amount of iron added as NaFeEDTA produces discoloration only very slowly. Of perhaps even greater importance is the fact that NaFeEDTA has been shown in certain instances to be better absorbed than the common pool of dietary nonheme iron.

The effect of EDTA on iron absorption has not always been found favorable. For example, in one study from this laboratory, increasing doses of Na_2EDTA impaired iron assimilation from the STD meal (Cook and Monsen, AJCN, 29:614, 1976). The disparity with subsequent reports could be accounted for in two ways. First of all, the amount of Na_2EDTA added was much larger in the study by Cook and Monsen than the amount proposed for iron fortification. Second, Na_2EDTA was used in the study by Cook and Monsen whereas preformed NaFeEDTA was used in studies reporting favorable absorption of NaFeEDTA.

The present study was undertaken as the first in a series to better define the availability of NaFeEDTA when used for iron fortification. The objectives were two-fold. First, we wanted to determine whether or not NaFeEDTA exchanges completely with the nonheme pool of dietary iron. Some studies have demon-

strated complete exchange while others have suggested that the complex is better absorbed than the common pool of nonheme dietary iron. The second question was whether the disparity in previous reports was due to the fact that preformed NaFeEDTA was used in some studies whereas a mixture of Na_2EDTA and iron was used in others.

DESIGN

In the present study only two test meals were administered (meals 1 and 2), but each of the meals was doubly tagged with ^{59}Fe and ^{55}Fe. Four separate iron absorption measurements were therefore obtained: A and B with meal 1 and C and D with meal 2. Both test meals consisted of the SS meal with half protein and no added calcium or phosphate. This is the same meal as that used in studies 12 and 13. The meals were fed to 11 male volunteer subjects.

In both meals 1 and 2, the common pool of nonheme iron was tagged extrinsically by adding 2 mg iron as $^{59}FeCl_3 \cdot 6H_2O$. This extrinsic tag was thoroughly mixed with the meals before adding the second label. Absorption of this radioiron is designated 'test B' for meal 1 and 'test D' for meal 2. Note that this radioiron labeling technic is the same as that used in all previous studies to measure nonheme iron absorption from the SS meal.

Meal 1 was designed to measure absorption when NaFeEDTA is not preformed chemically. An aqueous solution of Na_2EDTA (5.2 mg) was mixed with $^{55}FeCl_3$ (1 mg iron) and added in 1 ml volume to the meal. Note that the $FeCl_3$ and Na_2EDTA were premixed in solution before adding them to the SS meal. Absorption of this radioiron is designated as 'test A'.

For meal 2, radioiron was added as preformed NaFeEDTA in an amount that provided 1 mg iron and 5.3 mg EDTA, the same total quantity as in meal 1. Absorption of this radioiron is referred to as 'test C'.

NaFeEDTA in this study was synthesized by the method of Sawyer and McKinney (J. Amer. Chem. Soc. 82:4191, 1960). This procedure entails mixing equimolar $Fe(NO_3)_3 \cdot 9H_2O$ with $Na_2EDTA \cdot 2H_2O$; $^{55}FeCl_3$ is added at this point for radiolabeling. The pH of the mixture is then raised to 5.0 with sodium bicarbonate and the yellow-gold iron complex is precipitated by adding ethanol. After discarding the supernatant, the precipitated NaFeEDTA is dissolved in a minimal amount of water and reprecipitated with ethanol three times. The final precipitate was dried in an oven at 60°C overnight.

RESULTS

The absorption data is listed in table 14-1. Absorption of the extrinsic tag in the SS meal averaged 5.88% in meal 1 (column B) and 6.06% in meal 2 (column D). These values are almost identical to those observed in studies 12 and 13. The 3% difference in mean absorption between meals 1 and 2 (B and D) is not significant indicating that the form in which EDTA is added does not alter absorption of nonheme iron.

When $FeCl_3$ and Na_2EDTA were simply mixed in the solution before adding it to the SS meal (A), absorption averaged 5.42% as compared with 5.88% from the extrinsic tag (B); this small difference (mean ratio A/B=0.92) was statistically significant at the 0.1% level. A difference of only 8% would not be detected as significant if the two isotopes are administered on two successive days. However, when ^{55}Fe and ^{59}Fe are added to the same meal, day-to-day variations are eliminated thereby allowing detection of much smaller differences as significant; this

is reflected in the correlation coefficient between A and B of 0.987. While the difference in absorption of A and B was statistically significant, the difference is not regarded as meaningful. When preformed NaFeEDTA was added to the SS meal in meal 2 (C), absorption was exactly equal to the absorption of nonheme iron (D); mean ratio C/D=1.00.

COMMENT

There are two important observations in this study and both are negative. In the first instance, NaFeEDTA, whether preformed or simply added together as $FeCl_3$ and Na_2EDTA at the time of administration, are absorbed identically to the common pool of nonheme iron. Thus, although a higher absorption of NaFeEDTA has been reported in some studies, this was not observed in this report.

The second observation is that there is no difference in absorption when NaFeEDTA is added as a preformed complex or simply added as EDTA and iron. The previous report by Cook and Monsen in which increasing levels of Na_2EDTA were shown to impair iron absorption cannot be explained by use of the Na_2EDTA rather than NaFeEDTA. Also note that the amount of EDTA used in this study (5.2 mg) is far less than the minimal level of 25 mg EDTA shown in the previous study by Cook and Monsen to inhibit iron absorption.

TABLE 14-1

STUDY 14. Comparison of Pre-Formed NaFeEDTA With $FeCl_3$ and Na_2EDTA.

Subject	Age	Packed Cell Volume (%)	Serum Ferritin (ng/ml)	IRON ABSORPTION (% of dose) Meal 1: $FeCl_3 + Na_2EDTA$ (A)	Meal 1: $FeCl_3$ (B)	Meal 2: NaFeEDTA (C)	Meal 2: $FeCl_3$ (D)	Absorption Ratio A/B	Absorption Ratio C/D
1	26	42	115	2.67	2.81	2.12	2.22	0.95	0.95
2	24	46	88	3.00	3.73	3.12	3.28	0.80	0.95
3	19	41	55	4.12	4.17	5.77	5.48	0.99	1.05
4	27	45	144	4.63	4.57	8.01	8.25	1.01	0.97
5	31	48	82	5.55	6.53	7.03	7.98	0.85	0.88
6	26	45	110	5.63	5.97	6.40	5.97	0.94	1.07
7	30	46	95	6.38	6.77	5.31	5.58	0.94	0.95
8	24	47	32	6.57	7.30	8.17	8.11	0.90	1.00
9	31	45	77	6.85	7.66	9.37	8.80	0.89	1.06
10	24	46	108	7.16	7.85	4.36	4.22	0.91	1.03
11	27	47	45	12.27	12.75	17.57	16.50	0.96	1.06
MEAN	26	45	80*	5.42*	5.88*	6.09*	6.09*	0.92*	1.00*
			-1 SE	4.77	5.18	5.13	5.18	0.90	0.98
			+1 SE	6.17	6.68	7.22	7.18	0.94	1.02

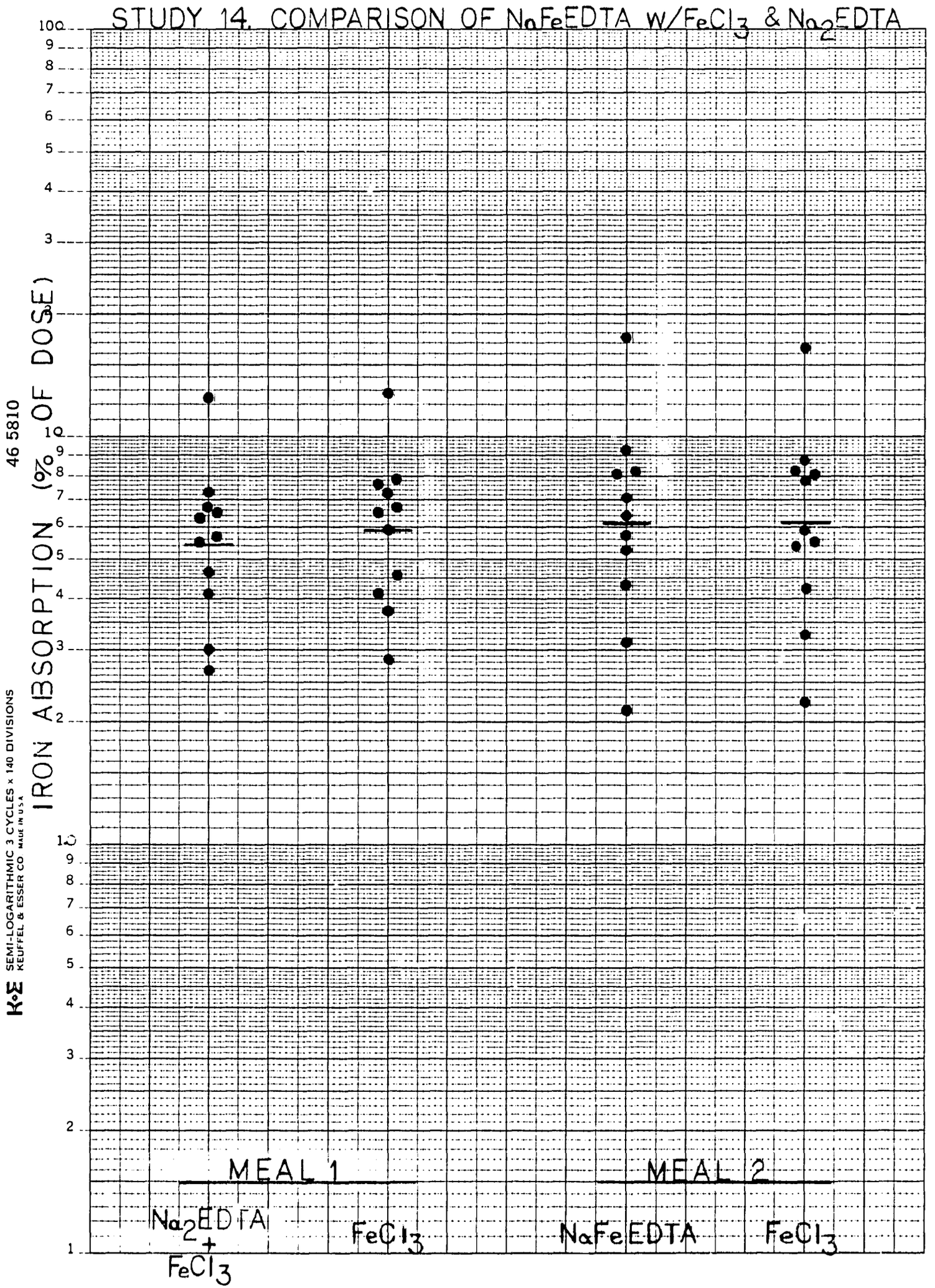
* geometric mean

TABLE 14-2

STUDY 14. Comparison of Pre-Formed NaFeEDTA With $FeCl_3$ and Na_2EDTA.

	ABSORPTION RATIOS					
	A/B	A/C	A/D	B/C	B/D	C/D
Mean Ratio	0.92	0.89	0.89	0.97	0.97	1.00
-1 SE	0.90	0.81	0.81	0.88	0.88	0.98
+1 SE	0.94	0.98	0.98	1.07	1.06	1.02
1/ Ratio	1.09	1.12	1.12	1.03	1.04	1.00
Student's *t*	4.06	1.24	1.27	0.34	0.36	0.07
P value	0.001	0.121	0.117	0.370	0.362	0.472
Correlation* (r)	0.987	0.842	0.826	0.814	0.803	0.994
P value	$<10^{-6}$	0.0006	0.0009	0.0011	0.0014	$<10^{-6}$

*Log scale


STUDY 14. COMPARISON OF NaFeEDTA w/FeCl3 & Na2EDTA
IRON ABSORPTION (% OF DOSE)
100
10
1
MEAL 1
MEAL 2
Na2EDTA + FeCl3
FeCl3
NaFeEDTA
FeCl3
46 5810
K+E SEMI-LOGARITHMIC 3 CYCLES x 140 DIVISIONS
KEUFFEL & ESSER CO. MADE IN U.S.A.

STUDY 15

IRON ABSORPTION FROM FILIPINO LOW COST MEALS

SUMMARY

Iron absorption was measured from morning, noon, and evening meals believed to be typical for the lower socioeconomic class in Manila, Philippines. Mean absorption from breakfast, lunch, and dinner averaged 1.71, 1.31, and 3.49% respectively. The total iron content in these meals was 10.5 mg. Based on these observations, the total amount of iron absorbed daily from this diet was only 0.28 mg, far below the amount required to maintain normal iron balance.

RATIONALE

An essential preliminary step in developing an iron fortification program in a given country is to first measure iron availability from that diet. If the diet is relatively low in iron content but has a high availability, iron fortification is likely to be effective in combating iron deficiency. On the other hand, if the diet contains a high amount of iron but that iron is poorly assimilated, the addition of a substance to the diet which enhances iron availability is likely to be more effective than the addition of more iron.

This study and the one that follows were undertaken to measure iron availability from two types of Filipino diets. The present study was performed with a diet believed to be typical for the low socioeconomic group and therefore has been designated the 'low cost meal'. The selection of this diet was made by Mrs. Anita Marzan from Manila, Philippines (Philippine Atomic Energy Commission). The purchase of the foods and their preparation was supervised by Mrs. Marzan and Ms. Mely Ong, a Filipino technician working in our laboratory. The composition of the three meals is listed in table 15-1. All food items were purchased in a local Chinese grocery store.

It is planned that Mrs. Marzan, on her return to the Philippines will study iron absorption from these identical meals in Filipino volunteer subjects using foods purchased in Manila. Therefore, there are four major objectives to the present study. First the data will provide a measure of iron availability from a typical Philippine diet. Second, it will test the validity of performing iron absorption measurements in normal American volunteers rather than in subjects in the LDC. Theoretically, any differences in iron status will be eliminated by expressing food iron absorption as a ratio of absorption

from a reference dose of inorganic iron. Reference dose measurements were performed in this study (meal D) and will also be performed in studies in the Philippines. The third objective is to compare iron absorption with foods purchased in the USA rather than the LDC. The fourth objective is to monitor and validate measurements of ^{55}Fe and ^{59}Fe blood radioactivity performed in the Philippines in Mrs. Marzan's laboratory. A portion of the blood samples obtained in the Philippines will be shipped to the University of Kansas Medical Center for replicate measurements.

DESIGN

A total of four iron absorption tests were performed in each of 10 male volunteer subjects. Three of the test meals were chosen to represent typical Philippine meals (Table 15-1). Breakfast consisted of rice, fried whole fish, tomatoes, black coffee, and sugar. Lunch consisted of a mungo bean dish containing pork, tomatoes, and seasonings. The dinner consisted of a soup-like dish of sweet potatoes, cabbage, bean sprouts, and a small quantity of pork. All meals were served with 150 g rice. The fourth meal consisted of a solution containing 3 mg iron as radioactive ferrous sulfate and a 2:1 molar ratio of freshly prepared ascorbic acid (reference dose). All the meals were prepared on the morning of their administration to the volunteers, all of whom had fasted overnight. The subjects encountered no difficulty in ingesting the meals with the exception of subject 7 who refused to eat fried whole fish for breakfast.

RESULTS

The absorption data is outlined in Table 15-2. Mean absorpton from the breakfast, lunch, and dinner meals averaged 1.71, 1.13, and 3.49% respectively. Absorption from the reference dose averaged 19.24%. Differences between each of the meals and

the reference dose were all highly significant. In addition, absorption from the dinner meal (C) was significantly higher than absorpton from either breakfast (mean ratio A/C=.49, p=.013) or lunch (mean ratio B/C=.32, $p < .001$).

Iron absorption ratios for each of the meals relative to reference dose absorption are listed in Table 15-3; mean ratios for breakfast, lunch, and dinner were .09, .06, and .18 respectively.

COMMENT

See study 16.

TABLE 15-1. Composition of Low Cost Filipino Meals.

	Weight (g)	Energy (kcal)	Protein (g)	Fat (g)	CHO (g)	Fiber (g)	Iron (mg)	Vitamin C (mg)
BREAKFAST								
Rice	150	233	3.8	0.6	51.3	0.15	0.90	--
Fried dilis	25	82	17.1	1.0	--	--	0.60	3.6
Tomato	100	20	1.2	0.3	4.2	0.70	0.60	23.0
Coffee	2	3	--	--	0.7	--	0.11	--
Sugar	10	35	--	--	9.8	--	0.01	--
Oil	7	62	--	7.0	--	--	--	--
Total	294	435	22.1	8.9	66.0	0.85	2.22	26.6
LUNCH								
Rice	150	233	3.8	0.6	51.3	0.15	0.90	--
Pork	30	137	3.6	0.2	--	--	0.54	--
Mungo beans	56	17	2.3	0.1	2.8	0.50	0.66	10.0
Tomato	17	3	0.2	0.1	0.1	0.12	0.11	3.9
Onion	11	3	0.2	--	0.7	0.09	0.12	2.1
Garlic	1	2	0.1	--	0.4	0.01	0.02	0.1
Oil	6	53	--	--	--	--	--	--
Salt	2	--	--	--	--	--	--	--
Total	273	448	10.2	1.0	55.3	0.87	2.35	16.1
DINNER								
Rice	150	233	3.8	0.6	51.3	0.15	0.90	--
Pork	21	96	2.5	9.5	--	--	0.38	--
Sweet potato	57	66	0.7	0.2	15.6	0.52	0.52	17.2
Cabbage	83	18	1.3	0.3	3.7	0.67	0.67	38.3
Bean sprouts	54	60	7.1	0.4	10.9	0.33	3.38	3.8
Onion	5	1	0.1	--	0.3	0.04	0.05	0.8
Garlic	1	2	--	7.5	--	--	0.02	--
Oil	8	66	--	--	--	--	--	--
Salt	2	--	--	--	--	--	--	--
Soy sauce	3	--	--	--	--	--	--	--
Total	384	542	15.5	18.5	81.8	1.71	5.92	60.1
Combined Total	951	1425	47.8	28.4	203.1	3.43	10.49	102.8

TABLE 15-2

STUDY 15. Iron Availability from Filipino Low Cost Meals.

Subject	Age	Packed Cell Volume (%)	Serum Ferritin (ng/ml)	IRON ABSORPTION (% of dose) Breakfast (A)	Lunch (B)	Dinner (C)	REF (D)	Absorption Ratio A/D	B/D	C/D
1	20	42	133	0.22	0.51	1.47	10.01	0.02	0.05	0.14
2	22	45	140	0.35	0.40	1.66	9.40	0.03	0.04	0.17
3	30	41	52	0.65	0.16	1.42	15.78	0.04	0.01	0.08
4	19	50	93	2.51	1.28	2.56	17.17	0.14	0.07	0.14
5	35	47	78	2.70	0.83	2.43	15.17	0.17	0.05	0.16
6	28	46	36	3.41	3.21	7.83	22.21	0.11	0.10	0.26
7	18	48	63	3.51	3.90	13.32	28.17	0.12	0.13	0.47
8	18	48	18	5.30	3.82	8.36	39.51	0.13	0.09	0.21
9	18	43	52	5.97	1.88	4.15	28.85	0.20	0.06	0.14
MEAN	23	46	63*	1.71*	1.13*	3.49*	19.24*	0.09*	0.06*	0.18*
			-1 SE	1.14	0.78	2.64	16.25	0.07	0.05	0.16
			+1 SE	2.57	1.64	4.61	22.78	0.12	0.08	0.21

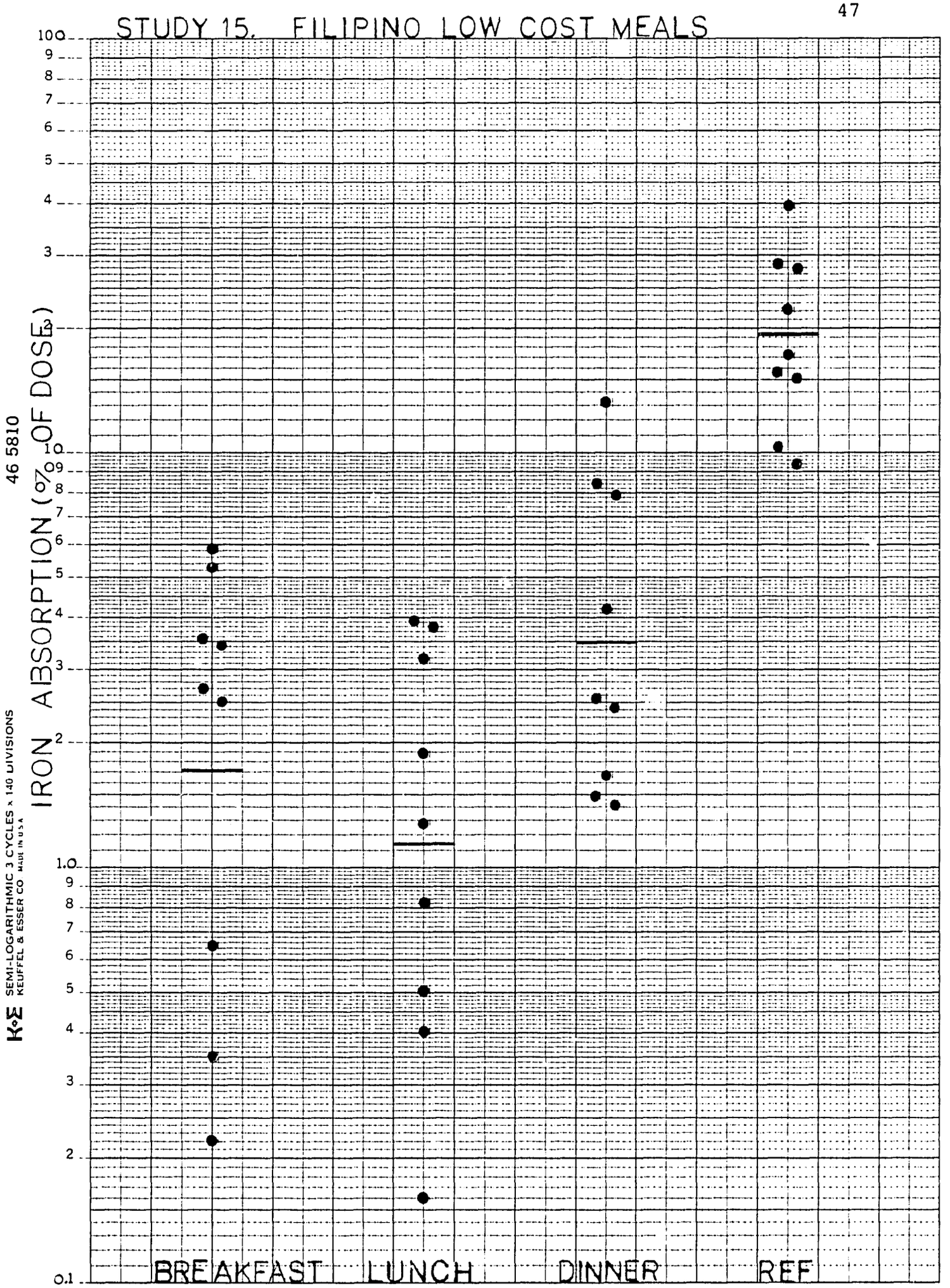
* geometric mean

TABLE 15-3

STUDY 15. Iron Availability From Filipino Low Cost Meals.

	ABSORPTION RATIOS					
	A/B	A/C	A/D	B/C	B/D	C/D
Mean Ratio	1.51	0.49	0.09	0.32	0.06	0.18
-1 SE	1.18	0.38	0.07	0.28	0.05	0.16
+1 SE	1.94	0.64	0.12	0.38	0.08	0.21
1/ Ratio	0.66	2.04	11.22	3.08	16.96	5.50
Student's *t*	1.66	2.73	9.08	7.33	11.13	10.98
P value	0.068	0.013	$<10^5$	.00004	$<10^5$	$<10^5$
Correlation* (r)	0.799	0.770	0.892	0.929	0.816	0.870
P value	0.0049	0.0075	0.0006	0.0001	0.0036	0.0011

*Log scale


STUDY 15. FILIPINO LOW COST MEALS
IRON ABSORPTION (% OF DOSE)
100
9
8
7
6
5
4
3
2
10
9
8
7
6
5
4
3
2
1.0
9
8
7
6
5
4
3
2
0.1
BREAKFAST
LUNCH
DINNER
REF
46 5810
K+E SEMI-LOGARITHMIC 3 CYCLES x 140 DIVISIONS
KEUFFEL & ESSER CO. MADE IN U.S.A.

STUDY 16

IRON ABSORPTION FROM FILIPINO MODERATE COST MEALS

SUMMARY

Iron absorption was measured from morning, noon, and evening meals believed to be typical for the people in the middle socioeconomic class in the Philippines. Mean absorption from breakfast, lunch, and dinner averaged 1.55, 1.49, and 1.46% respectively as compared with a reference dose absorption of 12.6%.

RATIONALE

The rationale for this study is outlined in study 15. The present study was performed to evaluate food iron availability from typical Filipino meals eaten by the middle socioeconomic class in that country.

DESIGN

The design of this study was exactly the same as that outlined in study 15. Eight normal male volunteers were given a total of four separate iron absorption tests; the meals consisted of breakfast (A), lunch (B), and dinner (C). The composition of the meals is shown in Table 16-1. The total caloric content of the three meals was 2,093 kcal with a total iron content of 15.61 mg. The fourth test meal consisted of the reference dose containing 3 mg iron as ferrous sulfate.

RESULTS

Mean absorption values are listed in Table 16-2. Absorption from breakfast, lunch, and dinner averaged 1.55, 1.49, and 1.46% respectively as compared to a reference dose absorption of 12.58%. Absorption from all Filipino meals was significantly lower than the reference dose. However, none of the differences in absorption between the three Filipino meals were significant. All three mean absorption ratios for test meal/reference dose averaged 0.12.

COMMENT

One interesting point in these studies (15 and 16) is the fact that although the meals were believed to be typical for low and moderate income groups, iron absorption from all six meals was remarkably similar. In fact, the only meal that appeared to have a slightly improved availability is dinner for the low income family. Clearly, when measured on the basis of nonheme iron availability, no advantage was observed between the two types of diet.

If one makes certain assumptions, the amount of iron absorbed from these two diets can be calculated. The first assumption required is that for meat products, approximately half the total iron content is in the form of heme iron. (There is ample precedent for this assumption in the existing literature). Second, absorption of this heme iron is assumed to be 40%. The assumption is valid because heme iron absorption, unlike nonheme iron, is little affected by the diet or the iron status of the subject.

The calculations are outlined in table 16-4. The total nonheme iron content of each meal was obtained from food composition tables; all meat and fish products were assumed to contain 50% heme iron. For the low income diet, nonheme iron content totaled 9.90 mg of which .27 mg was absorbed. The total heme iron content is only .76 mg but because of much higher assimilation, 0.31 mg heme iron is absorbed daily to give a total daily absorption of .58 mg. The total iron intake of 10.66 mg is far below the recommended intake of 18 mg iron daily in the U.S. It is interesting to calculate the amount of nonheme iron required in this diet as fortification to achieve a higher amount of 1.0 mg absorbed iron daily. An average percentage absorption of nonheme iron from the low cost and moderate cost diets in these studies is around 2%. Thus, to obtain another .4 mg iron daily from nonheme iron, an additional .4/.02=20 mg iron would be required. Thus, the nonheme iron content of the diet would have to be increased three-fold in order to achieve an absorption level consistent with current estimates of minimal iron requirements.

Calculations are also listed for the moderate cost diet which has a much higher total iron intake of 15.61 mg daily (vs. 10.66 mg for the low cost meal) of which 2.39 mg is heme iron and 13.22

nonheme iron. Due largely to the higher content of heme, the estimated total daily absorption from this diet is 1.17 mg, nearly twice that of the low cost diet. Note that the moderate cost diet is more advantageous not because of higher availability and/or higher content of iron but because of the higher proportion of dietary iron in the form of heme. Indeed, most of the absorbed iron in this diet is derived from heme.

The findings in this study should be extrapolated with some caution. Nevertheless, they indicate that iron availability for a typical diet in the Philippines is very low, at least when normal adult males in the U.S. are used as volunteer subjects. Second, although the heme content of the Philippine diet is low, it still provides a major source of absorbed iron--over 50% with a low cost diet and more than 80% with a moderate cost diet. It will be very interesting to see the extent to which the results measured in Filipino volunteer subjects with foods purchased in that country will differ from the data presented here.

TABLE 16-1. Composition of Moderate Cost Filipino Meals.

	Weight (g)	Energy (kcal)	Protein (g)	Fat (g)	CHO (g)	Fiber (g)	Iron (mg)	Vitamin C (mg)
BREAKFAST								
Rice	150	233	3.8	0.6	51.3	0.15	0.90	--
Sausage	32	114	6.8	9.4	2.1	--	0.96	--
Egg, boiled	60	98	7.7	6.9	0.5	--	1.92	--
Tomato	85	17	1.0	0.3	3.6	0.60	0.51	19.5
Coffee	2	3	--	0.7	--	--	0.11	--
Orange	100	40	0.8	0.2	9.9	0.40	0.30	43.0
Oil	7	62	--	7.0	--	--	--	--
Evap. milk	12	16	0.8	1.0	1.1	--	0.02	0.1
Total	448	583	20.9	26.1	68.5	1.15	4.72	62.6
LUNCH								
Rice	150	233	3.8	0.6	51.3	0.15	0.90	--
Bamboo shoots	130	26	2.0	0.7	4.6	1.56	--	--
Brd. Shrimp	70	61	12.3	0.6	0.6	--	1.12	0.7
Banana	115	115	1.4	0.3	30.0	0.69	0.92	16.1
Oil	7	62	--	7.0	--	--	--	--
Total	472	497	19.5	9.2	86.5	2.40	2.94	16.8
DINNER								
Rice	200	310	5.0	0.8	68.4	0.20	1.20	--
Spare ribs	210	410	36.1	28.3	--	--	2.70	--
Cabbage	80	18	1.3	0.2	3.5	0.64	0.64	36.8
Potato	170	122	3.2	0.2	27.7	0.51	1.36	18.7
Beans	24	83	5.1	0.4	15.3	1.06	2.03	0.5
Sugar	21	73	0.4	--	20.4	--	0.02	--
Total	705	1016	51.1	29.9	135.3	2.41	7.95	56.0
Combined Total	1625	2096	91.5	65.2	290.3	5.96	15.61	135.4

TABLE 16-2

STUDY 16. Iron Availability From Filipino Moderate Cost Meals.

Subject	Age	Packed Cell Volume (%)	Serum Ferritin (ng/ml)	IRON ABSORPTION (% of dose) Breakfast (A)	Lunch (B)	Dinner (C)	REF (D)	Absorption Ratio A/D	B/D	C/D
1	41	42	179	0.46	0.92	1.05	7.06	0.06	0.13	0.14
2	51	44	115	0.52	0.52	0.32	10.35	0.05	0.05	0.03
3	50	44	153	0.96	0.63	0.67	7.88	0.12	0.07	0.08
4	40	45	212	1.33	0.37	1.58	9.05	0.14	0.04	0.17
5	27	45	48	1.81	2.56	1.56	16.51	0.10	0.15	0.09
6	18	47	28	2.01	2.27	0.95	25.18	0.07	0.09	0.03
7	18	46	35	4.87	7.23	6.08	11.40	0.42	0.63	0.53
8	31	46	35	6.20	5.31	6.71	25.53	0.24	0.20	0.26
MEAN	35	45	76*	1.55*	1.49*	1.46*	12.58*	0.12*	0.12*	0.12*
			-1 SE	1.11	1.00	1.01	10.54	0.10	0.09	0.08
			+1 SE	2.15	2.21	2.11	15.03	0.16	0.16	0.16

* geometric mean

TABLE 16-3

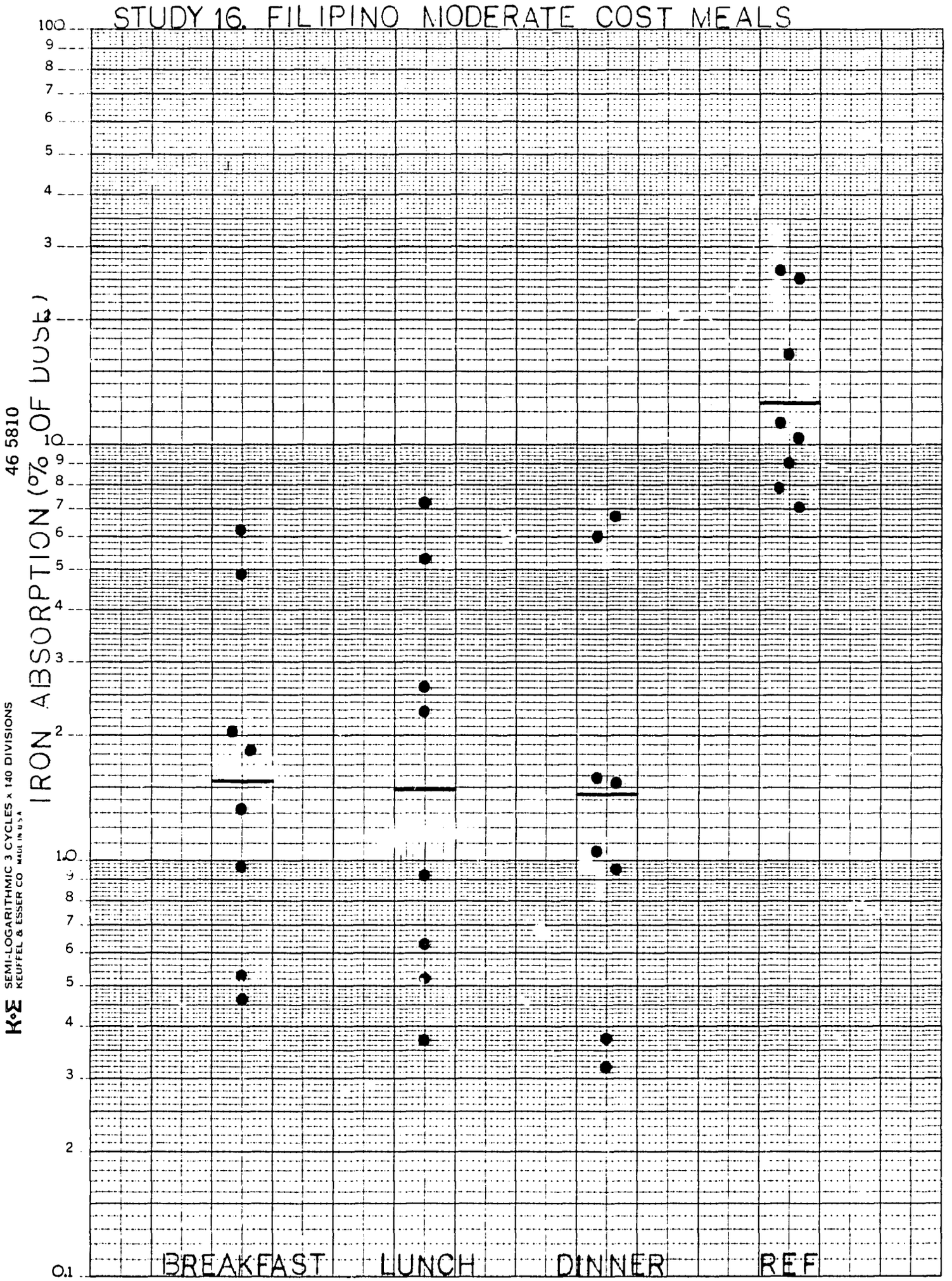
STUDY 16. Iron Availability from Filipino Moderate Cost Meals.

	ABSORPTION RATIOS					
	A/B	A/C	A/D	B/C	B/D	C/D
Mean Ratio	1.04	1.06	0.12	1.02	0.12	0.12
-1 SE	0.84	0.89	0.10	0.79	0.09	0.08
+1 SE	1.29	1.26	0.16	1.31	0.16	0.16
1/ Ratio	0.96	0.94	8.11	0.98	8.42	8.58
Student's *t*	0.17	0.32	8.43	0.07	6.92	6.37
P value	0.434	0.379	0.00003	0.4718	0.0001	0.0002
Correlation* (r)	0.835	0.881	0.593	0.788	0.648	0.405
P value	0.0049	0.0019	0.0603	0.0101	0.0409	0.159

*Log scale

TABLE 16-4. Iron Absorption from Philippine Diets

	Iron Content		Nonheme	Absorbed Iron		
	Nonheme	Heme	Absorption	Nonheme	Heme	Total
	(mg)		(%)		(mg)	
LOW COST MEAL						
Breakfast	1.92	0.30	1.71	0.03	0.12	0.15
Lunch	2.08	0.27	1.31	0.03	0.11	0.14
Dinner	5.90	0.19	3.49	0.21	0.08	0.29
Total	9.90	0.76		0.27	0.31	0.58
MODERATE COST MEAL						
Breakfast	4.24	0.48	1.55	0.07	0.19	0.27
Lunch	2.38	0.56	1.49	0.04	0.22	0.26
Dinner	6.60	1.35	1.46	0.10	0.54	0.64
Total	13.22	2.39		0.22	0.95	1.17


STUDY 16. FILIPINO MODERATE COST MEALS
IRON ABSORPTION (% OF DOSE)
100
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0.1
BREAKFAST
LUNCH
DINNER
REF
K+E SEMI-LOGARITHMIC 3 CYCLES x 140 DIVISIONS
KEUFFEL & ESSER CO. MADE IN U.S.A.
46 5810

STUDY 17

EFFECT OF COOKING SOY PROTEIN ISOLATES

SUMMARY

This study was undertaken to determine whether a difference in the methods of preparing soy products would account for the discrepancy between previous studies showing high iron absorption from soy products and our recent studies showing low iron availability. Baking isolated soy protein increased mean absorption from 0.64 to 1.2% and this two-fold increase was highly significant ($p < 0.001$). Baking of whole soybean similarly increased mean absorption from 1.06 to 1.60% and this 50% increase was also statistically significant ($p < .05$). When given in protein equivalent quantities, there was no significant difference in iron absorption from isolated soy protein and whole soybean.

RATIONALE

Several previous studies in this series have demonstrated a marked inhibition in iron absorption when the test meal contains soy protein; mean absorption has been consistently less than 1%. These findings conflict with previous studies of iron availability from soy products when administered as a single food item. Mean iron absorption from biosynthetically labeled soybean was in the order of 18%. Soybean was prepared in that study by shelling, grinding, diluting with water to obtain a porridge-like consistency, and then baking in an oven at 350-400°F for one hour. The present study was undertaken to determine whether prolonged baking of either whole soybean or isolated soy protein affects iron availability.

DESIGN

Studies were performed in nine male volunteer subjects. The first pair of test meals (A and B) examined the effect of baking ISP while the second set (C and D) examined the effect of baking whole soybean. In the first pair of meals ISP was substituted for egg albumin in the SS meal with no added calcium or phosphate. Meals C and D contained only soybean. Two milligrams iron as $FeCl_3$ was added to all test meals to raise the total iron content to 4.1 mg/meal; however, the iron content of whole soybean used in meal C and D was lower than anticipated.

The recipe for meals A and B is as follows:

	gram/person
ISP (Supro 710)	16.9
Dextrimaltose	66.8
Corn oil	34.8
Vanilla flavoring	--
Water	221.5
Total	340.0

In meal A, ISP as obtained from the manufacturer was added to the SS meal. For meal B, the amount of ISP required for the study was mixed with three volumes water and spread to a 1/4" thickness on a lightly greased cooking sheet. After baking at 400° for 1 hour and allowing the water to evaporate, the dry residue was finely ground with a mortar and pestle and then added to the other SS meal constituents. The baked ISP had a definite grittiness because it was no longer water-dispersible and it tended to settle to the bottom of the cup from which the dose was taken. The taste was not altered appreciably.

To prepare meal C, 1 kg whole soybean (Arrowhead Mills) was mixed with 2 L water. After soaking about 10 hours the water was poured off to remove any dirt and extraneous hulls. A further 3 L water was then added along with 4 medium size onions (diced), 4 bay leaves, 10 g salt, and 10 g monosodium glutamate. The beans were boiled for 2 hours and the supernatant discarded. Individual 100 g portions of the boiled beans were refrigerated overnight; servings were heated for 20 seconds in a microwave oven and then eaten with plastic spoons. Deionized water (100 ml) containing radioiron in 0.01 N HCl and 0.1 mg iron as $FeCl_3$ was taken with the meal.

Aliquots (100 g) of boiled soybean used in meal C were prepared for meal D as follows. Soybean were baked on a lightly oiled cooking sheet at 400°F for 45 minutes with periodic turning. The boiled beans turned to a light brown, crunchy bean. The 100 g aliquots were scraped quantitatively from the sheets, weighed, and served with a glass of water containing radioiron with 0.1 mg carrier iron as for meal C. The average weight of the baked soybean was 38 g, indicating a moisture loss of 62%.

The iron content of the boiled and baked soybean was measured by wet digestion. The soybean used in meal C provided 1.3 mg

iron/meal (1.3 mg iron/100 g) while baked soybean used for meal D contained 0.56 mg iron/meal or 1.4 mg iron/100 g.

RESULTS

The results are shown in the accompanying tables. For ISP, absorption averaged 0.6% when added directly to the SS meal as compared to 1.28% when the ISP was baked prior to administration. The mean ratio of 1.99 indicated a highly significant increase in absorption ($t=4.81$, $p<0.001$). Iron absorption also increased when whole soybean was baked, but this difference was less pronounced. Mean absorption from boiled (unbaked) soybean averaged 1.06% an increase to 1.60% when baked. The mean absorption ratio of 1.50 was significant ($t=2.01$, $p=0.04$).

This study also provided a comparison in iron absorption from ISP and whole soybean although the SS meal was served with ISP whereas whole soybean was consumed alone. The carbohydrate and fat in the SS meal have been found in previous studies to be relatively neutral with respect to iron absorption. With both baked and unbaked products, absorption was slightly higher from whole soybean than from ISP. Thus, the mean ratio for uncooked soy products (C/A) averaged 1.65 ($t=1.62$, $p=0.07$). Slightly higher absorption was also observed with the baked products; absorption ratio D/B averaged 1.25 but this 25% difference was not statistically significant ($t=0.75$, $p=0.24$).

COMMENT

While iron absorption with ISP remained very low, meal B is the first in which mean absorption from a meal containing soy has been greater than 1%. With both ISP and whole soybean, a significant loss of inhibition occurs when the product is baked prior to administration. It is obvious, however, from the relatively small two-fold increase in iron absorption that the

inhibitory effect of soybean is essentially heat stable. Baking ISP would not be feasible because this destroys the physical properties of water dispersibility for which this particular product was designed (Supro 710, Ralston Purina).

TABLE 17-1

STUDY 17. Effect on Iron Absorption of Cooked Isolated Soy Protein and Whole Soybeans.

Subject	Age	Packed Cell Volume (%)	Serum Ferritin (ng/ml)	IRON ABSORPTION (% of dose): Isolated Soy Protein, Uncooked (A)	Isolated Soy Protein, Cooked (B)	Whole Soybeans, Boiled (C)	Whole Soybeans, Baked (D)	Absorption Ratio B/A	D/C	C/A	D/B
1	22	41	14	0.26	0.51	0.63	0.96	1.96	1.52	2.42	1.88
2	27	46	172	0.37	1.16	1.37	0.72	3.13	0.52	3.70	0.62
3	20	45	55	0.52	1.08	1.38	2.63	2.07	1.90	2.65	2.43
4	29	46	132	0.53	1.48	0.85	0.97	2.79	1.14	1.60	0.65
5	19	45	61	0.68	2.45	0.18	0.92	3.60	5.11	0.26	0.37
6	35	44	35	0.70	1.15	4.06	7.07	1.64	1.74	5.80	6.14
7	18	46	32	0.85	1.67	1.65	1.65	1.96	1.00	1.94	0.98
8	25	46	139	0.87	0.83	1.02	1.82	0.95	1.78	1.17	2.19
9	20	46	56	2.10	2.61	1.43	2.02	1.24	1.41	0.68	0.77
MEAN	24	45	46*	0.64*	1.28*	1.06*	1.60*	1.99*	1.50*	1.65*	1.25*
			-1 SE	0.53	1.08	0.80	1.26	1.73	1.23	1.21	0.93
			+1 SE	0.78	1.52	1.41	2.02	2.30	1.84	2.25	1.67

* geometric mean

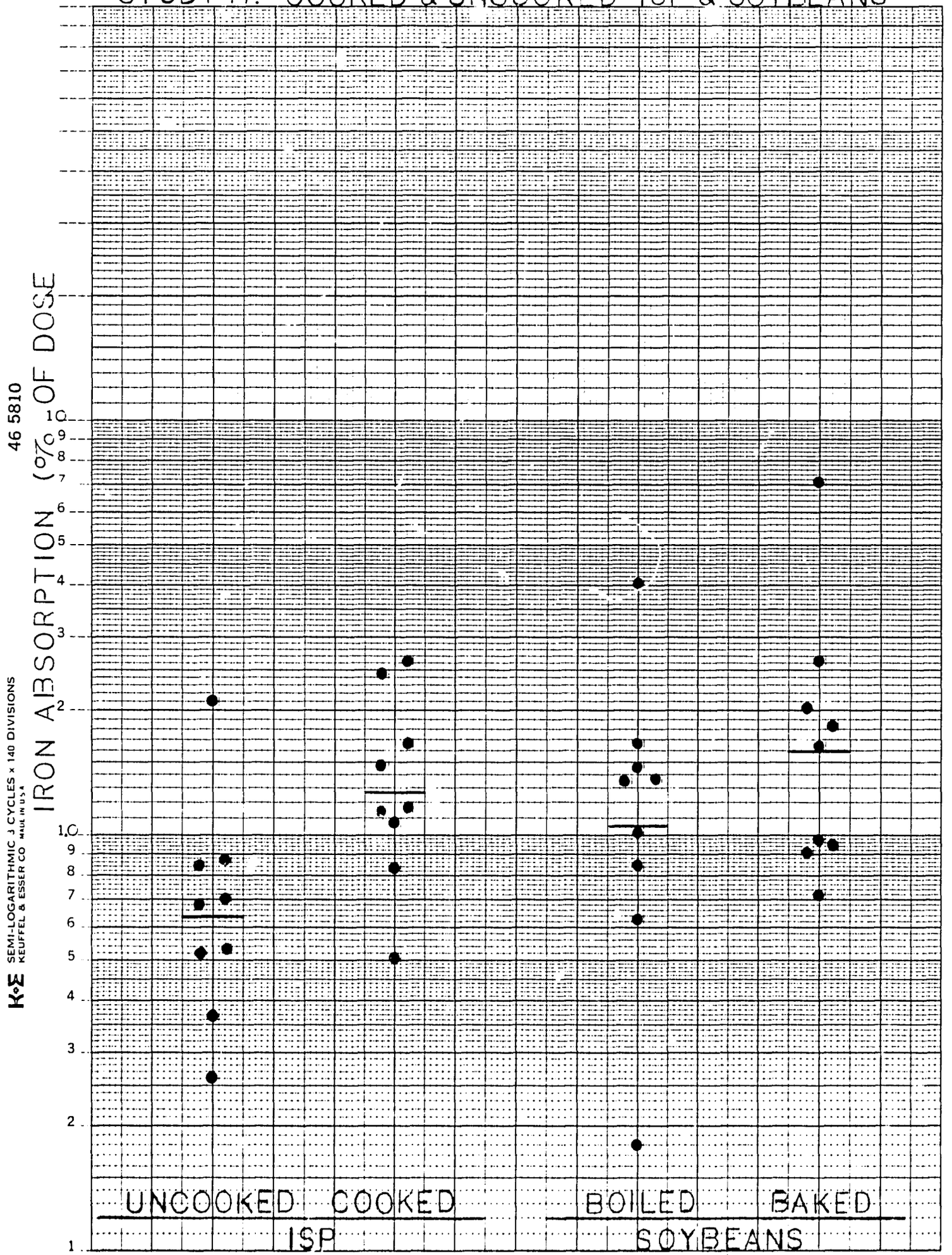
TABLE 17-2

STUDY 17. Effect on Iron Absorption of Cooked Isolated Soy Protein and Whole Soybeans

	A/B	A/C	A/D	B/C	B/D	C/D
Mean Ratio	0.50	0.61	0.40	1.21	0.80	0.67
-1 SE	0.44	0.44	0.32	0.85	0.60	0.54
+1 SE	0.58	0.83	0.51	1.72	1.07	0.81
1/ Ratio	1.99	1.65	2.48	0.83	1.25	1.50
Student's *t*	4.81	1.62	3.79	0.53	0.75	2.01
P value	0.0007	0.072	0.0027	0.304	0.236	0.040
Correlation* (r)	0.705	0.200	0.394	-0.164	-0.002	0.705
P value	0.017	0.303	0.147	0.336	0.497	0.017

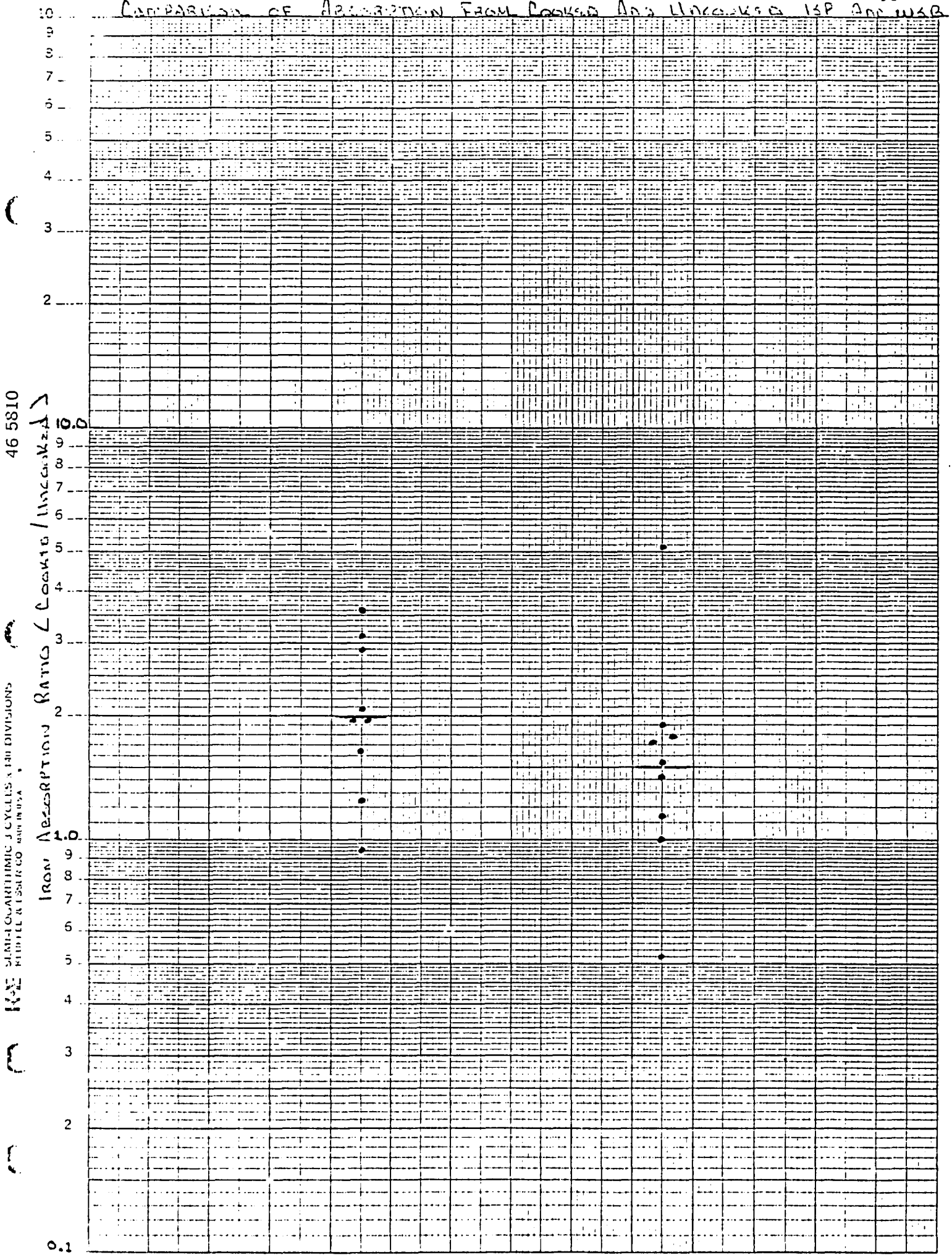
* log scale

STUDY 17. COOKED & UNCOOKED ISP & SOYBEANS



Study 17.

Comparison of Absorption from Cooked and Uncooked ISP and WSB



STUDY 18

INTERACTION OF NaFeEDTA AND ASCORBIC ACID

SUMMARY

The addition of 200 mg ascorbic acid to a modified SS meal produced the expected increase in mean absorption from 8.07 to 23.3%. However, when iron was added to the SS meal as NaFeEDTA rather than $FeCl_3$, mean absorption increased from 6.78 to 7.34%, a difference that was not statistically significant. It is concluded that the enhancing effect of ascorbic acid is largely ablated when iron in the meal is in the form of NaFeEDTA.

RATIONALE

The rationale for studying NaFeEDTA has been outlined in previous studies; there is now widespread interest in using this form of iron for fortification. In study 14 no difference could be detected when preformed NaFeEDTA was added rather than Na_2EDTA and $FeCl_3$. This and several subsequent studies will examine the interaction of Na_2EDTA with substances that either enhance or impair the assimilation of nonheme iron.

Many of the factors that influence iron availability are believed to do so by their ability to form an iron chelate complex. The effect of a given chelate on iron absorption depends on its relative affinity for iron. For example, a highly potent iron chelate, desferrioxamine, is capable of completely ablating assimilation of iron from the gastro-intestinal tract, presumably by failing to release its iron to the intestinal mucosa. Ascorbic acid, on the other hand, forms a chelate with iron that greatly enhances the assimilation of dietary iron. The present study was designed to determine the relative affinity for iron of EDTA and ascorbic acid. For example, if EDTA was added to a meal and complexed much of the iron in the nonheme pool, subsequent addition of ascorbic acid may show little or no further enhancement.

DESIGN

Eight male volunter subjects participated in this study. All received four separate test meals. All meals consisted of the modified SS meal (one-half protein, no added calcium or phosphate). To meal B, which served as the control, iron was added as $FeCl_3$. In meal A, the same amount of iron was added as preformed NaFeEDTA. Meals C and D were identical to meals A and B respectively except that 200 mg ascorbic acid was added. Both ascorbic acid and radioiron were added to the meals immediately prior to ingestion.

In summary, the four test meals were as follows:

Meal	
A	NaFeEDTA
B	$FeCl_3$
C	NaFeEDTA + AA
D	$FeCl_3$ + AA

RESULTS

The results of the study are presented in Table 18-1. Mean absorption from the modified SS meal (meal B) averaged 8.07%, slightly higher than has been observed in previous studies. When iron was added as NaFeEDTA rather than $FeCl_3$ (meal A), absorption averaged 6.78%; the difference was not statistically significant (mean ratio A/B=0.84, p=.23).

When ascorbic acid was added to the SS meal containing $FeCl_3$, there was a marked increase in absorption to 23.23% (mean ratio D/B=2.88, p=.004). When the same quantity of ascorbic acid was added to the SS meal containing NaFeEDTA (meal C), there was only a slight increase in absorption to 7.34% and the ratio was not statistically significant (mean ratio C/A=0.92, p=.39).

COMMENT

This study shows very clearly that a large dose of ascorbic acid is unable to enhance the absorption of iron from a meal when the form of iron is NaFeEDTA. However, extrapolation of these results to a normal diet or iron fortification with NaFeEDTA should be made with caution. The native iron content of the SS meal employed in these studies is extremely low (0.2 mg), and therefore virtually all of the iron contained in the SS meal was derived from $FeCl_3$ or NaFeEDTA. If used for iron fortification, the ratio of NaFeEDTA:nonheme dietary iron would be 0.3-0.4 rather than the ratio of over 20 in this study.

The present findings may have some relevance in iron fortification programs. Numerous studies have demonstrated that ascorbic acid is an extremely potent enhancer of iron absorption. If the enhancing effect of ascorbic acid is eliminated in the presence of EDTA, this form of iron may not be desirable for fortification with regard to iron bioavailability. Additional studies are required to determine if this blocking effect of ascorbic acid by NaFeEDTA persists when the proportion of NaFeEDTA and native iron is more nearly balanced.

TABLE 18-1

STUDY 18. Effect of Ascorbic Acid on Absorption of Iron as NaFeEDTA.

Subject	Age	Packed Cell Volume (%)	Serum Ferritin (ng/ml)	IRON ABSORPTION (% of dose) Without Ascorbic Acid NaFeEDTA (A)	Without Ascorbic Acid $FeCl_3$ (B)	With Ascorbic Acid NaFeEDTA (C)	With Ascorbic Acid $FeCl_3$ (D)	Absorption Ratio A/B	C/D	C/A	D/B
1	25	44	68	4.00	7.06	4.55	22.92	0.56	0.19	1.14	3.25
2	19	47	34	4.98	9.00	12.37	38.82	0.55	0.31	2.48	4.31
3	23	45	124	5.65	9.05	6.07	6.65	0.62	0.91	1.07	0.73
4	22	48	42	5.92	4.62	17.12	39.02	1.28	0.43	2.89	8.45
5	21	45	25	6.92	13.78	11.33	41.41	0.50	0.27	1.64	3.01
6	26	45	53	7.51	6.78	1.97	17.70	1.10	0.11	0.26	2.61
7	21	47	35	9.77	16.41	9.08	18.61	0.59	0.48	0.93	1.13
8	21	45	20	13.30	4.43	7.12	26.90	3.00	0.26	0.54	6.07
MEAN	22	46	43*	6.78*	8.07*	7.34*	23.23*	0.84*	0.32*	1.09*	2.88*
			-1 SE	5.92	6.84	5.77	18.74	0.68	0.25	0.82	2.17
			+1 SE	7.76	9.52	9.33	28.78	1.05	0.40	1.43	3.84

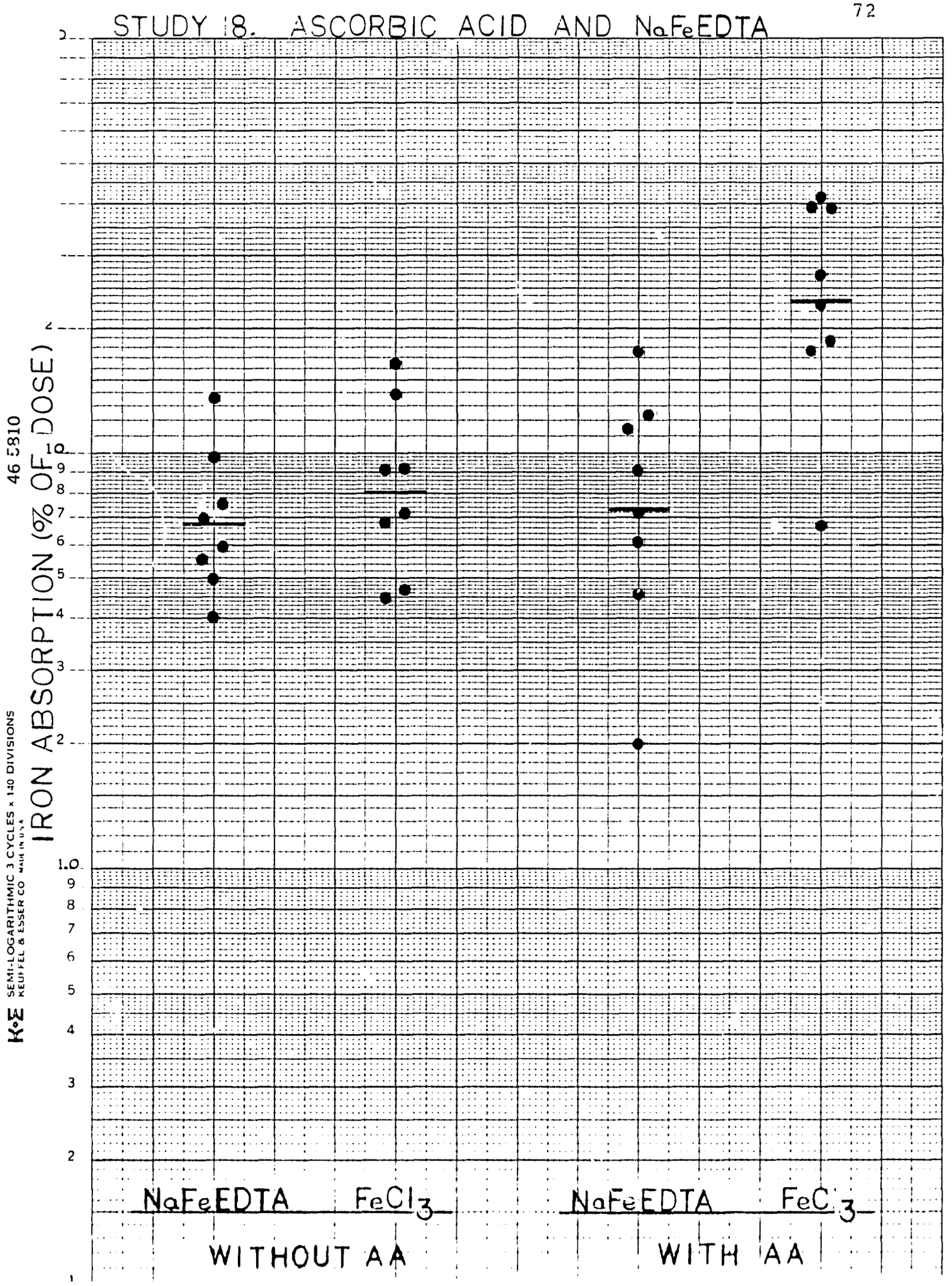
* geometric mean

TABLE 18-2

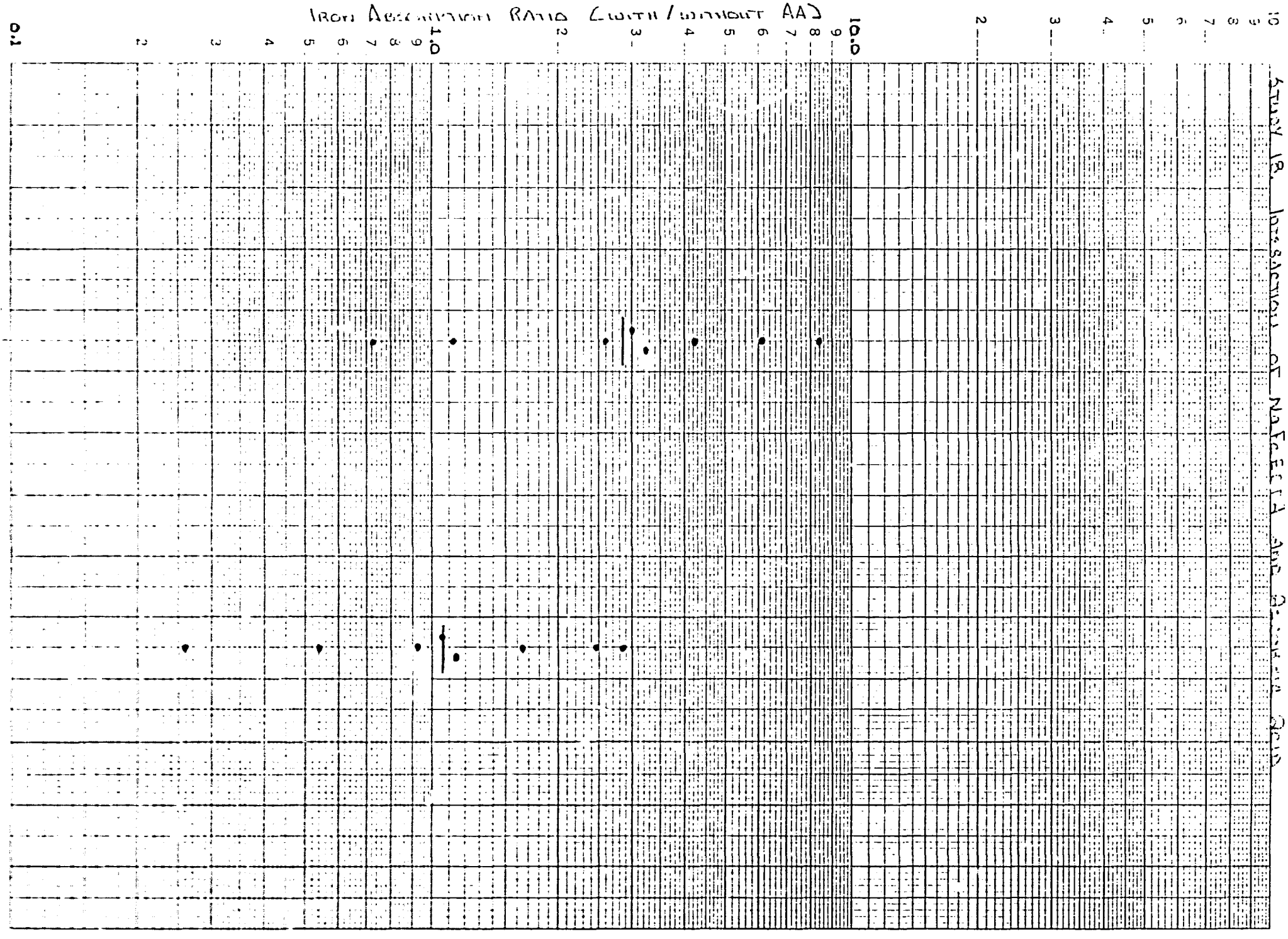
STUDY 18. Effect of Ascorbic Acid on Absorption of Iron as NaFeEDTA.

	ABSORPTION RATIOS					
	A/B	A/C	A/D	B/C	B/D	C/D
Mean Ratio	0.84	0.92	0.29	1.10	0.35	0.32
-1 SE	0.68	0.70	0.23	0.83	0.26	0.25
+1 SE	1.05	1.22	0.38	1.48	0.46	0.40
1/ Ratio	1.19	1.08	3.42	0.91	2.88	3.16
Student's *t*	0.79	0.28	4.87	0.34	3.67	5.17
P value	0.227	0.392	0.0009	0.37	0.004	0.0006
Correlation* (r)	0.047	0.030	0.005	0.114	0.132	0.523
P value	0.455	0.472	0.495	0.393	0.377	0.092

*Log scale


STUDY 18. ASCORBIC ACID AND NaFeEDTA
IRON ABSORPTION (% OF DOSE)
NaFeEDTA
FeCl3
NaFeEDTA
FeCl3
WITHOUT AA
WITH AA
K+E SEMI-LOGARITHMIC 3 CYCLES x 140 DIVISIONS
KEUFFEL & ESSER CO. MADE IN U.S.A.
46 5810

SEMI-LOGARITHMIC 3 CYCLES x 140 DIVISIONS
KEUFFEL & ESSER CO. MADE IN U.S.A.
46 5810



STUDY 19

INTERACTION BETWEEN NaFeEDTA AND COFFEE

SUMMARY

The present study examines the interaction between NaFeEDTA and coffee taken without food. When taken with water, the absorption of NaFeEDTA was slightly lower than that of $FeCl_3$ (3.83 and 5.04% respectively). When taken with coffee, absorption of $FeCl_3$ and NaFeEDTA fell significantly to 1.57 and 1.14% respectively. Whether taken with water or coffee, the differences in the absorption of $FeCl_3$ and NaFeEDTA were not statistically significant.

RATIONALE

Several previous studies in this series have demonstrated the inhibiting effect of coffee on the absorption of nonheme dietary iron. In certain developing countries, sugar may serve as a practical vehicle for iron fortification. One problem with sugar is that the fortification iron may discolor hot beverages such as tea or coffee. This problem can be overcome to some extent if iron is supplied in sugar in the form of NaFeEDTA. The present study was performed to determine whether the inhibiting effect of coffee on iron absorption prevailed when iron was ingested as NaFeEDTA rather than $FeCl_3$.

DESIGN

Studies were performed in 10 adult male volunteers. Four separate test doses were administered to each subject. In meals A and B, radioiron was administered in 200 ml water whereas in meals D and C, the same forms of radioiron were added to 200 ml coffee (1.5 g coffee per subject). No food was administered with these beverages. The total iron content of each test meal was 3.0 mg iron. Iron was added as $FeCl_3$ to meals B and D and as NaFeEDTA in meals A and C. Summary of the test meals is as follows:

Meal	
A	NaFeEDTA + H_2O
B	$FeCl_3$ + H_2O
C	NaFeEDTA + Coffee
D	$FeCl_3$ + Coffee

RESULTS

When $FeCl_3$ was taken with water (B) absorption averaged 5.04%. When $FeCl_3$ was taken with coffee (D), mean absorption decreased sharply to 1.57%, a difference that was highly significant

(mean ratio B/D =.31, t=5.44, p=.0002).

Mean absorption of NaFeEDTA when ingested with water (A) averaged 3.83%. Although slightly lower than absorption of $FeCl_3$ with water, the difference was not statistically significant (mean ratio A/B=.76, p=.089). When NaFeEDTA was ingested with coffee (C), there was again a striking decrease in mean absorption to 1.14%, a difference that was highly significant (absorption ratio C/A=.30, $p<.0001$). When taken with coffee, absorption of NaFeEDTA was slightly lower than $FeCl_3$ but this was not statistically significant (mean ratio C/D=.73, p=.100). It is interesting that the reduction in iron absorption by coffee was virtually identical for $FeCl_3$ and NaFeEDTA (69% for $FeCl_3$, meals B/D; 70% for NaFeEDTA, meals A/C).

COMMENT

While these test meals contain no foods, the findings have nutritional significance in countries that might use NaFeEDTA and sugar for iron fortification. Much of the iron contained in sugar would be consumed with coffee, at least in adult subjects. In the present study, NaFeEDTA did not prohibit the reaction of iron with coffee that reduces the availability of nonheme iron. Indeed, the mean absorption ratio with/without coffee averaged 0.30 with EDTA and 0.31 with water. This 70% reduction in iron absorption by coffee is somewhat greater than the 40% inhibition observed in Study 9 but not as marked as the 84% inhibition observed in Study 13. It is interesting that the inhibiting effect of coffee seems to be similar whether the beverage is consumed alone or with a meal; the previous ratios were obtained when coffee was consumed with a STD meal. The conclusion from this study is that NaFeEDTA confers no advantage over inorganic iron with respect to the inhibiting effect of coffee.

TABLE 19-1

STUDY 19. Effect of Coffee on Absorption of Iron as NaFeEDTA.

Subject	Age	Packed Cell Volume (%)	Serum Ferritin (ng/ml)	IRON ABSORPTION (% of dose) H_2O NaFeEDTA (A)	H_2O $FeCl_3$ (B)	Coffee NaFeEDTA (C)	Coffee $FeCl_3$ (D)	Absorption Ratio A/B	C/D	A/C	B/D
1	23	47	395	1.42	1.60	0.40	0.17	0.89	2.35	0.28	0.11
2	32	46	141	2.55	4.15	0.72	0.92	0.61	0.78	0.28	0.22
3	31	44	179	2.61	7.38	0.63	0.95	0.35	0.66	0.24	0.13
4	19	45	60	3.30	1.73	1.08	1.02	1.91	1.06	0.33	0.59
5	30	47	55	3.57	1.98	0.83	0.90	1.80	0.92	0.23	0.45
6	18	45	31	4.10	5.97	0.72	4.50	0.69	0.16	0.18	0.75
7	28	46	122	4.13	3.23	1.75	1.50	1.28	1.17	0.42	0.47
8	28	45	102	4.78	9.82	1.01	1.66	0.49	0.61	0.21	0.17
9	25	44	65	5.68	11.53	4.95	5.48	0.49	0.90	0.87	0.48
10	27	49	27	13.60	29.30	3.71	11.02	0.46	0.34	0.27	0.38
MEAN	26	46	86*	3.83*	5.04*	1.14*	1.57*	0.76*	0.73*	0.30*	0.31*
			-1 SE	3.18	3.74	0.88	1.08	0.63	0.58	0.26	0.25
			+1 SE	4.62	6.79	1.47	2.28	0.92	0.92	0.34	0.39

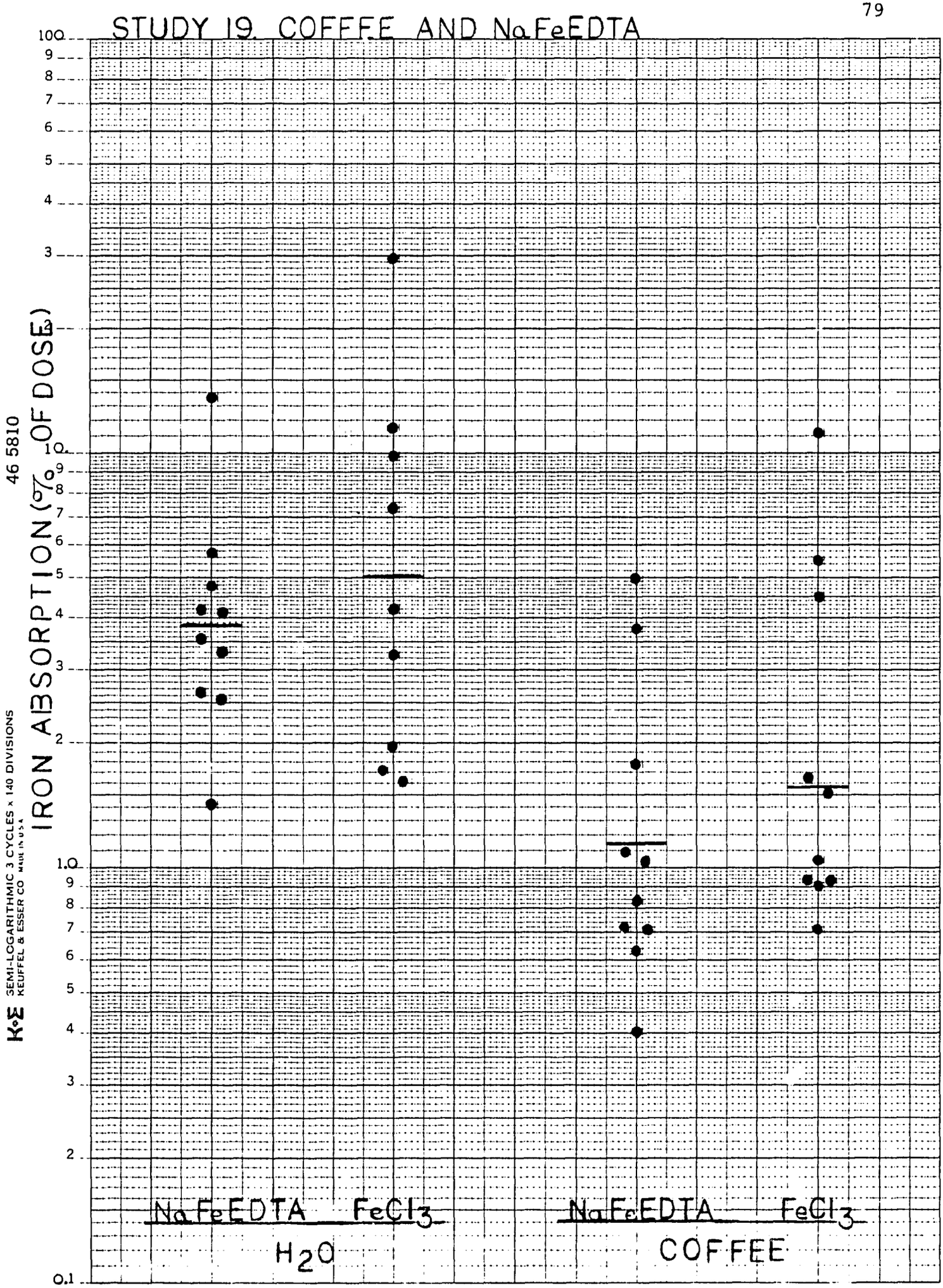
* geometric mean

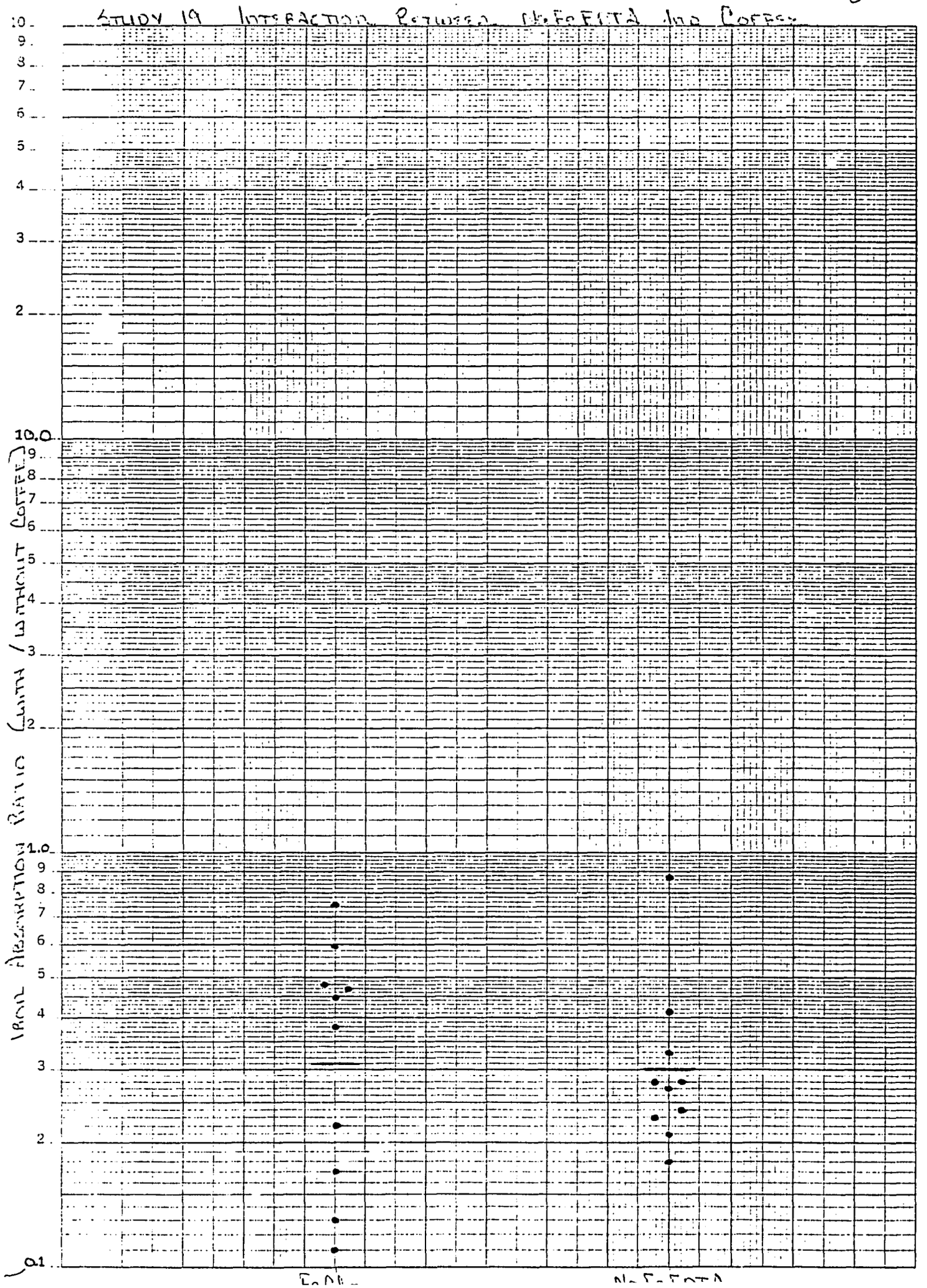
TABLE 19-2

STUDY 19. Effect of Coffee on Absorption of Iron as NaFeEDTA.

	ABSORPTION RATIOS					
	A/B	A/C	A/D	B/C	B/D	C/D
Mean Ratio	0.76	3.36	2.44	4.42	3.21	0.73
-1 SE	0.63	2.92	1.97	3.49	2.59	0.58
+1 SE	0.92	3.87	3.02	5.59	3.98	0.92
1/ Ratio	1.31	0.30	0.41	0.23	0.31	1.38
Student's *t*	1.46	8.56	4.20	6.32	5.44	1.38
P value	0.089	$<10^5$	0.001	0.00007	0.0002	0.100
Correlation* (r)	0.794	0.832	0.920	0.645	0.817	0.79
P value	0.0030	0.0014	0.00008	0.0218	0.0019	0.0031

*Log scale


STUDY 19. COFFEE AND NaFeEDTA
IRON ABSORPTION (% OF DOSE)
100
10
1.0
0.1
NaFeEDTA
FeCl3
H2O
NaFeEDTA
FeCl3
COFFEE
K+E SEMI-LOGARITHMIC 3 CYCLES x 140 DIVISIONS
KEUFFEL & ESSER CO. MADE IN U.S.A.
46 5810


STUDY 19 INTERACTION BETWEEN NaEDTA AND COFFEE
10
9
8
7
6
5
4
3
2
10.0
9
8
7
6
5
4
3
2
1.0
9
8
7
6
5
4
3
2
.01
TRAIL ABSORPTION RATIO (WITH / WITHOUT COFFEE)
46 5810
EDTA
NaEDTA

STUDY 20

EFFECT OF IRON LEVEL ON ABSORPTION OF NaFeEDTA

SUMMARY

A five-fold increase in the iron content of a modified SS meal produced a borderline increase in percentage absorption of both nonheme dietary iron and NaFeEDTA. At both iron levels, absorption of NaFeEDTA was about 10% higher than nonheme iron.

RATIONALE

The present study was undertaken to further examine the assimilation of NaFeEDTA when taken with food. It is anticipated that the effect on iron absorption will depend to some extent on the level of iron EDTA contained in the meal. For example, in a previous studies the addition of Na_2EDTA in amounts greater than 25 mg EDTA to a meal containing 4.1 mg iron progressively inhibited iron absorption. Yet smaller quantities of NaFeEDTA have been shown by other workers to provide equal if not better assimilation than the common pool of nonheme dietary iron. The present study examines absorption at two levels of NaFeEDTA.

A second question in this study is the possible interaction between EDTA and calcium/phosphate salts. Calcium and phosphate have been shown in previous studies to inhibit the absorption of nonheme iron. EDTA has a greater affinity for calcium than it does for iron. It was considered possible that NaFeEDTA might enhance absorption from the SS meal by binding calcium and phosphate.

DESIGN

Two separate meals, both of them tagged with double iron isotopes, were fed to 14 normal male volunteers. Both meals consisted of the SS meal containing one-half the usual quantity of egg albumin but <u>with</u> calcium and phosphate added. The iron and chelate content of both test meals is listed in Table 20-1. Meal 1 contained 0.5 mg iron as $^{55}FeEDTA$ and 0.5 mg iron as $^{59}FeCl_3$ to give a total of 1.18 mg iron. A solution of Na_2EDTA was also added to this meal to obtain an equimolar ratio with iron. Meal 2 was the same as meal 1 except that the quantities of added iron were much larger,5.0 mg iron as NaFeEDTA and 5.0 mg as $FeCl_3$; Na_2EDTA was added separately. In meals 1 and 2, NaFeEDTA was added first, followed by Na_2EDTA, and finally $FeCl_3$.

RESULTS

The results are summarized in Table 20-2. Mean absorption from both test meals was lower than in previous studies, presumably because of calcium and phosphate salts added to the meal. Mean absorption of NaFeEDTA at a low iron level averaged 1.61% and $FeCl_3$ 1.49%. This study showed a slight advantage in absorption of preformed NaFeEDTA; the mean ratio A/B averaged 1.08 and was statistically significant ($t=2.07$, $p=.029$). It should be noted that ordinarily it would be impossible to detect a 10% difference in absorption. However, because both tracers were contained in the same meal, day-to-day variability in absorption was eliminated and this small difference can be recognized as significant.

Similar results were observed with the meal of higher iron content. Mean absorption of NaFeEDTA averaged 2.18% (meal C) as compared to 1.95% for $FeCl_3$ (meal D). As in the case for meal 1, the absorption ratio C/D was significant (mean ratio $C/D=1.12$, $p=.001$) but this small difference is not believed to have biological significance. The slightly higher absorption of both tracers from a meal of higher iron content achieved borderline statistical significance; mean absorption ratio $C/A=1.35$, $p=.059$ and mean ratio $D/B=1.30$, $p=.08$.

COMMENT

As in previous studies, the absorption of preformed NaFeEDTA was essentially the same as separate addition of $FeCl_3$ and Na_2EDTA. Absorption of the preformed chelate was slightly higher with both the low and high iron content meal, but the difference was minimal. These findings provide further evidence in support of the concept of a common pool of nonheme iron.

It was of interest in this study that a ten-fold increase in iron content of the test meal did not reduce percentage absorption as anticipated, but actually led to a borderline increase in iron assimilation. This study would support the notion that in meals containing both Fe and EDTA it is the iron:chelate ratio that determines absorption rather than the total iron content of the meal.

There was no control meal in which calcium and phosphate were not added to the meal. Nevertheless, the presence of NaFeEDTA did not appear to alter the inhibiting effect of calcium and phosphate salts on iron absorption. For example, when no calcium and phosphate were added to this modified SS meal (studies 12 and 13), mean absorption was on the order of 5-6%. Absorption values of 1-2% observed in the present study are consistent with pronounced inhibition by calcium and phosphate, similar if not identical in degree to that observed in earlier studies.

TABLE 20-1. Iron and EDTA Content

		IRON (mg)	EDTA (mg)
MEAL 1			
	$FeCl_3$	0.59	--
	NaFeEDTA	0.59	3.09
	Na_2EDTA	--	3.45
	Total	1.38	6.54
		EDTA/Fe = 4.74	
MEAL 2			
	$FeCl_3$	5.0	--
	NaFeEDTA	5.0	26.20
	Na_2EDTA	--	29.39
		10.0	55.59
		EDTA/Fe = 5.45	

TABLE 20-2

STUDY 20. Absorption of Iron as Na*FeEDTA or $FeCl_3 + Na_2EDTA$ Added to a Semisynthetic Meal.

Subject	Age	Packed Cell Volume (%)	Serum Ferritin (ng/ml)	IRON ABSORPTION Meal 1 (0.59 mg Fe) Na*FeEDTA (A) (% of dose)	Meal 1 (0.59 mg Fe) $Na_2EDTA + FeCl_3$ (B) (% of dose)	Meal 2 (5.0 mg Fe) Na*FeEDTA (C) (% of dose)	Meal 2 (5.0 mg Fe) $Na_2EDTA + FeCl_3$ (D) (% of dose)	Absorption Ratio A/B	C/D	C/A	D/B
1	23	44	69	0.20	0.23	1.41	1.26	0.86	1.11	7.05	5.47
2	23	44	169	0.62	0.57	1.23	1.13	1.08	1.08	1.98	1.98
3	19	48	84	0.63	0.72	2.41	2.41	0.87	1.00	3.82	3.34
4	21	46	29	0.97	0.92	0.97	0.75	1.05	1.29	1.00	0.81
5	19	44	84	1.30	1.16	1.16	1.07	1.12	1.08	0.89	0.92
6	21	44	52	1.57	1.43	1.98	1.72	1.09	1.15	1.26	1.20
7	19	46	71	1.73	1.41	1.13	0.83	1.22	1.36	0.65	0.58
8	24	46	104	1.86	1.41	2.10	1.80	1.31	1.16	1.12	1.27
9	19	48	115	2.11	2.13	1.98	1.68	0.99	1.17	0.93	0.78
10	19	47	19	2.13	1.82	4.38	4.06	1.17	1.07	2.05	2.23
11	21	48	127	3.06	2.45	3.36	2.76	1.24	1.21	1.09	1.12
12	46	54	54	3.86	2.98	2.65	2.63	1.29	1.00	0.68	0.88
13	37	46	52	4.82	4.83	4.26	4.11	0.99	1.03	0.88	0.85
14	18	48	30	6.63	7.30	7.62	8.08	0.90	0.94	1.14	1.10
MEAN	22	46	64*	1.61*	1.49*	2.18*	1.95*	1.08*	1.12*	1.35*	1.30*
			-1 SE	1.26	1.18	1.85	1.62	1.04	1.09	1.13	1.10
			+1 SE	2.06	1.89	2.56	2.33	1.12	1.15	1.61	1.54

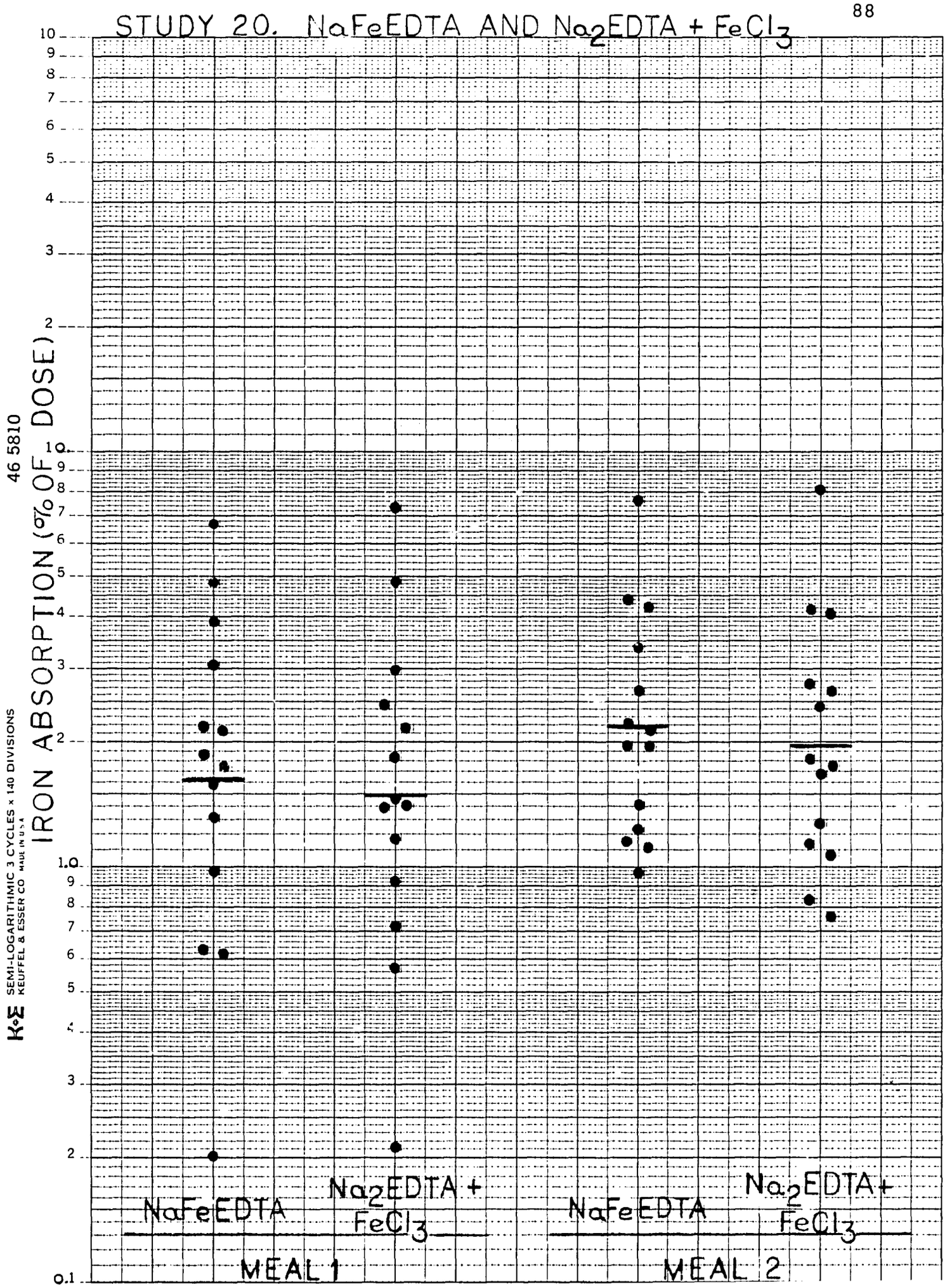
* geometric mean

TABLE 20-3

STUDY 20. Absorption of Iron as Na*FeEDTA or $FeCl_3 + Na_2EDTA$ Added to a Semisynthetic Meal.

	ABSORPTION RATIOS					
	A/B	A/C	A/D	B/C	B/D	C/D
Mean Ratio	1.08	0.74	0.83	0.69	0.77	1.12
-1 SE	1.04	0.62	0.69	0.59	0.65	1.09
+1 SE	1.21	0.89	1.00	0.80	0.91	1.15
1/ Ratio	0.93	1.35	1.21	1.46	1.30	0.89
Student's *t*	2.07	1.67	1.00	2.37	1.59	4.11
P value	0.029	0.059	0.168	0.017	0.068	0.001
Correlation* (r)	0.988	0.688	0.651	0.739	0.709	0.993
P value	$<10^6$	0.003	0.006	0.001	0.002	$<10^6$

* log scale


STUDY 20. NaFeEDTA AND Na2EDTA + FeCl3
IRON ABSORPTION (% OF DOSE)
10
9
8
7
6
5
4
3
2
1.0
9
8
7
6
5
4
3
2
0.1
NaFeEDTA
Na2EDTA + FeCl3
NaFeEDTA
Na2EDTA + FeCl3
MEAL 1
MEAL 2
46 5810
K+E SEMI-LOGARITHMIC 3 CYCLES x 140 DIVISIONS
KEUFFEL & ESSER CO. MADE IN U.S.A.

STUDY 21

EFFECT OF SOY PROTEIN ON IRON ABSORPTION FROM A MEAL CONTAINING MEAT

SUMMARY

The present study examined the effect of a soybean product (textured soy protein) on iron absorption from the STD meal. When soy protein was mixed in a 1:3 ratio with meat, there was a highly significant (50-60%) reduction in assimilation of nonheme iron from the meal. This observation confirms and extends previous studies showing a marked inhibition on iron absorption by soy products.

RATIONALE

Several previous studies have documented in various ways the inhibiting effect of soy products on the absorption of nonheme iron but none of these studies were performed with meals containing meat. The present study was designed to assess the effect of soybean protein on such a meal. The use of meat extenders such as textured soy protein has increased rapidly as the cost of beef has increased. The maximum substitution that is feasible for consumer acceptance is 30% soy. The present study examined the effect of adding soy protein to ground beef ata level of 30% by weight.

DESIGN

Eleven male volunteer subjects participated in this study. Four separate test meals were administered. Meals A, B, and C each consisted of some variation of the STD meal consisting of a hamburger, french fries, and a vanilla milkshake. Meal A contained 100 g beef (uncooked weight) and was identical to the STD meal used in previous studies except that the beef was not purchased at McDonald's (usual uncooked weight of beef in STD meal is 113 g). In meal B, 30 g textured soy protein was added to 100 g ground beef; the amount of beef was not reduced in proportion. In meal C, on the other hand, only 70 g beef was mixed with 30 g soy protein. To prepare meals A and B containing soy, the soy was first moistened with an equal quantity of water and then mixed with the beef to form a patty. All meat patties (including that for meal A) were broiled for 5-7 minutes per side, refrigerated overnight, and then heated in a microwave oven immediately before serving. The radioiron label consisted of 0.1 mg iron as $FeCl_3$ in 1 ml 0.1 N HCl added to the milkshake. Meal D consisted of the reference dose of 3 mg ferrous sulfate containing a 2:1 molar ratio of freshly prepared ascorbic acid.

RESULTS

Mean iron absorption values are listed in Table 21-1. Mean absorption from the STD meal (A) averaged 3.2%. When 30 g textured soy protein was added to the meal, iron absorption fell to 1.24%. Mean absorption from meal C containing only 70 g beef (and 30 g soy) averaged 1.51%. These reductions in iron absorption with meals containing soy were highly significant. The mean absorption ratio with:without soy was 0.39 for meal B ($t=7.47$, $p<.0001$) and 0.47 for meal C ($t=4.32$, $p=.0008$) which represent 61% and 53% decreases in iron absorption respectively. There was only a small difference in absorption from a soy-containing meal when 100 g and 70 g meat was used; mean absorption ratio B/C=1.22, $p=.04$. This difference is presumably due to the higher content of the meat in meal B which is known to facilitate nonheme iron absorption.

Mean reference dose absorption in these subjects averaged 19.9%, a value similar to that observed in previous studies. Absorption ratios for meals A, B, and C averaged 16, 6, and 8% of the reference dose respectively.

COMMENTS

The finding that 30 g soy protein reduces iron absorption by 50-60% from a meal containing 70-100 g beef is in keeping with prior studies showing a marked inhibition in iron absorption by soy products. Actually, the textured soy protein used in the present study is the product that gave the highest iron absorption value (1.91%) of any of the soy proteins studied previously by substitution in the SS meal. These studies again demonstrate that soy products contain a potent inhibitor of iron absorption in a meat-containing meal of relatively high iron availability. It is also apparent that the advantage of a high meat intake with respect to food iron availability may be lost by extending meat products with soy protein.

TABLE 21-1

STUDY 21. Effect of Soy Protein on Iron Absorption From a Standard Meal.

Subject	Age	Packed Cell Volume (%)	Serum Ferritin (ng/ml)	IRON ABSORPTION (% of dose) 100g meat (A)	30g soy + 100g meat (B)	30g soy + 70g meat (C)	REF (D)	Absorption Ratio B/A	C/A	B/C	A/D	B/D	C/D
1	36	44	60	0.48	0.13	0.25	17.80	0.27	0.52	0.52	0.02	0.001	0.01
2	22	44	70	1.43	1.16	1.08	14.05	0.81	0.75	1.07	0.10	0.08	0.07
3	18	39	33	2.30	1.16	1.22	17.91	0.50	0.53	0.95	0.12	0.06	0.06
4	19	44	59	2.41	0.78	0.60	7.73	0.32	0.24	1.30	0.31	0.10	0.07
5	18	48	41	3.10	1.06	0.85	31.95	0.34	0.27	1.24	0.09	0.03	0.02
6	18	46	61	3.47	1.55	3.25	23.43	0.44	0.93	0.47	0.14	0.06	0.13
7	19	49	60	3.76	1.31	1.75	13.71	0.34	0.46	0.74	0.27	0.09	0.12
8	37	41	51	3.81	0.88	0.91	7.88	0.23	0.23	0.96	0.48	0.11	0.11
9	18	48	61	3.91	0.82	0.91	19.46	0.20	0.23	0.90	0.20	0.04	0.04
10	20	46	31	10.76	6.47	8.08	36.85	0.60	0.75	0.80	0.29	0.17	0.21
11	29	49	27	14.61	7.87	15.62	95.83	0.53	1.06	0.50	0.15	0.08	0.16
MEAN	23	45	48*	3.20*	1.24*	1.51*	19.88*	0.39*	0.47*	0.82*	0.16*	0.06*	0.08*
			-1 SE	2.43	0.89	1.06	16.02	0.34	0.40	0.73	0.13	0.05	0.06
			+1 SE	4.21	1.72	2.17	24.68	0.44	0.56	0.91	0.20	0.08	0.10

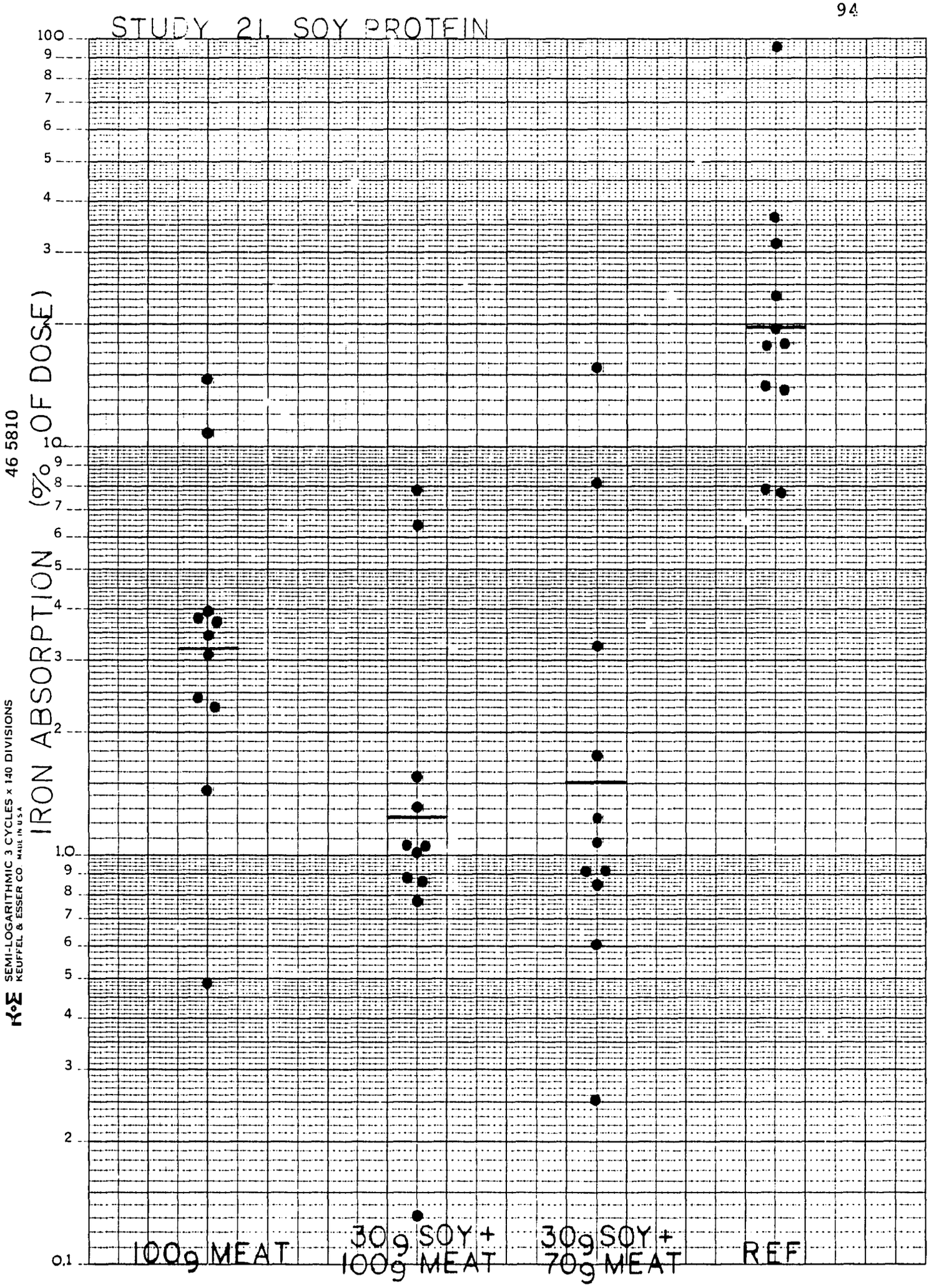
* geometric mean

TABLE 21-2

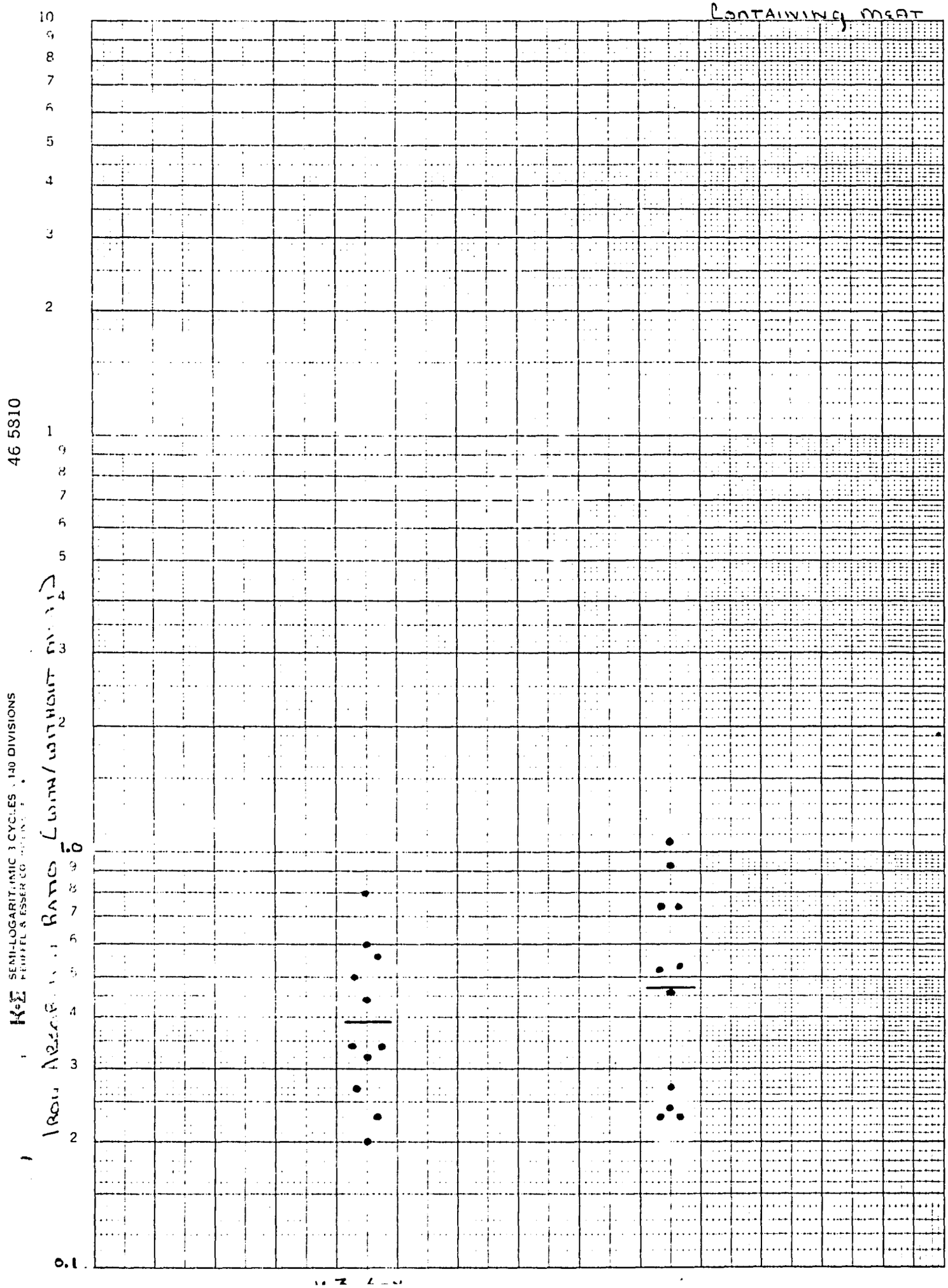
STUDY 21. Effect of Soy Proteins on Iron Absorption From a Standard Meal.

	ABSORPTION RATIOS					
	A/B	A/C	A/D	B/C	B/D	C/D
Mean Ratio	2.58	2.10	0.16	0.82	0.06	0.08
-1 SE	2.27	1.77	0.13	0.73	0.05	0.06
+1 SE	2.93	2.50	0.20	0.91	0.08	0.10
1/ Ratio	0.39	0.47	6.21	1.22	16.02	13.08
Student's *t*	7.47	4.32	7.79	1.89	10.89	10.41
P value	0.00001	0.0008	$<10^{-5}$	0.044	$<10^{-6}$	$<10^{-6}$
Correlation* (r)	0.988	0.688	0.651	0.739	0.709	0.993
P value	$<10^{-6}$	0.003	0.006	0.001	0.002	$<10^{-6}$

* Log scale


STUDY 21, SOY PROTEIN
IRON ABSORPTION (% OF DOSE)
100
9
8
7
6
5
4
3
10
9
8
7
6
5
4
3
2
1.0
9
8
7
6
5
4
3
2
0.1
100g MEAT
30g SOY + 100g MEAT
30g SOY + 70g MEAT
REF
46 5810
K+E SEMI-LOGARITHMIC 3 CYCLES x 140 DIVISIONS
KEUFFEL & ESSER CO. MADE IN U.S.A.

STUDY 21. EFFECT OF SOY PROTEIN ON ABSORPTION FROM A MEAL CONTAINING MEAT



STUDY 22

INTERACTION OF ASCORBIC ACID AND NaFeEDTA

SUMMARY

The purpose of the present study was to determine whether complete isotopic exchange occurs between NaFeEDTA and iron ascorbate. $^{59}FeCl_3$ (1.95 mg) and NaFeEDTA (1.95 mg iron) were added to a modified SS meal containing 200 mg ascorbic acid. Absorption of both forms of radioiron was virtually identical, in keeping with the concept of common pool of nonheme dietary iron. Slightly higher absorption was obtained when ascorbic acid was added before radioiron rather than after, but the difference was not statistically significant.

RATIONALE

Both EDTA and ascorbic acid form chelates with iron that maintain its solubility at physiologic pH. In study 18, ascorbic acid failed to enhance the absorption of NaFeEDTA added to the SS meal. The results suggested that one form of chelated iron (e.g., NaFeEDTA) can block the enhancing effect of a second chelate. This conclusion argues against the hypothesis of a common pool of nonheme dietary iron which states that in whatever form iron is ingested, it will exchange completely with other forms of iron in the same meal. The present study was designed to examine the extent of radioiron exchange between two iron chelates (NaFeEDTA and ascorbic acid) by determining the effect of the order in which they were added to the SS meal.

DESIGN

Two doubly tagged test meals were fed to eight male volunteer subjects. Both meals consisted of the modified SS meal containing half quantities of protein and no added calcium or phosphate. The two test meals were identical and differed only in the order in which radioiron and ascorbic acid were added. The following were added to both meals: $^{59}FeCl_3$, 1.9 mg iron (test B in meal 1 and D in meal 2); NaFeEDTA, 1.95 mg iron (test A in meal 1 and C in meal 2); ascorbic acid, 200 mg. All of these compounds were added in a volume of 1 ml 0.1 N HCl to the SS meal immediately prior to administration.

The order of addition was as follows:

Meal 1	Meal 2
1. $FeCl_3$	1. Ascorbic acid
2. NaFeEDTA	2. $FeCl_3$
3. Ascorbic acid	3. NaFeEDTA

RESULTS

The findings are presented in Table 22-1. They indicate that complete isotopic exchange occurred regardless of the order in which radioiron was added. When ascorbic acid was added after NaFeEDTA (meal 1), absorption from NaFeEDTA and $FeCl_3$ averaged 8.86 and 9.08% respectively, a difference that was not statistically significant. When ascorbic acid was added before radioiron (meal 2) absorption of NaFeEDTA and $FeCl_3$ averaged 13.50 and 13.05% respectively. Again, this small difference is not statistically significant. A comparison of absorption from meals 1 and 2 indicated higher absorption values when the ascorbic acid was added first but the difference was only marginally significant; mean ratio A/C = 0.66 ($t=1.80$, $p=.057$) and mean ratio B/D = 0.70 ($t=1.50$, $p=0.088$).

COMMENT

The somewhat higher absorption from meal 2 is not explained. If complete isotopic exchange occurs between NaFeEDTA and nonheme iron ($FeCl_3$), then the order of addition should not influence iron absorption. It is interesting that subjects 6-8 with high absorption values showed no change in iron absorption with early ascorbate addition, whereas those with low levels of iron absorption absorbed much better from the second meal. It is possible that blood drawn for the first pair of absorption measurements enhanced iron absorption from the second meal.

TABLE 22-1

STUDY 22. Effect of Order of Addition of Ascorbic Acid and Iron Salts on Iron Absorption From a Semisynthetic Meal.

Subject	Age	Packed Cell Volume (%)	Serum Ferritin (ng/ml)	IRON ABSORPTION (% of dose): AA Added Last, Na*FeEDTA (A)	AA Added Last, $FeCl_3$ (B)	AA Added First, Na*FeEDTA (C)	AA Added First, $FeCl_3$ (D)	Absorption Ratio A/B	C/D	A/C	B/D
1	25	41	89	1.45	1.43	7.78	7.48	1.01	1.04	0.18	0.19
2	35	44	22	5.02	5.05	11.02	10.43	0.99	1.05	0.45	0.48
3	22	43	95	6.12	6.41	4.31	4.11	0.95	1.04	1.41	1.55
4	20	48	20	10.88	11.06	14.68	14.26	0.97	1.02	0.73	0.77
5	35	49	70	11.53	11.63	28.23	27.93	0.99	1.01	0.40	0.41
6	29	45	93	15.13	15.36	14.40	13.98	0.98	1.03	1.05	1.09
7	20	48	13	17.48	18.48	17.00	16.41	0.94	1.03	1.02	1.12
8	20	46	21	26.08	27.38	29.58	28.82	0.95	1.02	0.88	0.95
MEAN	26	46	40*	8.86*	9.08*	13.50*	13.05*	0.98*	1.02*	0.66*	0.70*
			-1 SE	6.43	6.54	10.77	10.37	0.97	1.03	0.52	0.55
			+1 SE	12.59	12.59	16.94	16.43	0.98	1.04	0.83	0.89

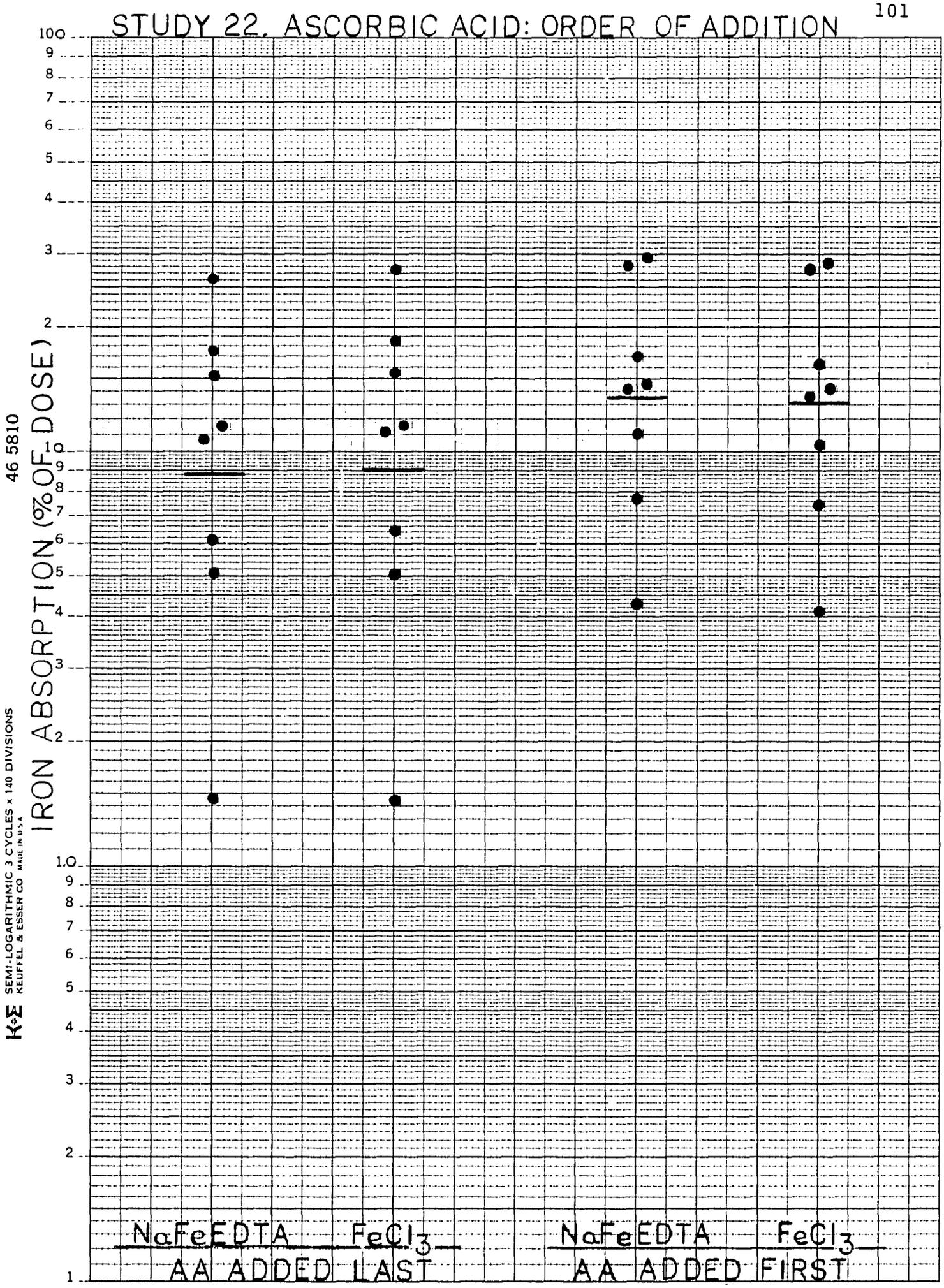
* geometric mean

TABLE 22-2

STUDY 22. Effect of Order of Addition of Ascorbic Acid and Iron Salts on Iron Absorption From a Semisynthetic Meal.

	ABSORPTION RATIOS					
	A/B	A/C	A/D	B/C	B/D	C/D
Mean Ratio	0.98	0.66	0.68	0.67	0.70	1.03
-1 SE	0.97	0.52	0.54	0.53	0.55	1.03
+1 SE	0.98	0.83	0.86	0.86	0.89	1.04
1/ Ratio	1.02	1.52	1.47	1.49	1.44	0.97
Student's *t*	2.78	1.80	1.65	1.65	1.50	7.05
P value	0.013	0.057	0.071	0.072	0.088	0.001
Correlation* (r)	0.99	0.68	0.68	0.68	0.67	0.99
P value	$<10^6$	0.031	0.031	0.033	0.033	$<10^6$

* log scale


STUDY 22. ASCORBIC ACID: ORDER OF ADDITION
IRON ABSORPTION (% OF DOSE)
100
9
8
7
6
5
4
3
2
10
9
8
7
6
5
4
3
2
1.0
9
8
7
6
5
4
3
2
1
NaFeEDTA
FeCl3
AA ADDED LAST
NaFeEDTA
FeCl3
AA ADDED FIRST
K+E SEMI-LOGARITHMIC 3 CYCLES x 140 DIVISIONS
KEUFFEL & ESSER CO. MADE IN U.S.A.
46 5810

STUDY 23

INTERACTION OF NaFeEDTA AND MEAT

SUMMARY

When egg albumin in the SS meal was substituted for beef, a pronounced increase in mean absorption from 1.11 to 6.24% was observed (mean ratio, 5.60). However, when iron in these meals was NaFeEDTA rather than $FeCl_3$, the increase in absorption by beef was less pronounced; mean absorption increased from 2.05 to 5.4% (mean ratio, 2.63). Thus, as with ascorbic acid, the enhancing effect of meat is less pronounced when iron is present as NaFeEDTA.

An important additional finding in this study was that NaFeEDTA is better absorbed than dietary nonheme iron with a low availability meal (SS) but less well absorbed with a high availability meal. The relative availability of NaFeEDTA therefore depends on basal availability of the meal.

RATIONALE

In study 18, the presence of NaFeEDTA in the SS meal was shown to block the enhancing effect of ascorbic acid on absorption of nonheme iron. Meat is another compound that is known to enhance the absorption of nonheme dietary iron. The present study was designed to determine whether the enhancing effect of meat is also lost when the iron in the meal is in the form of NaFeEDTA.

DESIGN

Four separate test meals were fed to 10 normal male volunteers. The first pair of meals (A and B) consisted of the original SS meal (full protein) plus added calcium and phosphate salts. Meals C and D consisted of the SS meal in which lean beef (136 g) was substituted for egg albumin. Ground beef was broiled 6-7 minutes on each side and ingested with the other components of the SS meal.

All of the meals differed with regard to the type of iron that was added. In meal A, 3.7 mg iron was added as $FeCl_3$ in 0.1 N HCl. In meal B, the same quantity of iron was furnished as NaFeEDTA. In meals C and D, the amount of added iron was reduced to adjust for the iron content of beef. The latter value, determined prior to the study from food composition tables, was 37 ug iron/g beef. Subsequent chemical analysis gave a value of only 28 ug iron/g beef; as a result the total iron content of meals C and D was less than anticipated. To meal C, 0.5 mg iron as NaFeEDTA was added and the same amount of iron as $FeCl_3$ to meal D. These iron additions brought the total iron content of meals C and D to 2.9 mg iron rather than 4.1 mg as planned prior to the study.

The meal composition was as follows:

Meal A	SS + NaFeEDTA
B	SS + $FeCl_3$
C	SS/Beef + NaFeEDTA
D	SS/Beef + $FeCl_3$

Nutrient composition of the meals is outlined in Table 23-1.

RESULTS

Iron absorption values are listed in Table 23-1. Mean absorption from the SS meal averaged 1.11% when iron was added as $FeCl_3$ and 2.05% when added as NaFeEDTA. The very low value of 1.11 mean absorption is consistent with previous values with the SS meal. There was a significantly higher absorption when iron was added as NaFeEDTA; mean ratio A/B=1.84, p=0.039.

As anticipated, absorption was substantially higher when beef was substituted for egg albumin in the SS meal. Mean absorption with $FeCl_3$ was 6.24% as compared to 5.41% with NaFeEDTA. The slightly higher mean absorption with $FeCl_3$ as compared with NaFeEDTA (mean ratio D/C=1.15) did not attain statistical significance (t=1.38, p=0.10).

Regardless of the form of added iron, meat significantly enhanced absorption from the SS meal. With meals containing $FeCl_3$, mean absorption increased from 1.11 to 6.24%, giving a mean ratio (D/B) of 5.60 (t=5.58, $p < 0.001$). The increase with meat was less pronounced with NaFeEDTA; mean absorption rose from 2.05 to 5.41%, giving a mean ratio C/D of 2.63 (t=11.22, $p < 0.001$). These studies indicate that NaFeEDTA also impairs the enhancement of nonheme iron absorption produced by meat and agrees with the findings obtained with ascorbic acid. Meat produces a 5.6-fold increase in absorption of nonheme iron

($FeCl_3$ but an increase of only 2.6 with NaFeEDTA.

COMMENT

A very pronounced effect of meat on nonheme iron absorption was observed in this study (5.6-fold increase). There are several factors other than meat per se that may account for this dramatic effect. In the first instance, deletion of egg albumin from the SS meal may produce an artifactual increase in iron absorption; egg albumin has been shown in other studies to inhibit iron absorption. Second, to balance the phosphate content of meat, greater amounts of inorganic phosphate were added to meals A and B than to the beef- substituted meals; inorganic phosphate produces a greater inhibition of iron absorption than organic phosphate. Finally, the nonheme iron content of meals C and D (1.7 mg) was substantially lower than meals A and B (4.1 mg). Despite these considerations, the higher absorption observed in beef-substituted meals (C and D) is for the most part due to the enhancing effect of meat.

As in the case of ascorbic acid, the enhancing effect of meat was significantly blunted when iron was present as NaFeEDTA rather than $FeCl_3$, although the enhancing effect of meat was less affected by EDTA than was ascorbic acid. In contrast to the study of ascorbic acid and NaFeEDTA interaction (study 18), calcium phosphate salts were added to the meals in the present study; the enhancing effect of meat may have been more apparent because of binding of free calcium by this chelate. Other factors listed above may explain the fact that meat further enhanced absorption of NaFeEDTA. For example, the iron content of meal C was lower than meal A and what appeared to be an enhancing effect of meat may have been due in part to a

reduction in the pool of nonheme iron.

One key observation relevant to the action of NaFeEDTA was seen in this study. When absorption was low, as with the SS meal (1.11%), a significant, nearly twofold higher absorption, was observed with the NaFeEDTA as compared with $FeCl_3$. This enhancement agrees with some of the observations reported by Dr. Viteri and Dr. Layrisse, both of whom have indicated that with certain types of foods, NaFeEDTA may promote absorption. However, at the much higher level of absorption observed with the beef-substituted SS meal, iron absorption was actually lower with the meal containing NaFeEDTA (5.41%) than with $FeCl_3$ (6.24%); this difference was only 15%, but it approached statistical significance. It appears that the relative absorption of NaFeEDTA depends on basal availability of iron from the meal. When iron absorption is very low, NaFeEDTA may promote absorption whereas with a high availability meal, NaFeEDTA may be less well absorbed than dietary nonheme iron. This may well explain the discrepancy in the literature between earlier studies by Cook and Monsen showing an inhibition in absorption by EDTA and subsequent studies in Guatemala and Venezuela suggesting an equal or better absorption of NaFeEDTA. In the former study, absorption was measured from a high availability meal (STD); one could predict that increasing amounts of EDTA would impair iron absorption. On the other hand, many studies by Viteri and Layrisse were performed with low availability meals and, as observed in the present study, NaFeEDTA may promote absorption in this setting.

TABLE 23-1. Composition of Test Meals

	Weight	Energy	Protein	CHO	Fat	C	P	Fe
SS Meal (A, B)								
Ovalbumin	37.0	136	29	2	-	24	40	0.4
Dextrimaltose	67.0	254	-	66	-	-	-	-
Corn Oil	35.0	308	-	-	-	-	-	-
$CaHPO_4$	0.6	-	-	-	35	178	138	-
K_2HPO_4	1.3	-	-	-	-	-	236	-
Iron*	-	-	-	-	-	-	-	3.7
Total		698	29	68	35	202	414	4.1
Beef Substituted SS Meal (C, D)								
Beef	94.0	178	29	-	6	12	252	2.4
Dextrimaltose	69.0	262	-	68	-	-	-	-
Corn Oil	29.0	258	-	-	29	-	-	-
$CaHPO_4$	0.6	-	-	-	-	199	147	-
K_2HPO_4	0.9	-	-	-	-	-	162	-
Iron*	-	-	-	-	-	-	-	0.5
Total		698	29	68	35	211	561	2.9

* as $FeCl_3$ or NaFeEDTA

TABLE 23-2

STUDY 23. Effect of Beef Protein on Iron Absorption From Na*FeEDTA in a Semisynthetic Meal.

Subject	Age	Packed Cell Volume (%)	Serum Ferritin (ng/ml)	IRON ABSORPTION (% of dose) SS Meal Na*FeEDTA (A)	SS Meal $FeCl_3$ (B)	SS + Beef Na*FeEDTA (C)	SS + Beef $FeCl_3$ (D)	Absorption Ratio C/A	D/B	A/B	C/D
1	22	44	71	0.76	0.08	1.98	2.72	2.60	34.00	9.50	0.72
2	51	46	83	0.76	0.23	3.02	1.78	3.97	7.73	3.30	1.69
3	25	42	84	0.83	0.67	1.82	2.60	2.19	3.88	1.23	0.70
4	33	44	300	1.36	0.58	2.92	2.23	2.14	3.84	2.34	1.30
5	24	44	56	1.57	0.23	4.70	6.56	2.99	28.52	6.82	0.71
6	30	44	31	1.97	3.10	6.36	10.57	3.22	3.40	0.63	0.60
7	34	44	33	3.92	3.55	8.68	10.96	2.21	3.08	1.10	0.79
8	23	42	8	4.96	4.50	17.75	16.80	3.57	3.73	1.10	1.05
9	25	48	49	5.50	11.82	14.57	20.17	2.64	1.70	0.46	0.72
10	29	46	75	6.36	3.11	10.23	12.46	1.60	4.00	2.04	0.82
MEAN	30	44	55*	2.05*	1.11*	5.41*	6.24*	2.63*	5.60*	1.84*	0.87*
			-1 SE	1.57	0.66	4.18	4.67	2.41	4.11	1.36	0.78
			+1 SE	2.69	1.86	7.01	8.33	2.87	7.62	2.51	0.96

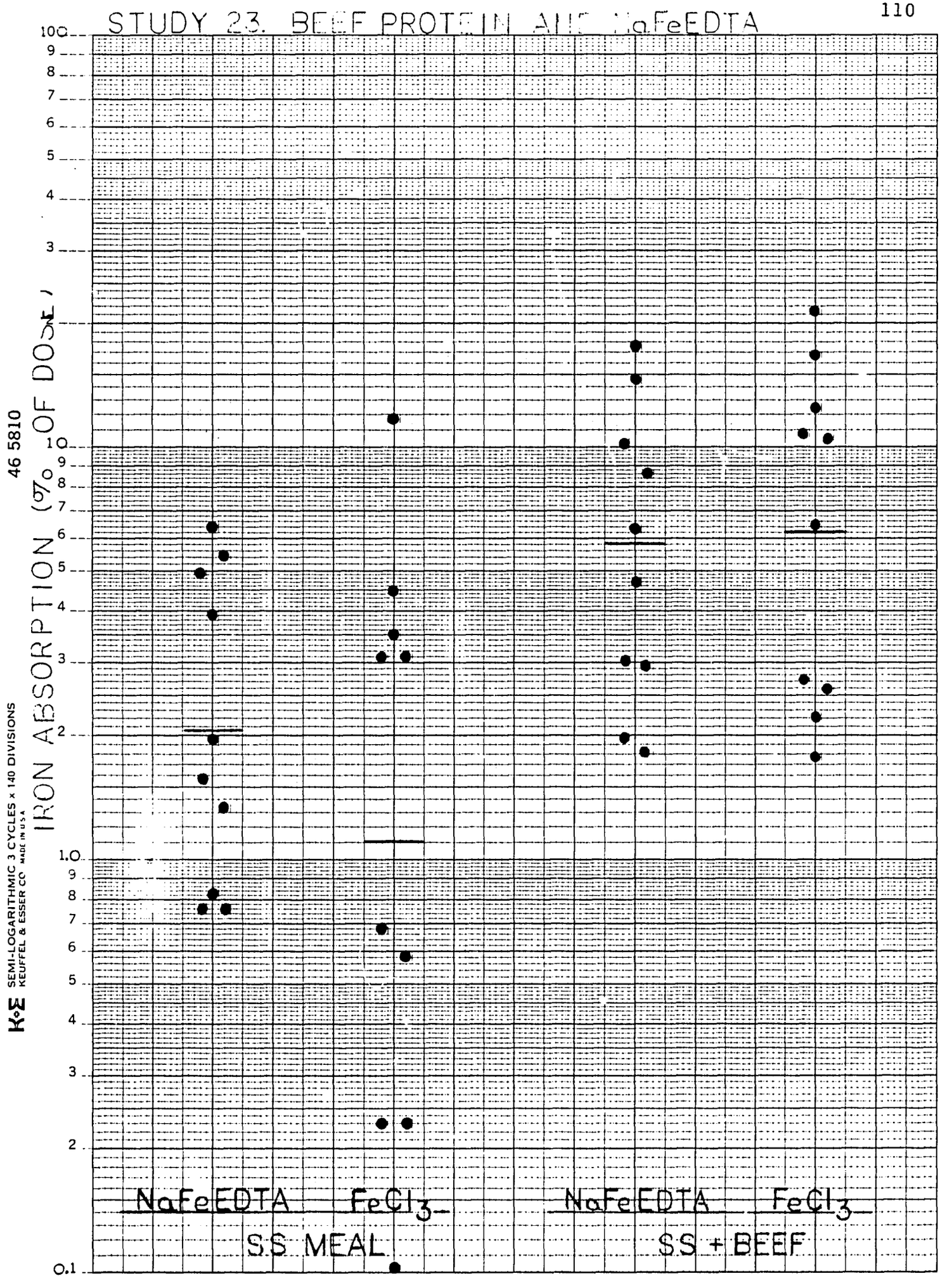
* geometric mean

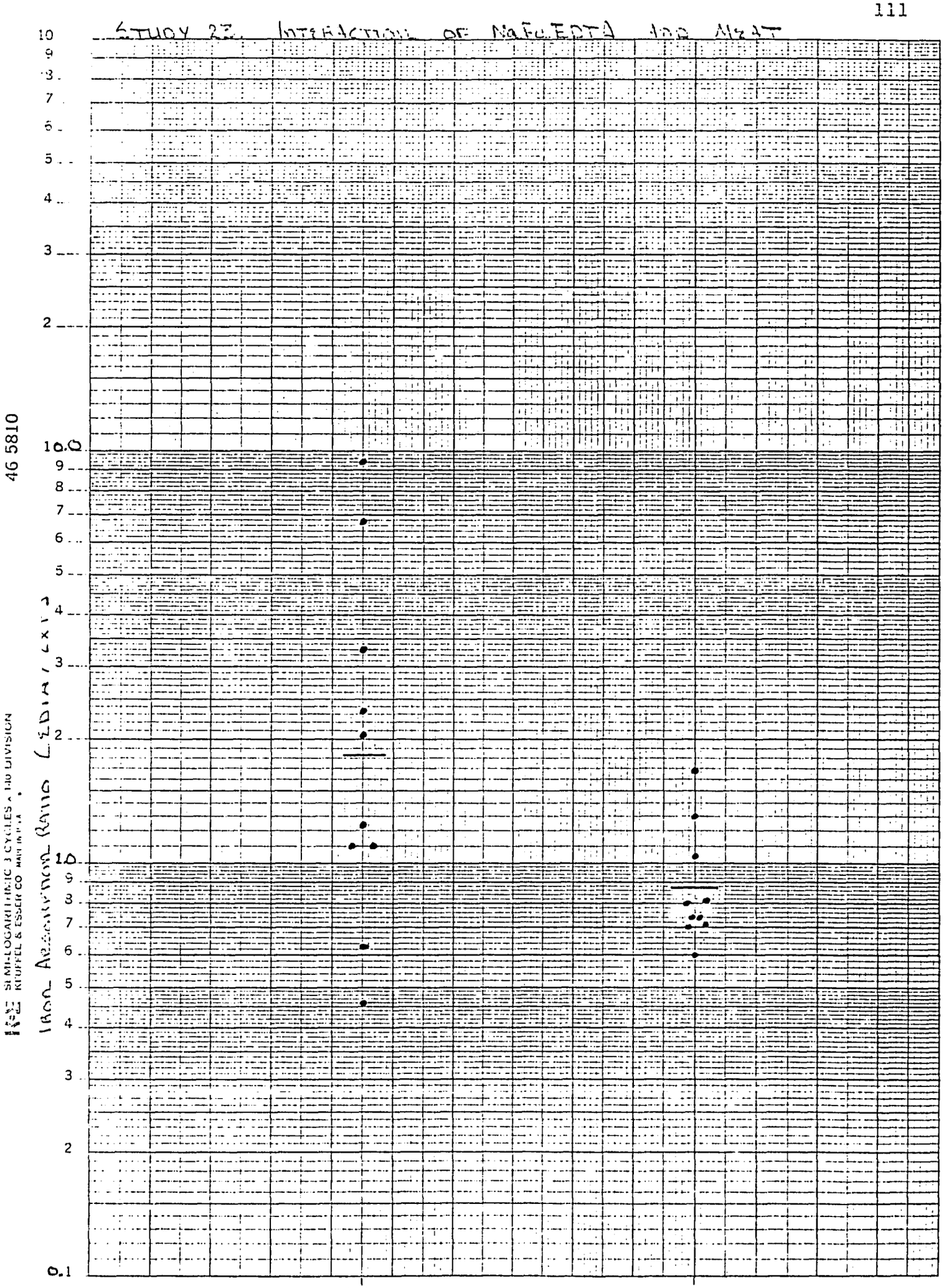
TABLE 23-3

STUDY 23. Effect of Beef Protein on Iron Absorption From Na*FeEDTA in a Semisynthetic Meal.

	ABSORPTION RATIOS					
	A/B	A/C	A/D	B/C	B/D	C/D
Mean Ratio	1.85	0.38	0.33	0.21	0.17	0.87
-1 SE	1.36	0.35	0.29	0.15	0.13	0.78
+1 SE	2.51	0.41	0.37	0.28	0.24	0.96
1/ Ratio	0.54	2.63	3.03	4.86	5.60	1.15
Student's *t*	1.99	11.22	9.82	4.91	5.58	1.38
P value	0.039	$<10^{6}$	$<10^{5}$	0.0004	0.0002	0.100
Correlation* (r)	0.87	0.95	0.92	0.85	0.85	0.94
P value	0.0005	0.00002	0.00008	0.0009	0.0010	0.00003

* log scale


STUDY 23. BEEF PROTEIN AND NaFeEDTA
IRON ABSORPTION (% OF DOSE)
100
9
8
7
6
5
4
3
10
9
8
7
6
5
4
3
2
1.0
9
8
7
6
5
4
3
2
0.1
NaFeEDTA
FeCl3
NaFeEDTA
FeCl3
S.S MEAL
S.S + BEEF
46 5810
K+E SEMI-LOGARITHMIC 3 CYCLES x 140 DIVISIONS
KEUFFEL & ESSER CO. MADE IN U.S.A.


STUDY 23. INTERACTION OF NaFeEDTA AND MEAT
IRON ABSORPTION RANGE (LEDIA / CXVI)
10
9
8
7
6
5
4
3
2
10.0
9
8
7
6
5
4
3
2
1.0
9
8
7
6
5
4
3
2
0.1
46 5810
SEMI-LOGARITHMIC 3 CYCLES X 180 DIVISION
KEUFFEL & ESSER CO. MADE IN U.S.A.

STUDY 24

EFFECT OF COFFEE BEFORE, DURING, AND AFTER A MEAL

SUMMARY

In previous studies coffee has been shown to inhibit absorption of nonheme iron when ingested with a meal. The present study compared the effect of drinking coffee one hour before the meal, with the meal, and one hour after the meal. A 44% reduction in nonheme iron absorption was observed when coffee was ingested before and during a meal, but no effect when taken one hour later.

RATIONALE

In previous studies tea was shown to inhibit absorption of nonheme iron when ingested simultaneously with a meal. However, much of the coffee consumed in both the U.S. and developing countries is taken between meals. The present study was performed to determine the effect of coffee served before, during, and after a hamburger meal.

DESIGN

Seven male volunteer subjects were given separate test meals, all of which consisted of a quarter-pound hamburger plus bun. This meal varied from the STD meal in that the milkshake and french fries were not included. The four meals differed only in regard to the beverage consumed. Meal A was taken with water. Meals B, C, and D were taken with hot coffee prepared by mixing 200 ml boiling water with 1.5 g instant coffee (Taster's Choice). In meal B, the coffee was given one hour before the meal, in meal C the coffee was consumed during the meal, and in meal D the coffee was ingested one hour later. In all four test meals dietary nonheme iron was tagged by adding 0.1 mg iron as $FeCl_3$ in 1 ml 0.1 N HCl to the hamburger after it had been heated in a microwave oven. Meals B, C, and D were identical except for the time the coffee was ingested.

RESULTS

Mean iron absorption from the meal containing no coffee (A) averaged 8.1%. When coffee was taken one hour before the meal (B), absorption averaged 6.3% and this decrease was not statistically significant; mean ratio B/A=0.78, p=0.092.

Virtually identical mean absorption values were obtained when the coffee was taken either with the meal (C) or one hour

following the meal (D); mean absorption was 4.6% in both cases. The mean ratio relative to absorption with no coffee averaged 0.56 in both cases; the difference was also statistically significant in both cases (p=0.034, 0.036).

COMMENT

The findings in the present study were very similar to study 9 when tea and coffee were served with a quarter-pound hamburger. Mean absorption without coffee was somewhat higher in the present study but the difference can probably be accounted for by differences in iron status. The mean serum ferritin in the present study was 36 ng/ml as compared with 60 ng/ml in study 9.

In the present study an inhibiting effect of coffee on iron absorption was observed when the beverage was taken with or following a meal, but not when it was taken one hour before. The findings agree with what is known about gastric emptying time. When the beverage is taken one hour prior to the meal it will have left the stomach by the time of the meal. On the other hand, most of the hamburger would remain in the stomach one hour later and therefore still be inhibited by coffee. It should be noted that although coffee ingested one hour before the meal did not produce a statistically significant decrease in absorption, mean absorption, nevertheless, was 20% lower.

The important finding in this study is that the inhibition of iron absorption produced by coffee is not confined to simultaneous ingestion. Since much of the coffee taken in this country is with a meal or one to two hours following, coffee may inhibit the absorption of a large portion of dietary iron.

TABLE 24-1

STUDY 24. Effect of Time of Administration of Coffee on Iron Absorption From a Standard Meal.

Subject	Age	Packed Cell Volume (%)	Serum Ferritin (ng/ml)	IRON ABSORPTION (% of dose): STD Meal (A)	Coffee Added: Before (B)	With (C)	After (D)	Absorption Ratios: B/A	C/A	D/A	B/C	B/D	C/D
1	24	46	28	1.86	3.00	1.73	2.72	1.61	0.93	1.46	1.73	1.10	0.63
2	19	49	51	3.98	3.18	4.37	3.90	0.79	1.09	0.97	0.72	0.81	1.12
3	25	44	95	5.48	2.20	3.33	2.53	0.40	0.60	0.46	0.66	0.86	0.31
4	25	46	48	9.81	8.67	2.00	2.02	0.88	0.20	0.20	4.33	4.29	0.99
5	19	48	30	10.61	8.78	2.76	2.76	0.82	0.26	0.26	3.18	3.18	1.00
6	18	50	22	19.95	17.70	10.16	13.93	0.88	0.50	0.69	1.74	1.27	0.72
7	28	44	22	27.72	14.06	30.13	19.52	0.50	1.08	0.70	0.46	0.72	1.54
MEAN	23	47	37*	8.12*	6.30*	4.58*	4.55*	0.78*	0.56*	0.56*	1.38*	1.38*	1.01*
			-1 SE	5.70	4.62	3.12	3.23	0.66	0.44	0.43	1.00	1.06	0.89
			+1 SE	11.57	8.60	6.72	6.41	0.92	0.73	0.73	1.89	1.81	1.13

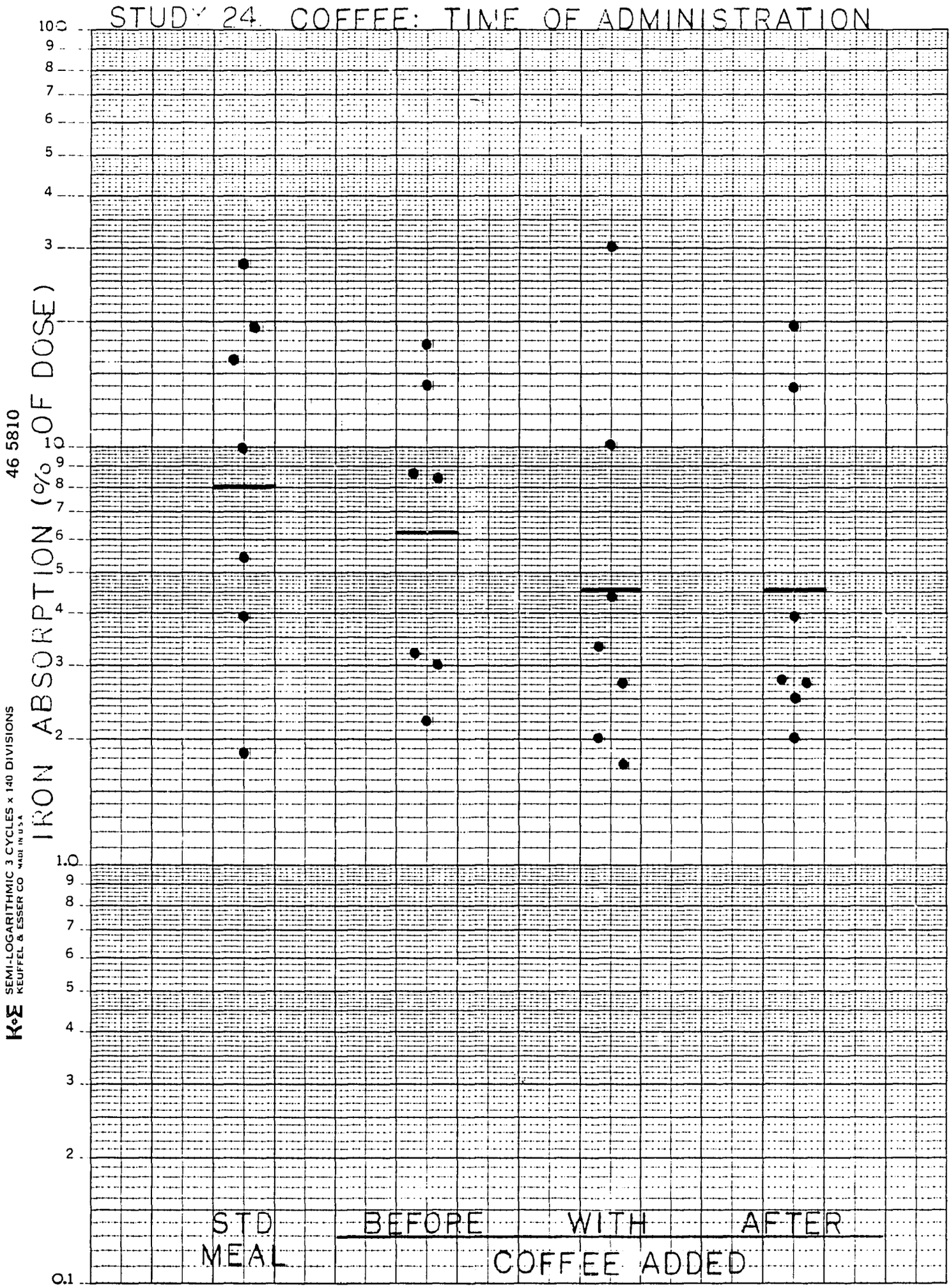
* geometric mean

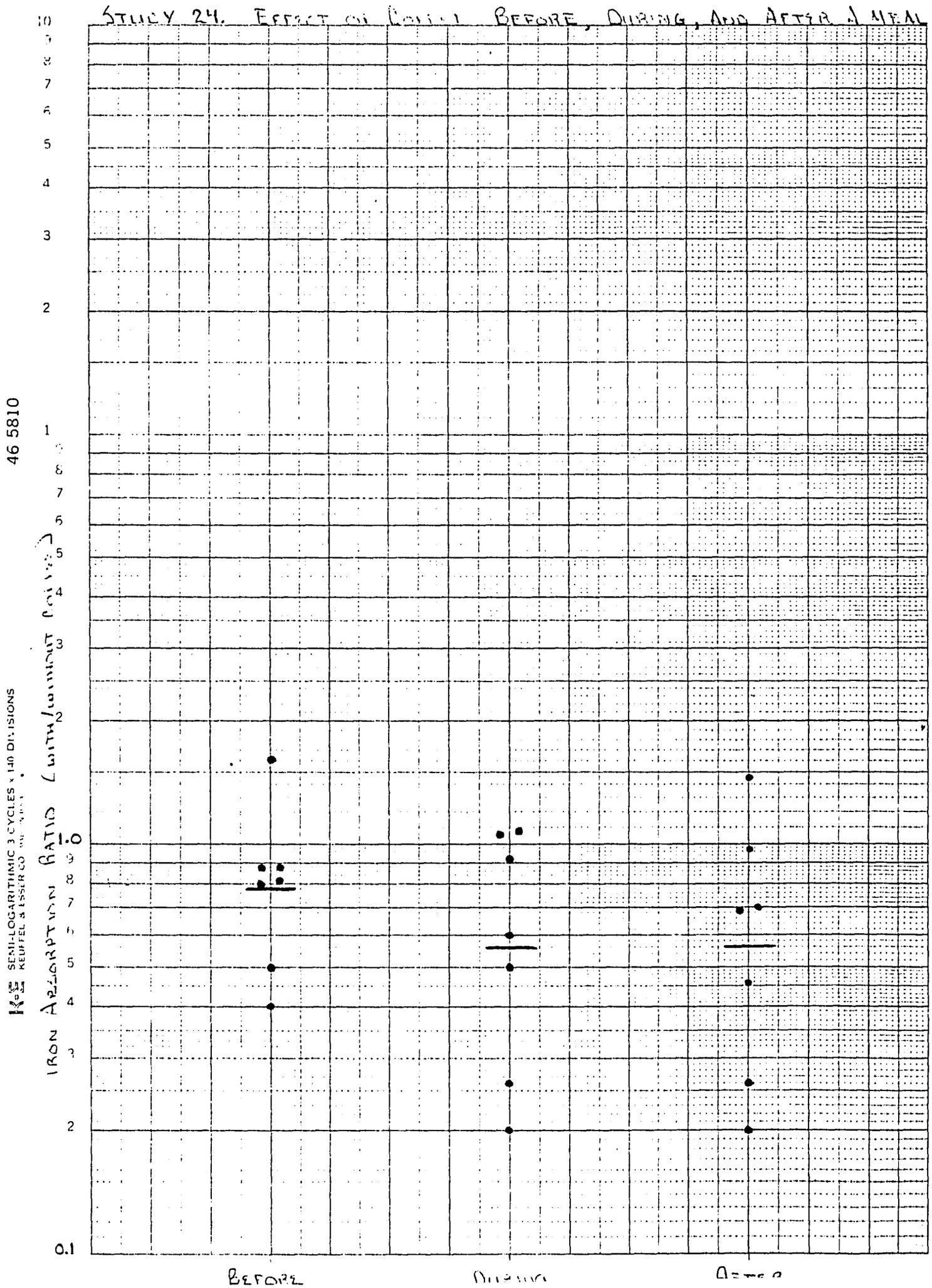
TABLE 24-2

STUDY 24. Effect of Time of Administration of Coffee on Iron Absorption From a Standard Meal.

	ABSORPTION RATIOS					
	A/B	A/C	A/D	B/C	B/D	C/D
Mean Ratio	1.29	1.77	1.78	1.38	1.38	1.01
-1 SE	1.09	1.37	1.37	1.00	1.06	0.89
+1 SE	1.52	2.29	2.34	1.89	1.81	1.13
1/ Ratio	0.78	0.56	0.56	0.73	0.72	0.99
Student's *t*	1.50	2.23	2.18	1.00	1.23	0.05
P value	0.092	0.034	0.036	0.177	0.133	0.479
Correlation* (r)	0.879	0.759	0.709	0.598	0.673	0.953
P value	0.005	0.024	0.037	0.078	0.049	0.0004

* log scale


STUDY 24 COFFEE: TIME OF ADMINISTRATION
IRON ABSORPTION (% OF DOSE)
100
9
8
7
6
5
4
3
2
10
9
8
7
6
5
4
3
2
1.0
9
8
7
6
5
4
3
2
0.1
STD MEAL
BEFORE
WITH
AFTER
COFFEE ADDED
46 5810
K+E SEMI-LOGARITHMIC 3 CYCLES x 140 DIVISIONS
KEUFFEL & ESSER CO. MADE IN U.S.A.


STUDY 24. EFFECT ON COFFEE BEFORE, DURING, AND AFTER A MEAL
IRON ABSORPTION RATIO (WITH/WITHOUT COFFEE)
10
7
5
4
3
2
1
8
7
6
5
4
3
2
1.0
9
8
7
5
4
3
2
0.1
BEFORE
DURING
AFTER
46 5810
K&E SEMI-LOGARITHMIC 3 CYCLES x 140 DIVISIONS
KEUFFEL & ESSER CO.

STUDY 25

INTERACTION OF SOY PROTEIN AND ASCORBIC ACID

SUMMARY

The addition of 100 mg ascorbic acid to the SS meal containing egg albumin increased iron absorption from 5.1 to 10.2% (mean absorption ratio, 2.0). Addition of the same quantity of ascorbic acid to an SS meal containing soy protein increased iron absorption from 0.56 to 3.2% (mean absorption ratio, 5.69). These results indicate that the inhibitory effect of soy protein on nonheme iron absorption can be partly abolished by ascorbic acid.

RATIONALE

Several studies during the past two years in this laboratory have demonstrated a pronounced inhibiting effect of soy protein on nonheme iron absorption. This inhibition exists for all soybean fractions, including whole soybeans, full fat soy flour, textured soy protein, and isolated soy protein. In study 21 this inhibition was also shown to exist when textured soy protein was added to hamburger as a meat extender. The present study was designed to determine the interaction between soy protein and ascorbic acid.

DESIGN

The present study was performed in 11 male volunteer subjects. All four test meals consisted of the SS meal or some modification thereof. Meals C and D were the SS meal with half protein (egg albumin) and no added calcium or phosphate. Meals A and B consisted of the SS meal in which isolated soy protein (ISP) was substituted for egg albumin. Thus, all meals contained dextrimaltose, corn oil, water, and either ISP (A and B) or egg albumin (C and D). One-hundred milligrams freshly prepared ascorbic acid was added to meals B and D but not to A and C. Since the iron content of egg albumin is very low (0.2 mg) as compared to ISP (2.1), 2.0 mg iron ($FeCl_3$) was added to meals A and B and 3.9 mg to meals C and D; the total iron content of all meals was 4.1 mg. All meals were tagged by adding radioiron (1 ml in 0.1 N HCl) to the carrier iron and mixing thoroughly with the meal prior to serving. In meals B and D the ascorbic acid was added after addition of the carrier iron.

In summary, the meals for this study were as follows:

Meal	A	ISP
	B	ISP + AA
	C	ALB
	D	ALB + AA

RESULTS

The data in this study is summarized in Table 25-1. Mean absorption of the SS meal (egg albumin) averaged 5.05% as compared to 0.56% from the SS meal containing ISP. This inhibitory effect of soy protein was highly significant (mean ratio A/B=0.11, p 10^{-6}).

A significant increase in iron absorption was observed with both test meals with the addition of 100 mg ascorbic acid. In the SS meal with egg albumin, absorption increased to 10.19% (mean absorption ratio D/C=2.02, $p < 0.001$). Even greater relative enhancement was seen when ascorbic acid was added to the SS meal containing ISP; mean absorption increased to 3.20%, giving an absorption ratio B/A=5.68. It is interesting that mean absorption with the SS meal (egg albumin) <u>without</u> ascorbic acid (5.1%) was similar to absorption with ISP <u>with</u> ascorbic acid (mean absorption 3.2%); the mean ratio B/C=0.63, p=0.06. Thus, the inhibiting effect of soy protein nearly balanced the enhancing effect of 100 mg ascorbic acid.

COMMENT

The enhancing effect of ascorbic acid is clearly demonstrated in this study regardless of the basal absorption or the source of protein. However, the degree of enhancement was greater when basal absorption was low. When basal absorption was high (egg albumin), the absorption ratio averaged 2.02. When basal absorption was low (soy), the ratio averaged 5.69.

It is of interest to compare these absorption ratios with those reported previously with 100 mg ascorbic acid; the studies are compared in table 25-3. When the five studies with 100 mg ascorbic acid are taken together, the results indicate that the relative enhancement of iron absorption is dependent on the basal level of absorption. Thus, in a previous study when the SS meal was given with full quantities of egg albumin and inorganic calcium and phosphorus, mean absorption averaged only 0.8%; with the addition of 100 mg ascorbic acid the absorption ratio was 4.14. This ratio is very similar to the present study with ISP where iron absorption increased from 0.6 to 3.2% giving a ratio of 5.69. When basal absorption is higher, the enhancement ratio is less. For example, in a previous study, a ratio of only 1.67 was observed when 100 mg ascorbic acid was added to a STD meal with a basal absorption of 4.1%. This is similar to an absorption ratio of 2.0 observed in the present study with a modified SS meal containing half quantities of egg albumin. Therefore, there appears to be nothing unique about the interaction of the inhibiting effect of soy and the enhancing effect of ascorbic acid.

TABLE 25-1

STUDY 25. Effect on Iron Absorption of 100 mg Ascorbic Acid Added to Semisynthetic Meals Made With Isolated Soy Protein and With Egg Albumin.

Subject	Age	Packed Cell Volume (%)	Serum Ferritin (ng/ml)	IRON ABSORPTION (% of dose): Isolated Soy Protein Alone (A)	Isolated Soy Protein +AA (B)	Egg Albumin Alone (C)	Egg Albumin +AA (D)	Absorption Ratio B/A	D/C	A/C	B/C	B/D
1	24	41	2	0.23	4.91	5.77	11.53	21.34	1.99	0.03	0.42	0.85
2	26	42	38	0.33	1.93	6.37	8.67	5.84	1.36	0.05	0.22	0.30
3	33	42	49	0.36	1.25	8.93	11.75	3.47	1.31	0.04	0.10	0.13
4	50	43	72	0.43	1.56	3.36	7.65	3.62	2.27	0.12	0.20	0.46
5	24	45	15	0.46	11.01	3.53	10.33	23.93	2.92	0.13	1.06	3.11
6	19	47	29	0.48	3.68	2.06	5.17	7.66	2.50	0.23	0.71	1.78
7	24	47	130	0.80	3.40	4.90	12.43	4.25	2.53	0.16	0.27	0.69
8	25	44	72	0.82	2.47	3.20	8.15	3.01	2.54	0.25	0.30	0.77
9	26	42	24	0.82	2.97	13.38	19.82	3.62	1.48	0.06	0.14	0.22
10	20	44	32	0.90	4.32	4.90	14.86	4.80	3.03	0.18	0.29	0.88
11	23	45	36	1.45	4.53	6.63	8.68	3.12	1.30	0.21	0.52	0.68
MEAN	27	44	31*	0.56*	3.20*	5.05*	10.19*	5.69*	2.02*	0.11*	0.31*	0.63*
			-1 SE	0.47	2.66	4.31	9.14	4.55	1.83	0.09	0.26	0.49
			+1 SE	0.66	3.84	5.91	11.37	7.10	2.23	0.14	0.39	0.83

* geometric mean

TABLE 25-2

STUDY 25. Effect on Iron Absorption of 100 mg Ascorbic Acid Added to Semisynthetic Meals Made With Isolated Soy Protein and Egg Albumin.

	ABSORPTION RATIOS					
	A/B	A/C	A/D	B/C	B/D	C/D
Mean Ratio	0.18	0.11	0.06	0.63	0.31	0.50
-1 SE	0.14	0.09	0.05	0.49	0.26	0.49
+1 SE	0.22	0.14	0.07	0.83	0.39	0.55
1/ Ratio	5.69	8.96	18.10	1.58	3.18	2.02
Student's *t*	7.82	10.12	16.14	1.70	5.68	7.04
P value	$<10^5$	$<10^6$	$<10^6$	0.060	0.0001	0.00002
Correlation* (r)	0.180	0.089	0.181	0.234	0.094	0.779
P value	0.297	0.396	0.297	0.235	0.391	0.002

* log scale

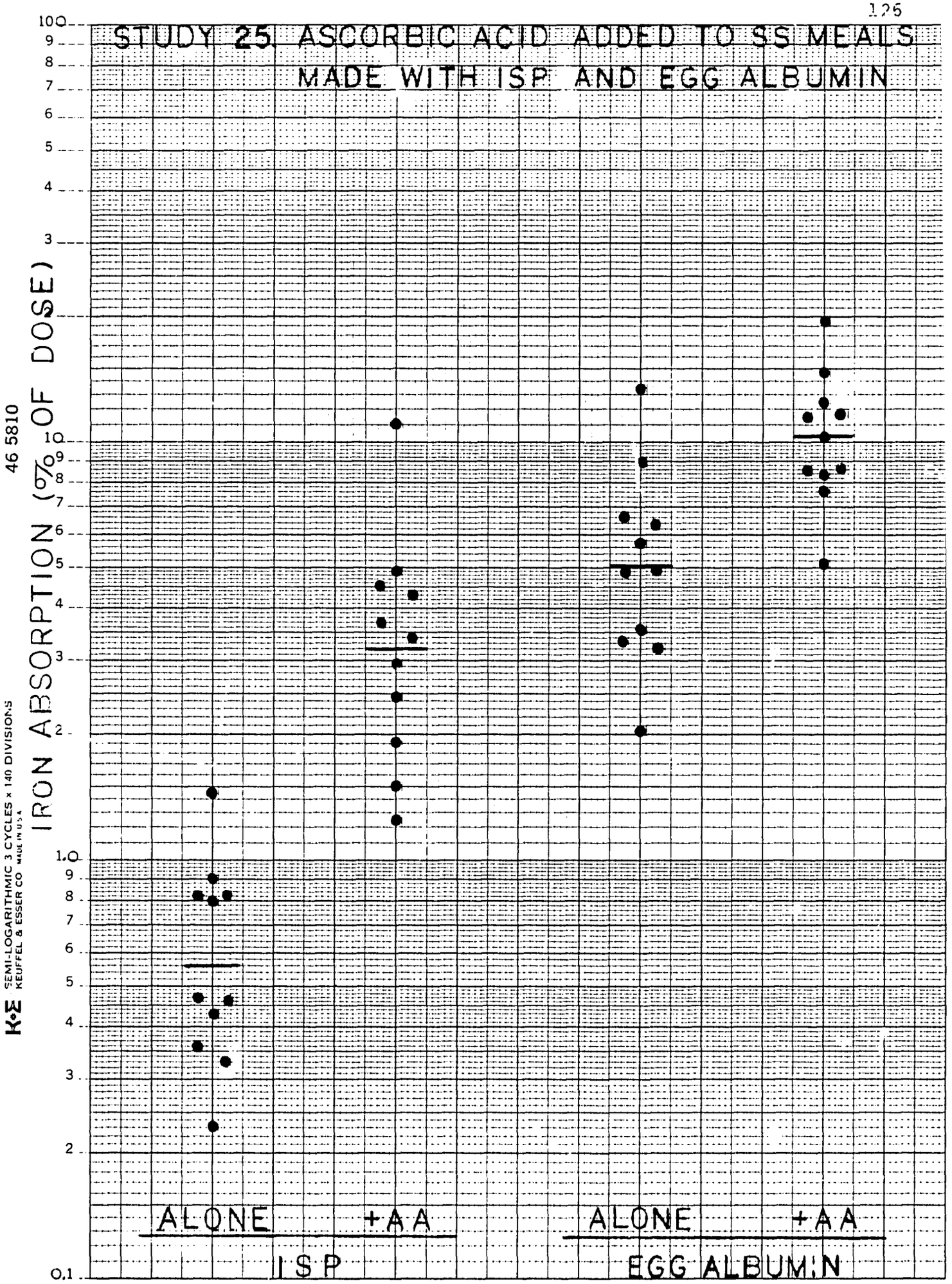
TABLE 25-3. Effect of Ascorbic Acid (100 mg) on Iron Absorption.

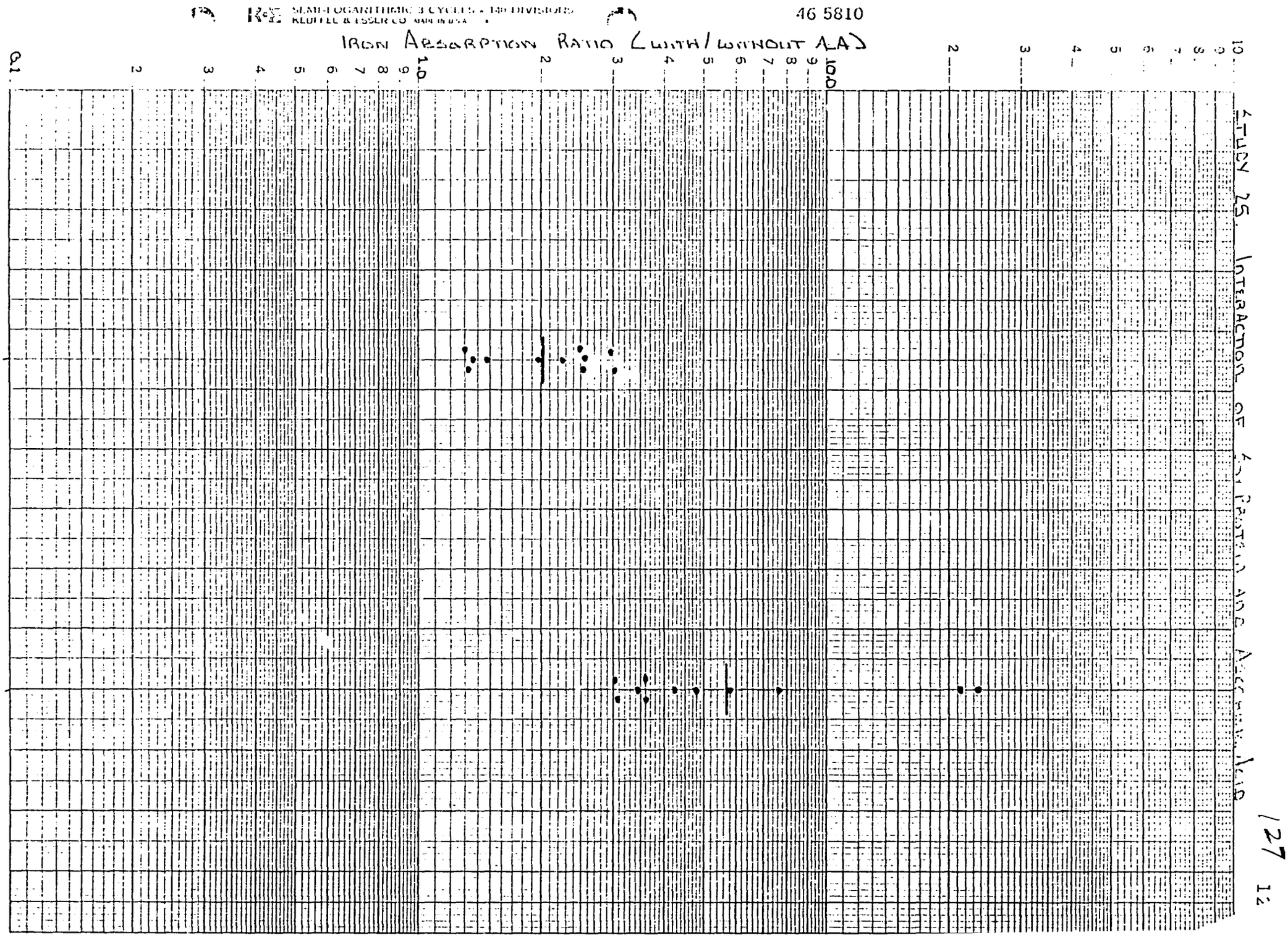
	Meal	Iron Absorption -AA	Iron Absorption +AA	Iron Absorption Ratio
Previous[+]				
	SS + Ca/P	0.8	3.2	4.14
	SS - Ca/P	2.1	7.9	3.77
	STD	4.1	6.9	1.67
Present				
	MSS* + EA[†]	5.1	10.2	2.02
	MSS + ISP	0.6	3.2	5.69

[+] AJCN 30:235, 1975

* modified SS (1/2 protein)

[†] egg albumin


STUDY 25. ASCORBIC ACID ADDED TO SS MEALS
MADE WITH ISP AND EGG ALBUMIN
IRON ABSORPTION (% OF DOSE)
100
9
8
7
6
5
4
3
2
10
9
8
7
6
5
4
3
2
1.0
9
8
7
6
5
4
3
2
0.1
ALONE
+AA
ALONE
+AA
ISP
EGG ALBUMIN
K+E SEMI-LOGARITHMIC 3 CYCLES x 140 DIVISIONS
KEUFFEL & ESSER CO. MADE IN U.S.A.
46 5810


SEMI-LOGARITHMIC 3 CYCLES X 180 DIVISIONS
KEUFFEL & ESSER CO. MADE IN U.S.A.
46 5810
IRON ABSORPTION RATIO (WITH/WITHOUT ALA)
0.1
1.0
10.0
STUDY 25. INTERACTION OF ALUMINATED AND ASCORBIC ACID

STUDY 26

INTERACTION BETWEEN SOY PROTEIN AND MEAT

SUMMARY

The addition of meat to a meal containing isolated soy protein increased mean absorption from 0.36 to 1.44% (mean ratio, 3.90; p 0.0001). The addition of meat to a meal containing egg albumin increased iron absorption from 5.94% to 7.47% (mean ratio, 1.26; p=0.12). It is concluded that the inhibiting effect of soy can be partly neutralized by the addition of foods known to enhance the assimilation of nonheme iron.

RATIONALE

In study 25 it was shown that the addition of ascorbic acid to an SS meal containing soy produced a marked increase in assimilation. In the present study the effect of meat on absorption of a soy-containing meal was similarly determined.

DESIGN

Four separate test meals were fed to seven male volunteer subjects. The design of the study was the same as study 25 except that meat was examined as an enhancing agent rather than vitamin C. All test meals consisted of variations of the SS meal. Meals C and D were the SS meal with egg albumin (half quantity) and no added calcium or phosphate salts. Meals A and B contained isolated soy protein (ISP) as a protein source, rather than egg albumin. Meals A and C were administered without meat whereas meals B and D were consumed with a hamburger (precooked weight, 100 g). Radioactive iron (0.1 ml iron as $FeCl_3$ in 1 ml N HCl) was added to the liquid portion of each meal. Additional iron was added as $FeCl_3$ to maintain the same total iron content for each meal as follows:

Meal		IRON (mg)			
		Intrinsic	Meat	$FeCl_3$	Total
A	ISP	2.1	--	3.5	5.6
B	ISP + Meat	2.1	1.5	2.0	5.6
C	ALB	0.2	--	5.4	5.6
D	ALB + Meat	0.2	1.5	3.9	5.6

RESULTS

Mean absorption from the modified SS meal containing egg albumin averaged 5.94%, a value similar to that seen in previous studies. When the meal included 100 g beef, absorption rose to 7.47% but the increase was not statistically significant; mean ratio D/C=1.26, p=0.12. The failure of meat to enhance iron absorption significantly probably relates to the high basal absorption from the SS meal as compared with previous studies where full doses of protein and both calcium and phosphate salts were added to the meal.

Mean absorption when ISP was substituted averaged only 0.36%, the lowest percentage absorption observed to date. When the meal was eaten with 100 g beef, mean absorption increased to 1.44%. While absorption remained low, the relative increase was pronounced; mean absorption ratio B/A=3.90, $p < 0.0001$.

COMMENT

The enhancing effect of beef in the present study was less pronounced than the enhancing effect of ascorbic acid observed in study 25. In fact, the increase in absorption when meat was added to the SS meal containing ovalbumin was not statistically significant. Furthermore, the enhancement when meat was added to ISP was only 3-4 times as compared with 5-6 times observed with ascorbic acid. Nevertheless, both ascorbic acid and meat are able to counteract the inhibiting effect of ISP to a significant extent.

This study demonstrates once again the importance of the level of basal absorption when assessing the effect of an enhancing agent. As with ascorbic acid, the relative increase by an enhancing agent is greater when basal absorption is low. For

example, when meat was fed in a previous study with the SS meal containing full protein and calcium and phosphate (AJCN 28: 1289, 1975), nonheme iron absorption increased from 1.2 to 4.3%, giving an absorption ratio of 3.55. This is very similar to the enhancing effect of meat when added to a meal containing ISP in the present study (mean ratio, 3.90). Clearly, the effect of an enhancing agent is more obvious when basal absorption is low.

TABLE 26-1

STUDY 26. Effect on Iron Absorption of 100 g Beef Added to Semisynthetic Meals Made With Isolated Soy Protein and Egg Albumin.

Subject	Age	Packed Cell Volume (%)	Serum Ferritin (ng/ml)	IRON ABSORPTION (% of dose): Isolated Soy Protein Alone (A)	Isolated Soy Protein Beef (B)	Egg Albumin Alone (C)	Egg Albumin Beef (D)	Absorption Ratio B/A	D/C	A/C	B/D
1	23	45	89	0.11	0.28	2.05	4.12	2.54	2.00	0.05	0.06
2	28	44	76	0.12	1.01	3.90	6.68	8.41	1.71	0.03	0.15
3	23	50	39	0.18	0.75	4.02	8.67	4.16	2.15	0.04	0.08
4	21	44	80	0.35	0.88	1.65	1.27	2.51	0.76	0.21	0.69
5	24	46	94	0.75	3.45	6.22	4.07	4.60	0.65	0.12	0.84
6	19	44	45	0.77	2.36	17.48	20.71	3.06	1.18	0.04	0.11
7	19	45	18	1.95	8.48	45.61	51.03	4.34	1.11	0.04	0.16
MEAN	22	45	55*	0.36*	1.44*	5.94*	7.47*	3.90*	1.26*	0.06*	0.19*
			-1 SE	0.24	0.94	3.79	4.75	3.33	1.05	0.05	0.13
			+1 SE	0.55	2.20	9.30	11.76	4.58	1.50	0.08	0.28

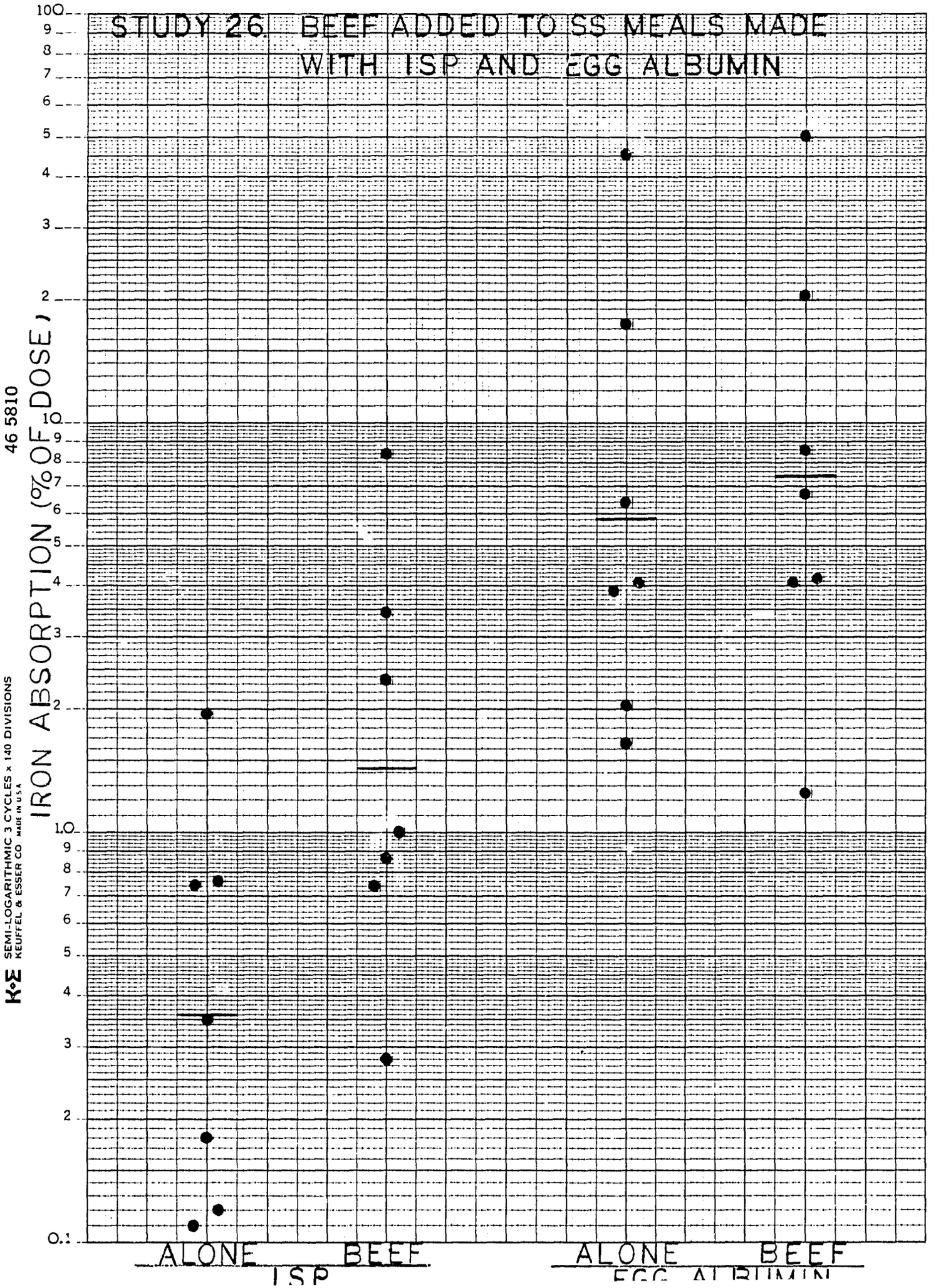
* geometric mean

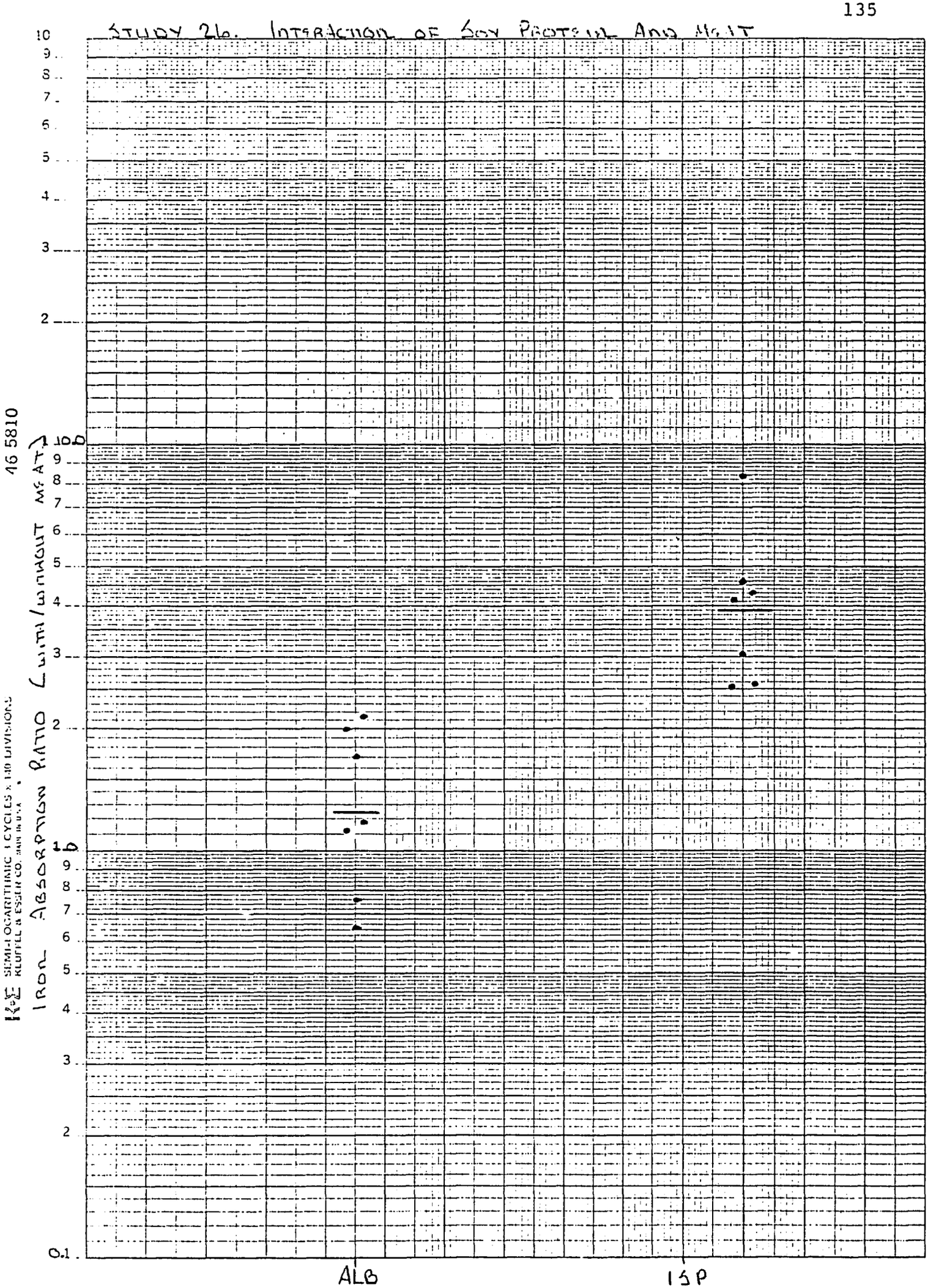
TABLE 26-2

STUDY 26. Effect on Iron Absorption of 100 g Beef Added to Semisynthetic Meals Made With Isolated Soy Protein and Egg Albumin.

	ABSORPTION RATIOS					
	A/B	A/C	A/D	B/C	B/D	C/D
Mean Ratio	0.26	0.06	0.05	0.24	0.19	0.80
-1 SE	0.22	0.05	0.03	0.19	0.13	0.67
+1 SE	0.30	0.08	0.07	0.30	0.28	0.95
1/ Ratio	3.90	16.10	20.24	4.13	5.19	1.26
Student's *t*	8.54	10.71	7.37	6.31	4.39	1.30
P value	0.00007	0.00002	0.0002	0.0003	0.002	0.120
Correlation* (r)	0.93	0.82	0.56	0.89	0.64	0.92
P value	0.0013	0.012	0.097	0.006	0.062	0.0014

* log scale


STUDY 26. BEEF ADDED TO SS MEALS MADE WITH ISP AND EGG ALBUMIN
IRON ABSORPTION (% OF DOSE)
100
10
1.0
0.1
ALONE
BEEF
ISP
ALONE
BEEF
EGG ALBUMIN
K+E SEMI-LOGARITHMIC 3 CYCLES x 140 DIVISIONS
KEUFFEL & ESSER CO. MADE IN U.S.A.
46 5810


STUDY 26. INTERACTION OF SOY PROTEIN AND MALT
IRON ABSORPTION RATIO (WITH/WITHOUT MALT)
10
9
8
7
6
5
4
3
2
1.0
0.1
ALB
ISP
46 5810
K&E SEMI-LOGARITHMIC 3 CYCLES X 140 DIVISIONS
KEUFFEL & ESSER CO. MADE IN U.S.A.

APPENDIX B

PUBLICATIONS

THE INHIBITORY EFFECT OF BRAN ON IRON ABSORPTION IN MAN[1,2]

Karen M. Simpson, B.S., Eugene R. Morris, Ph.D.,
and James D. Cook, M.D.[3]

1 From the Division of Hematology, Department of Medicine, University of Kansas Medical Center, Kansas City, Kansas and Vitamin and Mineral Nutrition Laboratory, Nutrition Institute, U.S. Department of Agriculture, Beltsville, Maryland.

2 This work was supported by cooperative agreement 12-14-100-1209 from U.S. Department of Agriculture, Grant AM-19011 from U.S. Public Health Service, and Contract DSAN-C-0045 from U.S. Agency for International Development.

3 Reprint requests: James D. Cook, M.D., University of Kansas Medical Center, Division of Hematology, 39th and Rainbow Boulevard, Kansas City, Kansas 66103.

SUMMARY

The effects of whole wheat bran and its components on the absorption of nonheme dietary iron were measured with a double isotope technic in human volunteers. When 12 g bran was added to a light meal, absorption decreased by 51-74 per cent; this inhibitory effect of bran was shown for meals of both high and low iron availability. Inhibition was not explained by monoferric phytate, the major form of iron in bran, because labeled iron from monoferric phytate was absorbed at least as well as the common pool of nonheme dietary iron. Furthermore, removal of phytate from bran by endogenous phytase did not alter the bran's inhibitory effect on iron absorption. Studies in which dephytinized bran was separated into a soluble, phosphate-rich fraction and an insoluble, high-fiber fraction indicated that the soluble fraction was more inhibitory tan the insoluble fraction.

INTRODUCTION

When several food items are ingested simultaneously, a common pool of nonheme iron forms from which absorption is measurable by extrinsic radioiron tagging (1-3). Recent studies of the dietary factors that influence iron absorption from this iron pool have focused on the importance of enhancing substances such as ascorbic acid and animal tissue (4,5) rather than the effect of inhibitory substances. Identification of inhibitory substances is important becaue their elimination from the diet might improve iron status.

The effect of wheat bran on iron absorption is important because whole wheat is a staple food for much of the world's population. Bran is known to inhibit iron absorption but the nature of this inhibition is unknown; it has been attributed in different reports to phytate, to phosphate, and to dietary fiber. In the series of studies reported here, we re-examined the inhibitory effect of bran and attempted to define the mechanism of this effect.

METHODS

Subjects. Multiple iron absorption tests were performed in 60 male and female volunteers ranging in age from 19-42 years. A total of 5 separate studies were conducted in groups of 9-18 subjects each. All subjects gave informed consent prior to participation in the study. None of the

volunteers gave a history of hematologic abnormalities or disorders known to influence the gastrointetinal absorption of iron. Iron status in these subjects was evaluated by measurements of hematocrit, serum iron (6), iron-binding capacity by a colorimetric magnesium carbonate technic (7), and serum ferritin by a 2-site immunoradiometric assay (8) These tests indicated that storage iron was marginal in some of the subjects, but none had iron deficiency anemia.

Iron absorption measurements. In most studies each subject was given four separate iron absorption tests as two pairs of parellel, double isotope measurements. On day 1 of each study blood samples were drawn between 8-10 a.m. from fasting subjects for measurement of blood background radioactivity. The subjects ate meal A labeled with either 2 uCi ^{59}Fe or 5 uCi ^{55}Fe; nothing further was ingested for at least 3 hours. The next morning each subject ate meal B tagged with the alternate form of radioiron. The subjects returned to the laboratory 14 days later, blood was drawn for measurements of circulating ^{55}Fe and ^{59}Fe radioactivity, and meal C was given. As with meals A and B, meals C and D were labeled separately with ^{59}Fe and ^{55}Fe and administered on 2 successive days. Two weeks later a final blood sample was drawn and the rise in ^{55}Fe and ^{59}Fe whole blood radioactivity was

determined. Unless otherwise stated, all test meals were labeled by adding 1 ml 0.01 N HCl containing carrier-free radioiron and 0.1 mg iron as $FeCl_3$.

Levels of ^{55}Fe and ^{59}Fe rradioactivity were measured simultaneously in duplicate 10 ml samples of whole blood by a modification of the Eakins and Brown method (9). Radioactivity was also measured simultaneously in aliquots of each test meal. From the net increase in the rdioactivity of circulating whole blood, iron absorption in each subject was calculated as a percentage of the radioactivity consumed; total blood volume of the subject was estimated on the basis of sex, height, and weight (10). We assumed that 80% of the absorbed radioactivity was incorporated into circulating whole blood 14 days after eating the test meal (11).

Test meals. The contents of the test meals are shown in Table 1. All meals in studies 1, 2, 4, and 5 consisted of two muffins which provided the vehicle for added bran or bran fraction and a 150 g milkshake. Plain muffins weighing 60 g each were prepared with enriched white flour, baking powder, granulated sugar, table salt, egg, whole milk and vegetable oil. These were given in meal A in studies 1, 2, 4, and 5 and in meal C in study 2, and served as a control in assessing the effect of bran. All other test meals, with

the exception of those in study 3, consisted of a milkshake and two bran muffins. The latter were prepared with the identical recipe except that whole bran in an amount sufficient to provide 12 g per meal or its equivalent was added. This quantity was considered to be the maximum amount of bran that might be contained in a normal meal. All muffins were prepared one day ahead and reheated in a microwave oven before serving. The total nutrient content of the test meals including the milkshake was as follows: (12)

	Energy (kcal)	Protein (gm)	Fat (gm)	Carbohydrate (gm)	Calcium (mg)	Phosphorus (mg)	Iron (mg)
Plain	632	16	20	96	521	337	3.1
Bran	656	18	20	104	531	491	4.8

Because of the higher iron content of bran muffins, 1.7 mg of $FeCl_3$ was added to the milkshake in meals containing plain muffins to maintain the same total iron content. Radioiron was added to the milkshake and thoroughly mixed just before the meals were served. The milkshake and muffins were served alone in study 1, with 100 g beef in meals A and B of study 2, and with 100 mg freshly prepared ascorbic acid solution in all remaining meals.

Two other test meals were used in study 3; both have been used extensively in this laboratory in studies of food iron absorption (13). The first, chosen as a typical American meal, contained beef, cornmeal, potatoes, peaches, ice milk, bread, and margarine. The nutrient composition of this standard (STD) meals was as follows: 690 kcal, 29 g protein, 68 g carbohydrate, 35 g fat, 202 mg calcium, 414 mg phosphorus, and 4.1 mg iron. The STD meal was labeled by mixing the radioiron with the potatoes and was given as meals C and D in study 3. The second test meal, called the semisynthetic (SS) meal, was developed in tandem with the STD meal and had the same total nutrient composition. It contained dextrimaltose, corn oil, and egg albumin as the sources of carbohydrate, fat, and protein respectively. Sufficient dibasic calcium phosphate and potassium phosphate were added to bring the contents of calcium and phosphorus to the same levels as those in the STD meal. Two mg iron was added to meals A and C as labeled $FeCl_3$ and to meals B and D as labeled monoferric phytate (MFP), the predominant iron-containing compound in wheat bran (14). We prepared synthetic MFP from sodium phytate and $FeCl_3$ by the method of Morris and Ellis (14), using Na_{12} phytate (sodium inositol hexaphosphate) from Sigma Chemical Co., St. Louis, Missouri. MFP tagged with ^{55}Fe was prepared by the same method except that $^{55}FeCl_3$ was the iron source. Radiolabeled MFP was then used to tag a larger quantity of synthetic MFP.

Bran fractions. The effect on iron absorption of components of whole wheat bran were evaluated in studies 4 and 5. All wheat bran used in these studies was purchased as a single lot (Golden Harvest Pure Bran, Natural Sales Company, Pittsburgh, PA 15230). To identify the nature of bran inhibition, we added various bran fractions to muffins in amounts equivalent to 12 g whole bran. The first fraction was lyophilized dephytinized bran (LDB) that was prepared by endogenous enzymatic hydrolysis. Bran was incubated for 16 hours at 37°C with constant shaking in 10 times its weight of deionized water. This material was freeze-dried before using it in the muffins. As a control for the dephytinization and lyophilization processes, another fraction of whole bran was suspended in deionized water at 4°C, frozen, and then lyophilized rather than incubated at 37°C; this prevented enzymatic hydrolysis of the phytate. This material will be referred to as lyophilized whole bran (LWB). Whole bran (WB), LWB, and LDB were all equal in weight because no part of the material was discarded. Phytic acid analysis showed that 94% of the bran phytate in the dephytinized material had been destroyed, whereas phytic acid values for WB and LWB were identical.

In study 5, we tried to determine whether the factor in bran responsible for inhibiting iron assimilation was located in the soluble or insoluble fraction. Bran was dephytinized as described above; it was allowed to settle for 30 minutes and

then the supernatant was decanted into another vessel. The sediment was washed three times in deionized water, with 30-min. settling times, and each decanted wash was combined with the original supernatant. The supernatant, or soluble fraction, could have contained some fine particulate matter. Both the sediment and supernatant were then lyophilized separately to obtain a soluble and insoluble bran. During this procedure quantitative recovery of each bran was carefully measured on the basis of the weight of starting material; from 1.0 g original bran, 0.65 g was recovered as insoluble material and 0.35 g as soluble.

Chemical measurements. Chemical measurements were made on duplicate or triplicate aliquots of each bran preparation. Iron contents of bran and its various fractions were measured by flame atomic absorption spectroscopy with the method of Elwell and Gidley (15). Phytic acid was measured by the method of Ellis, et al (16), and total phosphorus by the method of Fiske and Subbarow (17). Phytic acid phosphorus was then calculated on the basis of molar ratios. The calculated iron and phytate and total phosphorus contributed to the muffins by bran and bran fractions is given in Table 2. Dephytinization reduced the phosphorus present as phytate from 139 mg to 9 mg/meal with no change in the total phosphorus content. Phosphorus content was 13 mg in the insoluble bran fraction and 166 mg in the soluble fraction. Thus, phosphorus content, exclusive of that present as residual phytic acid,

was 23-fold higher from the soluble fraction than from the insoluble fraction.

Statistical analysis. Because the distribution of iron absorption measurements is highly skewed when expressed as a percentage, data were converted to logarithms for statistical analysis and then converted to antilogarithms to recover the original units (18). To compare absorption between any pair of test meals eaten by the same subject, we used a paired *t*-test to determine whether the mean difference in log absorption differed significantly from 0, which is equivalent to testing whether the mean ratio of percentage absorption was different than 1.

RESULTS

Effect of whole bran on iron absorption. In the first two studies we measured the effect of whole bran on iron absorption from meals with different levels of iron availability. In study 1, performed in 10 subjects, iron absorption averaged 2.39% with plain muffins and only 0.62% with bran muffins (Table 3). The mean ratio with:without bran was 0.26 (Fig. 1), reflecting a highly significant effect of bran on iron absorption ($t=6.38$, p 0.0001).

In study 2 the effect of bran was examined with meals of high iron availability. In meals A and B, 100 g cooked lean beef was ingested with the milkshake and muffins. Absorption in 13 subjects averaged 3.46% with plain muffins and only

1.69% with bran muffins. The mean ratio with:without bran of 0.49 was also highly significant (t=4.33, p 0.001). In meals C and D, 100 mg ascorbic acid was added to the milkshake to enhance absorption. Iron absorption averaged 9.29% with plain muffins and 3.89% with bran muffins. The absorption ratio with:without bran averaged 0.42 and was highly significant (t=7.30, p 0.0001). These studies indicated that bran inhibits iron absorption regardless of the basal level of iron availability in the meal although the inhibitory effect may be more pronounced with meals of low iron availability (Fig. 1).

Absorption of iron from MFP. The 12 g bran added to each test meal contained about 2 mg iron; previous studies have shown that at least half of this iron is in the form of MFP (14). In study 3 we mesured absorption of this biological form of iron from test meals of both high and low iron availability.

In the first pair of absorption tests, with 9 volunteers, 2 mg iron was added to the SS meal as either MFP or $FeCl_3$ (Table 4). Previous studies established that $FeCl_3$ undergoes complete isotopic exchange with nonheme dietary iron (19). Iron absorption from the SS meal averaged 0.59% with $FeCl_3$ and 0.73% with MFP. The mean absorption ratio $FeCl_3$:MFP was 0.81 which was not statistically significant (t=0.73, p=0.24). In the same study when the subjects ate the STD meal rather than the SS meal (meals C and D), iron absorption averaged 4.73% with $FeCl_3$ and 3.66% with MFP. The mean absorption

ratio $FeCl_3$:MFP of 1.29 was not statistically significant ($t=1.33$, $p=0.11$). Apparently the iron in MFP was no less well absorbed than nonheme dietary iron (Fig. 2).

Effect of bran fractions on iron absorption. In the remaining two studies we tried to identify the factor in bran that is responsible for inhibiting iron absorption. Ascorbic acid was added to the milkshake in all of these meals in an attempt to improve sensitivity for identifying an inhibitory effect.

It is widely believed that phytate is the factor responsible for the inhibitory effect of bran. In study 4 we examined this point in normal subjects using four test meals containing different types of muffins (Table 5). Absorption averaged 2.43% with plain muffins (meal A) and only 0.99% with bran muffins (meal B). The absorption ratio with:without bran of 0.41 ($t=8.17$, p 0.0001) approximated the ratio of 0.49 obtained for this ascorbic acid meal in study 2 despite the fact that absorption with plain muffins was much higher (9.29%). These same subjects were then given two additional test meals containing muffins prepared with either LWB (meal C) or LDB (meal D); iron absorption averaged 1.29 and 1.37% respectively. These nearly identical values indicate that the phytate content of bran is not responsible for its inhibiting effect. When compared to absorption from plain muffins, highly significant mean ratios with:without bran were observed: 0.53 for LWB ($t=3.19$, $p=0.005$) and 0.56 for LDB ($t=2.90$,

p=0.009). When the meals containing LWB or LDB muffins were compared, the mean ratio WB:LWB of 0.77 was not significant (t=1.36, p=0.10), whereas the similar ratio WB:LDB of 0.73 was marginally significant (t=2.18, p=0.029). These results suggest that a small amount of the inhibitory activity of bran may be lost during lyophilization.

In a final study, with 18 volunteers, we tried to determine whether the inhibitory effect of LDB was located in the soluble or insoluble fraction (Table 5). Absorption from the test meal with plain muffins averaged 3.02%, and with LDB muffins 2.23%. The absorption ratio with:without bran of 0.74 did not reach statistical significance at the 5% level (t=1.70, p=0.053), in contrast with study 4 in which the ratio of 0.56 was statistically significant.

Failure to establish a significant inhibition with LDB made it difficult to compare inhibition by insoluble and soluble fractions of LDB, particularly because the total effect of 12 g bran was distributed between the two meals. Indeed, mean absorption with insoluble bran muffins was actually higher (3.22%) than with LDB. Of the two fractons studied, inhibition was greater with soluble bran muffins for which absorption averaged 2.43%. The absorption ratio insoluble:soluble bran of 1.32 was statistically significant (t=2.41, p=0.013). Absorption is compared between plain muffins and muffins prepared with various bran fractions in Fig. 3.

DISCUSSION

The inhibitory effect of bran on iron absorption was first described by Widdowson and McCance (20) who determined by chemical iron balance that normal subjects retained less iron from a diet containing brown bread than from one containing white bread. This bran effect was studied by Bjorn-Rasmussen who demonstrated by a dual isotope method that the addition of 7% bran to white wheat flour decreased iron absorption from baked rolls by a factor of two (21). He reported a significant linear relationship between the ratio of iron absorption with:without bran and the log of the amount of bran added. In our study muffins containing 12 g bran reduced absorption by 74 per cent when added to a meal of low iron availability and by 51-58% when added to meals containing either meat or ascorbic acid. These data are consistent with those of Bjorn-Rasmusen but are not strictly comparable because of differences in the size of the meals and the amounts of added bran. Bjorn-Rasmussen did not examine the mechanism of the bran effect but he speculated that it was related to the phytate content of the bran. Morris and Ellis (14) reported that over half of the iron in wheat bran is present as MFP, a compound that in contrast to saturated ferric phytate, is soluble at neutral pH but insoluble at acid pH. In rats, this iron complex had high bioavailability (14). In dogs, complete isotope exchange of MFP with nonheme dietary iron was demonstrated (22). In our study, this isotopic exchange was confirmed in human subjects given

meals of both low (SS) and high (STD) iron availability. With both meals, iron absorption did not differ between MFP and $FeCl_3$.

Since MFP accounts for les than 5% of the total phytate content of bran, the effect of bran phytate on iron absorption is more important. Most workers report that phytate significantly inhibited iron absorption (20, 23-26) but others report that the effect of phytate on iron absorption was negligible (27-29). Some discrepancies could be attributed to differences among species in the assimilation of iron phytate, but often the inhibitory effect was studied only indirectly by adding purified phytate to food. We approached the question of the phytate effect on iron absorption directly by measuring the effect of phytate-free bran. This dephytinized bran produced the same degree of inhibition in iron absorption as whole bran did, indicating that the inhibitory effect of bran should not be attributed to its phytate content.

Reinhold, et al. (30) questioned the phytate theory as the sole explanation for bran inhibition because purified phytate was less effective at decreasing zinc and calcium absorption than its equivalent of whole meal bread. Reinhold, et al. (31) later demonstrated <u>in vitro</u> that dephytinized whole meal bread bound bivalent metals at least as well as the original phytate-containing products and our data support this conclusion. In a later study (32), the binding of

bivalent metals increased when bran was dephytinized and decreased when fiber was destroyed by hydrolysis in boiling acid, and the authors concluded that fiber rather than phytate was primarily responsible for the inhibitory effect of bran on iron absorption. From a balance study in human subjects, Reinhold, et al. (33) concluded that both fiber and phosphate bind metals such as magnesium and zinc in the small intestine and thereby impair their absorption, but they did not evaluate iron absorption. Other workers report that inorganic phosphate impairs iron absorption (34-36).

In our study, we administered the fiber- and phosphate-rich components of dephytinized bran separately in an attempt to identify the fraction that inhibits iron absorption. Chemical analysis determined that this separation was effective. The total phosphorus content was 13 mg in insoluble bran and 166 mg in soluble bran; neutral detergent fiber* was 4.6 in insoluble bran and was undetectable in he soluble fraction. None of the lyophilyzed bran components inhibited iron absorption significantly, but inhibition was greater with soluble than with insoluble bran, suggeting that phytate is more inhibitory than fiber.

Several factors could account for our failure to clearly identify the bran fraction that inhibits iron absorption.

*Kindly performed by Doris Baker, Nutrient Composition Laboratory, Nutrition Institute, Beltsville, Maryland, using the method of the American Association of Cereal Chemists.

In an effort to achieve high baseline absorption from which inhibition could be readily recognized, we chose a test meal that contained 100 mg ascorbic acid. However, the inhibitory effect of bran apparently was greater for meals without meat or ascorbic acid (ratio with:without bran 0.26) than for meals with either meat or ascorbic acid (ratios, 0.49 and 0.42 respectively). Evaluation of the effects of dietary components from which iron is absorbed at low levels is not always reliable because precision is low at levels of 1-2% absorption. However, our data suggest that the meals designed to achieve relatively high baseline absorption supplied dietary components that ablated the inhibitory effect under study.

Another possible explanation for the failure to establish a cause of the inhibitory effect of bran components relates to the lyophilization process. With meals containing 100 mg ascorbic acid the absorption ratios with:without <u>whole</u> bran were 0.49 (Table 3) and 0.41 (Table 5) but the ratios with:without <u>lyophilized</u> bran were only 0.53 and 0.59 (Table 5). Lyophilization may have altered some component of bran and reduced its inhibitory effect. For example, bran fiber exists in the form of hemicellulose and cellulose and these branched and long chain polysaccharides might have physical properties that are altered by freeze-drying.

Use of the double isotope technic has greatly enhanced the sensitivity of food iron absorption tests because multiple comparisons within individual subjects eliminate the problem of individual differences in absorption. Precision is enhanced when the subjects eat test meals on several days, thus reducing the effect of day-to-day variability on iron absorption. Even with these refinements in methodology, however, the effect, particularly inhibitory, of a dietary variable that changes iron absorption by less than a factor of two is still difficult to measure. The magnitude of inhibition demonstrated with lyophilized bran, whether dephytinized or not, was too small for definitive evaluation of the specific components of bran.

TABLE 1. Contents of test meals.

Study	Number of Subjects	Test Meals A/B	Test Meals C/D
1	10	MS	--
2	13	MS + Beef	MSC
3	9	SS	STD
4	10	MSC	MSC
5	18	MSC	MSC

Abbreviations: MS = milkshake and two muffins
MSC = milkshake containing 100 mg ascorbic acid and two muffins
SS = semisynthetic meal
STD = standard meal

TABLE 2. Chemical composition of bran fractions.[a]

Fraction	Iron Content (mg)	Phosphorus Content As Phytate (mg)	Phosphorus Content Total (mg)
Whole bran, untreated[b]	2.0	139	153
Dephytinized			
Whole	1.6	9	155
Soluble	0.9	6	166
Insoluble	0.9	3	13

[a] Values are contents per 12 g bran or the equivalent fraction in the two muffins in each meal.

[b] Same values were obtained for lyophilized whole bran (LWB).

TABLE 3. Effect of whole bran on iron absorption

Subject	Sex/Age	Packed Cell Volume (%)	Serum Iron (μg/dl)	TS[a] (%)	Serum Ferritin (ng/ml)	Iron Absorption (% of dose) -bran (A)	Iron Absorption (% of dose) +bran (B)
Study 1							
1 PP	M23	46	141	36	69	0.43	0.27
2 KD	M22	48	104	25	52	1.07	0.43
3 RM	M22	50	226	59	143	1.73	0.12
4 MB	F25	43	147	34	35	1.98	0.56
5 CH	M22	44	89	25	104	2.73	0.97
6 JW	M24	42	83	22	86	2.78	0.70
7 TS	M22	51	162	39	118	2.96	1.18
8 CB	F24	40	108	28	35	4.97	0.48
9 MC	M22	49	133	31	39	5.81	1.70
10 MD	M22	48	144	31	63	6.01	1.72
MEAN	23	46	134	33	66[b]	2.39[b]	0.62[b]

Subject	Sex/Age	Packed Cell Volume (%)	Serum Iron (μg/dl)	TS[a] (%)	Serum Ferritin (ng/ml)	Meat -bran (A)	Meat +bran (B)	Ascorbic Acid -bran (C)	Ascorbic Acid +bran (D)
Study 2									
1 JT	F21	49	145	27	67	0.90	0.45	7.90	2.03
2 PP	F33	44	207	48	58	1.03	0.21		
3 JH	F24	47	95	30	34	1.22	0.88	18.42	9.62
4 SL	F20	51	151	39	73	1.50	1.07	6.10	4.30
5 PM	F26	49	144	36	104	1.60	1.10	1.93	0.96
6 KH	F23	44	121	36	40	2.48	1.55	19.88	6.28
7 PM	M21	46	275	100	323	2.48	4.26	7.88	2.11
8 DW	M20	50	135	43	147	2.66	1.00	2.65	0.95
9 DV	M30	46	89	20	8	5.55	3.08	21.25	12.35
10 AF	F35	48	165	42	73	7.05	1.97		
11 MK	F42	48	81	24	66	12.93	2.28		
12 TS	F19	44	100	25	16	17.01	8.61	40.28	26.10
13 TC	F28	45	50	13	15	27.25	12.40	7.95	2.47
MEAN	25	47	135	37	51[b]	3.46[b]	1.69[b]	9.29[b]	3.89[b]

[a]TS = transferrin saturation

[b]geometric mean

TABLE 4. Absorption of ferric chloride and monoferric phytate (Study 3)

Subject	Sex/Age	Packed Cell Volume (%)	Serum Iron (μg/dl)	TS[a] (%)	Serum Ferritin (ng/dl)	Iron Absorption (% of dose) Semisynthetic $FeCl_3$ (A)	Semisynthetic MFP (B)	Standard $FeCl_3$ (C)	Standard MFP (D)
1 LJ	M23	49	69	20	90	0.18	1.50	4.83	3.65
2 SM	M24	45	127	38	108	0.22	0.17	1.53	1.91
3 JB	M29	49	168	50	184	0.30	0.31	2.80	2.23
4 PH	M22	49	162	37	47	0.38	0.18	14.15	7.78
5 BK	M23	49	120	30	73	0.78	1.65	9.22	12.33
6 MG	M24	49	184	57	114	1.17	1.70	5.92	3.00
7 KD	M25	48	148	35	59	1.21	0.71	4.87	9.56
8 LD	F20	44	138	33	62	1.27	1.08	3.82	2.35
9 WE	M29	49	122	34	71	1.46	1.97	4.05	1.18
MEAN	24	48	138	37	83[b]	0.59[b]	0.73[b]	4.73[b]	3.66[b]

[a] TS = transferrin saturation

[b] geometric mean

TABLE 5. Effect of dephytinized bran on iron absorption

Subject	Sex/Age	Packed Cell Volume (%)	Serum Iron (μg/dl)	TS[a] (%)	Serum Ferritin (ng/ml)	Iron Absorption (% of dose)			
Study 4						-bran (A)	WB (B)	LWB (C)	LDB (D)
1 KK	F28	39	89	23	61	0.90	0.67	0.50	0.70
2 SM	F20	44	182	48	47	1.32	0.60	0.75	1.17
3 LB	M25	45	99	39	149	1.43	0.75	1.50	1.73
4 MP	M33	46	105	26	142	1.76	0.80	1.55	1.35
5 UB	M27	50	204	63	187	1.96	0.65	1.06	0.98
6 JG	M27	47	166	43	157	2.46	1.27	0.55	0.77
7 SB	M21	48	89	24	67	3.10	0.76	1.58	0.63
8 DC	F24	43	90	15	73	3.50	1.01	0.57	1.47
9 JG	F33	41	88	22	24	3.83	1.11	2.18	1.16
10 JO	M20	47	150	46	43	12.02	5.72	13.70	15.22
MEAN	26	45	126	35	79[c]	2.43[c]	0.99[c]	1.29[c]	1.37[c]

Study 5						-bran (A)	LDB (B)	LDB Fraction insoluble (C)	LDB Fraction soluble (D)
1 GR	M24	48	94	33	87	0.66	1.16	0.91	0.72
2 TS	F22	40	140	37	79	1.03	0.41	1.51	1.56
3 KH	M23	41	126	40	112	1.22	0.78	2.12	2.45
4 EV	M23	45	114	32	166	1.52	0.55	1.02	0.80
5 MV	F23	42	134	35	39	1.71	2.95	5.82	4.00
6 DD	F22	41	40	14	29	1.82	5.88	3.98	1.65
7 PJ	M25	47	173	56	200	2.03	0.61	1.98	0.97
8 EH	M23	48	99	27	113	2.31	1.98	2.56	2.08
9 TW	F20	43	105	27	85	3.31	1.58	3.68	5.01
10 AD	M25	50	86	22	128	4.02	1.07	1.93	1.53
11 LH	F19	40	77	27	34	4.55	8.38	4.53	3.47
12 WO	M24	43	96	30	103	3.15	1.20	4.66	1.36
13 CT	M23	47	97	28	16	4.96	2.73	6.40	5.38
14 PB	M28	43	99	22	13	5.05	4.15	6.63	7.37
15 SB	F24	43	94	23	132	5.20	4.75	2.60	1.37
16 CL	F22	46	51	12	55	5.87	10.90	6.17	2.72
17 JK	F23	36	51	13	10	6.76	5.56	14.32	8.70
18 BD	F22	38	142	54	19	18.42	7.27	2.81	6.85
MEAN	23	43	101	30	57[c]	3.02[c]	2.23[c]	3.22[c]	2.43[c]

[a] TS = transferrin saturation
[b] LWB = lyophilized whole bran; LDB = lyophilized dephytinized bran
[c] geometric mean

LEGENDS

Figure 1. Effect of bran on food iron absorption (studies 1 and 2). Iron absorption ratios of with:without bran are plotted on semilogarithmic coordinates for test meals containing only milkshake and muffins (ALONE) and the same meal with beef or 100 mg ascorbic acid.

Figure 2. Absorption of iron in MFP (study 3). The iron absorption ratios for $FeCl_3$:MFP are plotted for the semisynthetic and standard meal. The ratios did not reflect a significant difference.

Figure 3. Iron absorption ratios for with:without bran fractions are plotted for dephytinized (WHOLE) bran and insoluble and soluble bran components (studies 4 and 5).

FIGURE 1.

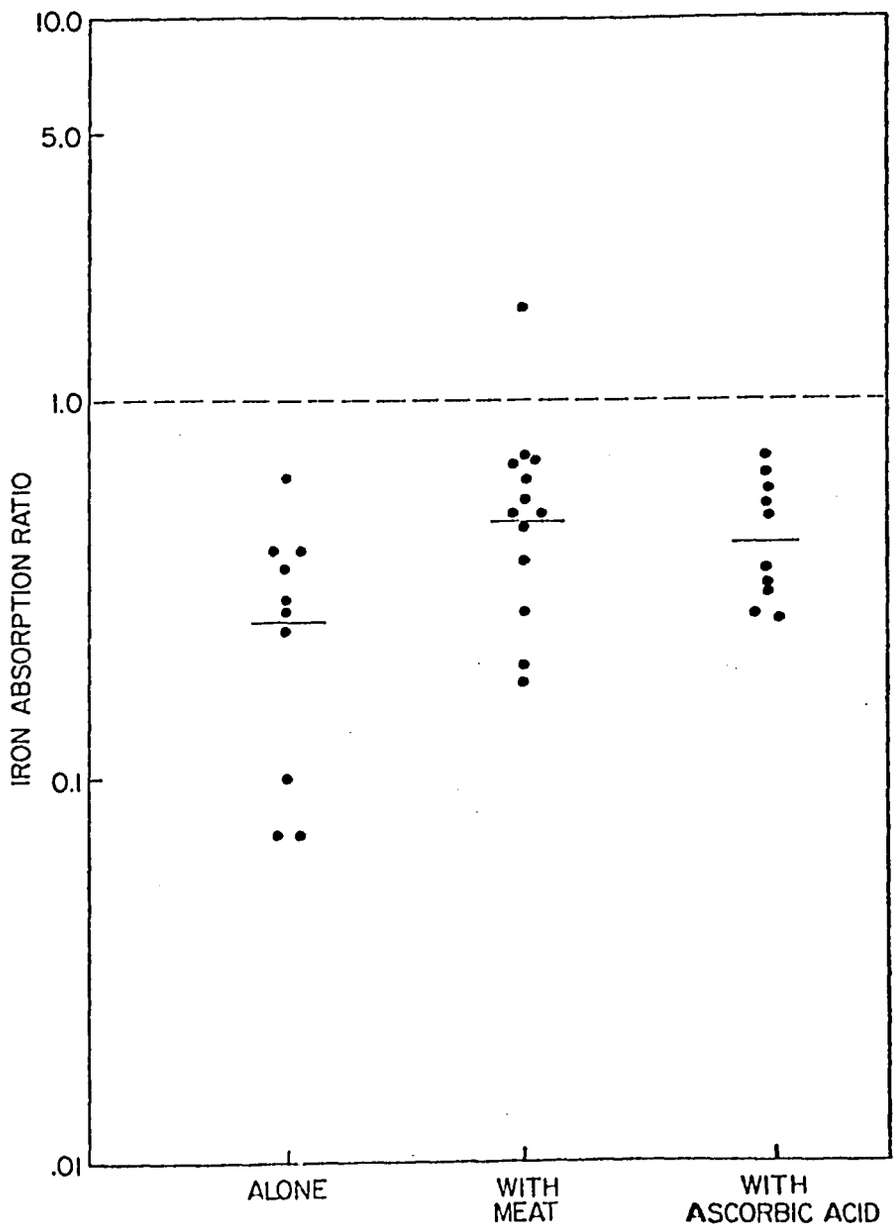

10.0
5.0
1.0
0.1
.01
IRON ABSORPTION RATIO
ALONE
WITH MEAT
WITH ASCORBIC ACID

FIGURE 2.

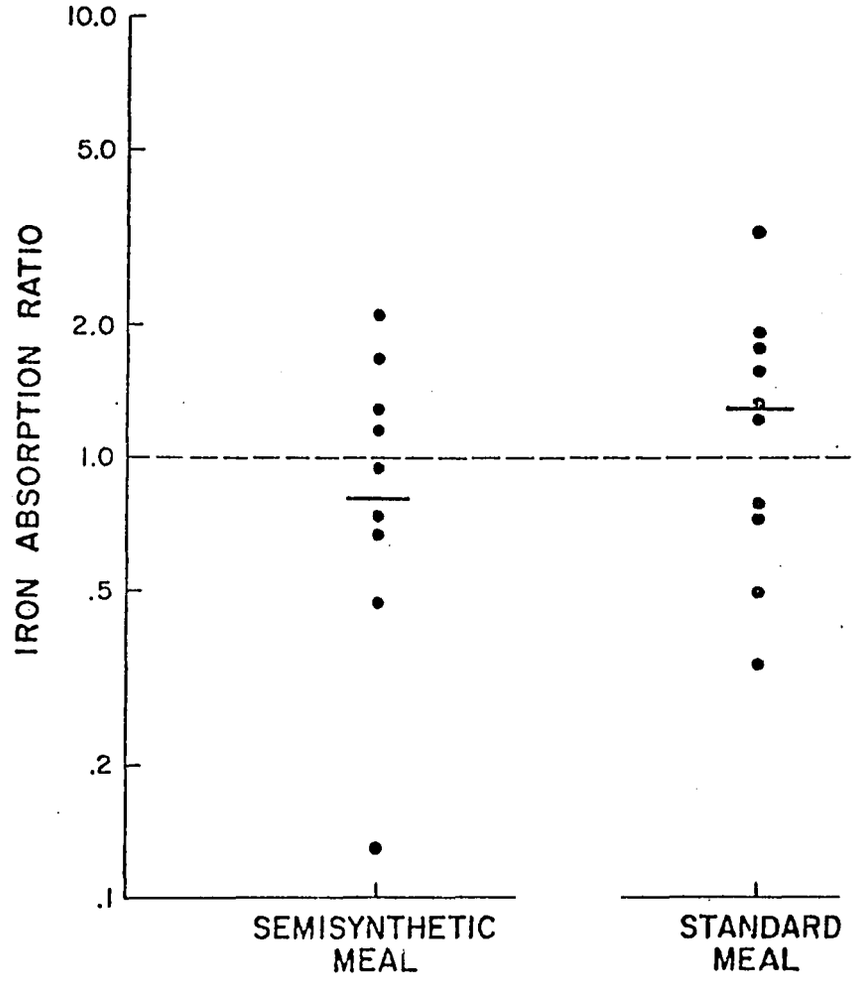
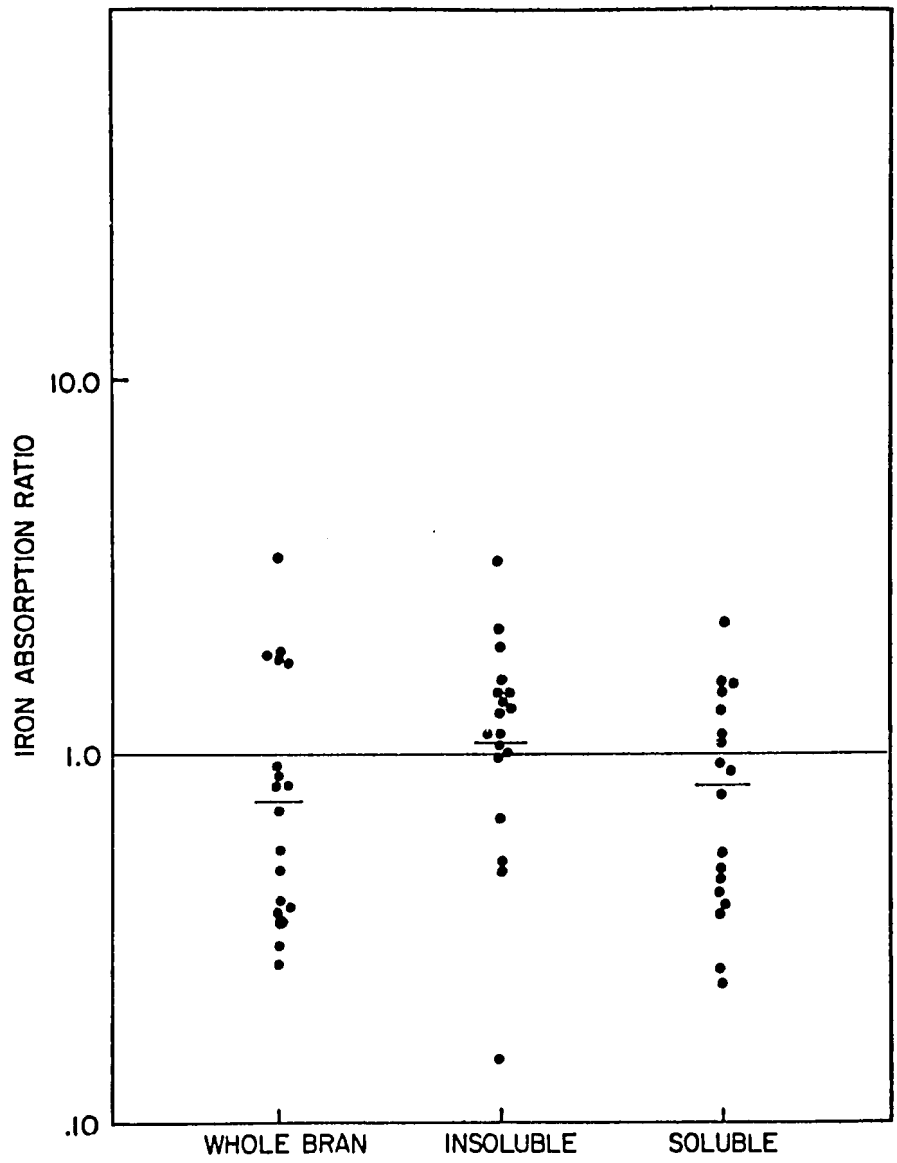

10.0
5.0
2.0
1.0
.5
.2
.1
IRON ABSORPTION RATIO
SEMISYNTHETIC MEAL
STANDARD MEAL

FIGURE 3.


10.0
1.0
.10
IRON ABSORPTION RATIO
WHOLE BRAN
INSOLUBLE
SOLUBLE

INTERACTION OF VITAMIN C AND IRON

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In early studies of food iron absorption, test meals consisted of single foods that had been biosynthetically labeled with radioiron.[1] Absorption varied considerably with individual food items and, in general, iron derived from animal tissue was more available than iron of vegetable origin.[1-3] More recently, it has been shown that the percentage absorption of iron from a given food item differs substantially when it is eaten in a meal containing 2 or more additional foods.[2-4] For example, soybean iron is much better absorbed than black bean iron if each is eaten separately, but a similar percentage of iron is absorbed from both bean sources when they are eaten together.[3] Absorption therefore appears to be a property of the overall composition of the meal rather than the single food item. These studies led to a very important observation. If a small quantity of soluble inorganic iron is added to a biosynthetically labeled vegetable food just before it is eaten, percentage absorption of the two forms of iron is virtually identical.[5] The same is true of meals containing more than one vegetable food.[5-8] Therefore, it appears that when several foods are eaten together, nonheme iron destined for absorption behaves as though it is derived from a single common pool. Soluble inorganic iron salts used in fortification also enter this pool.[7,8] Iron absorption from it can be measured by determining the size of the pool and the percentage absorption of a soluble radioiron tracer added to the meal.[5-8]

Similar observations have been made for heme iron which comes principally from hemoglobin and myoglobin of meat. Heme compounds enter a second common pool and share a set of properties different from those

that govern nonheme iron absorption.[9] For example, heme is not degraded to any degree in the lumen of the gut but is taken up by the mucosal cells with iron still within the porphyrin ring.[10] Heme iron absorption is relatively independent of other components of the meal; ascorbic acid has no effect.[11,12] Absorption of heme iron can be measured by adding a small quantity of labeled hemoglobin to a meal just before it is eaten.[9] Virtually all dietary iron is absorbed from these two pools of heme and nonheme iron.[13]

In attempting to combat iron deficiency, most investigators have focused their attention on nonheme iron which makes up over 90% of the iron in an average diet.[13] The percentage absorption of nonheme iron is only about one-tenth that of heme. Unlike heme iron, the availability of nonheme iron is critically dependent on several factors, some of which enhance and others of which inhibit its uptake.[14] Many chemical ligands present in food are known to have a powerful influence. For example, carbonates, oxalates, phosphates, fiber components and tannates impair absorption while ascorbic acid, citric acid, tricarboxylic acids, amino acids, and sugars promote absorption. Of these, ascorbic acid has the greatest influence on iron assimilation.

Initial evidence suggesting that ascorbic acid can enhance iron absorption from certain foods was obtained in studies using meals eaten with and without citrus fruits.[1,15-20] For example, when 100 ml orange juice containing 40-50 mg ascorbic acid was added to a meal consisting of bread, butter, jam, tea or coffee, and an egg which had been biologically labeled with iron[59], absorption increased from 3.7 to 10.4%.[18] More

recently, Rossander et al[20] studied a common Swedish "continental breakfast" and demonstrated that when orange juice containing 70 mg ascorbic acid is consumed with coffee and 2 wheat rolls, absorption is 2½ times higher than that from the same meal without orange juice.

Moore and Dubach[1] were the first to identify ascorbic acid as the cause of the increased absorption seen with meals containing fruit. They demonstrated a similar increase in absorption with equivalent quantities of ascorbic acid. Their findings have subsequently been confirmed by others. Layrisse et al[19] demonstrated that nonheme iron absorption from a maize meal was increased several fold when papaya was added to the meal. The same degree of enhancement was achieved by adding ascorbic acid equivalent in amount to that contained in the papaya. Thus, although it is possible that there are factors other than ascorbic acid in fruit juices that enhance absorption, there is little doubt that ascorbic acid accounts for the major effect.

Ascorbic Acid as a Nonheme Common Pool Ligand

Early observations involving fruit juices led to a series of studies of the effect of ascorbic acid on the absorption of various forms of nonheme iron.[19, 21-31] In two of the more recent investigations ascorbic acid was added to meals prepared from maize, wheat soya and rice.[24,25] The cereal component of each meal had been labeled intrinsically with Fe^{55} by hydroponic cultivation. Iron[59] chloride, used as the extrinsic label for the nonheme pool, was added to the meal in trace quantities either immediately before it was eaten or to one of the ingredients before cooking. Supplemental iron in the form of a soluble iron salt such as

2-5 mg ferric ammonium citrate, or 4 mg as ferrous sulfate was incorporated into some of the meals. Ascorbic acid produced the same increase in absorption of both extrinsic and intrinsic radioiron labels over a wide range of absorption values and ascorbic acid concentrations (Figure 1). The composite mean absorption ratio of extrinsic to intrinsic radioiron was 1.13, a value very close to that of 1.10 reported by Cook et al[5] for meals containing no ascorbic acid. These observations strongly support the conclusion that ascorbic acid acts as a ligand for the common nonheme iron pool and thus enhances absorption of all iron compounds in this pool to an equal extent. An important corollary is that extrinsic tagging allows valid comparisons to be made between meals eaten with and without supplementary ascorbic acid.

A number of ligands in food have a powerful inhibitory effect on absorption from the nonheme iron pool. The interaction of ascorbic acid with two of these, tannates[32] and calcium/phosphate,[31] has been studied. Ascorbic acid reverses the strong inhibitory effect that tea exerts on nonheme iron absorption from cereal-based meals. The degree of reversal of inhibition is directly proportional to the quantity of added ascorbic acid; absorption in the presence of ascorbic acid and tea may exceed absorption from the basal meal containing neither. The effect of ascorbic acid on the inhibitory influence of calcium/phosphate on iron absorption was studied using a semisynthetic meal.[31] When inorganic calcium/phosphate were omitted from this meal, absorption increased about twofold, but the mean ratio of the meal with to that without ascorbic acid was similar whether or not calcium/phosphate were present. Apparently, ascorbic acid and known inhibitors of iron absorption have an opposite but additive effect on

absorption from the nonheme common pool and can therefore be considered to act as competitive ligands.

The influence of ascorbic acid on iron absorption from meals containing other absorption promoters is less well defined. For example, meat and fish increase nonheme iron absorption when added to a vegetable meal. Cook and Monsen compared iron absorption from a standard meal containing beef, potatoes, corn meal, peaches, ice milk, bread, and margarine with that of a semisynthetic meal of similar composition prepared with dextrimaltose, corn oil, and ovalbumin.[31] Nonheme iron absorption from the standard meal was much higher owing to its content of meat. Absorption from both these meals was enhanced by adding 100 mg of ascorbic acid. However, the mean absorption ratio of the meal with to that without ascorbic acid was 3.19 for the semisynthetic meal but only 1.67 for the standard meal, indicating that the enhancing effects of meat and ascorbic acid are not additive. A similar observation has been made with fish and ascorbic acid. Whereas 100 gm fish increased the absorption of nonheme iron about threefold,[33] the percentage absorption of iron from a maize meal eaten with 100 g fish and fresh papayas containing approximately 70 mg ascorbic acid was the same as that from the meal eaten with the papayas alone.[19] Although it is not possible to draw definitive conclusions from this evidence, these two studies suggest that ascorbic acid and beef or fish are complimentary rather than additive in their enhancing properties and that the addition of ascorbic acid to a meal containing meat or fish may have a relatively small influence on the quantity of nonheme iron absorbed.

Accumulating evidence indicates that the total intake of dietary iron is less important from a nutritional standpoint than the availability

of that iron. A model has recently been proposed for estimating dietary iron absorption from any given meal based on the iron status of the individual, the content of heme and nonheme iron and the content of enhancing constituents.[34] Of the many factors that have been said to influence iron absorption, only two were considered important enough for inclusion in the method for estimating available dietary iron. Meals were defined as having low, medium, or high iron availability based on the content of meat, poultry, or fish and of ascorbic acid. In this model, 1 g meat was considered roughly equivalent in its enhancing effect to 1 mg ascorbic acid. Estimates of nonheme iron absorption in subjects with 500 mg iron stores are shown in TABLE 1. The model defines the effect of these substances when added separately to a meal. It is less clear whether their effect is additive when both are contained in the meal. Presumably, the effect of 30-90 g meat, fish, or poultry and 25-75 g ascorbic acid would be roughly additive and therefore place the meal in the high iron availability category, but as previously discussed, this point is not clearly established.

Interaction of Ascorbic Acid and Contaminant Iron

Not all the iron in a meal is necessarily intrinsic to the food. Chemical assays of Indian diets have consistently revealed iron contents considerably in excess of those determined from food composition tables.[14] The disparity is believed to represent either iron contamination from the soil or from cooking utensils. The high prevalence of iron deficiency in these geographic areas of the world suggests that this iron is poorly absorbed. Therefore, the potential influence ascorbic acid has on these less soluble forms of iron could be of considerable nutritional

importance. Evaluation of its effect is difficult because the chemical nature of the extraneous iron has not been identified and because suitable radiolabeled material is difficult to obtain. One study has addressed the problem indirectly.[30] Two likely food contaminants, ferric oxide which is the major component of rust derived from cooking utensils, and ferric hydroxide, which forms much of the iron found in soil and unpurified water sources, were radiolabled and added to a maize porridge meal; both were poorly absorbed with mean values of 0.01% and 1.5%, respectively. The addition of 100 mg ascorbic acid increased absorption from ferric oxide to 0.5% and from ferric hydroxide to 6.7%. Although the relative increase was pronounced, absorption was still inappropriately low in the context of the meal and the iron deficient population studied. This suggests that a significant proportion of each compound remains insoluble and unavailable to the common nonheme iron pool. Moreover, ferric hydroxide was absorbed only half as well as the intrinsic iron contained in maize porridge. Presumably most of the contaminating iron contained in food remains relatively unavailable for absorption despite the presence of ascorbic acid.

Quantitative Aspects

While many observers have noted that percentage nonheme food iron absorption rises with increasing ascorbic acid content of a meal, the quantitative effect of ascorbic acid has been examined rigorously in only two studies which yielded similar results.[27,31] In one of them ascorbic acid, ranging in amounts from 25 to 1,000 mg, was added to a semisynthetic meal containing 4.1 mg elemental iron. A linear relationship

was observed between the absorption ratio of the meal with to that without ascorbic acid and the quantity of added ascorbic acid when both values were plotted on a logarithmic scale. A somewhat different relationship is seen when percentage absorption is plotted against the molar ratio of ascorbic acid to iron using linear scales (Figure 2). The response curve has two components. With the molar ratios below 7.5, which corresponds to approximately 100 mg ascorbic acid, there is a proportionately greater effect than with molar ratios above 7.5. It is interesting that when ascorbic acid is taken with pharmacologic doses of ferrous sulfate in the fasting state, a similar two-phase dose response pattern occurs although the degree of enhancement and the actual quantity of iron absorbed is very different in the two studies (Figure 3).[35]

The factors responsible for the two components of the dose response curve are unknown, but it is tempting to speculate that the initial component may represent the effect that ascorbic acid has in rendering ferric iron soluble at the alkaline pH of the duodenum (see below). It would account for the relatively small increase in absorption seen when ascorbic acid was added to ferrous sulfate since a large quantity of iron taken in this form would be expected to arrive at the absorptive surface of the duodenum as the soluble ferrous salt even in the absence of ascorbic acid.

Most of the published evidence dealing with the effect of ascorbic acid on vegetable iron absorption is derived from meals containing maize or rice. If the effect of varying iron status in these studies is removed by relating food iron absorption to a reference dose of ferrous

sulfate and ascorbic acid, it is possible to compare the relative absorption of iron in different studies and in different population groups.[36] We have used the results obtained in a number of different investigations[19,24-26,29,30] to construct a dose response curve for iron absorption against molar ratio of ascorbic acid to iron in the meal (Figure 4). All values have been adjusted to a reference absorption of 40%, a level which was considered indicative of iron deficiency. Although there is considerable variability between studies, the increase in percentage absorption is again directly proportional to the molar ratio of ascorbic acid to iron in the meal, emphasizing the singular importance of the quantity of ascorbic acid to nonheme iron absorption from vegetable meals.

Mechanism of Action

Ascorbic acid is effective only if it is present in the meal. In one study, 500 mg ascorbic acid taken with the meal increased absorption about sixfold whereas the same quantity had little effect when taken 4 and 8 hours prior to the meal.[31] In most investigations, ascorbic acid has been contained in an uncooked component of the meal or has been added just before consumption. Under certain circumstances, enhancement was also seen when it was incorporated into one of the ingredients prior to cooking. After boiling, approximately 70% of 50 and 100 mg supplements of ascorbic acid remained in the reduced form and significant enhancement of iron absorption was seen.[24] However, in baked foods such as soy biscuits and wheat bread, very little ascorbic acid remained in the reduced state and no enhancement of iron absorption occurred.

A better understanding of the probable mode of action of ascorbic acid has been provided by the studies of Conrad and Schade.[37] When

reducing agents are absent from food, iron in the common pool exists in ferric form. Ferric iron has a coordinating valency of 6 and in an aqueous solution metal ions are bound to each other through water bridges. Since increases in pH make more hydroxyl ions available, metallic polymers or precipitated metallic hydroxides are formed and the iron becomes inaccessible to the mucosal cell.[14] Conrad and Schade demonstrated that the addition of ascorbic acid to a ferric chloride solution at an acid pH causes displacement of hydrogen ions from ascorbate and the formation of a purple-colored complex that can be isolated by alcoholic precipitation. This iron chelate remains in solution over a wide pH range of 2-11. The reaction however must be initiated at acid pH (Figure 5). Both ferric chloride alone and ferric chloride plus ascorbate are insoluble at alkaline pH. Both become progressively more soluble as the pH is reduced by adding hydrochloric acid and are totally soluble at a pH of approximately 2. As pH is again raised with sodium hydroxide, ferric chloride is precipitated and very little remains soluble at a pH above 4. In contrast, the addition of ascorbic acid to ferric chloride yields a complex of iron which remains completely soluble at a higher pH. The importance of solubility to iron absorption was confirmed in gastrectomized rats. Radioiron absorption was measured from oral test doses of ferric chloride to which ascorbic acid had been added. Mean percentage absorption for test doses containing ascorbic acid added before and after alkalization was 17 and 5.3, respectively. Similar results were obtained in experiments in which the test doses were instilled directly into the duodenum. When the two iron solutions were introduced into the stomach, approximately equal quantities of iron were absorbed.

Ascorbic Acid Fortification

In the past, attempts to improve iron nutrition in populations at risk for iron deficiency have relied on iron fortification. The efficacy of this approach has been questioned since added iron enters the nonheme pool of dietary iron and is affected by inhibitors of iron absorption prevalent in this type of diet. If the diet consists predominantly of cereals such as wheat, maize and rice which are poor sources of available iron, fortification iron is also poorly absorbed. Improvement in iron status may only be possible if fortification iron is added in a form less affected by dietary constituents or if a substance is added to the diet that renders both food iron and fortification iron more available. The former approach has led to the use of iron with EDTA in pilot fortification studies. The latter might be made feasible by adding ascorbic acid to the diet.

Although theoretically attractive, a number of obstacles have been encountered in using ascorbic acid for dietary fortification. The initial investigations were based on the premise that optimal results would be obtained by adding both ascorbic acid and fortification iron to the diet to ensure a sufficiently large pool of nonheme iron on which ascorbic acid could act.[24] However, in selecting a suitable food vehicle for dual fortification, a number of practical problems were encountered. It is desirable for such a vehicle to be eaten in approximately the same quantities by all individuals. Ideally, the vehicle should not be added prior to cooking since ascorbic acid is largely inactivated when subjected to the high temperatures used for baking. Common salt seemed to be the most suitable vehicle but discoloration occurred when ascorbic acid and

various iron compounds were added to it simultaneously.[25,26] The discoloration was particularly pronourced when cheaper grades of salt were used and after storage in hot, humid conditions. Nevertheless, it is possible to fortify common salt with 0.1% iron as ferric phosphate ($FePO_4$) and 1.25% ascorbic acid. This product can be stored under all but the most extreme tropical conditions provided that 2.5% starch is incorporated to retard the development of discoloration. However, $FePO_4$ is a relatively insoluble compound, and the influence of ascorbic acid on its absorption is variable. When added to rice and maize meals prior to cooking, it appears to enter the common pool completely and the enhancement achieved by the addition of ascorbic acid is the same for $FePO_4$ as for intrinsic iron or added ferrous sulfate.[26] In contrast, when $FePO_4$ is added to a maize meal immediately before it is eaten, it remains relatively unavailable for absorption even when large quantities of ascorbic acid are present.[29] Cane sugar has been examined as a fortification vehicle,[29] but it is also unsatisfactory since it is commonly taken in tea and coffee and any soluble iron present produces a black discoloration due to the precipitation of tannins.[32] Finally, ascorbic acid fortification is expensive when compared with iron fortification and this is an important consideration in developing countries.

Ascorbic Acid Supplementation

The recommended dietary allowance for ascorbic acid is currently set at 45 mg daily. However, it has been suggested that this quantity is not adequate for optimal health and that very large doses of ascorbic

acid are desirable. Intakes as high as 2-3 gm daily have been reported to be beneficial in reducing the frequency and severity of the common cold and possibly reducing the risk of cancer.[31]

The long term effects of such megadose quantities of ascorbic acid on iron balance in man are not known. In a limited survey among Americans living in the Northwestern United States, Cook and Monsen found ascorbic acid intakes ranging between 0 and 2,000 mg with a mean of 280 mg.[31] Based on studies with a semisynthetic meal containing no meat, they concluded that 280 mg ascorbic acid would produce a twofold increase in iron absorption if taken with breakfast and a threefold increase if taken in divided doses during the day.

These estimates probably represent the maximum possible effect of ascorbic acid and the actual increase in iron absorption may be considerably less for several reasons. Ascorbic acid is effective in promoting food iron absorption only if taken with the meal; some or all of the supplementary ascorbic acid may be taken separate from meals. Population groups eating large quantities of ascorbic acid are drawn more commonly from the affluent sections of society where the consumption of animal foods is relatively high. The estimates of the enhancing effect of ascorbic acid on iron absorptionwere based on a diet containing no meat or fish.[31] However, as indicated above, ascorbic acid may have a much smaller effect on percentage iron absorption from meals containing animal foods. The final consideration in estimating the potential hazards of megadose levels of ascorbic acid intake relates to the dose-response effect of ascorbic acid. From the relationship between iron absorption and the molar ratio of ascorbic acid to iron (Figure 2), 100 mg ascorbic acid may

represent a near maximal effect. If this is so, the estimated increase in absorption produced by a daily intake of 280 mg ascorbic acid in a meat-containing diet would be approximately 20% if the supplement is eaten with breakfast and slightly less than 40% if taken in three divided doses with each meal of the day. Support for this argument has been obtained in pilot studies which have shown remarkably little long-term effect on iron stores in volunteer subjects taking 2 gm ascorbic acid over 24 months.[38]

Summary

Food iron is absorbed by the intestinal mucosa from two separate pools of heme and nonheme iron. Heme iron derived from hemoglobin and myoglobin is well absorbed and relatively little affected by other foods eaten in the same meal. On the other hand, the absorption of nonheme iron, the major dietary pool, is greatly influenced by meal composition. Ascorbic acid is a powerful enhancer of nonheme iron absorption and can reverse the inhibiting effect of substances such as tea and calcium/phosphate. Its influence may be less pronounced in meals of high iron availability containing meat, fish, or poultry. The enhancement of iron absorption from vegetable meals is directly proportional to the quantity of ascorbic acid present. The absorption of soluble inorganic iron added to a meal increases in parallel with the absorption of nonheme iron but ascorbic acid has a much smaller effect on insoluble iron compounds such as ferric oxide or ferric hydroxide which are common food contaminants. Ascorbic acid facilitates iron absorption by forming a chelate with ferric iron at acid pH that remains soluble at the alkaline pH of the duodenum. High cost and instability during food storage are the major obstacles to using ascorbic acid in programs designed to combat nutritional iron deficiency anemia.

LEGENDS FOR FIGURES

Figure 1. Nonheme iron absorption from test meals tagged simultaneously with intrinsic and extrinsic radioiron labels (see text for explanation).

Figure 2. The effect of ascorbic acid on iron absorption from a semi-synthetic meal. Geometric mean percentage iron absorption is plotted against the molar ratio of ascorbic acid to iron in the meal. (From the data of Cook and Monsen.[31])

Figure 3. The effect of ascorbic acid on iron absorption from 30 mg elemental iron as ferrous sulphate. Percentage iron absorption is plotted against the molar ratio of ascorbic acid to iron. Since there were differences in basal absorption between groups of subjects receiving varying levels of ascorbic acid, the percentage absorption values plotted arethose predicted from the absorption ratio with/without ascorbic acid for each group and the geometric mean basal absorption without ascorbic acid of the first group of subjects receiving 50 mg ascorbic acid. (Data from Brise and Hallberg.[35])

Figure 4. Enhancing influence of ascorbic acid on vegetable nonheme iron absorption. Mean percentage iron absorption corrected for reference absorption is plotted against the molar ratio of ascorbic acid to iron in the meals. Solid circles represent maize meals and open circles, rice meals (see text for explanation).

Figure 5. Effect of pH on the solubility of $FeCl_3$ with and without ascorbate. Both $FeCl_3$ (interrupted line) and $FeCl_3$ to which an equimolar quantity of ascorbic acid had been added were relatively insoluble in NaOH. As the pH was reduced by adding HCl both became soluble. $FeCl_3$ was again precipitated by realkalization with NaOH while $FeCl_3$ with ascorbic acid remained soluble . (After Conrad and Schade.[37])

TABLE 1

DIETARY IRON AVAILABILITY*

Meal Iron Availability	Meat, Fish Poultry (g)		Ascorbic Acid (mg)	Nonheme Iron Absorption (%)
Low	<30	and	<25	3
Medium	30-90	or	25-75	5
High	>90	or	>75	8

*Based on data of Monsen et al[34]

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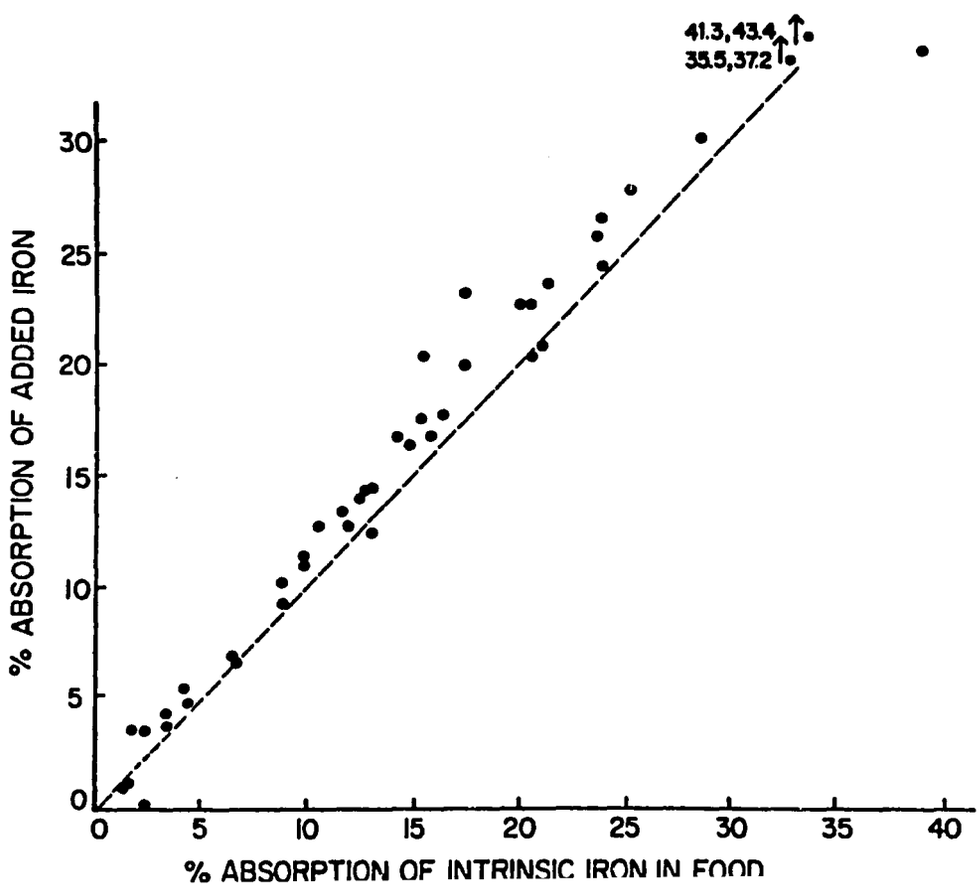
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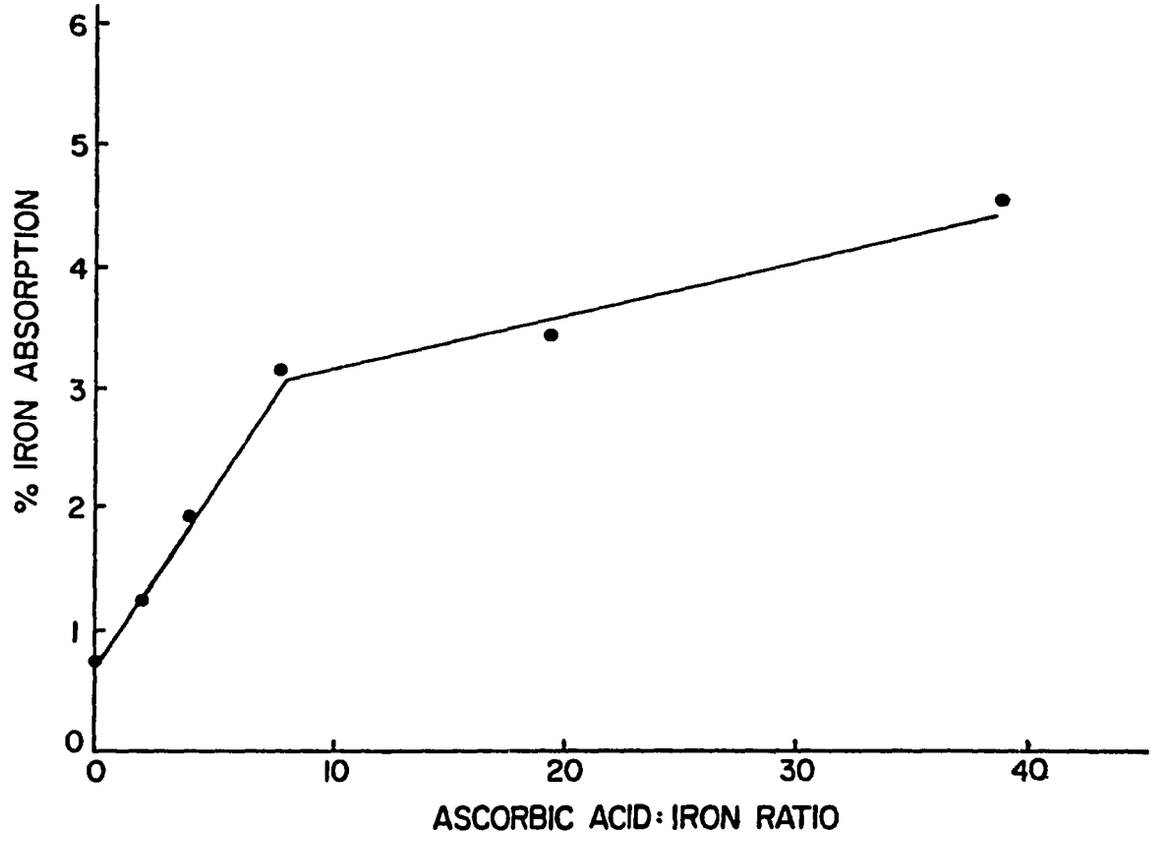
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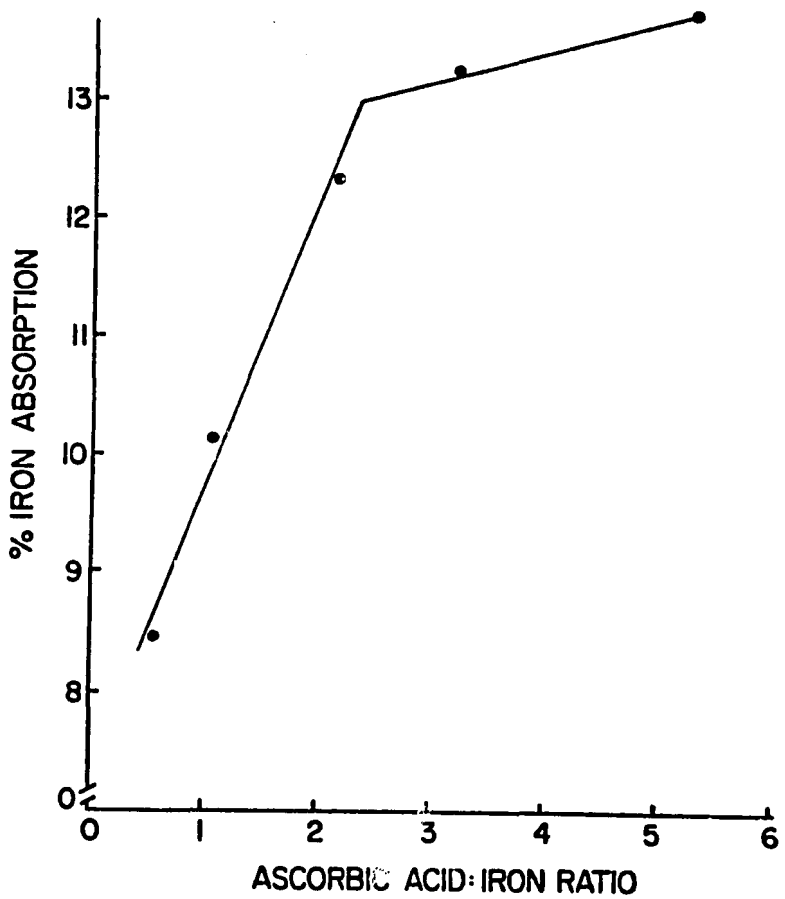
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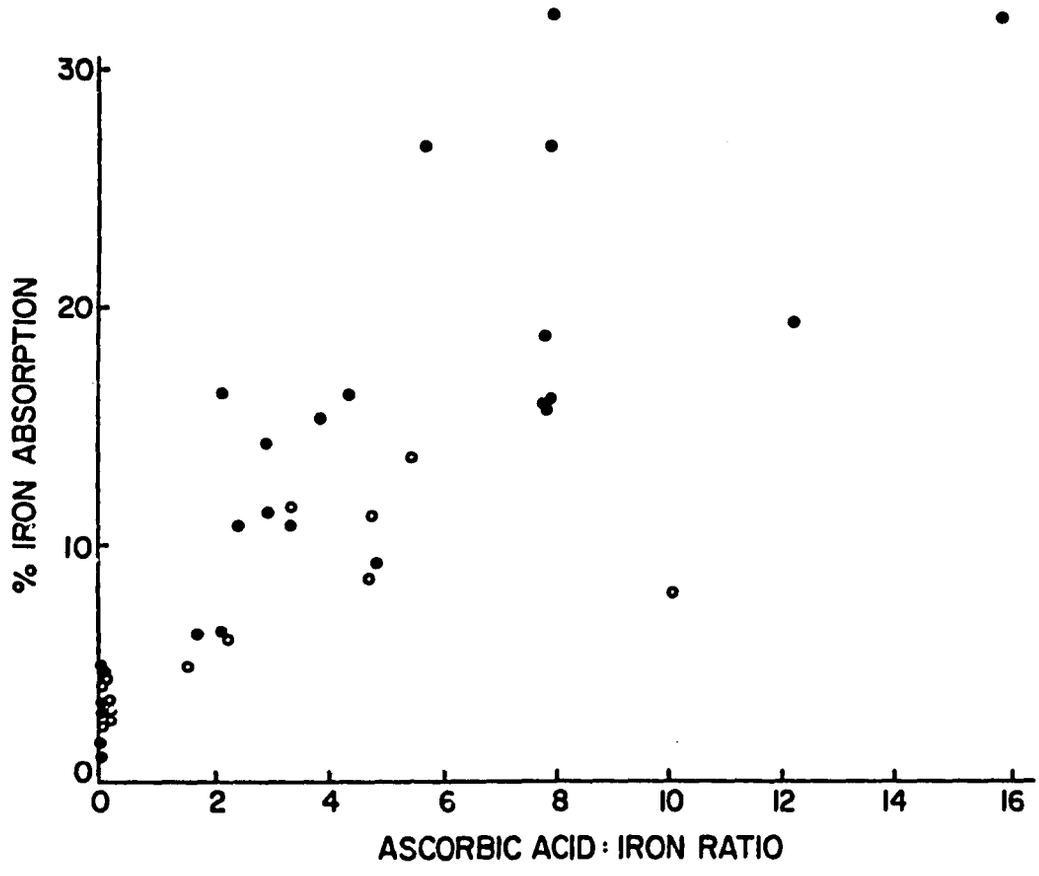
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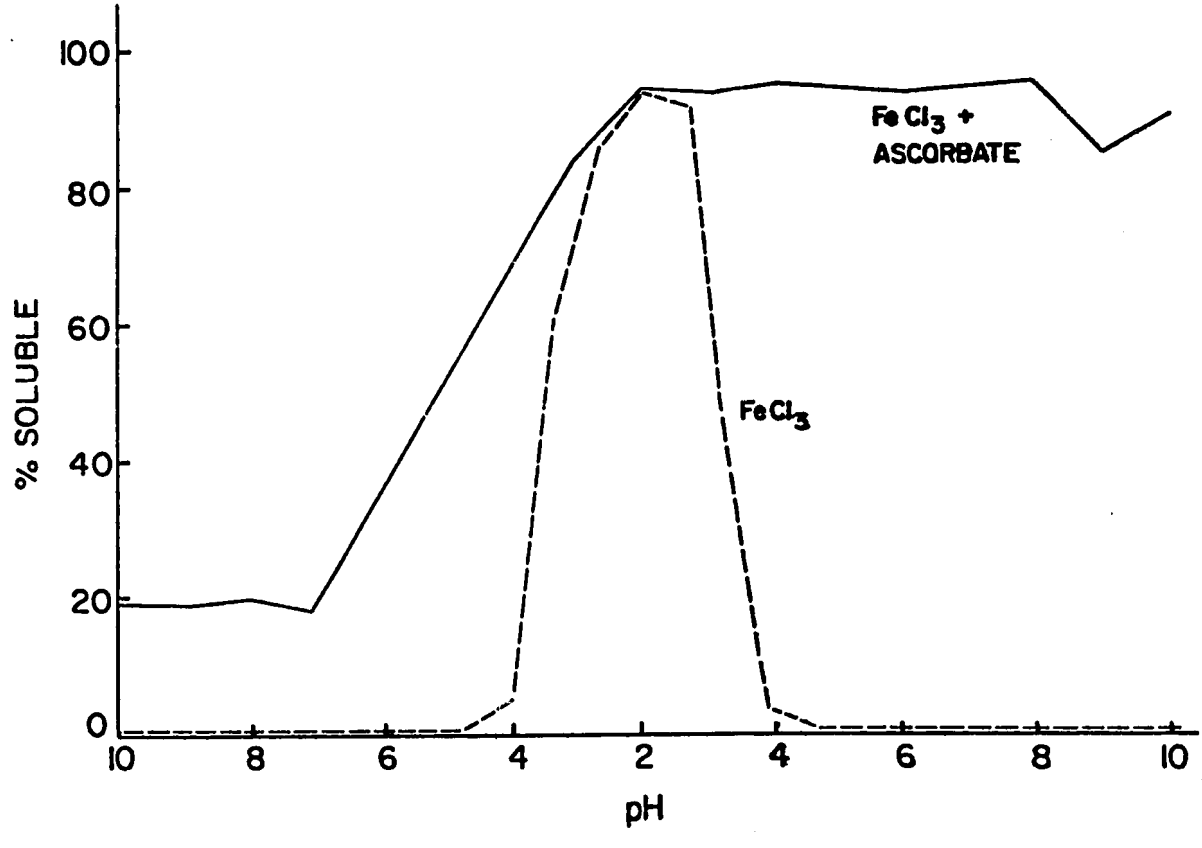
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THE IMPORTANCE OF ANIMAL PRODUCTS IN HUMAN IRON NUTRITION

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THE IMPORTANCE OF ANIMAL PRODUCTS IN HUMAN IRON NUTRITION

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I INTRODUCTION

Long before iron deficiency was recognized as the major cause of hypochromic anemia, many clinicians believed that a high intake of meat would produce a hematologic reponse. Increasing the intake of meat in the diet is not a satisfactory method of treating patients with iron deficiency anemia. Nevertheless, there is mounting evidence that the intake of animal foods is a major determinant of iron status. Recognition of the importance of animal protein in iron nutrition is based largely on the results of food iron absorption studies in human subjects. Because these measurements form the cornerstone of our present knowledge of iron balance, they will be reviewed before discussing the importance of animal foods in iron nutrition.

II MEASUREMENTS OF IRON ABSORPTION IN MAN

Early studies of food iron absorption were based largely on chemical balance measurements; absorption was calculated as the difference between iron intake and fecal iron loss. This approach proved to be unreliable because the small differences between iron intake and iron loss are often obscured by iron contamination. Most of our present knowledge of iron nutrition is based on isotopic measurements of iron absorption which have been used extensively since their introduction in the late 1940's.

A. Radioiron Absorption

There are several ways of determining percentage absorption of orally administered radioiron: fecal radioiron balance, plasma radioiron

tolerance curves, whole-body counting, and measurements based on incorporated red cell radioactivity. When assessing iron balance in patients with various clinical disorders, whole-body counting is the optimal method because the results are not affected by the distribution of absorbed radioactivity. However, when studying food iron absorption in normal individuals, measurements of red cell radioactivity are preferable because as many as four separate iron absorption tests can be performed in the same subject using dual isotopes of iron (^{55}Fe and ^{59}Fe). By calculating the ratio of absorption from different meals in the same subject, the effect of differences in iron status between subjects can be eliminated. Moreover, any errors in estimating blood volume from height and weight or assuming that all subjects incorporate the same proportion of absorbed radioactivity into circulating red cells are eliminated by calculating the ratio of iron absorption in a series of tests in the same subject.

One drawback in food iron absorption studies is that percentage absorption of iron may vary markedly from study to study depending on the iron status of the subjects (Cook et al., 1969). This problem can be circumvented by measuring absorption in all subjects from a standard reference dose consisting of 3 mg inorganic iron as ferrous sulfate. By relating food iron absorption to reference dose absorption in each subject, the effect of differences in iron status of the subjects can be eliminated.

B. Biosynthetic Tagging

The most important aspect of food iron absorption is not the method

for determining isotopic retention but rather the technic used for tagging dietary iron. Early studies were performed with single food items that had been biosynthetically labeled with radioiron (Moore, 1964). Vegetable foods were labeled by growing them in hydroponic media containing radioiron and animal foods were tagged by injecting radioiron intravenously several months before sacrifice. Studies with these foods provided the first evidence that iron bioavailability of animal foods is much higher than vegetable foods (Layrisse et al., 1969; Martinez-Torres and Layrisse, 1974). Thus, iron absorption from staple foods (maize, black beans, and wheat) averaged 3-5% while absorption from animal foods, (meat, liver, and fish), ranged from 15-30%.

A major limitation of studies with biosynthetically labeled foods became apparent when two foods tagged separately with ^{59}Fe and ^{55}Fe were administered in the same meal. It was found that iron absorption of one food item could be markedly affected by the presence of a second food. For example, when labeled meat was administered with biosynthetically tagged maize, absorption of meat iron was diminished while absorption of maize iron was greatly enhanced (Martinez-Torres and Layrisse, 1971). It was obvious from these mixing studies that data obtained with biosynthetically tagged foods are of limited importance because of the complexity of the average diet. It was necessary to develop a technic to measure absorption from a complete meal.

C. Dietary Nonheme Iron

A major advance in studies of food iron absorption was the development in the early 1970's of extrinsic tagging to measure absorption of dietary

heme and nonheme iron. This approach was based on the observation that if a small quantity of inorganic radioiron is simply added to a vegetal food at the time of its administration, absorption is nearly identical to that of a biosynthetic, or intrinsic, tag. Moreover, if a meal containing several vegetal foods is labeled extrinsically after thorough homogenization, absorption of the extrinsic tag is nearly identical to absorption of a food item in the meal that has been biosynthetically labeled (Cook et al., 1972). These observations provide convincing evidence that a common pool of absorbable nonheme iron is formed with foods ingested in the same meal and that iron absorption from this pool can be measured by extrinsic tagging.

Although most studies have shown that extrinsic labeling is a valid means of measuring nonheme iron absorption, there have been a few exceptions. When biosynthetically tagged whole grain rice was tagged extrinsically with a second form of radioiron, absorption of the two labeled foods differed markedly whereas this difference was not seen when rice was administered as flour; apparently the extrinsic tag does not permeate the whole rice kernel (Björn-Rasmussen et al., 1973). Some forms of fortification iron, such as ferric orthophosphate and sodium iron pyrophosphate, do not exchange completely with dietary iron because of their high degree of insolubility (Cook et al., 1973) and it is likely that iron contamination of food by dirt and/or food handling processes also results in an insoluble form of dietary iron that does not exchange with native food iron. Iron in ferritin and hemosiderin have been shown to exchange incompletely with an extrinsic tag, presumably because the protein coat of these storage iron proteins inhibits complete isotopic exchange. (Layrisse et al., 1975; Martinez-Torres et al., 1976). Nevertheless,

with these few exceptions, extrinsic tagging has proven to be a valid method of measuring dietary nonheme iron absorption.

D. Dietary Heme Iron

It is well known that iron enters the mucosal cell from the intestinal lumen not only as ionic iron but also as an intact porphyrin complex. This heme iron is rapidly degraded within the mucosal cell by heme oxygenase and the liberated iron enters the same pathway as absorbed ionic iron. Thus, it is either transported to the serosal surface where it enters the circulation or it is incorporated into ferritin as storage iron within the mucosal cell. It is important to distinguish between heme and nonheme iron because they are affected quite differently by the composition of the meal. Absorption of nonheme iron is markedly affected by substances that enhance iron absorption, such as ascorbic acid, and by inhibitors that bind nonheme iron and render it unavailable to the mucosal cell, such as tea or fiber. The absorption of heme iron, on the other hand, is little affected by the composition of the meal because this iron remains sequestered within the porphyrin complex until it enters the mucosal cell.

The same principle of extrinsic tagging for measurement of nonheme iron absorption can also be used to measure heme iron absorption; heme iron is labeled by adding a small quantity of radioactive hemoglobin to the test meal. Because there are two separate isotopes of iron available, ^{55}Fe and ^{59}Fe, it is possible to separately tag heme and nonheme iron in the same meal and thereby provide a measure of total iron absorption from a complete meal. This two-pool model is particularly relevant in studies of animal foods since animal tissue contains both forms of iron.

The validity of the two-pool model for studying iron absorption has been evaluated by comparing iron absorption measurements with predicted values of absorption based on measurements of body iron loss. In one study, a meal composed of aliquots of all foods contained in a typical 6-week diet was tagged with heme and nonheme radioiron and administered to 32 normal male subjects (Björn-Rasmussen et al., 1974). Dietary iron intake based on this composite meal was estimated at 17.4 mg iron daily of which 1 mg was in the form of heme iron. Absorption of nonheme iron averaged 5.3% or 0.8 mg/day while absorption of heme iron averaged 37% or 0.37 mg/day. The combined absorption of 1.2 mg iron daily is in close agreement with estimates of iron loss in normal men. Similar absorption data have been obtained by Layrisse and Martinez-Torres (1972) who studied a meal of meat, black beans, maize, and rice containing a total of 4.5 mg iron of which 1.5 mg was in the form of heme. In normal subjects, absorption of heme iron averaged 27% or 0.34 mg as compared with a value for nonheme iron of 6% or 0.12 mg; total absorption was 0.46 mg iron. In iron deficient subjects, absorption of heme and nonheme iron increased to 37% (0.52 mg) and 14% (0.43 mg), respectively, to give a total absorption of 0.95 mg. These two studies not only validate double extrinsic tagging, but also underscore the importance of heme as a source of dietary iron.

III BIOAVAILABILITY OF IRON IN ANIMAL FOODS

A. Meat

The use of biosynthetically tagged animal products in human iron absorption studies provided the first clear evidence of the beneficial

effect of these foods on iron balance. Early studies were performed with chicken (Moore, 1964) and rabbit muscle (Heinrich et al., 1969), but the most extensive observations have been made using veal (Layrisse et al., 1969). Radioactive iron was injected into the jugular veins of 3-month old calves; two to four months later, the animals were sacrificed, and the biosynthetically labeled muscle and liver were removed and kept frozen until administered in test meals to human subjects.

Veal muscle was cooked and served as hamburger to 107 fasting adult subjects, many of whom were iron deficient (Martinez-Torres and Layrisse, 1971). Geometric mean absorption in the composite group was 21.5%. In 58 of these subjects, absorption was also measured from a reference dose of ferrous sulfate; absorption averaged 23.8% giving a mean absorption ratio veal:reference of 1.12. The high bioavailability of veal muscle in this study was due at least in part to the high content of heme iron. Eighty-eight to ninety-three percent of the radioactivity was recovered in the supernatant of thoroughly hemogenized meat and 93-97% of this radioactivity was present as hemoglobin or myoglobin. Further evidence of the high heme iron content of veal was that substances that are known to alter the absorption of nonheme iron had little effect on the absorption of iron from veal muscle. For example, when 500 mg ascorbic acid was added to veal muscle, a slight increase (16%) was observed but this rise was not statistically significant. Similarly, the addition of 500 mg desferrioxamine to the labeled food produced a relatively modest decrease (27%) in absorption. These results are in striking contrast to the profound effect that ascorbic acid and desferrioxamine have on the absorption of nonheme iron.

B. Liver

Similar studies were performed with labeled veal liver (Martinez-Torres et al., 1974). When cooked veal liver was fed to 74 adult subjects, absorption averaged 19.8% with a typically broad range of 2.8 to 55.4%. In 27 of these subjects, reference dose absorption was measured simultaneously and averaged 16.8%; the liver:reference absorption ratio averaged 1.00. Analysis of the labeled veal showed that 78% of the radioactivity was in the form of hemoglobin while the remaining 22% was contained in either hemosiderin or ferritin. As with veal muscle, the addition of compounds such as ascorbic acid or desferrioxamine, which markedly influence nonheme iron absorption, had little or no effect on the assimilation of labeled liver. When 500 mg ascorbic acid was added to the labeled liver, iron absorption in 10 subjects actually decreased from a mean of 15.3 to 13.8%. When 500 mg desferrioxamine was added to the same food, mean absorption decreased from 20.6 to 8.6%. Although this difference was statistically significant, the effect was far less striking than the effect of this chelate on nonheme iron absorption.

C. Other Foods

Apart from veal muscle and liver, fish is the only other food that has been studied extensively by biosynthetic labeling (Martinez-Torres and Layrisse, 1970). Radiolabeled fish muscle was fed to 19 adult subjects who absorbed a mean of 18.3%; reference dose absorption averaged 44.8%. The iron in fish muscle is apparently less available than the iron in either veal muscle or liver. Iron absorption from eggs has also been studied by biosynthetic labeling (Moore, 1964; Chodas et al., 1957; Callendar et al., 1970). Mean absorption from iron in tagged eggs

was consistently less than 5%; this low iron availability is in striking contrast to results obtained with animal tissues.

IV EFFECT OF ANIMAL PROTEIN ON FOOD IRON ABSORPTION

A. Effect on Nonheme Iron Absorption

Animal foods are an important constituent of our diet, not only because of high intrinsic iron availability but also because of their ability to increase the absorption of all nonheme iron ingested simultaneously. This enhancing effect was first demonstrated in a series of studies by Layrisse et al., (1968). Vegetable and animal foods biosynthetically tagged with different radioiron labels were fed both separately and simultaneously to volunteer subjects to determine the extent to which the absorption of one food was affected by the other. Because both foods were tagged with separate radioiron labels, it was possible to detect any increase in iron absorption from the staple food as well as any decrease from the animal food.

Representative mixing studies with biosynthetically tagged foods are listed in Table I. Maize or black beans were used as the staple food and veal muscle, veal liver, or fish as the animal food. In the first study iron absorption from a meal containing only maize (4 mg iron) averaged 4.1% but when maize (1 mg iron) was fed together with veal muscle (3 mg iron), absorption of maize iron increased to 7.6%; the absorption ratio with:without animal protein averaged 1.87, an effect that was highly significant. Indeed, in all six studies shown in Table I, the increase in iron absorption from staple foods when taken with

veal muscle (3 mg iron), veal liver (2 mg iron), or fish (1 mg iron) was highly significant and veal liver was at least as effective as veal muscle.

While these studies identify the enhancing effect of animal tissue on nonheme iron absorption, it is difficult to estimate their quantitative effect on iron absorption from a normal diet. In order to obtain a constant total iron content in all meals in the studies listed in Table I, a smaller quantity of the staple food was given with animal tissue than when it was given alone. Since the quantity of food in a meal may have a more important effect on absorption than its iron content, it may be preferable to make substitutions on the basis of protein or caloric content rather than on iron content.

A final point in these mixing studies is the relatively minor effect of maize and black beans on the absorption of iron from veal muscle, veal liver, or fish. For example, absorption of veal muscle fell by only 14% and 17% when eaten with maize and black beans, respectively. Absorption of veal liver was reduced by 25 and 44% when consumed with 1 and 2 mg maize iron, respectively. The enhancing effect of animal tissue is much greater than the inhibitory effect of vegetal foods.

In a more recent study, several animal foods were evaluated with regard to their effect on nonheme iron absorption by substituting them in two types of test meals (Cook and Monsen, 1975). The first, considered to be a <u>standard</u> meal, was a meal containing beef, potatoes, corn, bread,

margarine, ice milk, and peaches. This meal contained 696 calories and 4.1 mg total iron; beef provided most of the protein. The second was a semisynthetic meal containing semipurified ingredients in which ovalbumin was the only source of protein. This meal was designed to match exactly the composition of the standard meal in respect to food energy, fat, protein, carbohydrate, calcium, phosphorus, and iron. Several animal foods were substituted in both meals including pork, lamb, beef, chicken, fish, whole milk, cheese, and powdered egg. These foods were substituted for beef in the standard meal and for ovalbumin in the semisynthetic meal in amounts that would provide the same quantity of protein. When the animal foods were substituted for beef in the standard meal, no adjustments were made for differences in nutrient content. With the semisynthetic meal, however, the other constituents were varied so that the substituted meal had the same total nutrient content as the original meals. Four iron absorption test meals were given to each volunteer subject: standard, semisynthetic, substituted standard, and substituted semisynthetic meals.

The results of the study are summarized in Table II. When pork, lamb, liver, chicken, and fish were substituted for beef in the standard meal, absorption ratios (substitute:beef) ranged from 0.82-1.03; in no case did the ratio reflect a significant difference. However, with dairy products, which contain no animal tissue, absorption ratios ranged from 0.22-0.39, all of which were statistically significant. Comparable results were obtained with the semisynthetic meal. Thus, when animal tissue was substituted for ovalbumin, the increase in absorption ranged from 2.11 to 3.77. Some enhancement was also observed with chicken

and fish (2.43 and 2.11, respectively), although the increase was less pronounced than with other animal tissues. On the other hand, milk, cheese, and eggs were all similar to ovalbumin in their effect on the absorption of nonheme iron; ratios ranged from 0.88 to 1.22. These results indicate that dairy products have little or no enhancing effect on the absorption of dietary iron, and should not be considered as animal products with respect to food iron availability.

B. Effect on Ferritin and Hemosiderin Iron Absorption

Nonheme iron in animal tissue is comprised of ferritin and hemosiderin, the two major forms of storage iron in tissue. Nonheme iron in animal foods averages between 20 and 60% depending in part on the body organ; muscle, for example, has a much higher nonheme iron content than liver. Early studies in which purified ^{59}Fe labeled ferritin alone was administered to human subjects indicated an absorption of about 7% in normal subjects, values that were distinctly lower than absorption from other animal foods (Hussain et al., 1965). More extensive studies with radiolabeled ferritin and hemosiderin were reported subsequently by Layrisse et al., (1975); and Martinez-Torres, et al., (1976). Iron absorption of purified ferritin in 108 subjects ranged from 0.1 to 13.5% with a geometric mean of 1.9%, a much lower absorption than was observed in earlier studies. When purified ferritin was administered with biosynthetically tagged maize, iron absorption from maize was actually higher than from ferritin (2.0 and 0.9%, respectively); similar results were obtained with wheat and soybeans. However, when purified labeled ferritin was fed with animal tissue, a striking increase in absorption occurred. Absorption

increased from 1.3 to 12% when 2 mg ferritin iron was administered with veal muscle containing the same quantity of iron, and in a second study using veal liver, absorption increased from 1.7 to 6.6%. These absorption levels were comparable to those observed in meals containing both vegetable and animal foods. Since low absorption of ferritin iron may be an artifact related to purification of the protein, it seems unnecessary to regard ferritin and hemosiderin as separate pools of dietary iron.

C. Effect on Heme Iron Absorption

Animal tissue consists predominantly of heme iron and therefore is little affected by factors such as ascorbic acid or desferrioxamine which have a profound effect on nonheme absorption. It would seem unlikely that other foods would have any effect on the absorption of purified hemoglobin. Surprisingly, however, iron absorption from labeled hemoglobin is much less when the hemoglobin is given alone than when given with meat. Martinez-Torres and Layrisse (1971) observed an increase in absorption from 12 to 22% when labeled hemoglobin was mixed with meat prior to administration and this finding was recently confirmed by Hallberg et al., (1979). The enhancing effect of meat on hemoglobin iron absorption is more important from a physiologic than from a nutritional standpoint because, with the exception of heme iron fortification, dietary heme iron is consumed as a component of animal tissue.

D. Mechanism of Meat Effect

One of the vexing questions in iron nutrition is what mechanism accounts for the enhancing effect of meat and liver on nonheme iron absorption. Some inferences can be drawn from studies of the enhancing

effect of ascorbic acid, the only other dietary substance that is known to be as effective as meat in increasing iron absorption. There is evidence that the enhancing effects of meat and ascorbic acid are not additive. When 100 mg ascorbic acid was added to a semisynthetic meal (Cook and Monsen, 1977), the absorption ratio with:without ascorbic acid was 3.19. When the same quantity of vitamin C was added to a standard meal, a lower ratio of 1.67 was observed. In another study, the addition of 100 mg fish to a meal containing fresh papaya (70 mg ascorbic acid), produced no further enhancement in iron absorption (Layrisse et al., 1974).

A series of iron absorption studies designed to identify the effect of animal protein on iron absorption were recently reported by Björn-Rasmussen and Hallberg (1979). The possibility that meat acts by stimulating gastric secretion was ruled out when it was shown that meat increased iron absorption of maize from 0.7 to 2.1% in patients with histamine-fast achlorhydria. Furthermore, when betazole, a drug that stimulates gastric secretion, was administered to normal subjects, absorption did not increase significantly. A modest increase in absorption was observed when a mixture of amino acids in amounts corresponding to that contained in beef muscle was added to maize, but contrary to previous observations (Martinez-Torres and Layrisse, 1970), no increase was observed when cystine alone was added to maize. When beef was fractionated into a water extract (broth) and an insoluble residue and fed with a meal of maize, the residue increased iron absorption from 1.4 to 2.4% whereas the broth had no enhancing effect; the meat factor therefore does not appear to be water soluble.

The possibility that meat acts by stimulating mucosal uptake, or iron transport, would explain the similar enhancing effect on nonheme iron, ferritin iron, and heme iron. However, a mucosal cell effect should also exist with inorganic iron and meat does not increase the absorption of ferrous chloride (Layrisse and Martinez-Torres, 1972). Meat might act by stimulating gastric secretion or altering gastrointestinal motility in favor of iron absorption but it seems more likely that animal tissue promotes the solubility of both heme and nonheme iron, presumably by separate biochemical mechanisms. Whether a "meat factor" is present in meat when administered or only formed during digestion of the food in the gastrointestinal tract is still a matter of speculation.

V EFFECT OF INTAKE OF ANIMAL FOODS ON IRON STATUS

A. Estimates of Food Iron Availability

Little or no association has been found in various nutritional surveys between total iron intake and iron status. This may indicate that blood loss due to menstruation or pregnancy are more important determinants of iron nutrition than is the diet. Another possible explanation is that the total intake of dietary iron is less important nutritionally than the type of diet consumed. A model has recently been developed for estimating food iron availability and predicting absorption from the composition of the meal (Monsen et al., 1978). It is of interest to review this model since the content of animal food is an important parameter in estimating iron availability.

The first step in estimating iron absorption from a given meal is to calculate the total content of heme and nonheme iron. It is assumed for this purpose that pork, liver, fish, beef, lamb, and chicken all

contain 40% heme iron although, as noted earlier, this percentage may vary appreciably. Because heme iron absorption is not affected by the nature of the meal, iron absorption from heme is calculated by multiplying heme iron content by 25%, the estimated level of percentage absorption of heme iron in an individual with 500 mg body iron stores.

Estimating nonheme iron absorption from a meal is more complex because of food interactions. Two enhancing substances in the diet were considered to have overriding importance: ascorbic acid and animal foods including fish and poultry. One gram animal food was assumed to be roughly equivalent in its enhancing effect to 1 mg ascorbic acid. Meals were divided on this basis into those of low, medium, and high iron availability (Table III). Absorption of nonheme iron from these meals was estimated at 3, 5, and 8%, respectively, in a subject with 500 mg iron stores.

This model for estimating absorption of dietary iron is undoubtedly oversimplified and is a preliminary step at best in determining the importance of the diet in iron nutrition. Other dietary factors that are known to influence iron absorption, such as tea or EDTA, were not included in the estimates because precise quantitative data on their effect is not available. It is also not clear what factor should be used when the meal contains both ascorbic acid and animal food. Even with these limitations, however, this model will come much closer to determining the importance of diet in maintaining iron balance.

B. Epidemiologic Studies

Many attempts have been made to establish a correlation between iron intake and iron status of a population, but the correlation has been poor when based on total iron intake without regard to food iron availability. However, a recent study of 7,000 people in Finland showed that the intake of meat products does indeed correlate with iron status (Tukkanen and Seppänen, 1975). The intake of animal products was found to be significantly lower in both male and female anemic subjects as compared with those having a normal hemoglobin level. A correlation was also observed in this study between meat consumption and iron status as measured by either the serum iron or total iron-binding capacity (Figure 1). A much clearer picture of the importance of meat products should emerge when the model described above is used in future studies of dietary iron intake and its relation to iron status.

VI IMPORTANCE OF ANIMAL FOOD IN IRON FORTIFICATION

A. Nonheme Iron Fortification

The only practical long-term approach for combating iron deficiency anemia in a population is to increase dietary iron intake by fortifying with iron a common item in the diet such as bread, sugar, or rice. However, recent studies suggest that the success of iron fortification will largely depend on the intake of meat, fish, and poultry, and that without these animal foods, even heavy fortification is unlikely to be effective. For example, Layrisse et al., (1973) added increasing amounts of ferric chloride to meals containing either maize (1 mg iron), meat alone (3 mg iron), or a mixture the two foods in the ratio 2 mg meat iron:1 mg maize iron. Ferric chloride was chosen as the iron salt

because it undergoes complete exchange with dietary nonheme iron. Absorption values when 5, 20, and 60 mg iron were added are shown in Figure 2. When 60 mg iron, a level far above that which would be technically feasible on a national scale, was added to the maize meal, only 0.3 mg iron was absorbed. When this same amount of iron was added to a meal containing either meat alone or a mixture of meat and maize, an average of 3.27 mg and 1.22 mg, respectively, was absorbed.

Similar findings were observed in a later study by Hallberg et al., (1978). When 5 mg fortification iron as ferrous sulfate was added to a rice-based meal containing only vegetables, absorption in an iron-deficient individual was estimated at 0.03 mg iron. When 60 gm fish was added to the same meal, net absorption of fortification iron increased to 0.32 mg. The authors concluded that iron fortification may be feasible with a rice-based diet, but that a certain minimal threshhold level of fortification iron would be required to achieve a reasonable level of absorption. The relatively high level of 5 mg fortification iron per meal was considered realistic even with diets containing only negligible amounts of fish or animal foods.

B. Heme Iron Fortification

The low bioavailability of inorganic iron added to meals containing little or no animal products has prompted the suggestion that heme iron in animal blood recovered from the slaughterhouse could be employed for iron fortification (Reizenstein, 1975). Hemoglobin iron has the advantage that it is less inhibited by dietary substances that impair the absorption of nonheme iron. The dose response curve also may be more favorable with hemoglobin iron than with inorganic iron, at least in patients with

latent iron deficiency. Vehicles that have been suggested for heme iron fortification include blood sausage, pudding, meat products, and baking products.

The major limitation of fortification with animal blood is the risk of iron overload in iron replete individuals. Because heme iron absorption is less affected by iron status than nonheme iron absorption, heme iron might be well absorbed in iron replete individuals which could conceivably create a risk of iron excess in susceptible segments of the population. In addition, the dark color of blood greatly limits the number of food items that could serve as vehicles for fortification. The costs of recovering hemoglobin and processing it to avoid bacterial contamination are undoubtedly high. It is therefore unlikely that slaughterhouse blood will be extensively used for iron fortification.

VII SUMMARY

Studies employing double extrinsic tags of heme and nonheme iron to measure absorption of dietary iron have clearly established the importance of animal foods in iron nutrition. The major factor that accounts for high assimilation of iron from animal foods is their content of myoglobin and hemoglobin. These iron complexes enter the mucosal cell of the intestinal tract as intact molecules and therefore are little affected by dietary substances that impair the absorption of nonheme iron. Animal foods also play an essential role in iron nutrition because of their ability to enhance assimilation of all forms of dietary nonheme iron ingested simultaneously. It is unlikely that iron fortification programs would be effective in populations consuming only minimal quantitites of meat, fish, or poultry.

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Table I. Effect of animal tissue on the absorption of nonheme iron from a staple food.

Animal Food	Staple Food	No. of Subjects	Iron Content Of Staple Food (mg)		Iron Absorption From Staple Food (% of dose)		Mean Absorption Ratio (with:without animal food)
			Fed Alone	Fed With Animal Food	Fed Alone	Fed With Animal Food	
Veal Muscle[a] (3mg iron)							
	Maize	15	4	1	4.1	7.6	1.87
	Black beans	11	4	1	4.8	10.7	2.21
Veal Liver[b] (2mg iron)							
	Maize	12	2	2	4.9	10.9	2.22
	Maize	14	3	1	2.2	7.0	3.17
Fish[c] (1mg iron)							
	Maize	6	3	2	4.2	7.5	1.78
	Black beans	8	3	2	1.0	1.7	1.70

[a] Martinez-Torres and Layrisse, 1971
[b] Martinez-Torres *et al.*, 1974
[c] Layrisse *et al.*, 1968

TABLE II. Effect on nonheme iron absorption of substituting various animal products in meals containing either albumin (semisynthetic) or beef (standard) as the source of protein.†

SUBSTITUTE		SEMISYNTHETIC MEAL: Mean Iron Absorption ALB (% of dose)	SEMISYNTHETIC MEAL: Mean Iron Absorption SUB (% of dose)	SEMISYNTHETIC MEAL: Absorption Ratio (SUB:ALB)	STANDARD MEAL: Mean Iron Absorption BEEF (% of dose)	STANDARD MEAL: Mean Iron Absorption SUB (% of dose)	STANDARD MEAL: Absorption Ratio (SUB:BEEF)
Beef	(7)*	1.7	5.1^{+++}	2.96^{a}	--	--	--
Pork	(7)	1.6	5.2^{+++}	3.21^{a}	9.7	9.3	0.95^{a}
Lamb	(7)	1.6	5.2^{+++}	3.23^{a}	7.6	7.8	1.03^{a}
Liver	(8)	1.4	5.4^{+++}	3.77^{a}	3.8	3.9	1.01^{a}
Chicken	(7)	1.4	3.4^{++}	2.43^{ac}	11.5	10.4	0.90^{a}
Fish	(8)	1.9	3.9^{+}	2.11^{ac}	14.8	12.2^{+}	0.82^{a}
Milk	(8)	0.7	0.9	1.22^{bc}	5.5	1.6^{++}	0.29^{b}
Cheese	(8)	2.0	2.4	1.20^{bc}	9.4	3.6^{++}	0.38^{b}
Egg	(10)	0.8	0.7	0.88^{b}	8.2	1.8^{++}	0.22^{b}
Albumin	(7)	--	--	--	10.0	3.9^{++}	0.39^{b}

† Cook and Monsen, 1976

* number of subjects

$^{+}$ significant difference, $P < .05$; $^{++}$ significant difference, $P < .01$; $^{+++}$ significant difference, $P < .001$.

^{abc} mean ratios with differing superscripts are significantly different by Duncan's multiple range test.

TABLE III. Dietary Iron Availability.†

Meal Iron Availability	Meat, Fish, Poultry (gm)		Ascorbic Acid (mg)	Nonheme Iron Absorption (%)
Low	<30	and	<25	3
Medium	30-90	or	25-75	5
High	>90	or	>75	8

† Monsen *et al.*, 1978

FIGURE LEGENDS

Figure 1. Relationship between percent of population with TIBC greater than 400 ug/dl and daily intakes of meat in women (○) and men (●). (Takkunen and Seppänen, 1975)

Figure 2. Absorption of fortification iron (ferric chloride) added to meals containing maize alone (100 gm), meat alone (150 gm), and maize plus meat (50 and 100 gm, respectively). The enhancing effect of meat on nonheme iron absorption is apparent. (Layrisse et al., 1973)

Figure 1

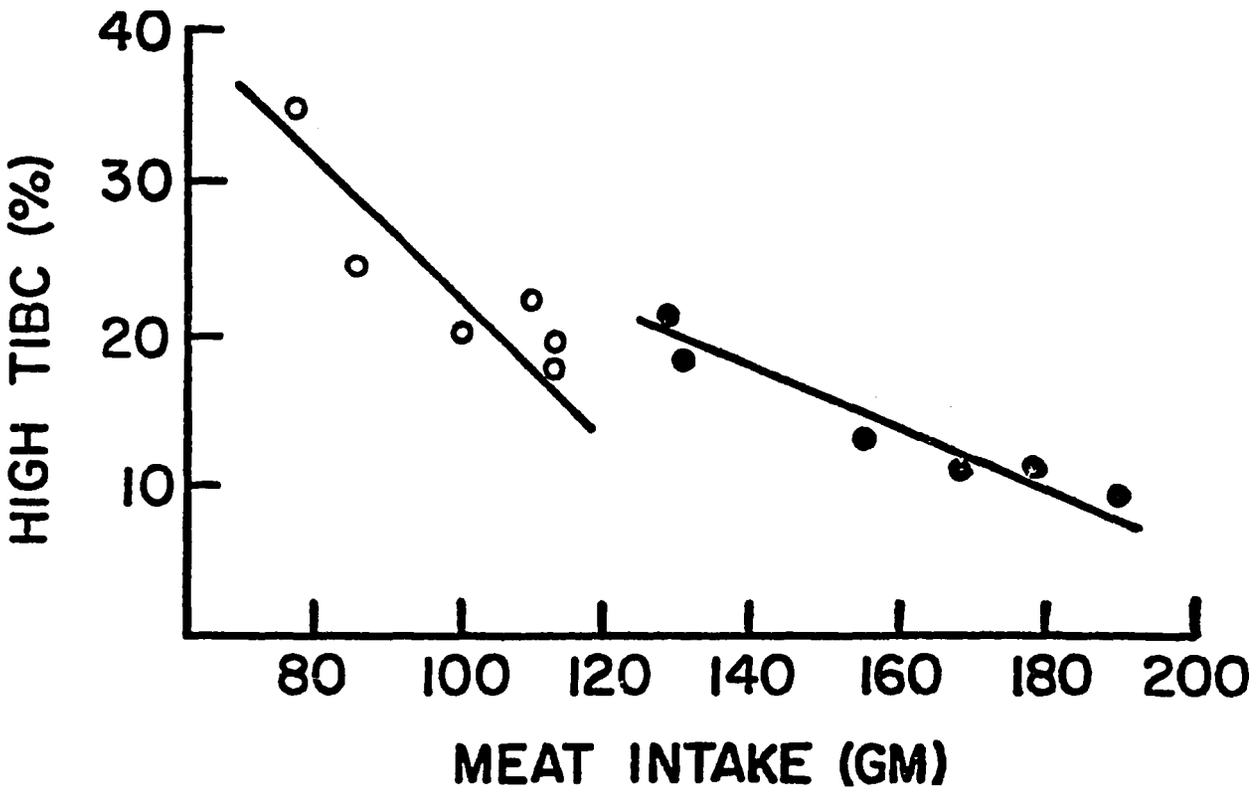
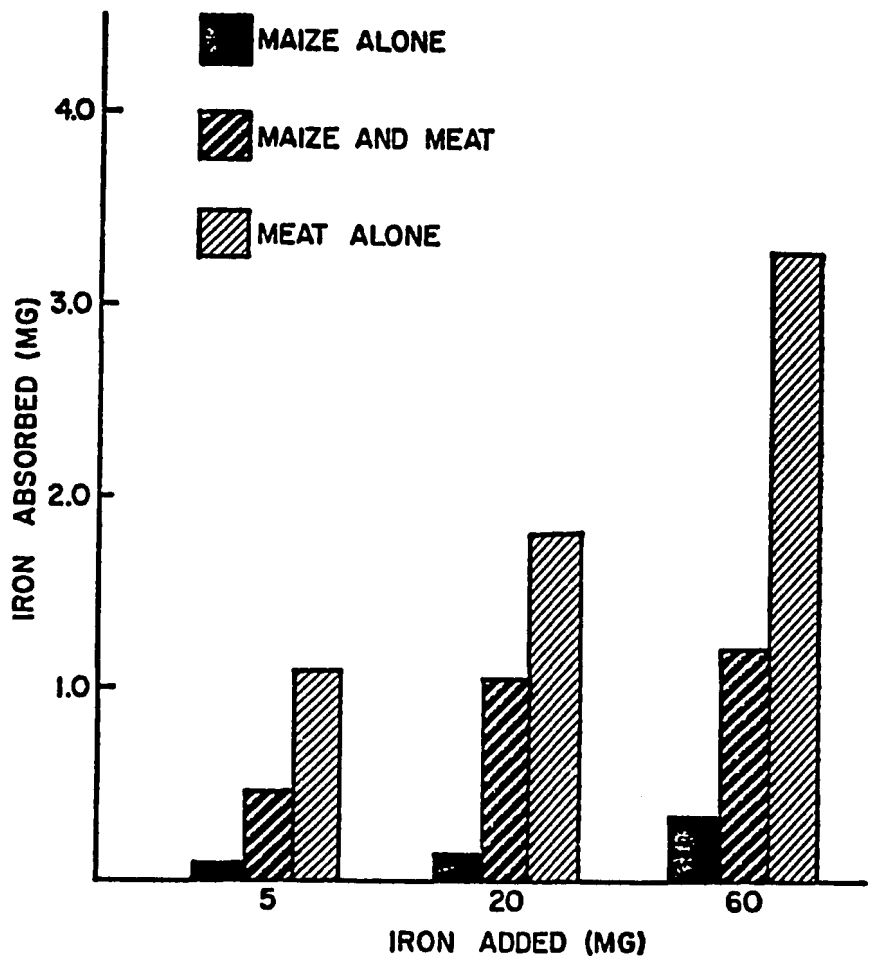


Figure 2


MAIZE ALONE
MAIZE AND MEAT
MEAT ALONE
4.0
3.0
2.0
1.0
IRON ABSORBED (MG)
5
20
60
IRON ADDED (MG)

APPENDIX C

LDC COLLABORATIONS

MANILA VISIT--March 8-15, 1980

The following is a summary of dicussions held at the Nutrition Center of the Philippines (NCP) and the Food and Nutrition Research Institute (FNRI) during the week of March 9th. The key individuals who were present during most if not all of these discussions were as follows:

Dr. Rodolfo Florentino	-	NCP (Deputy Director)
Dr. R. W. Engel	-	USAID, Philippines
Dr. J. D. Cook	-	K.U. Medical Center
Dr. S. R. Lynch	-	K.U. Medical Center
Dr. Fred Barrett	-	USDA
Romualdo M. Guirriec	-	NCP
Miriam Kuizon	-	FNRI
Lilly Lansangan	-	PAEC
Anita Marzan	-	PAEC
Benigna Roxas	-	FNRI
Trinidad Trinidad	-	FNRI

Our efforts during this visit were directed to two main research projects. The first is an extensive iron supplementation study in pregnant women and infants. This research project will require approximately 4 years for completion and will be jointly funded by WHO and the USAID mission. The second project concerned iron fortification and was therefore related more directly to our current USAID contract "Development of Strategies to Combat Iron Deficiency". Approximately 2 days each were devoted to the supplementation and fortification projects. During discussion of the iron supplementation study, Dr. Barrett made a tour of

facilities that might assist in developing a program of iron fortification with various vehicles. An extensive discussion on iron fortification was held during the first day of our meeting; progress during the week was reviewed and summarized on the final day. Because these programs involve quite different strategies and personnel, they will be reviewed separately.

IRON FORTIFICATION

The first day was spent reviewing six separate iron-related research projects, one of which is iron fortification. Since the last meeting in the Philippines a year ago, Anita Marzan has completed a very extensive and careful review of data on the various food vehicles that might be used for fortification. Those considered include rice, sugar, salt, MSG, corn, wheat, fish sauce (patis), and fish paste (bagoong). The tables prepared by Marzan for this meeting contain detailed information on these vehicles including production costs and per capita consumption in various economic segments of the population. The following is a brief description of these vehicles in decreasing order of their importance and/or potential for use in the Philippines.

Rice. Despite both political and economical problems in using rice as a vehicle for iron fortification in the Philippines, it still seems to be the best option. A historical review of rice fortification in the Philippines was made and a related publication not found at last year's conference was located and reviewed. In the so-called "Batan" project started in 1948, rice fortification alleviated problems of beri-beri and other B-vitamin deficiencies. There was a review published in 1952

indicating that hemoglobin determinations in pregnant women performed in 1948 and repeated in 1950 showed an increase in mean hemoglobin of 2.1 gm/100 ml, ostensibly as a result of rice fortification. However, this was not a randomly selected sample and some or all of the apparent improvement in hemoglobin may have been due to biased sampling. The related article was published in the Journal of Nutrition, 1952.

Despite the evidence of improved nutrition of B vitamins resulting from rice fortification in the Philippines, and the fact that it had been required by law since 1950, rice fortification has never been instituted. The reason is that no adequate system for policing rice fortification has been implemented and rice millers as a group have ignored the legislative requirements for fortification. Part of their reluctance is due to the fact they are taxed on the quantity of rice milled and to reduce this, they were able to avoid having their product regularly analyzed by government officials. The technology for rice fortification is not felt to be a major problem although it is important to note that sodium iron pyrophosphate was employed as the iron salt in this program.

A number of workers felt that rice is still not a suitable vehicle for iron fortification, although it remains high on the list because of problems associated with other food vehicles. A meeting was scheduled with the Director of the National Grain Association (NGA) but was canceled because the Director was out of the country.

One option for rice fortification in the Philippines would be to link it with a food subsidy program that will be implemented within the next few months under joint support from USAID and the Philippine government. Rice and cooking oil will be provided to lower income groups at a 30% discount. It seems feasible that the rice supplied through this program could be fortified with iron; this would amount to about 10% of the total rice consumed in the Philippines. It would have been reassuring to obtain a tentative agreement from the Director of NGA but that was not possible at this visit. Dr. Engel encouraged us to proceed with plans for rice fortification and worry about its implementation at some later stage.

One of the positive aspects of rice fortification was the finding of a rice enrichment facility in Manila that could perform the type of food technology studies required to develop an effective iron fortification program. Dr. Barrett visited this facility and was greatly impressed by the competency of the personnel and the adequacy of their facilities. Some or all of the food technical problems will probably be tackled in collaboration with FNRI. Once the technology in iron fortification of rice is solved, absorption studies could be started shortly thereafter. In summary, rice fortification with a highly available iron salt appears to be technically feasible, and this avenue should be pursued because of the highly constant intake of this food in the population as a whole.

<u>Bagoong (fish paste)</u>. A very close second candidate as a vehicle for iron fortification is fish paste, the use of which is somewhat unique to the Philippines. In contrast with fish sauce which is only consumed by

the higher income segment of the population, relatively constant amounts of fish paste are consumed by the majority of individuals in the low economic groups. (See Dr. Marzan's tables for details of intake and production.) The estimated intake of fish paste is approximately 6 gm/day/person, and there is a remarkably stable intake of this substance over a wide economic range of the population. Fish paste is prepared by mixing one part salt with three parts fish and allowing the mixture to ferment for a period of 2-3 months. It can be made with many kinds of fish but anchovy and shrimp are the most commonly used. After fermenting, the insoluble material is allowed to sediment, and the supernatant is harvested and sold as fish sauce. The heavy, dark residual is then sold either in individual packages or in bulk at local food stores. Because of its intense odor and dark color, iron fortification with a soluble iron salt would pose no major problem. Sodium iron EDTA would be a natural choice but it would be of interest to determine whether other forms of iron will remain soluble when added either before or at the end of the fermentation process. FNRI was encouraged to conduct preliminary studies of iron fortification and to determine the effect on color and taste of adding approximately 1 mg elemental iron/gm fish paste. Dr. Engel suggested that the small quantity of fish sauce which is regularly used to "prime" the fermentation of the fish paste could be used as a premix; if large quantities of iron are added to the premix, this alone might bring the iron content of the fish paste to an adequate level Bagoong is unique to the Philippines but because it is targeted to the low economic income groups and because its intake in different segments of the population is relatively uniform, this vehicle deserves continued study. Because the product varies substantially depending on the fermentation process and exact ingredients, feasibility studies with fish paste will have to be performed in the Philippines.

Sugar. Refined sugar remains an option for iron fortification in the Philippines but not a very attractive one. The major reason is that the intake of refined sugar varies greatly and is often sharply decreased in low economic groups. Intake patterns as summarized by Dr. Marzan indicate that its elasticity is quite high; that is to say, there is progressive increase in consumption with an increase in family income. We did not consider the enrichment of brown sugar feasible because of its high moisture content. Because the problems of using sugar for fortification appear almost insurmountable, the use of this vehicle was not explored at length. It was noted that the technology for fortifying sugar with sodium iron EDTA is already established (Guatemala project). If the high economic burden with this approach to iron fortification was acceptable (sodium iron EDTA), fortification could proceed with minimal research efforts.

Salt. Much of our discussion concerned salt but it was concluded that this vehicle does not seem feasible in the Philippines. The source of salt consumed in the Philippines was discussed at some length. Roughly 80% of it is sea salt that is dried locally, almost on a household basis, and is used directly by other households for preparing dried fish. The use of iron fortified salt for preparing dried fish would be very attractive, but it would not be practical because most salt does not pass through a refinery. Additional discussion concerned fortification of the remaining 20% of the salt which is supplied as a relatively refined material to the rest of the population. However, the intake of this refined salt is inversely proportional to income which limits its appeal for iron fortification. Moreover, the technical problems related

to the fortification of salt with iron seem insurmountable at present There was general agreement by the group that this vehicle should not be pursued further unless there is a breakthrough in the technology of salt fortification.

Wheat. There is some intake of wheat in certain segments of the population, but on the whole, it supplies less than 10% of the protein consumed in the Philippines and intake is localized to certain geographic "pockets" It was emphasized that technology already exists for high level fortification of wheat flour, and this is a program that the government could undertake at any time. It was felt that additional food technology studies would not be needed in this area.

Corn. As in the case of flour, Dr. Barrett could see no technical problems in fortifying corn which is usually ingested as corn grits. However, the intake of this food is low. It was felt that detailed technical studies are not required and that iron fortification of corn could be performed with the knowledge that this would solve the iron problem for only a very small segment of the Philippine population

MSG. The fortification of MSG with vitamin A has met intense opposition by nutritionists in the Philippines who believe that continued ingestion of this condiment poses a significant health risk, particularly to children. Although this is unlikely to decrease the use of MSG in the Philippines, it would be very difficult to justify its use for iron fortification. The vitamin A trial with MSG is proceeding but it is unlikely that it will be implemented on a national level. It was agreed

by all that MSG would be a "loser" with respect to iron fortification and further studies of this vehicle are ill-advised.

In summary, there are a number of food vehicles that could be used for iron fortification in the Philippines. All of these have significant drawbacks not the least of which is a pattern of food intake that does not favor the lower socioeconomic groups. Despite all its shortcomings, the use of rice for iron fortification remains an attractive option and it was agreed that studies of this food vehicle should proceed immediately. Bagoong should be explored at a pace determined by investigators at FNRI; it is hoped that they will pursue this condiment because of its wide-spread use in the lowest income segments. Incidentally, the addition of iron to NUTRIPACK was discussed at this conference, but there was doubt as to whether adequate levels of iron could be added (to one of the constituents) without serious technical problems. To examine this, a 60 mg iron tablet was crushed and added to the boiled gruel. The gruel took on a shiny gray color with a strong metallic taste that would be totally unacceptable. If NUTRIPACK is used as a vehicle for iron, it may be preferable to include in the package a 30 mg iron tablet that could be given to the infant or child along with the gruel but not mixed with it.

IRON SUPPLEMENTATION

Preliminary discussion of the proposed iron supplementation studies in the Philippines revealed an ambitious but poorly defined project. The development of a more acceptable protocol was achieved after many hours of discussion.

The investigators were encouraged to set aside their plans for an iron supplementation study in children 1-6 years of age because of the scope of the proposed study in pregnant women. Dr. Engel participated actively in the development of the supplementation protocol because its support will require local AID funding.

A detailed protocol of the proposed supplementation study is being prepared and will be circulated to the group. The following are the major features of the proposed study: A total of at least 6 study groups would be randomly selected, and would consist of the following:

1. Placebo*
2. $FeSO_4$, 120 mg iron once daily, unsupervised
3. $FeSO_4$, 120 mg iron once daily, supervised
4. $FeSO_4$, 60 mg iron once daily, unsupervised (1 rather than 2 UNICEF tablets)
5. Durules, 100 mg iron once daily
6. Feosol capsule, 50 mg once daily

There was some uncertainty when designing this study whether the "standard" dose of iron should be 2 UNICEF tablets (120 mg iron) or a special iron tablet prepared by Astra for this specific study containing 100 mg iron as ferrous sulfate (rapid acting). It is planned to ask Dr. DeMaeyer for assistance in obtaining tablets for the placebo, 60 mg, and 100 mg iron (ferrous sulfate) in exactly the same form as Durules. This would mean that only the Feosol capsule would have a different appearance.

*all tablets including the placebo will contain 5 mg folic acid

It was decided that a total of 150 pregnant women should be placed in each group on a random basis. Randomization by village was strongly discouraged. It is planned to first register pregnant women when they present at the Health Clinic at approximately 12-16 weeks gestation. They would then be entered into the study at 24 ± 2 months gestation. Details will not be given here of the infrastructure in the Philippines that would be used for this supplementation study. Strong reliance would be placed on baryguy nutrition scholars and this would diminish the cost of the project. It should be noted that in selecting the above dose levels for this study, it was debated whether group 4 would be given twice or one-half the dose as group 2 and 3. The latter was selected because the side effects of 240 mg iron daily would probably be unacceptable in a national program.

The remaining details of the supplementation project will follow closely the guidelines developed for the Jakarta study. A period of 100 days supplementation will be attempted. Hemoglobin determinations will be performed as baseline and at 1,2, and 3 months following institution of the supplementation. Effort was made to outline in detail the method used for hemoglobin determinations. It is planned to draw capillary samples with a disposable 20 µl pipette and place these in pre-measured 5 ml volume Drabkin's solution. These samples will be transported to a central lab (probably FNRI) for hemoglobin measurements using a conventional spectrophotometer. Samples will be obtained at the same time each day (± 1 hour) and all subjects will be requested to recline for at least 10 minutes before the sample is taken. Hemoglobin measurements should be tested in the field before actually instituting the iron supplementation program using a system of blind duplicates prepared both in the field and replicated in the laboratory.

The performance of other measurements such as hematocrit, serum iron, iron-binding capacity, and red cell protoporphyrin were considered but discarded. The only measurement considered of value was serum ferritin, and it is planned to obtain baseline and 3-month follow-up samples that could be analyzed for ferritin at a later date if hemoglobin determinations do not provide a sufficiently clear answer to this trial. Capillary tubes will be used to obtain serum. The blood will be allowed to clot and will be centrifuged later on the same day at a central facility; the tube will be broken at the cell/serum interface, then resealed and stored at -20°C. It was emphasized that serum ferritin determinations may not be required on these samples. Decision to perform the analysis would be made after analyzing the hemoglobin data. If the trial does not improve iron nutrition in pregnant women, serum ferritin determinations may indicate whether this failure was due to an inadequate dosage or other factors such as complicating infection, etc. It should be noted that the design of this iron supplementation program and its supervision will be the responsibility of NCP, whereas the laboratory determinations (hemoglobin and/or ferritin) will be the responsibility of FNRI.

The form of the questionnaire that will be used in determining side effects in this study is similar to that proposed in Jakarta. Compliance will be assessed by providing 40 tablets in a childproof container every 30 days and counting the number of tablets remaining at each return visit.

In proposing 150 subjects for each study group, a 30% dropout rate was anticipated that would leave a total of 100 subjects in each group. The duration

of this study was of some concern in that it was calculated that approximately 1½ years will be required to accumulate all the subjects for the study. It was planned to conduct the study in 4 separate municipalities; this represents a sizable geographic area. However, the technical field problems did not appear insurmountable to NCP personnel. The study was designed to detect a 0.5 gm difference in hemoglobin level as significant. Data analysis would be performed by NCP.

Following the outline of this proposal study on considerable discussion focused on what iron supplementation studies might be conducted in infants or growing children. An obvious program that could be linked to the pregnancy study would be to supplement infants from 6 to 12 months of age from the same mothers participating in the pregnancy study. Thus, of 900 pregnant women entered into the initial study, only about one third of their infants will be required to furnish three study groups: placebo, supervised and unsupervised administration. An iron dosage of 20 mg iron/dose as liquid ferrous sulfate was decided upon. Hemoglobin determinations would be performed as a baseline and at 3 and 6 months following the institution of iron supplementation. Follow-up values would also be obtained at 18 and 24 months of age. As in the pregnancy study, samples would be obtained for serum ferritin determinations but these would not be analyzed at the outset. Mrs. Guirriec will supervise both supplementation programs and she seemed confident that adequate information could be obtained with a minimum of personnel and at a reasonable cost. As indicated above, the detailed protocols for iron supplementation in both pregnancy and infants will be circulated in the near future.

The final question focused on possible supplementation programs for preschool children aged 1-6 years. Many options were discussed but most of them were discarded because of awareness that iron supplementation in children is not likely to be maintained for a period of longer than 1 to 2 months without intensive reinforcement. It was emphasized that iron fortification is the optimal approach to alleviate iron deficiency in the preschool child. Some discussion was held about placing an iron tablet in NUTRIPAK and focusing on children with second and third degree malnutrition. There was no strong opposition to this move but it was decided that it should not cloud the design of the initial study and it could be initiated at a later date once the study of iron supplementation in pregnancy is underway.

CAIRO, EGYPT - Visit of Drs. Cook and Dallman, May 10-16, 1980

The following is a summary of the discussions held in Cairo, Egypt by Drs. Cook and Dallman. The main objective of this visit was to assist in redesigning a project entitled "Pilot Study to Combat Anemia" that will be conducted by the Nutrition Laboratory, Child Health Laboratory, National Research Center, Cairo. In the original proposal, the principal investigator is Dr. Osman Galal and the co-principal investigator is Dr. F. El Shobaki. The present visit was a follow-up to a meeting held in Washington, D.C. on February 21, 1980 with Drs. Galal, Chichester, Kahn, and Cook.

Initial discussions in Cairo, Egypt involved the detailed review of the proposed studies with various individuals who will be involved with the anemia project. Apart from the principal investigator, the key investigators are Dr. Shobaki and Dr. Metwalli. Dr. Shobaki obtained his Ph.D. in Cairo on work relating to iron absorption and he has retained an interest in iron metabolism since that time. He recently returned from 2 years in Germany where he studied mucosal transport in the rat (Dr. Rommel). Dr. Shobaki will conduct most of the hematologic evaluations and will supervise any special measurements of iron absorption either in the rat or in human subjects. Dr. Metwalli is a chemical pathologist and has worked in the village where the proposed anemia study will be conducted. He has extensive knowledge of the public health and laboratory aspects of schistosomiasis.

A number of changes in the anemia project were suggested, and these will be briefly outlined. It was proposed that the study be conducted in children 6-12 years of age who are attending school in a village that is approximately 90 minutes driving time from central Cairo (Kafr el-Kahdra). At the beginning of the fall semester in 1980, hemoglobin determinations will be performed on all children. All those with a hemoglobin below 7 gm% will be removed from the study and will undergo detailed hematologic evaluation to determine the cause of their anemia. The extent to which their anemia will respond to iron will be determined in a therapeutic trial of oral iron iron in full doses. All other children will be randomized into two groups: placebo and iron treated. The iron treated group will be given a single tablet containing 60 mg elemental iron as ferrous sulfate once daily under supervision of their teacher. The control group will receive a tablet containing no iron.

It is proposed to continue this supplementation program for 6 months although if a marked improvement in hemoglobin levels can be detected after 3 months of supplementation, supplementation could be stopped at that time. In any case, the supplementation would have to stop after 6 months because children attend school for roughly 6 months out of each year. Subsequent hemoglobin determinations will be performed at 3-6 month intervals for the following 2 years to determine the durability of the hemoglobin response.

It was suggested that laboratory measurements in the study be confined to hemoglobin determinations on capillary (finger stick) samples; this would avoid the need for a venipuncture. The optimal method for hemoglobin determinations in the field was discussed: a 20-50 ul sample of blood should be placed in a measured volume of Drabkin's solution. The sample could then be transferred to a central laboratory for spectrophotometric measurements any time up to 14 days later. It was also suggested that 150-200 ul serum be obtained and frozen in capillary tubes for later serum ferritin determinations. In the event that supplementation produced little or no change in hemoglobin, these samples could then be assayed to obtain additional information as to the cause of anemia. For example, if anemia was due to infection, initial serum ferritin levels would be elevated. Ferritin determinations would not be required if the supplementation trial was highly effective.

Some comment as to the major objective in this study should be made. The primary objective is to determine whether the relatively severe anemia commonly seen in children in Egypt is responsive to iron. Although the project may not serve as the basis for national intervention, it is nevertheless an important prerequisite to other iron intervention strategies. Thus, if anemia responds only poorly to iron supplementation, a program of food fortification with iron would be of little value. If the present study shows that iron supplementation is highly efficacious, the results might well serve as the basis of a national iron supplementation program.

A site visit was made during our visit to Kafr el-Kahdra; Dr. Metwali accompanied us on this trip. We had an extended conversation with the resident physician who provides all medical care in the village with the assistance of 5 nurses. The health care facility is immediately adjacent to the school but is equipped with nothing but the simplest of clinical measurements such as body weight or examination of urine for parasites. The physician believed that bilharzia is by far the most common health program in rural Egypt. Giardiasis is also very common but hookworm is seen infrequently and malaria is exceedingly rare. It seems likely from our discussions that anemia in children might well be multi-factorial. While anemia may occur in subjects with bilharzia due not only to blood loss (and iron deficiency), but also because of an impairment of intestinal absorption, a defect in RE cell release of iron, and by hypersplenism. Giardiasis may also be a common cause for intestinal malabsorption and failure of an iron supplementation program.

Subsequent discussions were held regarding the prospects for food iron fortification in Egypt although these were very preliminary in nature. There was a strong feeling among the group at National Research Center that neither salt nor sugar would be acceptable vehicles for fortification; attention was focused on wheat. Approximately 70% of wheat consumed in the country is imported, largely from the US and Australia. This wheat is milled in 4-5 central facilities that would simplify an iron fortification program. However, most of the imported wheat is distributed to higher income segments of the population and in urban areas. In rural villages, most of the wheat is grown and milled locally and this would its fortification very difficult to implement or regulate. Nevertheless, wheat appears to hold the greatest promise as a vehicle for iron fortification.

The development of an iron fortification program of wheat in Egypt should not be a major task since wheat and flour fortification in the US poses no serious technical problems. Some decision would have to be made as to the type of iron and level of fortification. Food technologists at NCR also had reservations because they believed that technical problems might arise with wheat fortification because of the type of diet consumed in Egypt. It was agreed that iron absorption studies from a typical Egyptian diet would be of great value in the design of an suitable iron fortification program. This would be facilitated if Dr. Shobaki were able to visit the US for 2-4 months to become acquainted with food iron absorption methods and technics for assessing iron status. This expertise could be acquired easily because of his past experience in this area.

In summary, we were impressed with the enthusiasm for developing intervention strategies to combat iron deficiency in Egypt. Iron supplementation holds promise for bringing early hemoglobin responses but all agreed that this approach is unacceptable as a long term intervention. Additional studies of the efficacy of iron supplementation should be designed for pregnant women and for children 2-6 years of age. Additional studies are currently underway to revive a weaning food (Supremene) that has been available in Egypt for some time but that has not been well accepted because of its taste and consistency. If this product could be altered to eliminiate its undesirable qualities, it may well serve as an optimal vehicle for iron fortification in infancy and possibly in older children as well.

Report on a Visit to Guyana
and
Recommendations for Preliminary Measures
to Control Anaemias in Guyana

by

Dr. Allan Johnson (Cornell University)

Dr. James Cook (University of Kansas)

Dr. Daphne Roe (Cornell University)

Dr. Michael Latham (Team Leader - Cornell University)

on behalf of

the United States Agency for International Development

at the request of

The Government of Guyana

Objectives

The objectives of the visit were to make recommendations concerning appropriate steps that might be taken to control nutritional anaemias which have been found to be prevalent in Guyana.

Arrangements for the Visit

A request by Dr. Samuel Kahn from the Office of Nutrition of A.I.D. in Washington was made to the Consultants to make a short visit to Guyana for the purposes stated above. This unpaid consultancy was believed to be the logical next step, after the short visit made by one of us (M.C.L.) in 1977. The report of that visit is attached as Appendix I for easy reference. It summarizes existing knowledge about the anaemia problem in Guyana.

The present visit lasted from July 31 until August 6, 1978. Local arrangements were made by the National Science Research Council and USAID.

Some officials and others with whom discussions were held in Guyana

Ms. Edna A. Boorady - Director, USAID, Guyana

Mr. D. Steen - Agricultural Adviser, USAID

Mr. N. Mariani - Programs Officer, USAID

Dr. Pat Munroe - Secretary General, National Science Research Council

Ms. June Henry - Research Officer, NSRC

Mr. Fitsroy Collins - Executive Director, NSRC

Dr. R. O. B. Wijesekara - Specialist Consultant, NSRC

Dr. Robert Baird - CMO, Ministry of Health

Mr. Charles Hinds - Permanent Secretary, Ministry of Health

Dr. Gustraro Mora - PAHO/WHO Country Representative

Ms. Raquel Nievas - PAHO/WHO Nutritionist

Dr. Eric Austin and staff - Childrens Ward, Public Hosiptal Georgetown

Ms. Laljee - Central Medical Laboratory

Mr. Subryan - Central Medical Laboratory

Mr. Ally - Central Medical Laboratory

Dr. Leslie Chin - Guyana Pharmaceutical Corp.

Mr. Vickram Oditt - Guyana Pharmaceutical Corp.

Dr. Philip Boyd - Chief Caricom Health Desk

Ms. Veronica Regis - Caricom Economist

Mr. Carl Goodchild - Ministry of Economic Development

Mr. A. R. K. Kahn - Ministry of Economic Development

Dr. Walter Chin - Medical Superintendent, Public Hospital Georgetown

Dr. C. Harry - Medex Program/MCH Services, MOH

Dr. Sookraj - Obstetrician/Gynecologist, PHG

Dr. G. Noel - Ministry of Health

Places Visited

U.S.A.I.D. Office

National Science Research Center

Two rural health centers north of Demerara River

Ministry of Economic Development

Ministry of Health

Central Medical Laboratory

Childrens Ward, Public Hospital Georgetown

Guyana Pharmaceutical Corporation

Market in central Georgetown

The team's visit to Guyana got quite extensive press coverage there, and two long interviews with Professor Latham were broadcast on Radio Guyana and Radio Demerara. This publicity stressed the importance of anaemia as a public health problem in Guyana.

Developing the Recommendations

As a result of discussions individually and with groups the Consultants together developed a series of possible measures that might be taken. These were fully discussed at a meeting held at the National Science Research Council on August 3, 1978. Attending the meeting were Drs. Munroe, W. Chin, Baird, Sookraj, Harry, Noel, Ally and Boyd, as well as the 4 Consultants. As a result of these discussions the Consultants prepared a document of Preliminary Recommendations for Consideration Concerning the Control of Anaemias in Guyana. This document was then provided to those who had attended the meeting on August 3. At a second meeting on August 5 attended by Drs. Munroe, W. Chin, L. Chin, Harry and Boyd, as well as the 4 Consultants, the document was thoroughly reviewed. In general, those present agreed that the recommendations were highly acceptable with some minor modifications and additions.

The Consultants then redrafted the recommendations which are contained below. It was agreed that Dr. Munroe in consultation with his colleagues would prepare a draft budget, and that he would hold discussions with Ms. Boorady, Director of USAID in Guyana, concerning the budget, and a request for funding.

RECOMMENDATIONS

Introduction

Anaemia has been shown to be a very prevalent condition in Guyana. This is clear from the CFNI Nutrition Survey carried out in 1971 and from the recent anaemia study of Dr. Allan Johnson. Dr. Johnson's research shows that overall, some 50% of persons had anaemia as defined by W.H.O. criteria. The prevalence ranged from 74% in pregnant females to 32% in adult males.

It is recognized that anaemia is only one of several important nutritional problems in the population. Undoubtedly, Protein Energy Malnutrition

(PEM) of young children is the most important form of malnutrition present. PEM has serious consequences in terms of morbidity, mortality and its effects on child development.

The U.S.A.I.D. team were asked to address the question of nutritional anaemias, and not other forms of malnutrition, however important. Their mission was to advise the Government of Guyana how it might best address this question, and to recommend to U.S.A.I.D. what assistance might be given in this endeavor. By doing so we do not suggest that control of anaemia deserves a higher priority than attempts to reduce the prevalence of PEM.

The team does not believe that any large scale new survey is needed to establish the prevalence of anaemia. They feel that steps should be taken towards alleviating a problem that has been clearly demonstrated to be prevalent. They favor the program described below which could lead quickly to appropriate national programs. These actions should be undertaken largely by Guyanese staff and Guyanese institutions.

The Government of Guyana may request that some link be formed between the Guyanese institution running the program and an institution in the United States. Certainly, there are American universities that would be willing to provide advice and technical assistance either on a formal or informal basis. The Consultants both from Cornell University and the University of Kansas expressed their willingness to assist with future activities if requested to do so. The team believes that Guyanese should have the ultimate right to decide on which U. S. institutions, if any, it wishes to work with in the future.

The program proposed here consists of three components which should be carried out concurrently. These are:

1) the better characterization of the anaemia found to be prevalent in Guyana;
2) an evaluation of the current iron-folate supplementation program in comparison with an improved supplementation program in urban and rural health centres; and
3) an investigation of the technical feasibility of fortifying wheat flour and sugar with iron at specified levels, so that one of these might be chosen for national fortification.

These activities would require a Guyanese scientist of high caliber with a Doctoral degree and some experience in anaemia work and in field studies to direct the activities as a full-time or near full-time activity. It would also be necessary to have two professional assistants. Each component of the program would require a commitment from one or more government ministries or Guyanese institutions. The National Science Research Council would be a logical body to coordinate the program activities.

Characterization of Anaemia

In the 1976 survey of nutritional anaemia performed in Guyana by Dr. Johnson, more than 70 percent of pregnant women were anaemic by WHO standards. Because this anaemia correlated well with transferrin saturation, a measure of iron status, the anaemia was undoubtedly due to iron deficiency. Other factors such as folate deficiency or chronic urinary tract infection might well have contributed.

In this same survey, a surprisingly high prevalence of anaemia was encountered in other segments of the population. In menstruating women, infants and school aged children, the cause of this anaemia was presumably iron lack, although measurements of transferrin saturation gave no clear evidence for this conclusion. Of particular concern was the high prevalence for anaemia of 32 percent in adult men.

Anaemia is uncommon in adult men, and in the few Latin American countries where it has been detected, the cause is invariably iron deficiency due to hookworm infestation. In the Guyana survey however, transferrin saturation was not significantly decreased in men and hookworm was only rarely identified by stool examination. The cause of anaemia in Guyanese men remains undefined and we consider it essential to characterize this anaemia further before considering intervention strategies that will reach the entire population.

One approach to this problem would be to undertake another full-scale prevalence survey on a randomly selected population sample and to include a battery of hematologic studies which would fully identify the cause of discovered anaemia. The expense of such an undertaking is likely to be prohibitive; we therefore recommend that those subjects identified as anaemic in the 1976 survey be studied further. In men, for example, 30% of the 78 subjects were anaemic or about 25 subjects. If two-thirds or about 15 of the men could be relocated for further study, it would provide a reasonable sample of randomly selected anaemic men for detailed study. A similar procedure will be used for obtaining a representative sample of non-pregnant women, pre-school children and school age children. Where this method does not yield 15 to 25 anaemic subjects, then adjacent households at each location will be used to provide subjects.

Either a hematocrit or hemoglobin determination should be performed initially in these men, to verify the presence of continuing anaemia. If present, an additional 15-20 ml whole blood will be obtained for further laboratory measurements and a stool sample examined for occult blood and parasites. Portions of whole blood or serum will be frozen immediately for later hematologic determinations including serum iron, iron-binding

capacity, red cell protoporphrin, red cell folate, sickle cell prep, G6 PD, and most importantly, serum ferritin levels. Iron deficiency would be clearly identified if the ferritin level was significantly decreased. This measurement could therefore be performed initially and other laboratory parameters obtained only if the serum ferritin is normal or elevated. Nevertheless, it would be preferable to include the entire battery of hematologic measurements on all subjects in an effort to identify contributing factors. The radio-isotope measurements (serum ferritin and serum folate) would have to be performed in a U.S. laboratory, but the remaining determinations could all be performed in Guyana. A similar approach should be used to fully characterize anaemia in children and menstruating women for a total sample not exceeding 100 persons.

Iron-Folate Supplementation for the Control of Pregnancy Anaemia

Statement of Need

Since evidence points to iron deficiency as the major cause of anaemia among pregnant women, risks of such an anaemia must be emphasized. Risks of anaemia in pregnancy include those pertaining to the mother and those relating to the developing infants.

While moderate anaemia has been the common form in pregnant women in Guyana, the additive effects of moderate anaemia in the antenatal period and blood loss at the time of delivery can cause severe anaemia in the postnatal period. Severe anaemia is particularly a hazard in women with closely spaced pregnancies. Symptoms of severe anaemia which most limit functional capacity are chronic fatigue and breathlessness on exertion due to hypoxia. The capacity for a woman to perform work, both during pregnancy and afterwards may be compromised also by moderate anaemia. Preliminary evidence suggests that iron deficiency may depress cellular

immune function making a woman more vulnerable to infection and may also adversely affect postnatal development of the infant.

Supplementation Trial

The present method for providing iron as an anaemia prevention measure for pregnant women attending the Guyana health centers is apparently not working. The aim of the proposed intervention trial is therefore to compare the advantages of a new method for delivery of iron supplements to these women with the existent method.

Five demonstration "clinics" and five "clinics" following the present system, hereafter to be designated demonstration health centers, and control health centers, will be selected from the total number of health centers in the coastal area such that the demographic characteristics of the women attending both groups of health centers will be similar. Urban and rural health centers will be included in the demonstration and control systems.

A total of about 20 women will be enrolled for study at each of the 10 health centers. This will provide about 100 subjects in the demonstration centers and 100 in the control health centers. Women will be enrolled only when they are being seen at the Health Centre for the first time during their current pregnancy. It should be made clear however that all women attending the demonstration health centers irrespective of whether they are, or are not, study subjects will receive identical treatment and attention. Similarly, study subjects and others in the control centers will receive similar treatment. The only difference will be in the frequency with which blood is taken, and the hematological tests that will be performed.

In the demonstration health centers, women will receive an iron-folic

acid preparation in pill form from the time of the first health center visit to the time of delivery. The formulation will be such as to supply 60 mg of elemental iron and 0.1 mg of folic acid per day. Consideration will be given to the further addition of ascorbic acid to maintain the stability of the folic acid. Justification for addition of folic acid to the tablets is that:

1) Folic acid deficiency is common in pregnancy and contributes to poor pregnancy outcome as well as impaired fetal development.
2) On present information, the prevalence and severity of folic acid deficiency in the pregnant women of Guyana is unknown though evidence suggests that it exists.
3) The need for folic acid may be increased by correction of the iron deficiency.

It is proposed that the iron-folic acid pills be prepared at the lowest possible cost by a pharmaceutical company in Guyana.

Pills would be dispensed by the nurse or nurse-midwife at the demonstration health centers in child-proofed containers to the women at each clinic visit. The numbers of pills dispensed on each occasion will be adjusted to the intervals between these clinic visits.

The program in the demonstration health centers will not only include the distribution of supplements, as detailed above, but also provision of nutrition education by trained clinic staff at a level appropriate to the comprehension of the recipients. Nutrition education will include group discussion and handout materials.

In the control health centers, provision of iron-folic acid supplements and nutrition education will be as at present.

Monitoring the effectiveness of the new intervention system will

be by hemoglobin and hematocrit determinations. In both demonstration and control health centers, these determinations will be performed at the initial clinic visit and thereafter at the last clinic visit prior to delivery, in the home/hospital at the time of delivery and at the follow-up visit to the health center. These hematologic tests will require the provision of simple equipment to the health centers including micro-hematocrit apparatus and portable spectrophotometers for hemoglobin determinations. Duplicates of blood serum obtained will be frozen and stored so that serum ferritin levels can be determined later if this is thought to be necessary.

Records will be kept both in the demonstration and control health centers including:

1) the number and frequency of pill distributions;
2) the number of pills consumed by the women (a check measure will be devised)
3) hemoglobin and hematocrit levels.

Data analysis will compare change (Δ) hemoglobin level and Δ hematocrit from first to subsequent visits for women by both systems.

Comparison will also be made between individual health centers in the demonstration and control systems and between Δ values for the total sample population in the demonstration and control systems.

Variables which characterize women who do not respond to the proposed intervention will be examined.

It is fully realized that a failure to improve hemoglobin or hematocrit levels during pregnancy in both the demonstration and control health centers may be due (a) to a failure of women to comply with instructions to take the pills supplied, and (b) to the fact that many women first

come for examination at the centers very late in pregnancy. These two factors are important in prenatal clinics in many parts of the world. It is important to know before making recommendations for a national program whether these factors are so common as to make a supplementation program completely ineffective. Therefore either a positive or negative result from this study will be important and affect future planning. In the case of compliance a simple set of questions will be used after delivery to obtain opinions from mothers about their use of the pills and their reasons for not taking them.

This supplementation program for pregnant women should be viewed as an integral component of the delivery of health services to women. It is a part of efforts to improve and increase the coverage of primary health care.

Food Fortification

It is felt that the most effective long-term intervention approach is food fortification of a suitable vehicle. The vehicle chosen should be one that is consumed in adequate amounts by the people in need; it should be available for fortification in relatively few centers so that quality can be adequately controlled and monitored; it should be suitable for fortification on a large scale; and it should result in a product which is stable under extreme conditions of storage. The two main vehicles which satisfy these requirements are flour and sugar.

At present all wheat is imported into Guyana where it is milled into flour by the National Milling Company. The wheat flour is then enriched with reduced iron (metallic iron powder) at a level of 13.0 mg iron per pound of flour. It is proposed that the level of iron in the flour be increased three or four times to a level of 40-50 mg iron per pound of

flour. This would necessitate further studies on the effect of increasing the level of iron fortification on the color, texture, and palatability of the flour, as well as radio-isotope studies to determine the availability of the iron. Because of the proposals to add cassava flour to the wheat flour, studies would also have to be carried out to determine what effect the addition of the cassava flour would have on the availability of the iron.

Very little is known about the fortification of "raw" or brown sugar with iron. Exploratory work would be carried out to determine the effect of iron-fortification at one or more levels on the color, texture, and taste of the sugar, as well as radio-isotope studies to determine the availability of the added iron. Additional studies would be necessary to determine if the added iron remains evenly distributed during storage.

Storage stability studies would be necessary for both vehicles. Isotope studies would also be carried out using typical Guyanese meals to determine the gain in iron absorption resulting from iron fortification.

Proposed Distribution of Work Responsibilities

The overall direction of the research will be the responsibility of the principal investigator, the Guyanese scientist working in Guyana. He and his professional assistants will work in the health centers, will be responsible for distribution of iron tablets, for maintenance of records for coding and analysis of data.

In the characterization of anaemias, the principal investigator will carry out hemoglobins and hematocrit and most other determinations. Stool samples for ova and parasites will be collected and transported by his assistants to the Central Medical Laboratory in Georgetown for examination by the parasitologist. Blood serum could be mailed to Professor

James Cook at the Kansas University Medical Center for ferritin determinations. Plasma and whole blood samples could be sent to Professor Daphne Roe in the Division of Nutritional Sciences at Cornell University for folacin determinations. Erythrocyte protoporphyrin measurements could be carried out at Cornell but may also be performed by the principal investigator in Guyana.

Responsibility for using equipment purchased under the contract for explaining the feasibility of using Guyanese wheat/cassava flour and Guyanese brown sugar as vehicles for iron in a fortification program will be carried out by Dr. Leslie Chin and his assistants at the Guyana Pharmaceutical Corporation. Dr. Chin will also take charge of the task of formulating and providing the iron-folic acid tablets for the supplementation trial. In the event that it seems advisable to purchase these tablets from overseas, Dr. Chin and the principal investigator will be responsible for making the purchase.

Finally, studies of the availability of iron from Guyanese foods, from fortified flour and sugar could be undertaken at the University of Kansas.

It is stressed that all the field work, and 80-85% of the laboratory determinations will be done in Guyana by the Guyanese Principal Investigator, his staff and other Guyanese institutions. The U. S. laboratories mentioned above have through the consultants mentioned a willingness to do the other determinations. However the choice of where these are done must rest with the Guyanese who could of course select other U. S. institutions with which to cooperate.

Conclusion

The information gained from each of the three activities described

above, will be used to design appropriate programs for the control of anaemias. If the studies to characterize anaemias in Guyana suggest that iron deficiency is unimportant, this will influence decisions concerning both national supplementation and fortification programs.

If technical problems with fortification of either wheat flour or sugar are found to be non-existent and if absorption of iron from the fortified product is found to be good, then a trial fortification program can be planned, and possibly begun within the three year life of the proposed project.

By the end of the project, clear recommendations will be available concerning a national program to control anaemia in Guyana.

Besides the three activities recommended here, the team suggests a series of other measures which may in different ways help to lower the prevalence of anaemia. None of these other measures deserve the priority we assign to the other recommendations, and all of them should be integrated into existing programs. The measures suggested are:

1) The fortification of the proposed weaning food with 20 mg of ferrous fumerate (or some similar iron salt) per pound.
2) Nutrition and health education to increase the consumption of iron rich foods and to improve public awareness of the problem of anaemia and the steps that people can take to control it.
3) The inclusion in planned surveys such as the Agricultural Sector survey of questions relating to food intake after adequate consultation with persons trained in nutrition.
4) Government programs that will reduce poverty and infectious and parasitic diseases which may contribute to anaemia and other forms of malnutrition.

5) The adoption and implementation of a national food and nutrition policy aimed at a coordinated approach to control all forms of malnutrition.

Because nutritional anaemias are particularly prevalent in women of child bearing age and in young children, it is clear that the major beneficiaries of a control program are likely to be women and children. As UNICEF has declared 1979 to be the year of the child and as USAID is encouraging programs likely to benefit women, we believe an anaemia control program to be most appropriate at this time.

MAJOR CAUSES OF ANEMIA IN CHILDHOOD AND PREGNANCY IN CAMEROON

A. F. Fleming

and

S. R. Lynch

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PREFACE

In conformance with an agreement with the United States Agency for International Development (USAID), AID/DSPE-C-0053, the American Public Health Association (APHA) invited the authors to undertake a consultant assignment for three weeks, to work with the Ministry of Health of the United Republic of Cameroon and the University of Yaoundé, in a field survey to determine the major causes of anemia in Cameroon.

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SUMMARY

Anemia is known to occur at a high prevalence in children in Cameroon (38.1 percent). Only a few previous studies of the etiology of anemia in Cameroon have been undertaken. They suggest that iron deficiency is an important factor in both pregnancy and childhood. The present work is of the nature of a pilot study into the etiology of anemia in childhood and pregnancy in the Eastern Province of Cameroon, where the prevalence in childhood is high (57.6 percent) and in the Northern Province, which has a different ecology, but also a relatively high prevalence of anemia in childhood (41.0 percent).

In the Eastern Province, eleven children and 10 women were studied. In the Northern Province, 17 children and 14 women were included. A brief clinical history was taken and a restricted physical examination was performed on all patients. Hematological investigations were confined to hemoglobin (Hb) concentration, blood-cell morphology, malarial parasites on thick films, serum ferritin concentrations and Hb-electrophoresis on capillary-blood specimens; bone-marrow was aspirated from the anterior iliac crests, and examined for cell morphology, malarial pigment and intracellular stainable iron.

Ten of the eleven children in the Eastern Province were anemic. All showed multiple etiology of their anemia, including malaria in nine, other infections in eight, iron deficiency in six and folate deficiency in six. Seven out of ten pregnant women in the East were anemic. The most severe anemias were encountered in primigravida. Again, multiple etiology was usual, including malaria in five, iron deficiency in six, folate deficiency in three and some other infection in one. Also, all three non-anemic women were iron-deficient and one was folate-deficient.

Fewer of the patients studied in the North were anemic. Ten only out of 17 children were anemic. Again, more than one etiological factor was usually present. They included malaria (four patients), other infections (seven patients), iron-deficiency (three patients), folate-deficiency (nine patients) and sickle-cell anemia (one patient). The seven non-anemic children also showed hematological abnormalities; four had malaria, five other infections, one iron-deficiency, and three folate-deficiency.

Eight out of the 14 pregnant women in the Northern Province were anemic. Four had malaria, three had iron-deficiency, three had folate-deficiency and one had another infection. In six non-anemic women, malaria was diagnosed in two, iron-deficiency in two and folate-deficiency in four.

It is not possible to make any accurate generalizations about the etiology or pattern of anemia in the population of Cameroon as a whole on the basis of the results obtained in this pilot study. The restrictions imposed by constraints of time and available facilities necessitated that studies were carried out on a highly selected group of patients. The value of the investigation, therefore, lies first in the demonstration that it is possible to evaluate anemia in the field using relatively inexpensive supplies and equip-

ment which is already available. The second important conclusion is that at least four factors known to be the most important causes of anemia in underdeveloped countries were immediately identified and that no other unexpected factor was encountered. Based on these findings the investigators consider it appropriate to recommend treatment for patients drawn from high risk groups (young children and pregnant women) in whom anemia is identified; further investigation being unnecessary unless the patient fails to respond. Furthermore, anemia is a sufficiently significant problem for it to be appropriate to consider the implementation of public health measures and nutritional planning specifically designed to alleviate the problem. However, before this is done the authors would urge that a carefully planned field study be conducted to identify the pattern of anemia in other segments of the population (men, non-pregnant women, and older children). Finally, since it was not possible to say with confidence which etiological factor was most important in any one patient or what impact on anemia the correction of one or more would have, it would be wise to attempt to define their individual contribution to the overall frequency of anemia by an investigation in which selective correction is undertaken.

RECOMMENDATIONS

Anemia is a cause of serious socio-economic problems. Work capacity is limited severely; if a farmer is incapacitated, his family suffers from deprivation of his produce or of his earnings, and the area of land which is cultivated effectively is reduced. Severe anemia in women has adverse effects on the home, especially on the younger children. Anemia in pregnancy results in maternal morbidity or mortality, and is associated strongly with low birthweight, prematurity and perinatal mortality. As a consequence, the live infants born to the anemic mothers are prone to complications of malnutrition and infection. Anemia from any cause in childhood is associated with retarded growth, delayed development and poor performance at school.

Therefore, financial investment into the diagnosis, prevention and treatment of anemia in the community, commands a high priority for both economic and humanitarian reasons. Specific recommendations are as follows.

Staff development in Cameroon

Investment should be made in staff-development in the discipline of hematology and blood-transfusion. This should be considered at several levels.

Training of medical laboratory assistants. Candidates entering the course would need to have completed primary education. The training which is visualized is in-service, and would be similar to that leading to the National Certificate for Medical Laboratory Assistants issued by the Institute of Medical Laboratory Technology of Nigeria.

Training of medical laboratory technologists. Candidates entering the course need to have completed secondary education. The instruction would be likely to be a combination of formal biological science-teaching and in-service training with the emphasis on practical procedures. This program should be an expansion of existing training courses. One other objective would be to organize and raise the prestige of the profession of medical laboratory technology.

In the training of laboratory assistants and technologists emphasis should be placed on the development and use of standard hematological techniques which are applicable in the field where a minimum of laboratory equipment is available.

Medical students. Hematology is part of the curriculum of the course leading to qualification as a medical practitioner in Cameroon. Teaching of the discipline could be strengthened in the short term by recruitment from overseas until such time as more Cameroonians have specialized in Hematology.

Post-graduate. Encouragement should be given to young Cameroonian physicians to specialize in Hematology, combining both the clinical and laboratory disciplines. Such encouragement could come from expansion of postgraduate training in Cameroon, and scholarships for training overseas for selected Cameroonian doctors.

Immediate intervention

On the evidence available and without further research, advice can be given now as to the prevention and treatment of anemia in childhood and pregnancy.

Anemia in children. There is currently no way of erradicating malaria in tropical Africa. Therefore, it is inadvisable to offer antimalarial prophylaxis to large numbers of children, so preventing them from acquiring immunity to malaria and exposing them to risk whenever prophylaxis is no longer available. Every attempt should be made to make available adequate supplies of chloroquine for the treatment of children suffering from febrile episodes. Such children will either be infected with malaria, or have their immunity to malaria suppressed by some other infection.

The laboratory investigation of an anemic child should include a minimum of Hb, thick film for malaria and examination of feces for ova of hookworm. Pending the results of investigations, the anemic child should be started on a regimen of:

(a) curative doses of chloroquine, followed by prophylactic proguanil for two to three weeks,

(b) oral iron therapy, in a dose calculated from weight and Hb,

(c) oral folic acid, 5 mg per day for two to three weeks,

(d) such antibiotics as are indicated by the presence of infection.

Whenever the diagnosis of sickle-cell disease is suspected, iron should be withheld until such time as hemoglobin-electrophoresis can be performed: if the physician remains in doubt as to the diagnosis, a trial of iron therapy is probably justified.

Prevention of anemia of pregnancy. Every woman presenting in pregnancy should receive the following regimen (Fleming, Hendrickse and Allan, 1968).

(a) Chloroquine 600 mg base in a single dose at first attendance.

(b) Proguanil 100 mg per day until after delivery.

(c) Ferrous sulphate 200 mg per day until after delivery.

(d) Folic acid 5 mg per day until after delivery.

The widespread application of this regimen would have probably a greater impact on the health of the community than any other single medical intervention.

Treatment of severe hookworm-anemia. The following regimen is recommended (Werblinska and Fleming, 1979).

(a) Chloroquine 600 mg base once, followed by proguanil 100 mg per day for two to three weeks.

(b) Folic acid 5 mg per day for two to three weeks.

(c) Bephenium hydroxynaphthoate 5 g per day for three days, followed by tetrachlorethylene 4 ml as soon as the Hb is above 6 g/dl. It is the general experience in West Africa that bephenium hydroxynaphthoate has limited usefulness. Tetrachlorethylene is highly effective and extremely cheap; however, its use leads to cerebral intoxication in severely anemic patients.

(d) Oral ferrous sulphate 200 mg twice a day until six weeks after the Hb has ceased to rise: despite its much greater cost, consideration should be given to the use of intramuscular iron when available, in view of the unlikelihood that oral therapy will be taken by some illiterate rural farmers.

The dose is calculated from the formula

$$\frac{\text{Wt in Kg} \times (14.0 - \text{Hb g/dl})}{10} \text{ ml, to give the dose (ml) of}$$

intramuscular iron using a preparation containing elemental iron 50 mg per ml.

Prophylaxis against malaria

Chloroquine is highly effective both in prophylaxis and treatment of malaria. However, resistance to chloroquine has been reported in strains of *P. falciparum* in both East and West Africa (Campbell *et al.*, 1979; Eke, 1979; Anonymous, 1979), and the continued use of chloroquine as prophylactic will favor the selection and spread of these resistant strains (Nguyen-Dinh and Strager, 1978; Jopling, 1979). For this reason, a distinction should be drawn between prophylaxis and treatment. The authors recommend proguanil as the drug of choice in the prevention of malaria: some authorities prefer the combinations of pyrimethamine and sulfonamide (Jopling, 1979), but costs are higher and the administration of sulfonamides for long periods of time may lead to microbiological and hematological problems. Chloroquine undoubtedly remains the first drug of choice for the treatment of malaria. However, it should be noted that this recommendation is at variance with that of both the Center for Disease Control in the U.S.A. and the World Health Organization, who favor the use of chloroquine prophylaxis.

Further investigations in selected villages

While the treatment of anemia is undoubtedly important, it is unlikely that the present prevalence will be significantly reduced unless preventive measures are implemented at a regional or national level. Neither the current investigation, nor investigations previously carried out in Cameroon, provide

sufficient information to recommend suitable preventive public health or nutritional measures. Two important pieces of information are missing. The first is the pattern of anemia in other segments of the population (men, non-pregnant women, older children); the second lies in the fact that the relative contribution of each of the etiological factors already identified to the genesis of anemia remains undefined. The following investigations are therefore recommended in selected villages.

(a) A survey should be conducted along the lines of the United Republic of Cameroon National Nutrition Survey, but on a much more limited scale with the aim of establishing the pattern of anemia in the population as a whole. This should not involve hospital patients, but should be conducted in selected villages and include men, non-pregnant women, and older children as well as the two groups already studied.

(b) The baseline investigations would,

(i) determine whether anemia is sufficiently widespread to suggest that preventive measures would be necessary for the whole population, or whether it occurs predominately in certain groups (such as children and pregnant women), at whom specific intervention might be aimed:

(ii) determine the frequency and density of malarial infections and relate these to hemoglobin concentrations:

(iii) determine frequency and density of hookworm infections, and relate these to hemoglobin concentrations:

(iv) determine the prevalence of iron deficiency.

(c) The initial survey outlined above should be designed to lead at an early stage into pilot intervention studies. The only accurate means of determining the relative contribution of each of the etiological factors already identified, would be byselective intervention, in which each factor or a combination of factors is eliminated. A study of this type would provide a basis for national planning aimed at combating anemia and would prevent the investment of time and funds in the treatment of factors which might be of secondary importance. The relative geographic stability of the population in Cameroon would facilitate this type of investigation. Particular emphasis should be placed on the impact of intervention measures which have already been shown to be effective in other countries and which would correct the etiological factors already identified in the anemias seen in Cameroon. The measures which could immediately be considered would include:

(i) malarial prophylaxis

(ii) treatment of acute malaria without prophylaxis

(iii) treatment of hookworm, and an attempt to interrupt transmission education

(iv) iron supplementation

(v) folate supplementation

(vi) combinations of the above.

This type of investigation would provide the correct focus for planned intervention measures at a national level in the future.

(d) While it would be appropriate to define the impact of iron supplementation on anemia in the Cameroon before examining the feasibility of food fortification, the vegetable based diet which the majority of people eat is likely to be a poor source of available iron. The Division of Hematology at the University of Kansas Medical Center has both a special interest in food iron availability and facilities for studying this problem and would be very willing to participate in any further investigations of this aspect of the problem.

(e) The folate content of foods could be assayed on the same samples as are used for the studies of iron availability.

INTRODUCTION

The United Republic of Cameroon National Nutrition Survey (1978) revealed that the prevalence of anemia was 38.1 percent in children aged six months to 59 months. Anemia was defined as a hemoglobin (Hb) concentration of less than 10 g/dl in the six to 23 months age group, and less than 11 g/dl in the 24 to 59 months age group, a correction being made for altitude (Sauberlich, Dowdy and Shela, 1971). The highest prevalence was encountered in the Eastern Province (57.6 percent), followed by the Central Southern Province (53.3 percent) (Table 1). The Nutrition Survey did not include any investigation of the etiology of anemia, except that sickle-cell disease was found to make only a small contribution to the overall incidence. The report of the Nutrition Survey included a recommendation that a study be mounted to identify the major causes of anemia.

Only a few studies have been conducted into the etiology of anemia in Cameroon. A high prevalence of iron deficiency in pregnancy has been reported in Yaoundé, associated with infestation by hookworm (Eya Eyong, 1975; Joseph, 1979), and lower than normal percentage saturation of transferrin was reported in 66 out of 92 children in the Northern Province (Delpeuch, Cornu and Chevalier, 1980). Investigations conducted in the neighboring state of Nigeria, have shown that anemia in childhood is commonly the result of combinations of acute Plasmodium falciparum malaria, bacterial or viral infection, iron deficiency and folate deficiency (Werblinska, Fleming and Atachakwa, 1975). Studies from Nigeria indicate also that anemia is particularly common during first pregnancies, as a result of a loss of resistance to malaria (Gilles et al., 1969) and of folate deficiency, largely secondary to the erythroid hyperplasia resulting from malarial hemolysis (Fleming, Allan and Stenhouse, 1969; Fleming, 1970). The most severe deficiencies of iron result from heavy infestations with hookworm (Gilles, Watson-Williams and Ball, 1964; Fleming, 1977; Azubuike, Izuora and Obi, 1977). Although it is dangerous to extrapolate from results in another environment, these findings influenced the design of the present work.

Due to the limited time available, it was decided to concentrate on two areas of Cameroon. The Eastern Province was chosen as it had the highest prevalence of anemia and is ecologically similar to the Central Southern Province, including Yaoundé and Douala, where anemia was also found to be most frequent (Table 1). The Northern Province, which is ecologically different from the East and Central South, was included since high prevalence rates for malnutrition and anemia (41.0 percent) were reported by the Nutrition Survey and interesting contrasts in the etiology of anemia might be expected in the two different environments.

It was thought that a maximum of information would be derived from a study in depth of a small number of children and pregnant women attending clinics in these areas. The laboratory investigations were confined to the estimation of the concentration of Hb, the study of blood-cell morphology, the search for malarial parasites, hemoglobin-electrophoresis, the examination of smears of bone marrow aspirates and the measurement of the concentration of ferritin in serum.

PATIENTS AND METHODS

Patients studied

Samples of blood and bone marrow were obtained from 28 children aged between two months and five years presenting at out-patient clinics with a variety of complaints and 24 women attending prenatal clinic for the first time in the present pregnancy (Table 2). Eighteen women were pregnant for the first time and six had had at least five previous pregnancies. Eleven children and ten women were living in the Eastern Province while the remainder were from the North. None was known to have received any medication during the previous month, except for one pregnant woman who was taking supplements of iron (no. 41, Table 10). One pregnant woman (no. 12) absconded immediately after the collection of the specimen of blood, so that information on her is incomplete (Table 5). Investigations were conducted on two older siblings (nos. 23 and 25) of one child, at the request of their mother; results are included in Table 4, but are not analyzed further.

Methods

A brief history was taken in each case and a limited physical examination performed. The period of gestation was estimated from the height of the fundus in pregnant women. Blood was obtained by finger-stick for the measurement of hemoglobin (Hb) and serum ferritin concentration, the preparation of thick and thin blood films and hemoglobin-electrophoresis. Bone marrow was then aspirated from the anterior iliac crest using 1% xylocaine for local anesthesia and slides made for routine morphological interpretation and assessment of marrow-iron stores.

Hemoglobin estimations were carried out by the cyanmethemoglobin technique (Dacie and Lewis, 1975); 0.02 ml blood was diluted in 5 ml Drabkin's solution in the field and transported to the hematology laboratory at CUSS, care being taken to avoid evaporation and exposure to light. Readings were made on an Eppendorf Photometer 1101M. Anemia has been defined as Hb less than 10 g/dl below 23 months of age, less than 11 g/dl in the 24 to 59 months age group and less than 11 g/dl in pregnant women. All areas visited in this study were at about 700 m above sea level, and no corrections have been made for altitude.

Thin peripheral blood films were dried and then fixed for 20 minutes in absolute methanol and thick films merely dried, before being transported to the hematology laboratory at CUSS for staining. The thin and thick peripheral blood films were stained by the May-Grunwald and Giemsa techniques respectively.

Blood for serum ferritin estimation was drawn into four plain microhematocrit tubes, allowed to coagulate and then centrifuged in a microhematocrit centrifuge in the field. The red cell and serum fractions were separated by breaking the tubes, which were immediately resealed and transported on ice to Yaounde, where they were deep frozen. The serum was sent to the Division of Hematology, University of Kansas Medical Center for estimation of serum ferritin concentration by the two site immunoradiometric method

(Miles et al., 1974). The frozen cells were used to prepare a hemolysate for hemoglobin-electrophoresis on cellulose acetate (Lehmann and Huntsman, 1975), performed in the hematology laboratory, CUSS, Yaounde.

Four bone marrow slides were obtained from each patient, dried and then fixed in absolute methanol for 20 minutes. Two were stained with May-Grunwald and Giemsa stain for routine morphological assessment and one by the Perl's method for examination of bone marrow iron stores; the iron stores in the bone marrow was quantitated on a scale 0-6 (Gale, Torrance and Bothwell, 1963). Megaloblastic erythropoiesis was assumed to be evidence of a deficiency of folates. The slides stained for the examination of iron were used also to record the presence of malarial pigment. The diagnosis of iron deficiency was based on the absence of stainable marrow iron and that of malarial infection on the finding of malarial pigment in the bone marrow (Edington and Gilles, 1969).

RESULTS

Eastern Province

Children

Seven children in Abong-Mbang and four in Bertoua were studied (Tables 2, 3 and 4). Splenomegaly was observed in four children in Abong-Mbang and in one of the children under five years of age in Bertoua. Ten were anemic, but one (no. 7) was included only at the insistence of her mother, who was a nurse. The Hb concentration in the ten anemic children ranged from 4.6 to 10.4 g/dl. Only the least anemic child (no. 24) had a single cause of anemia. Malaria was diagnosed in nine, other infections in eight, iron deficiency in six and folate-deficiency in six (Table 12); the bone marrow specimen from patient no. 2 was insufficient to judge iron and folate status. Patients nos. 23 and 25 were studied also at the request of their mother; they illustrated that malaria was a problem in this family even up to the age of 12 years.

Pregnant women

All ten women were seen in the third trimester of their pregnancies (Tables 5 and 6). One primigravida (no. 11) had palpable splenomegaly. Seven women were anemic, but one primigravida and two multigravida had Hb values in the normal range. The lowest Hb's were recorded in anemic primigravida (6.1 - 10.3 g/dl). Malarial pigment was present in five of the six anemic women in whom the marrow was examined, and in one of those with normal Hb values (Table 13). Only one patient (no. 11) had evidence of another infection. None of the nine women whose marrows were examined had stainable iron. Folate deficiency was present in four women, three of whom were anemic, two having concurrent malaria. The two most anemic patients on whom there were data had two or three causes of anemia.

It should be noted that iron deficiency was seen in all of the women with normal Hb, and that one was also folate-deficient.

Northern Province

Children

Seventeen children were studied in four centers in the Northern Province (Tables 2, 7, 8, 9); ten of these children were anemic. The severest anemias were in two children at Oura-Tada (Mokolo) (nos. 70 and 72; Table 8). Patient no. 70 (Hb 4.2 g/dl) had clinical evidence of having sickle-cell disease, and the diagnosis was confirmed on hematological investigation. Patient no. 72 (Hb 4.0 g/dl) showed large quantities of malarial pigment in the bone marrow and grossly megaloblastic erythropoiesis.

The predominant hematological diagnoses in these children were malaria, other infections and folate deficiency (Table 14). In this small sample, only four were iron-deficient, whereas 12 were folate-deficient.

Pregnant women

Nine primigravida were studied at Maroua, and four primigravida and one multigravida at Mokolo (Tables 2, 10, 11). All were in the second trimester, except one who was in the sixteenth week of pregnancy (no. 64). None had any complaint, clinical evidence of infection or splenomegaly. Five patients from Maroua and three from Mokolo were anemic, but in only one (no. 33) was the anemia severe (Hb 7.1 g/dl).

Iron deficiency appeared to be the sole cause of anemia in the patient with the lowest Hb, and was found in combination with malaria and folate deficiency respectively in two other patients (Table 15). Malaria appeared to be the sole cause in two and folate deficiency in one anemic patient. Myeloid hyperplasia suggested that patient no. 60 had some undiagnosed infection.

Hemoglobin Electrophoresis and Serum Ferritin Concentrations

Hemoglobin electrophoresis results were obtained from a total of 47 patients (Table 3-11). Four had sickle cell trait (Hb-AS) and one child aged 18 months had sickle cell anemia (Hb-SS) with some persistent Hb-F (no. 70, Table 8). All other patients had a normal Hb pattern.

Serum ferritin concentrations were assayed in 54 patients. The relationship between serum ferritin and bone marrow iron is shown in Figure 1 for the 45 patients in whom both measurements were available.

DISCUSSION

This pilot study has demonstrated the feasibility of carrying out a fairly sophisticated laboratory evaluation of anemia in patients located in the rural areas of Cameroon. The partial processing of samples in the field, combined with further measurements done in Yaoundé, yielded satisfactory results. It is also evident that most of the essential information required for a field study could be obtained from a single finger stick blood sample.

Bone marrow samples were collected in this study and the procedure was well accepted by all patients. Although this test may be important in the diagnosis of anemia in some patients admitted to hospitals, it would clearly not be appropriate in field studies. Since an electricity supply was available in most areas, the microhematocrit could be substituted for the hemoglobin estimation in future studies. As discussed below, although not as accurate as bone marrow examination, the observations made in this study suggest that the serum ferritin may have sufficient predictive value to be useful in field studies of iron deficiency in adults. Its value in children is less certain.

Inferences drawn from the observations made in this investigation are of necessity tentative, because the subjects studied were highly selected and included only pregnant women and children who were sick and attending hospital clinics. The groups chosen are amongst those in whom anemia is known to be particularly prevalent throughout the world, and generalizations for the whole population cannot be made.

The identification of etiological factors responsible for anemia was based largely on the interpretation of bone marrow morphology. The frequency of active malarial parasitemia has probably been under estimated because of technical errors in the preparation of thick peripheral blood smears. The films from the Eastern Province were fixed accidentally with methanol in the field, and a few of the unfixed films from the north were eaten partially by flies. The diagnosis of malarial infection in this study is based, therefore, mainly on the finding of malarial pigment in the bone marrow: whenever the species of parasite could be identified on thick blood film (Tables 3-11), *P. falciparum* predominated.

The current observations support the conclusions reached during the Nutrition Survey and indicate that anemia is common and more prevalent in the East than in the North of Cameroon. Of the 52 patients studied, 35 (67%) were anemic, the prevalence being higher in both children (10 of 11) and women (7 of 10) in the Eastern Province than in the North, where only 10 of the 17 children and 8 of the 14 women had low Hb concentrations. The importance of a combination of etiological factors is readily apparent. In only six of the 35 anemic patients was a single factor identified.

Malaria

It may be assumed that malaria is holoendemic in the rain forests, which include the areas of Abong-Mbang and Bertoua. The high frequency of splenomegaly observed in children in Abong-Mbang (4 of 6) is in agreement with

this. In the savanna and the Mandara Mountains, malaria is hyperendemic, and only one of the 17 children in the North had a palpable spleen. Malarial infection was present in nine of the 10 anemic children and five of the pregnant women in the Eastern Province. None of the children or women with normal Hb values had evidence of malaria. In the North, only eight of 14 children and six of thirteen women in whom complete data sets were available, were thought to be suffering from malaria. Furthermore, six of the twelve non-anemic individuals from the North were also suffering from malaria. It is evident, therefore, that the prevalence of malaria was higher and the correlation with the presence of anemia greater in the Eastern Province, although malaria appeared to be an important etiological factor contributing to anemia in both ecological areas.

It should not be overlooked that anemia is only one aspect of the pathology of malaria. In childhood, infections with P. falciparum disturb profoundly the functions of many organs and systems, notably the central nervous system (Edington, 1967). In pregnancy, malaria is associated with abortion, infection of the placenta, impaired fetal-nutrition, low birth-weight and perinatal mortality (Jelliffe, 1968; Gilles et al., 1969; Harrison and Ibeziako, 1973); the infants of low weight are, in turn, at hazard from infections and malnutrition.

Other infections

Significant infections other than malaria were common in all children in both areas surveyed and they were considered to be making an important contribution to the etiology of the anemias. It should be noted, however, that this finding is dependent on sample selection, since all studies were performed on children presenting at outpatient clinics. Infection would be expected to be the predominant cause of illness in such a group. Nevertheless, other studies have shown that infections are commonly associated with anemia among children in the tropics (Werblinska et al., 1975), and the importance of this factor should not be discounted.

Iron deficiency

An important nutritional component in the anemia was present in all groups. Iron-deficiency appeared to be a limiting factor at the time of study in six of nine anemic children and all the anemic women in the Eastern Province. These findings are in agreement with the earlier reports of a high prevalence of iron deficiency in pregnancy in Yaoundé (Eya Eyong, 1975; Joseph, 1979).

In the North, however, storage iron was absent in only three out of seven anemic children and three of the seven anemic women. This finding might lead to the conclusion that iron deficiency is less important in the anemias seen in the North. However, there are three observations which should temper this interpretation. First, a reduced percentage saturation of transferrin with iron was reported in 66 of 92 children in North Cameroon by Delpeuch et al (1980). Secondly, Dr. Jacques Steveny, who has worked at the Hopital

Adventiste, Koza for several years, was of the opinion that most anemias in children and pregnant women responded to iron and that its prevalence was related to hookworm infestation. Thirdly, only small quantities of iron were present in five of the eight women and three of the nine children who had stainable iron in the marrow. This would be insufficient to support the repair of even moderate anemia, implying that although iron-deficiency was not limiting Hb-production at the time of study, total body iron was reduced.

While serum ferritin concentration is closely correlated with the size of iron stores in health and pure iron deficiency (Jacobs et al., 1975), inflammation, liver disease, and neoplastic disorders disturb the relationship (Lipschitz et al., 1974). In uncomplicated iron deficiency the serum ferritin concentration is <12 μg/l. The fact that this was the case in only six of the 22 patients with an absence of stainable iron in the bone marrow provides further evidence that anemia was multifactorial in these patients, and demonstrates a serious limitation to the usefulness of the serum ferritin estimation as the sole measure of iron deficiency in populations where inflammatory diseases are prevalent. The serum ferritin estimation, which can be performed on a sample of blood obtained by finger-stick, may nevertheless still have some value in predicting iron deficiency in field studies where bone marrow specimens cannot be obtained. In the presence of inflammatory disease, a serum ferritin concentration of <50 μg/l indicates probable iron deficiency (Cook et al., 1980). Thirteen of the 18 women and seven of the 14 children with markedly reduced iron stores (0 or 1+) had serum ferritin values below 50 μg/l while only one of the three women and two of the 10 children with iron stores >1+ had values in this range (Table 16). The increase of serum ferritin associated with inflammation was more apparent in iron deficient children than in iron-deficient women.

Folate deficiency

In the Eastern Province, folate-deficiency was diagnosed in six anemic children and four pregnant women, of whom three were anemic. In no patient was folate-deficiency the sole cause of anemia. It was associated with malaria in five, other infections in five and iron-deficiency in four. In four of the children, there was hypoplasia of the erythroid series, associated with infections, with minimal evidence of any change towards megaloblastic erythropoiesis; however, there was granulocytic proliferation showing numerous giant metamyelocytes. In the four women with folate-deficiency, there was associated iron-deficiency in four and malaria in two: their bone marrows were characterized by erythroid hyperplasia with frank megaloblastic changes.

In the Northern Province, folate-deficiency was observed in twelve children, nine of whom were anemic, and in seven pregnant women, three of who were anemic. As in the East, folate-deficiency was never a solitary hematological abnormality in children, being associated with malaria (six), infection (eight), iron deficiency (four) and sickle cell anemia (one child). Megaloblastic erythropoiesis was obvious or even gross in nine children, possibly because erythroid activity was less depressed by infection than

was the case in the East. Folate deficiency was the sole hematological abnormality discovered in two pregnant women, one of whom was not anemic; the other was mildly anemic.

These observations suggest that folate deficiency was occurring as a secondary complication in most patients. Folate metabolism may be disturbed during infections by various mechanisms. They include (i) insufficient intake due to anorexia, (ii) malabsorption (Karney and Tong, 1972), (iii) increased requirement secondary to the erythroid hyperplasia following hemolysis (Fleming et al., 1968; Strickland and Kostinas, 1970; Mati, Hatima and Gebbie, 1971), and (iv) pyrexia leading to inactivation of the essential enzyme dihydrofolate-reductase at around 39°C (Chanarin and Davey, 1964; Panders and Rupert, 1965). Of these, the high demand for folates secondary to erythroid hyperplasia induced by malarial hemolysis is of supreme importance. Protection against malaria during pregnancy in Ibadan resulted in higher serum-folate activities than in the unprotected (Fleming et al., 1968), and was sufficient to prevent the development of megaloblastic erythropoiesis in the majority of patients in Zaria (Northern Nigeria), even without the addition of folate-supplements (Fleming, A.F. and others, unpublished observations).

This does not exclude an important role played by inadequacy of foods rich in folates. The results from the small numbers in this survey suggest that this may have been more important in the North than in the East. However, the prevalence of folate deficiency might show considerable seasonal variation. This study was conducted at the end of June and the beginning of July, a time when there is known to be a peak incidence of dietary deficiency of folates in Nigeria, both in the South (Fleming, 1970) and the North (Fleming, unpublished observations).

Hemoglobinopathies

The prevalence of sickle cell trait varies throughout Cameroon, ranging between 11.9 percent to 22.2 percent in the sample of the population tested in the United Republic of Cameroon National Nutrition Survey (1978). A much higher prevalence is reported from areas where malaria is hyperendemic; 32 percent in North West Province (Mbantenkhu, 1979), which is in agreement with the 32 percent reported at Obudu (Udeozo and Ele, 1975), just across the Nigerian frontier near to Ashunda in Cameroon. High frequencies are reported also in the Kanuri (27.9 percent) and Bede (32.6 percent) in Bornu State, Nigeria (Roberts, Lehmann and Boyo, 1960), and similar figures may be anticipated in the far north of Cameroon.

Hb-AC has a frequency of five percent in two isolated tribes, the Wakura and Hidkala (Roberts et al., 1960), who practice terrace agriculture on the hillsides above Gwoza in Northern Nigeria. It is possible that Hb-C will be found at similar frequency in Cameroon in areas of the Mandara Mountains around Koza.

Where sickle-cell trait occurs in the adult population at 20 percent prevalence, one percent of newborn infants will have sickle cell anemia (Hb-SS); where the prevalence of Hb-AS in adults is 30 percent, two percent of infants will have Hb-SS. About two percent of all newborns have sickle

cell disease in Zaria, Northern Nigeria, but amongst children aged three months to three years presenting at hospital with a PCV <30, 20 percent have sickle cell disease (Werblinska et al., 1975). Experience in Cameroon will certainly be similar once children's Outpatients Clinics become established. Hemoglobin-SC disease in pregnancy may be anticipated as a problem in the Mandara Mountains.

In the future, sickle cell disease will have increasing importance, but at present hemoglobinopathies as causes of anemia are overshadowed by malaria, other infections and iron deficiency.

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Table 1

PREVALENCE OF ANEMIA (w) - BY PROVINCE

AFTER ADJUSTING FOR ALTITUDE †
Children Aged 6-59 Months

PROVINCE	PERCENT	SAMPLE SIZE
Central South[x]	53.3	165
East	57.6	163
North	41.0	200
North-West/West	23.1	164
South-West/Littoral[x]	38.9	172
Total Provinces	37.6	864
Yaounde/Douala	42.8	156
TOTAL CAMEROON	38.1	1020
Special Group	37.9	225

[x]Excluding Yaounde/Douala

(w) Weighted Percent

† United Republic of Cameroon National Nutrition Survey (1978). p 107

Table 2

PATIENTS STUDIED

Location of Clinic	Children	Adult Women	
		Primigravida	Multigravida
Eastern Province			
Abong-Mbang	7	5	5
Bertoua	4	0	0
Northern Province			
Maroua	8	9	0
Mokolo[x]	6	4	1
Koza	3	0	0
Totals	28	18	6

[x]Two clinics

ABBREVIATIONS USED IN TABLES 3-11

Eosin	=	eosinophilia
GM	=	giant metamyelocytes
Hyper	=	hyperplasia
Hypo	=	hypoplasia
Meg	=	megaloblastic
Nil	=	unmarried mother
Norm	=	normoblastic
NR	=	not recorded
P.falc.	=	*Plasmodium falciparum*
Trans.lyc.	=	Transformed lymphocytes
Unemp.	=	unemployed
URTI	=	upper respiratory tract infection

Table 3

CLINICAL AND HEMATOLOGICAL RESULTS ON CHILDREN

AT ABONG-MBANG, EASTERN PROVINCE

Series number	1	2	3	4	5	6	7
Sex	M	M	F	M	F	M	F
Age (months)	24	27	36	36	23	8	28
Father's occupation	Nil	Laborer	Farmer	Nil	Farmer	Nil	Clerk
Clinical observations	Otitis media, edema	Conjunctivitis, Diarrhea	Fever, Scabies	Fever, Diarrhea	Diarrhea	URTI	Nil
Liver (cm below costal margin)	8	4	4	4	0	2	6
Spleen (cm below costal margin)	6	2	0	4	0	2	0
Hb (g/dl)	5.1	9.2	9.5	8.2	9.3	9.7	11.2
Blood cell morphology							
Anisocytosis	3	2	2	2	2	2	1
Macrocytosis	2	2	1	2	2	1	0
Microcytosis	2	2	1	1	2	2	0
Poikilocytosis	0	0	0	0	0	0	0
Target cells	0	1	0	0	0	0	0
Hypochromia	2	0	0	0	1	2	0
Other features	0	0	0	Eosin	*P. falc*	0	Eosin
Malaria							
P. falciparum asexual	NR	NR	NR	NR	NR	NR	NR
P. falciparum gametes,							
P. malariae							
P. ovale							
Bone-marrow							
Erythron.	Meg.	NR	Norm.	Norm.	Meg.	Hypo.	Norm.
Granulocytes	GM+		Hyper.	GM++	GM+	GM+	Norm.
Other elements	-		-	-	-	Lymphocytosis	-
Malarial pigment	2	2	2	3	0	0	0
Iron stores	0	NR	0	2	3	0	1
Serum ferritin (µg/l)	191	257	23	773	71	33	65
Hb electrophoresis	AA	AA	AS	AA	AA	AA	AA
Causes of anemia							
Malaria	+	+	+	+	+	0	0
Other infections	+	+	+	+	+	+	0
Iron deficiency	+	?	+	0	+	+	0
Folate deficiency	+	?	0	+	+	+	0
Other	0	0	0	0	0	0	0

Table 4

CLINICAL AND HEMATOLOGICAL RESULTS ON CHILDREN

AT BERTOUA, EASTERN PROVINCE

Series number	20	21	22	23*	24	25*
Sex	M	F	M	M	M	F
Age (months)	36	52	8	12 year	48	7 year
Father's occupation	Carpenter	Electrician	Gendarme	Clerk	Clerk	Clerk
Clinical observations	Malaria, Ascaris, treated 2 days	Kwashiorkor	Measles	Malaria, treated 2 days	Malaria, treated 2 days	Malaria, treated 2 days
Liver (cm below costal margin)	0	0	0	0	0	0
Spleen (cm below costal margin)	0	0	0	0	4	4
Hb (g/dl)	8.6	4.6	7.4	10.0	10.4	9.3
Blood cell morphology						
Anisocytosis	2	2	3	2	2	1
Macrocytosis	2	1	2	1	1	0
Microcytosis	2	1	3	1	2	0
Poikilocytosis	0	0	2	0	0	0
Target cells	0	0	2	0	0	0
Hypochromia	0	1	2	0	0	0
Other features	Polychromasia	Leukocytosis	Trans. lyc.	P.falc	P.falc	P.falc
Malaria						
P. falciparum asexual	NR	NR	NR	NR	NR	NR
P. falciparum gametes.						
P. malariae						
P. ovale						
Bone-marrow						
Erythron.	Norm	Norm	Norm	NR	Norm	NR
Granulocytes	GM+	Hyper	GM+		Norm	
Other elements	-	Lymphocytosis	-		-	
Malarial pigment	3	2	2		3	
Iron stores	2	0	0		3	
Serum ferritin (µg/l)	201	328+	3	300	85	148
Hb electrophoresis	AA	AA	AS	AA	AA	AA
Causes of anemia						
Malaria	+	+	+	+	+	+
Other infections	0	+	+	?	0	?
Iron deficiency	0	+	+	?	0	?
Folate deficiency	+	0	+	?	0	?
Other	0	0	0	0	0	0

*Sibling of patient No. 24; over age and excluded from further analyses

Table 5

CLINICAL AND HEMATOLOGICAL RESULTS ON PRIMIGRAVIDA

AT ABONG-MBANG, EASTERN PROVINCE

Series number	9	10	11	12*	14
Sex	F	F	F	F	F
Age	20	15	17		20
Husband's occupation	Unemp.	Nil	Soldier		Soldier
Clinical observations Para	0	0	0	0	0
Weeks of Gestation	32	34	28		28
Liver (cm below costal margin)	0	0	0		0
Spleen (cm below costal margin)	0	0	6		0
Hb (g/dl)	11.9	10.3	8.3	6.0	6.1
Blood cell morphology					
Anisocytosis	2	1	2	2	2
Macrocytosis	2	0	2	2	2
Microcytosis	1	0	0	1	2
Poikilocytosis	0	0	0	0	0
Target cells	0	0	1	0	0
Hypochromia	0	0	1	0	2
Other features	0	0	Trans. lyc.	0	0
Malaria	NR	NR	NR	NR	NR
P. falciparum asexual					
P. falciparum gametes.					
P. malariae					
P. ovale					
Bone-marrow					
Erythron.	Meg.	Norm.	Norm.		Norm.
Granulocytes	GM+	Norm.	Hyper.		Norm.
Other elements	-	-	-		-
Malarial pigment	0	2	1		1
Iron stores	0	0	0		0
Serum ferritin (µg/l)	62	20	146	276	66
Hb electrophoresis	NR	NR	NR	NR	NR
Causes of anemia					
Malaria	0	+	+		+
Other infections	0	0	+		0
Iron deficiency	+	+	+		+
Folate deficiency	+	0	0		0
Other	0	0	0		0

Table 6

CLINICAL AND HEMATOLOGICAL RESULTS ON GRANDE MULTIGRAVIDA

AT ABONG-MBANG, EASTERN PROVINCE

Series number	8	13	15	16	17
Sex	F	F	F	F	F
Age (years)	28	32	38	28	26
Husband's occupation	Catechist	Soldier	Laborer	Teacher	Unemployed
Clinical observations					
Para	5	5	6	7	5
Wks. of Gestation	32	32	32	32	26
Liver (cm below costal margin)	0	0	0	0	0
Spleen (cm below costal margin)	0	0	0	0	0
Hb (g/dl)	10.8	11.6	11.0	10.8	10.8
Blood cell morphology					
Anisocytosis	2	2	2	1	1
Macrocytosis	1	1	1	1	0
Microcytosis	2	1	1	1	0
Poikilocytosis	0	0	0	0	0
Target cells	0	0	0	0	0
Hypochromia	1	0	0	0	0
Other features	D. Perstans	0	D. Perstans	0	0
Malaria	NR	NR	NR	NR	NR
P. falciparum asexual					
P. falciparum gametes.					
P. malariae					
P. ovale					
Bone-marrow					
Erythron.	Meg.	Norm.	Norm.	Norm.	Meg.
Granulocytes	GM+	Norm.	Norm.	GM+	GM+
Other elements	-	Eosin.	-	-	P.falc
Malarial pigment	2	0	0	0	2
Iron stores	0	0	0	0	0
Serum ferritin (µg/l)	77	88	28	11	36
Hb electrophoresis	NR	NR	AA	AA	AA
Causes of anemia					
Malaria	+	0	0	0	+
Other infections	0	0	0	0	0
Iron deficiency	+	+	+	+	+
Folate deficiency	+	0	0	+	+
Other	0	0	0	0	0

Table 7

CLINICAL AND HEMATOLOGICAL RESULTS ON CHILDREN

AT MAROUA, NORTHERN PROVINCE

Series number	30	32	34	36	38	40	42	44
Sex	F	M	F	M	M	M	F	F
Age (months)	30	24	24	14	2	12	17	48
Father's occupation	Farmer	Trader	Mechanic	Trader	Mechanic	Driver	Mechanic	Driver
Clinical observations	Bronchitis	Malnutrition	Gastroenteritis	Fever	URTI	Gastroenteritis	Fever	Fever, URTI, Gastroenteritis
Liver (cm below costal margin)	2	5	0	0	0	4	0	4
Spleen (cm below costal margin)	0	0	0	0	0	0	0	0
Hb (g/dl)	12.6	9.7	11.7	8.2	8.9	11.5	10.6	9.7
Blood cell morphology								
Anisocytosis	1	2	2	2	1	2	1	2
Macrocytosis	0	1	2	2	0	1	0	1
Microcytosis	0	1	2	2	0	1	0	1
Poikilocytosis	0	0	0	0	0	0	0	0
Target cells	0	0	0	1	0	0	0	0
Hypochromia	0	0	0	1	0	0	0	0
Other features	Trans. lyc.	0	Trans. lyc.	0	0	0	0	Trans. lyc.
Malaria								
P. falciparum asexual	0	0	0	0	NR	0	0	++
P. falciparum gametes.	0	0	0	0		0	0	0
P. malariae	0	0	0	0		0	0	0
P. ovale	0	0	0	0		0	0	0
Bone-marrow								
Erythron.	Hyper.	Hyper.	Meg.	Meg.	NR	Norm.	Norm.	Meg.
Granulocytes	Norm.	GM+	GM+	GM+++		Norm.	Norm.	GM+
Other elements	-	-	-	-		-	-	-
Malarial pigment	1	NR	1	0		1	0	2
Iron stores	2	NR	2	0		3	1	1
Serum ferritin (µg/l)	25	46	184	6	17	40	150	342
Hb electrophoresis	AA	AA	AA	AA	AA	AA	AA	AA
Causes of anemia								
Malaria	+	?	+	0	?	+	0	+
Other infections	+	+	+	+	+	+	0	+
Iron deficiency	0	?	0	+	?	0	0	0
Folate deficiency	0	+	+	+	?	0	0	+
Other	0	0	0	0	0	0	0	0

Table 8

CLINICAL AND HEMATOLOGICAL RESULTS ON CHILDREN

AT MOKOLO, NORTHERN PROVINCE

Series number	63	70	71	72	73	74
Sex	F	M	F	M	F	M
Age (months)	12	18	9	7	36	32
Father's occupation	Farmer	Laborer	Cook	Farmer	Messenger	Farmer
Clinical observations	Marasmus	Fever, Diarrhea Sickle-celldisease	Measles	Fever	Fever	Fever
Liver (cm below costal margin)	0	6	4	0	0	4
Spleen (cm below costal margin)	0	10	0	0	0	0
Hb (g/dl)	8.8	4.9	10.5	4.0	11.2	6.[illegible]
Blood cell morphology						
Anisocytosis	2	3	1	3	2	2
Macrocytosis	3	3	1	3	1	1
Microcytosis	0	1	1	0	1	0
Poikilocytosis	0	2	0	0	0	0
Target cells	0	2	0	0	0	0
Hypochromia	0	2	0	0	0	0
Other features	0	Polychromasia Sickle cells Leukocytosis	0	Megalo-blastosis	0	P.falc
Malaria						
P. falciparum asexual	0	0	0	0	NR	+++
P. falciparum gametes.	0	0	0	0		0
P. malariae	0	0	0	0		0
P. ovale	0	0	0	0		0
Bone-marrow						
Erythron.	Meg.	Meg.	Norm.	Grossly Meg.	Norm.	Meg.
Granulocytes	Hyper	Hyper	Hyper	GM+++	Hyper	GM++
Other elements	GM++	NR	GM++	-	-	-
Malarial pigment	0	NR	1	3	0	3
Iron stores	0	NR	0	3	1	2
Serum ferritin (µg/l)	40	68	74	441	320	64
Hb electrophoresis	AA	SS+F	AA	AA	AA	AA
Causes of anemia						
Malaria	0	0	+	+	0	+
Other infections	0	+	+	0	+	0
Iron deficiency	+	?	+	0	0	0
Folate deficiency	+	+	+	+	0	+
Other	0	Sickle-cell dis.	0	0	0	0

*Megaloblastic from blood film

Table 9

CLINICAL AND HEMATOLOGICAL RESULTS ON CHILDREN

AT KOZA, NORTHERN PROVINCE

Series number	50	51	52
Sex	M	M	M
Age (months)	18	32	12
Father's occupation	Farmer	Farmer	Farmer
Clinical observations	Diarrhea, Marasmus	Marasmus	Marasmus
Liver (cm below costal margin)	0	0	0
Spleen (cm below costal margin)	0	0	0
Hb (g/dl)	9.1	8.7	11.2
Blood cell morphology			
Anisocytosis	1	2	2
Macrocytosis	0	2	2
Microcytosis	0	0	1
Poikilocytosis	0	0	0
Target cells	0	0	0
Hypochromia	0	0	0
Other features	Leukocytosis	0	Tran. lyc. Leukocytosis
Malaria			
P. falciparum asexual	0	0	0
P. falciparum gametes.	0	0	0
P. malariae	0	0	0
P. ovale	0	0	0
Bone-marrow			
Erythron.	Hypo.	Meg.	Meg.
Granulocytes	Hyper	GM+	GM+
Other elements	GM++	-	-
Malarial pigment	0	2	NR
Iron stores	3	0	NR
Serum ferritin (µg/l)	57	22	76
Hb electrophoresis	AA	AA	AA
Causes of anemia			
Malaria	0	+	?
Other infections	+	0	+
Iron deficiency	0	+	?
Folate deficiency	+	+	+
Other	0	0	0

Table 10

CLINICAL AND HEMATOLOGICAL RESULTS ON PRIMIGRAVIDA

AT MAROUA, NORTHERN PROVINCE

Series number	31	33	35	37	39	41*	43	45	46
Sex	F	F	F	F	F	F	F	F	F
Age (years)	16	17	19	18	18	15	20	14	18
Husband's occupation	Wizard	Driver	Driver	Trader	Driver	Village Head	Driver	Mason	Laborer
Clinical observations									
Para	0	0	0	0	0	0	0	0	0
Wks. of Gestation	32	32	32	28	28	36	36	28	32
Liver (cm below costal margin)	0	0	0	0	0	0	0	0	0
Spleen (cm below costal margin)	0	0	0	0	0	0	0	0	0
Hb (g/dl)	9.7	7.1	10.3	12.3	12.2	12.1	9.9	9.6	11.7
Blood cell morphology									
Anisocytosis	2	2	1	1	1	2	1	1	2
Macrocytosis	2	0	0	0	2	2	1	1	1
Microcytosis	1	2	0	0	0	0	1	1	1
Poikilocytosis	0	0	0	0	0	0	0	0	0
Target cells	1	0	0	0	0	0	0	0	0
Hypochromia	0	1	0	0	0	0	0	0	0
Other features	0	0	0	0	Poly-chromasia	0	0	0	0
Malaria									
P. falciparum asexual	0	0	0	0	0	0	0	+++	0
P. falciparum gametes.	0	0	0	0	0	0	0	0	0
P. malariae	0	0	0	0	0	0	0	0	0
P. ovale	0	0	0	0	0	0	0	+	0
Bone-marrow									
Erythron.	Hyper.	Hyper.	Norm.	Norm.	Meg.	Meg.	Meg.	Norm.	Hyper.
Granulocytes	GM+	Norm.	Norm.	GM+	GM+	GM++	GM+	Norm.	Norm.
Other elements	-	-	-	-	-	-	-	*P.falc*	-
Malarial pigment	0	0	1	0	0	1	0	2	1
Iron stores	0	0	0	0	0	1	1	2	1
Serum ferritin (µg/l)	8	2	8	26	29	45	8	410	31
Hb electrophoresis	AA	AS	AA	AA	AA	AA	AA	AA	AA
Causes of anemia									
Malaria	0	0	+	0	0	+	0	+	+
Other infections	0	0	0	0	0	0	0	0	0
Iron deficiency	+	+	+	+	+	0	0	0	0
Folate deficiency	+	0	0	+	+	+	+	0	0
Other	0	0	0	0	0	0	0	0	0

*Receiving oral iron therapy.

Table 11

CLINICAL AND HEMATOLOGICAL RESULTS ON PRIMIGRAVIDA AND ONE MULTIGRAVIDA AT MOKOLO, NORTHERN PROVINCE

Series number	60	61	62	64	65
Sex	F	F	F	F	F
Age	16	25	16	21	18
Husband's occupation	Vet. Nurse	Laborer	Farmer	Warden	Nurse
Clinical observations					
Para	0	5	0	0	0
Wks. of Gestation	28	28	28	16	36
Liver (cm below costal margin)	0	0	0	0	0
Spleen (cm below costal margin)	0	0	0	0	0
Hb (g/dl)	10.3	11.6	9.0	11.6	98
Blood cell morphology					
Anisocytosis	3	2	1	1	1
Macrocytosis	2	1	1	1	0
Microcytosis	2	1	1	1	0
Poikilocytosis	0	0	0	0	0
Target cells	0	0	0	0	0
Hypochromia	0	0	0	0	0
Other features	0	0	Nucleated RBC	0	0
Malaria					
P. falciparum asexual	0	0	+++	0	0
P. falciparum gametes.	0	0	0	0	0
P. malariae	0	0	0	0	0
P. ovale	0	0	0	0	0
Bone-marrow					
Erythron.	Hypo.	Norm.	Norm.	Norm.	Norm.
Granulocytes	Hyper.	Norm.	Norm.	GM+	Norm.
Other elements	GM++	-	-	-	-
Malarial pigment	NR	0	1	0	2
Iron stores	NR	1	2	2	1
Serum ferritin (µg/l)	18	29	418	20	4
Hb electrophoresis	AA	AS	AA	AA	AA
Causes of anemia					
Malaria	?	0	+	0	+
Other infections	+	0	0	0	0
Iron deficiency	?	0	0	0	0
Folate deficiency	+	0	0	+	0
Other	0	0	0	0	0

TABLE 12

HEMATOLOGICAL DIAGNOSES ON CHILDREN IN THE EASTERN PROVINCE

	Anemic (n=10)			Non-anemic (n=1)			All children (n=11)		
	Present	Absent	NR	Present	Absent	NR	Present	Absent	NR
Malaria	9	1	0	0	1	0	9	2	0
Other infectons	8	2	0	0	1	0	8	3	0
Iron-deficiency	6	3	1	0	1	0	6	4	1
Folate-deficiency	6	3	1	0	1	0	6	4	1
Sickle-cell anemia									

TABLE 13

HEMATOLOGICAL DIAGNOSES ON PREGNANT WOMEN IN THE EASTERN PROVINCE

	Anemic (n=7)			Non-anemic (n=3)			All patients (n=10)		
	Present	Absent	NR	Present	Absent	NR	Present	Absent	NR
Malaria	5	1	1	0	3	0	5	4	1
Other infectons	1	5	1	0	3	0	1	8	1
Iron-deficiency	6	0	1	3	0	0	9	0	1
Folate-deficiency	3	3	1	1	2	0	4	5	1

TABLE 14

HEMATOLOGICAL DIAGNOSES ON CHILDREN IN THE NORTHERN PROVINCE

	Anemic (n=10)			Non-anemic (n=7)			All children (n=17)		
	Present	Absent	NR	Present	Absent	NR	Present	Absent	NR
Malaria	4	4	2	4	2	1	8	6	3
Other infections	7	3	0	5	2	0	12	5	0
Iron-deficiency	3	4	3	1	5	1	4	9	4
Folate-deficiency	9	0	1	3	4	0	12	4	1
Sickle-cell anemia	1	9	0	0	7	0	1	16	0

TABLE 15

HEMATOLOGICAL DIAGNOSES ON PREGNANT WOMEN IN THE NORTHERN PROVINCE

	Anemic (n=8)			Non-anemic (n=6)			All patients (n=14)		
	Present	Absent	NR	Present	Absent	NR	Present	Absent	NR
Malaria	4	3	1	2	4	0	6	7	1
Other infectons	1	7	0	0	6	0	1	13	0
Iron-deficiency	3	4	1	2	4	0	5	8	1
Folate-deficiency	3	5	0	4	2	0	7	7	0

FIGURE 1

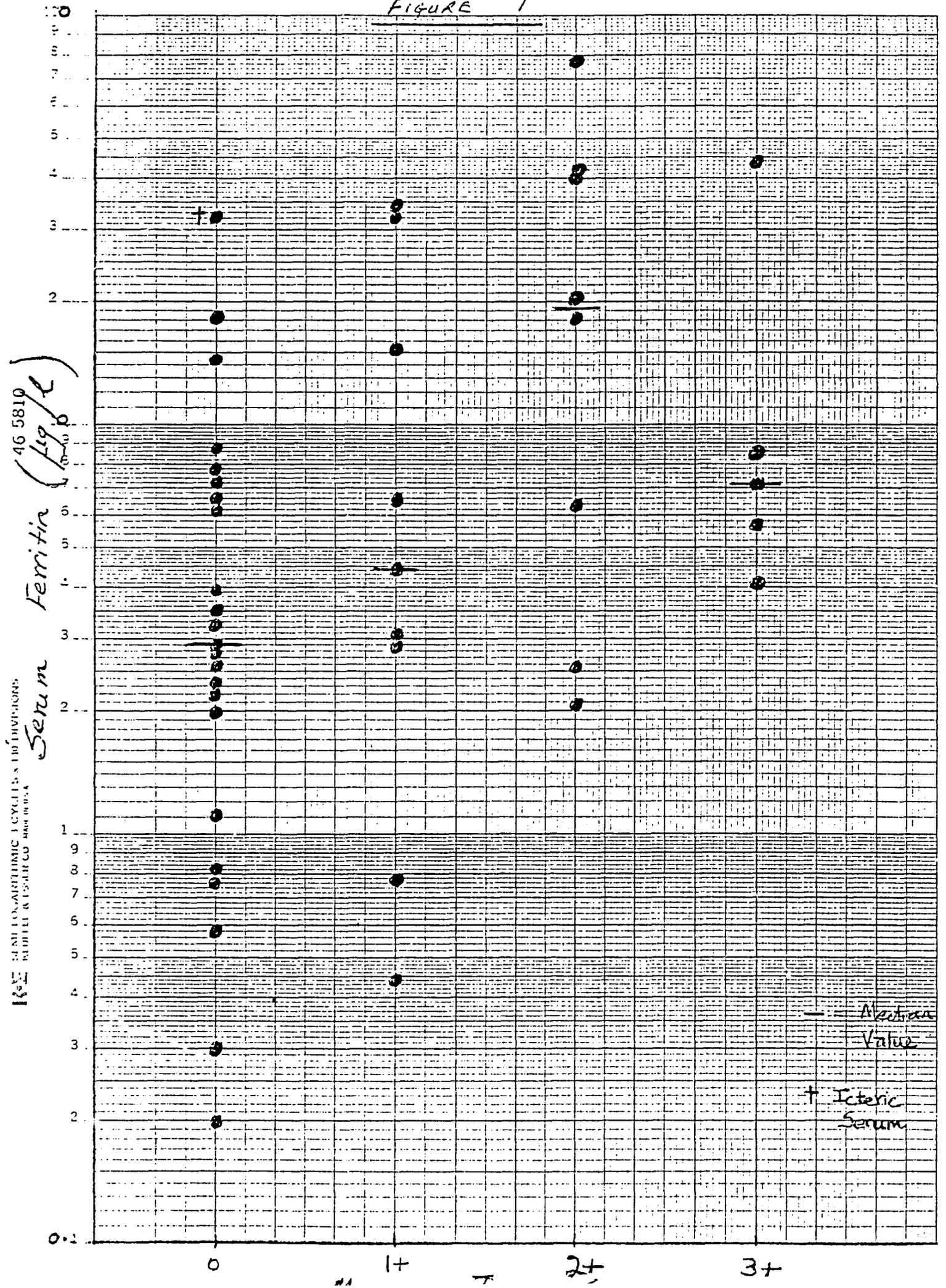

Table 16

COMPARISON OF SERUM FERRITIN AND BONE MARROW IRON

	CHILDREN Number of Subjects	
Marrow Iron	Serum Ferritin < 50 ug/l	Serum Ferritin > 50 ug/l
0 - 1+	7	7
2 - 3+	2	8
Total	9	15
	PREGNANT WOMEN Number of Subjects	
Marrow Iron	Serum Ferritin < 50 ug/l	Serum Ferritin > 50 ug/l
0 - 1+	13	5
2 - 3+	1	2
Total	14	7

APPENDIX A

Timetable of Assignment

Wednesday 18 June. AFF travelled Zaria, Kaduna, Lagos; obtained visa for Cameroon and attended to other administrative matters. SRL travelled Kansas, Paris, Douala, Yaoundé.

Thursday 19 June. AFF travelled Lagos, Douala, Yaoundé and reported at USAID Mission. SRL arrived Yaoundé.

Friday 20 June to Wednesday 25 June. Yaoundé. Briefing at USAID mission. Visits to the Department of Hematology at CUSS and the Blood Transfusion Center, preparing material for field trips and holding discussions with Cameroonian Hematologists.

Thursday 26 June. Travelled by road to Abong-Mbang.

Friday 27 June. Data and specimen collection in Hopital Départemental d'Abong-Mbang (Haut Nyong). Afternoon: travelled to Bertoua.

Saturday 28 June. Data and specimen collection in Hopital Provincial de Bertoua. Afternoon: returned to Yaoundé.

Monday 30 June, Tuesday 1 July. Processing and interpretation of specimens at CUSS, Yaoundé.

Wednesday 2 July. Flight to Maroua.

Thursday 3 July. Morning: data and specimen collection in Prevention Maternale et Infantile (PMI), Maroua. Afternoon: travelled by road to Mokolo.

Friday 4 July. Morning: travelled by road to Koza; data and specimen collection in Hopital Adventiste de Koza. Afternoon: returned to Mokolo; data and specimen collection in Hopital Départemental de Mokolo.

Saturday 5 July. Morning: data and specimen collection in Dispensaire Mission Catholique, Oura-Tada, Mokolo. Afternoon: travelled by road to Maroua.

Sunday 6 July. Flight to Yaoundé.

Monday 7 July to Thursday 10 July. Yaoundé. Processing and interpretation of specimens at CUSS. Debriefing USAID Mission, Yaoundé.

Thursday 10 July. SRL returned Yaoundé, Douala, Paris, Kansas.

Friday 11 July. AFF returned Yaoundé, Douala, Lagos.

Saturday 12 July. AFF travelled Lagos, Kaduna, Zaria.

APPENDIX B

List of Persons Met on Assignment

Lagos

His Excellency Alhadji Yerima Lamine, Ambassador of the United Republic of Cameroon to the Federal Republic of Nigeria.

Yaoundé

- Professor V. Ngu, Vice Chancellor of the University of Yaoundé.
- Professor L. Kaptué-Noche, Chef de Service d'Hematologie, Hopital Central de Yaoundé, Professeur d'Hematologie et Immunologie CUSS, Yaoundé.
- Dr. Fosi Joseph Mbatenkhu, Médecin Assistant en Hematologie, Hopital Central, Yaoundé.
- Professor Dan Lantum, Assistant Director, CUSS.
- Mr. Peter Lord, U.S. Chargé d'Affaire, Yaoundé.
- Mr. James Williams, Director USAID, Yaoundé.

Abong-Mbang

- Mr. John Acha, Prèfet du Haut-Nyong.
- Dr. Mbog Emmanuel, Chef de la Section Départementale de la Medécine Preventive et Rurale du Haut-Nyong.
- Souer Marie-Ange Privat, Infirmière Sage-Femme, D.E.

Bertoua

- Dr. Philippe Issom, Conseiller-Medical, Hopital Provincial de Bertoua.
- Dr. Otto Dondinjiki, Pediatre.
- Dr. Wefuan Jonah Ndifoa, Medécine Generale.

Maroua

- M. Oumarou Koué, Prefet.
- Dr. Pierre Le Jeannie, Conseiller-Medical, Hopital de Maroua.
- Dr. Abba Liman, Chef de Section Départementale de la Medécine Préventive et Rurale, et Chef de Service de Pediatrie à l'hopita de Maroua.
- Mme. Malmadi, Infirmière, PMI.
- Mme Tenguen, Sage-Femme.

Mokolo

- M. Mohamadou Labarang, Premier Adjoint Prefectural, Mokolo.
- Dr. Granier Jean-Claude, Pediatrie, Hopital Départemental de Mokolo
- Dr. Owona Essomba René, Medécine.
- Dr. Kedi à Nwatsok, SDNPR et Gynéco-obstétrique.

Koza

- Dr. Jaques Steveny, Hopital Adventiste de Koza.

Oura-Tada

- Souer Anuncia Morale, Dispensaire Mission Catholique, Oura-Tada.

APPENDIX D

IRON BIOAVAILABILITY PROTOCOL

IRON BIOAVAILABILITY STUDY PROTOCOL

INTERNATIONAL NUTRITIONAL ANEMIA
CONSULTATIVE GROUP (INACG).

IRON TASK FORCE

DR. ALLEN FORBES, CHAIRMAN

1980

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The following experiments are designed to measure the iron bioavailability of three compounds: ferrous sulfate ($FeSO_4 \cdot 7H_2O$); reduced iron (Fe°); and ferric orthophosphte, ($FePO_4 \cdot 2H_2O$). These compounds were chosen because they represent high ($FeSO_4 \cdot 2H_2O$), medium (Fe°) and low ($FePO_4 \cdot 2H_2O$) iron bioavailability. In addition, they represent the most common types of iron compounds used for food fortification.

The experiments are divided into three major parts: clinical studies; animal studies; and physical properties measurements. The clinical part is divided into two parts. The first part consists of administering the iron compound in conjunction with a standard meal (in this case a hamburger, fries and a milk shake from McDonald's). In the second part the iron is administered without the meal. The first part measures the iron uptake as a food fortification element while the second part measures the iron uptake as a dietary supplement. The animal experiments are conducted using the same iron compounds as were used for the clinical portion of the study. Measurement of some physical properties of the iron compounds such as surface area are necessary because the regulation currently being written in Canada base iron availability on solubility in hydrochloric acid.

A summary of the proposed specification for iron bioavailability to be used in Canada is enclosed in the Appendix.

Iron Bioavailability - Clinical Study

The first clinical study will be to determine the absorption or iron compounds which are given with the bulk part of the meal. The compounds to be studied are: reduced iron (Fe°); ferric orthophosphate ($FePO_4 \cdot 2H_2O$); and ferrous sulfate ($FeSO_4 \cdot 7H_2O$). Each compound will be studied individually, however, a small amount of ferric chloride ($FeCl_3$) will be administered as a reference standard. The amount of $FeCl_3$ given will not interefere with the iron compound under study. Each iron compound (eg: $FeCl_3$ and Fe°) will be tagged with a different isotope of iron (eg: ^{59}Fe and ^{55}Fe) in order to measure the absorption of iron from each compound. Ferrous sulfate (3 mg) will be given with each study to measure the iron-absorbing capacity of each individual. This will serve as a calibration standard.

For each iron compound tested approximately 15 subjects are required and the total time for each study is approximately 35 days.

Reduced Iron (Fe°)

The purpose of this study is to measure the iron absorption of reduced iron (Fe°) when given as food fortification.

Reduced iron (2-4 mg) is given with the bulk portion of a standardized meal. At the same time a small amount of ferric chloride (0.1 mg) is given along with the reduced iron. The ferric chloride is known as an extrinsic tag to measure food iron absorption. The reduced iron and the iron in the ferric chloride are labeled with different isotopes of iron so that individual absorption may be measured.

After fourteen days a sample of blood from each subject is taken and the absorption of iron is measured for each compound by determining the ratio of activities for each of the iron radionuclides.

On the fifteenth day another dose is given and again $FeCl_3$ (0.1 mg) is used as an extrinsic tag. However, on the sixteenth day a calibration dose 2-4 mg of ferrus sulfate ($FeSO_4 \cdot 7H_2O$) is given with water and ascorbic acid. The use of ferrous sulfate will enable a direct comparison with other experiments. By relating iron absorption from the other test meals to the absorption of the $FeSO_4 \cdot 7H_2O$ dose, variability in absorption due to differences in iron status of each subject can be reduced.

Again after fourteen days (29th to 30th day of the experiment) blood is taken from each subject and the relative absorption is determined by measuring the iron isotopes ratio.

The same experiment is repeated with ferric orthophosphte ($FePO_4 \cdot 2H_2O$). The following is a summary of the procedure to be used.

Table 1.

297 Study Design to Measure Bioavailability of Reduced Iron (Fe°)

Test Meal	Meal Type	Day of Administration	RADIOIRON Iron Source	Iron Dose (mg)	Radioactivity Dose (μCi)	Nature of Tag
1	Standard	0	^{55}Fe	3.0	5	Fortification Iron
			$^{59}FeCl_3$	0.1	1	Nonheme Dietary Iron (extrinsic tag)
2	Standard	15	$^{59}FeCl_3$	0.1	1	Noneheme Dietary Iron
3	Water	16	$^{55}FeSO_4$	3.0	5	Reference Dose

Table 2.

Study Design to Measure Bioavailability of Ferric Orthophosphate ($FePO_4 \cdot 2H_2O$)

Test Meal	Meal Type	Day of Administration	RADIOIRON Iron Source	Iron Dose (mg)	Radioactivity Dose (μCi)	Nature of Tag
1	Standard	0	$^{55}FePO_4 \cdot 2H_2O$	3.0	5	Fortification Iron
			$^{59}FeCl_3$	0.1	1	Noneheme Dietary Iron (extrinsic tag)
2	Standard	15	$^{59}FeCl_3$	0.1	1	Nonheme Dietary Iron
3	Water	16	$^{55}FeSO_4$	3.0	5	Reference Dose

Table 3.

Study Design to Measure Bioavailability of Ferrous Sulfate ($FeSO_4 \cdot 7H_2O$)

Test Meal	Meal Type	Day of Administration	RADIOIRON Iron Source	Iron Dose (mg)	Radioactivity Dose (μCi)	Nature of Tag
1	Standard	0	$^{55}FeSO_4 \cdot 7H_2O$	3.0	5	Fortification Iron
			$^{59}FeCl_3$	0.1	1	Nonheme Dietary Iron (extrinsic tag)
2	Standard	15	$^{59}FeCl_3$	0.1	1	Nonheme Dietary Iron
3	Water	16	$^{55}FeSO_4$	3.0	5	Reference Dose

Calculations of iron absorption are based on the total blood volume estimated from the sex, height, and weight of the subjects and by assuming that 80% of the absorbed radioactivity was incorporated into the circulating red cells.

All tests are administered between 8:00 a.m. and 9:00 a.m. following an overnight fast. Nothing by mouth is allowed for three hours following each test meal. Double isotope measurements of ^{55}Fe and ^{59}Fe are performed on duplicate 5-ml samples of whole blood drawn before the study and on the 14th and 30th days. These are counted by liquid scintillation technique used by Cook (J. Clin. Nutrition 28 1289-95 (1975)). Sufficient counts are obtained on each sample to reduce the net counting rate error to less than $\pm 2\%$ in subjects absorbing more than 1% of the test dose.

Four absorption studies are to be done on each test subject. The extrinsic tag is administered as 0.1 mg iron as labeled $FeCl_3$. The $FeCl_3$ measures the Fe contained in the meal. It represents the non-heme iron. The dose of Fe°, $FePO_4 \cdot 2H_2O$ or $FeSO_4 \cdot 7H_2O$ is administered with the bulk of the meal as an additive. The $FeSO_4 \cdot 7H_2O$ is administered as a reference dose in water and ascorbic acid.

SALIENT FEATURES OF CLINICAL STUDIES TO MEASURE IRON BIOAVAILABILITY

1. Three separate study groups (15 subjects each) will be employed - one for each form of fortification iron: $FePO_4 \cdot 2H_2O$, reduced iron (Fe°), and $FeSO_4 \cdot 7H_2O$.

2. Three separate test "meals" will be given to all subjects in each study. The meals will be given to fasting subjects on days 0,15, and 16 of each study. Meal 1 and Meal 2 will be identical apart form the forms of iron added and will consist of a hamburger, french fries, and a milkshake. The radiolabels will be added to the milkshake and thoroughly mixed prior to administration.

3. Meal 3 will contain no food but will consist of 50 ml in which 3 mg iron as ferrous sulfate and freshly prepared ascorbic acid at a 2:1 molar ratio of absorbate:iron have been dissolved. This meal is the reference dose and serves to "calibrate" each subject in terms of his iron-absorbing capacity or his iron status. Note that Meal 3 contains ferrous sulfate in all three studies and that this is different than the 3 mg ferrous sulfate that is added to Meal 1 when ferrous sulfate is the form

of fortification iron under study.

4. In meals 1 and 2, 0.1 mg iron as ferric chloride is added to the meal (milkshake) to measure total nonheme iron absorption from the meal, that is to measure the absorption of the iron in the milkshake, french fries, and meat. This tag is usaully referred to as the "extrinsic label". Thus, if the foods in this meal contain 3 mg nonheme iron, absorption of dietary iron from the meal can be calculated by multiplying percent absorption by the total amount of nonheme iron (Table 4).

5. Meal 1 contains <u>two</u> radioiron labels whereas meals 2 and 3 contain only one. The second label in Meal 1 consists of the fortification iron that has been labeled with ^{55}Fe. This tag contains 3 mg iron (as opposed to 0.1 mg for extrinsic tag). The availability of the fortification iron will be assessed by a direct comparison with absorption of the nonheme iron in the meal (extrinsic tag).

6. Blood is obtained on three occasions during each study: on day 0 immediately prior to the first meal for the determination of radioactive background, and then 14 days

following administration of each labeled meal (on day 15, just prior to Meal 2, and only on day 29, at the conclusion of the study).

7. The rationale for measuring absorption for the standard meal on two occasions is illustrated in Table 4 which shows a sample calculation. The absorption of nonheme dietary iron is determined in both Meal 1 and Meal 2 in order to measure the effect of fortification iron o the absorption of nonheme dietary iron. Higher percentage absorption of the nonheme iron is expected from Meal 2 because percentage absorption is inversely proportional to the total iron content of the meal and Meal 1 contains both dietary fortification iron. In the example outlined in Table 4, there is no net effect (or actually 0.01 mg iron) of added fortification iron to this meal.

Table 4.

Sample Calculation to Determine Absolute Iron Absorption.

	Total Iron	Absorption Percent	Absorption Amount (mg)
Meal 1 (Day 0)			
Dietary Iron (nonheme)	4	5	0.20
Fortification Iron	3	3	0.09
		Total	0.29
Meal 2 (Day 15)			
Dietary Iron (nonheme)	4	7	0.28
		Total	0.28

II. Iron Bioavailability Study - Animal Study

Concurrent with the clinical studies, animal studies will be conducted. The method to be used in essentially the same as given by Fritz and Pla (JAOAC, 53, 791-8 (1970); JAOAC, 54, 13-17 (1971); JAOAC, 55, 1128-1132 (1972). Copies of these papers are given in the Appendix for reference purposes. There will be two studies. One involves feeding different levels of iron (or iron compound) to iron depleted rats and measuring the rate and level of iron assimulation for each compound. The second study involves feeding different iron compounds to non-iron depleted rats to measure the uptake of three iron compounds. Since these are the same iron compounds used in the clinical study there will be a basis for comparison.

Purpose: To study the bioavailability of iron from $FeSO_4 \cdot 7H_2O$, Fe°, and $FePO_4 \cdot 2H_2O$ and to relate this study with clinical studies using the same iron compounds.

This first study requires that the weanling rats be depleted in iron before the feeding of the iron compound. This is accomplished by using a low iron diet about (5 mg Fe/kilo) for approximately 3 to 4 weeks until the rats reach a hemoglobin level of about 6 mg Fe/ml of blood. After depletion, the rats will be given iron compounds.

The time required for complete repletion is approximately 2 to 3 weeks. The reference standard for this work will be $FeSO_4 \cdot 7H_2O$ (reagent grade). Since this is the same iron compound that is used in the clinical study, a direct comparison is possible.

A diet containing different levels or iron is fed to the iron depleted rats and the repletion rate is measured by drawing blood from the tail of the rat and measuring the iron hemoglobin level with time. The diet is fortified with 0,10,20,30, or 40 mg Fe/kilogram of diet. The 0 fortification level will serve as the control level for each iron compound studied. Procedure summaries follow.

Animals: Rats (Sprague-Dawley)

Number of Animals per study: 8 rats per group.

Diet and Method of Analysis: See JAOAC 54, 13-(1971) 12th Edition AOAC Methods of Analysis. 43:188 - 43:190

Vitamin supplement modified according to: JAOAC 61, 709-714 (1978)

Ferrous Sulfate ($FeSO_4 \cdot 7H_2O$)

---

1). Five groups of iron depleted rats, each containing eight animals are fed a low Fe diet plus 0,10,20, 30, or 40 mg $FeSO_4 \cdot 7H_2O$ per kilogram diet (each group has a different level of fortification).

2). After feeding the rats with fortified diets for 2 weeks, individual blood samples are taken from the tail vein and analyzed for hemoglobin (hemoglobin contains 3.5 mg Fe/gm).

3). The amount of iron in blood is calculated from the hemoglobin values. The $FeSO_4 \cdot 7H_2O$ feeding will serve as the reference biological values for the other compounds. Also this study will serve

as a link between the animal and clinical experiments.

Reduced Fe (Fe°)

1). Four groups of iron depleted rats, each containing eight animals are fed a low iron diet plus 10,20, 30, or 40 mg added Fe per kilogram diet (each group has a different level of fortification).

2). After feeding the rats with the fortified diets for 2 weeks individual blood samples are taken from the tail vein and analyzed for hemoglobin as before.

3). The level of iron in the blood from this study is compared to the amount of iron in the blood from the reference $FeSO_4 \cdot 7H_2O$ study to obtain the relative biological value of the reduced iron.

Relative Biological Value =
for equal curative effect.

$$\frac{100x \text{ mg Fe/kg from } FeSO_4 \cdot 7H_2O}{\text{mg Fe/kg Fe}^\circ \text{ (reduced)}}$$

Ferric Orthophosphate ($FePO_4 \cdot 2H_2O$)

---

1). Four groups of iron depleted rats, each containing eight animals are fed a low iron diet plus 10,20,30, or 40 mg added $FePO_4 \cdot 2H_2O$ per kilogram diet (each group has a different level of fortication.

2). After feeding the rats with fortified diets for 2 weeks individual blood samples are taken from the tail vein and analyzed for hemoglobin as before.

3). The level of iron in the blood from this study is compared to the amount of iron in the blood from the reference $FeSO_4 \cdot 7H_2O$ study to obtain the relative biological value of $FePO_4 \cdot 2H_2O$.

Relative Biological Value =

for equal curative effect.

$$\frac{100x \text{ mg Fe/kg from } FeSO_4 \cdot 7H_2O}{\text{mg Fe/kg from } FePO_4 \cdot 2H_2O}$$

The second study involves the use on non-iron depleted rats which have been starved for a 24 hour period prior to the feeding of the fortified diet. This study is done so that comparisons between the animal studies and the clinical studies can be made. Since the human test subjects were not depleted in iron before receiving the labeled diet a more direct comparison can be made between the two studies.

Reduced Iron (Fe°)

1). A group of 8 rats are starved for 24 hours prior to feeding.

2). A low iron diet fortified with ^{55}Fe (1μCi) plus $^{59}FeSO_4$ $7H_2O$ (1μCi) solution (administered directly into the rats mouths) is given to the rats.

3). The rats are again starved for 24 hours.

4). Step 2 is repeated.

5). After 7-10 days blood is taken from the tail vein nd the ^{55}Fe and ^{59}Fe activity is measured.

6). The relative biological value is calculated from the iron nuclide activities.

Relative Biological Value=
for equal curative effect.

$$\frac{100x \text{ Activity } {}^{59}Fe \text{ (from } FeSO_4 \cdot 2H_2O)}{\text{Activity } {}^{55}Fe \text{ (from } Fe^{\circ})}$$

Ferric Orthophosphate ($FePO_4 \cdot 2H_2O$)

---

1). A group of 8 rats are starved for 24 hours prior to feeding.

2). A low Fe diet fortified with ${}^{55}FePO_4 \cdot 2H_2O$ (1μCi) plus ${}^{59}FeSO_4$ $7H_2O$ (1μCi) solution (administered directly into the rats mouths) is given to the rats.

3). The rats are again starved for 24 hours.

4). Step 2 is repeated.

5). After 7-10 days blood is taken from the tail vein

and the ^{55}Fe and ^{59}Fe activities are measured.

6). The relative biological value is calculated from the iron nuclide activities.

Relative Biological Value =
for equal curative effect.

$$\frac{100\text{x Activity of } ^{59}Fe \text{ (from } FeSO_4 \cdot 7H_2O)}{\text{Activity of } ^{55}Fe \text{ (from } FePO_4 \cdot 2H_2O)}$$

Iron Bioavailability - Supplemental Clinical Study

The second part of the clinical study is to determine iron bioavailability when iron is given as a dietary supplement rather than as food fortification. The experiment is accomplished in much the same way as the first part of this study. The difference is that the dietary iron is administered with water and not with the bulk of the meal. The other exception is that the administered dose will be raised from 2-4 mg Fe to 18 mg or the usual supplement dose for iron.

This study will use the same iron compounds as before: reduced iron (Fe°); ferric orthophosphate ($FePO_4 \cdot 2H_2O$); and ferrous sulfate ($FeSO_4 \cdot 7H_2O$). As before, each compound will be studied individually. A small amount of ferric chloride ($FeCl_3$) will be administered as a reference standard. The amount of $FeCl_3$ (0.1 mg) will not interfere with the iron compound under study. The $FeCl_3$ serves as a measure of the dietary non-heme iron. Each compound (eg: Fe° and $FePO_4 \cdot 2H_2O$) will be tagged with a different isotope of iron (eg: ^{59}Fe and ^{55}Fe) in order to measure the absorption of iron from each compound.

For each compound tested approximately 15 subjects are required and the total time for each study is approximately 35 days. This study is of secondary priority due to the

number of subjects required, amounts of labeled iron compounds, time and expense. It is included for completeness. Because the study is essentially the same, except for amounts and method of administration, only one example procedure will be given. The following is the protocol for reduced iron (Fe°).

Table 1.

Study Design to Measure Bioavailability of Reduced Iron (Fe^0)

Test Meal	Meal Type	Day of Administration	RADIOIRON			
			Iron Source	Iron Dose (mg)	Radioactivity Dose (uCi)	Nature of Tag
1	Water	0	^{55}Fe	18.0	5	Fortification Iron
			$^{59}FeCl_3$	0.1	1	Nonheme Dietary Iron (extrinsic tag)
2	Water	15	$^{59}FeCl_3$	0.1	1	Nonheme Dietary Iron
3	Water		$^{55}FeSO_4$	3.0	5	Reference Dose

SUGGESTED PROTOCOL FOR IN VITRO EXAMINATION OF IRON FORTIFICANTS -INACG STUDY-

The physical-chemical characterization of the three iron sources to be used in this study is necessary for reference when comparisons with other bioavailability studies are made. Sections I and II are included for this characterization. Correlations between biological activity and the parameters from I and II for the three sources cannot be expected because of the gross differences in characteristics of ferrous sulfate, ferric orthophosphate and electrolytically reduced iron.

Section III, the measurement of solubility, offers the best possibility of correlation with bioavailability. This method represents a modification of one used to evaluate reduced iron powders in a collaborative study (M. K. Austin, private Kellogg Communications; B. G. Shah).

Special care should be taken to assure representative sampling of the iron sources for the three series of tests (human, animal and physical). Similarly, within each test area precautions should be taken to avoid particle size segregation, especially with the reduced iron source, prior to use.

Protocol for physio-chemical characterization of the three iron fortificants to be used in the INACG work:

1. Determination of Purity

   % Iron in sample (as Fe^{+3}, Fe^{+2})

   Bathophenanthroline (colorimetric method attached)

   Contaminating elements

   Cu, Mn, Zn, Ni, Cr, Si, Al, Mg (atomic absorption), Cl^-, PO_4^-

   Anal. Methods of Atomic Absorption Spectrophotometry, Perkin-Elmer Co., Norwalk, Conn., 1976.

II. Physical Measurements

A. Surface area - gas adsorption (BET) (Brunauer, S., P.H. Emmett, E. Teller [1938] J Am Chem Soc 60, 309.)

B. Particle Size:

1. Median and distribution

Photomicrographs (Bjorn Rasmussen, E., L. Halleberg and L. Rossander British J of Nutr. 37, 375-388 [1976])

2. Scanning electron microscopy

C. Apparent density: weight of a mass of powder in a given volume. (ASTM B 212)

III. Solubility Measurements

Estimation of the rate of solubility of elemental iron powders.

Apparatus:

1. Shaker, orbital incubator, one or three-quarter inch stroke.
2. Stirring bars, magnetic, teflon coated.
3. Conical flasks, 500 ml size, glass stoppered.
4. Glass beads, 4 and 5 mm size.

Reagents:

1. Hydrochloric acid (HCl), 0.2% w/v, pH about 1.2. Made up with degassed, distilled water.

Procedure: Determine the solubility of each powder in triplicate.

A. Preparation of Sample

1. Cover bottom of a conical flask with single layer of the glass beads.
2. Mix sample thoroughly prior to weighing.
3. Weigh accurately ca 100 mg of iron powder.
4. Transfer into the flask and pre-warm at 37°C.
5. Add 250 ml of HCl which has been pre-warmed at 37°C.
6. Immediately place flask in shaker set at 37°C and at 150 revolutions per min. for 1" stroke or 200 rpm for 3/4" stroke.

B. Iron Determination

7. Remove aliquot from sample at 10, 30, and 90 minutes.
8. Immediately remove any undissolved iron particles from the aliquot by using a teflon coated magnetic stirring bar.

B. Iron Determination cont'd

9. Filter the aliquot using a syringe fitted with a 1μ Millipore filter.

10. Dilute aliquot appropriately and determine amount of iron by atomic absorption spectrophotometry as described in Sections 2.096, 2.097, and 2.099 in Reference 2.

C. Calculations:

11. Calculated percent solubility using the following equation:

$$\frac{C \times D \times 25}{S} = \% \text{ soluble iron}$$

Where C = concentration of iron in the appropriately diluted aliquot (ut/ml).

D = factor of dilution of aliquot.

S = the weight of iron powder (mg.)

12. Calculate the average and standard deviation for each powder at each time interval.

Reference:

1. Shah, B. G.; Giroux, A.; and Belonje, B.
Specifications for Reduced Iron as a Food Additive.
Journal Agric. Food Chem., Vol. 25, No. 3 (592-594) 1977

2. Official Methods of Analysis, Association of Official Analytical Chemist, Washington, D. C., Twelfth Edition, 1975

DETERMINATION OF IRON WITH 1,10-PHENANTHROLINE

Willard, H. H., L. L. Merritt, J. A. Dean. Instrumental Methods of Analysis, 5th Edition, D. Van Nostrand Co., N.Y. [1974], p. 113.

Standard iron solution Dissolve 0.7022 g of reagent grade $(NH_4)_2Fe(SO_4)_2$ $6H_2O$ in 100 ml of distilled water, add 3 ml of 18 M H_2SO_4 and dilute to 1 liter. One milliliter contains 0.100 mg of iron (II).

For a standard iron (III) solution, dissolve 0.864 g $NH_4Fe(SO_4)_2 \cdot 12\ H_2O$ water, add 3 ml of H_2SO_4, and dilute to 1 liter. Alternatively, the ferrous ammonium sulfate solution can be carefully oxidized with $KMnO_4$ added dropwise until a slight pink coloration remains after stirring well, before diluting to 1 liter. One milliliter contains 0.100 mg of iron.

1,10-Phenanthroline Dissolve 0.25 g of the monohydrate in 100 ml of water, warming if necessary.

Hydroxylamine hydrochloride, 10% (w/v) Dissolve 10 g in 100 ml of water.

Sodium acetate, 2M Dissolve 17 g of sodium acetate in 100 ml of water.

Procedure Take an aliquot portion of the unknown solution containing 0.1 to 0.5 mg of iron and transfer it to a 100-ml volumetric flask. Determine by the use of a similar aliquot portion containing a few drops of bromophenol blue, the volume of sodium acetate solution required to bring the pH to 4.0 (or use a pH meter). Add the same volume of acetate solution to the original aliquot and then 5 ml each of the hydroxylamine hydrochloride and 1,10-phenanthroline solutions. Dilute to the mark, mix well and measure the absorbance after 10 min in the region 460-520 nm.

Both iorn(II) and iron(III) can be determined simultaneously. Only the iron (II) complex absorbs at 515 mn, and both complexes have identical absorption at 396 nm, the amount being additive. The solution should be buffered at pH 3.9.