CHAPTER III

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This section presents an integrated account of the problem, its execution, the conclusions drawn from the study as a whole, and the suggestions offered for future work in this area.

It is cogent to point out here that the ensuing discussion has been arranged specially to determine and indicate to what extent the specific objectives spelled out at the start of the study (page 29) have been achieved. The arrangement that has been followed in this section therefore sticks to the four main criteria of suitability fixed for the fortificant, i.e. the physico-chemical stability, the consumer acceptability, the bioavailability and the industrial processability of the fortificant system, in that order. The present author hopes that this sequence would clarify better the situation in respect of this problem as it stands at present.

It is also clarified that the 'discussion' of the results obtained in the individual experiments has already been included in the "experimental section".

(1) The problem
(a) The genesis of the present study

The present study originated from a simple but astonishing fact of life, which emphasizes that the metal most precious to man is iron and not gold, because man can survive without gold, but, certainly, he cannot survive without iron, which, so to say, he must eat every day! Without adequate iron from his daily diet, he soon loses his capacity to carry and utilize oxygen, and his metabolic machinery, the essence of his
vital activity and the very basis of his existence, comes to a deadly halt (334). The reality, however, is that, despite his desperate and dire need for iron, man is relatively poorly equipped to capture iron from his food, and this odd imperfection and shortcoming in his life inevitably brings him on to a state of iron deficiency (334).

The iron deficiency: Iron deficiency has now been well recognized as the commonest deficiency state in man (50). Its fairly high frequency in most populations, certainly including even those in the affluent countries, has been traced to dietary iron as the ultimate/main cause. Dietary iron has conclusively been shown to be only a marginally adequate nutrient which, by itself, is wholly incapable of meeting the normal metabolic requirements of the concerned age groups (48). The organism's need at crucial periods of life which has to be met (334) emerges as the limiting factor. Iron deficiency during pregnancy reduces the tissue iron stores in the infants, a deficit which persists if subsequent iron intake remains marginal (335).

The fairly common prevalence of iron deficiency in India has recently been reviewed (336-338, 400). Inspite of the comparative affluence in our region, the number of anemic Punjabi children stands around 70% (339). Recent surveys in some developing tropical countries revealed moderate to severe anemia in more than 50% women (334). The minimum estimates of the overall prevalence of iron deficiency range from 20% in industrialized countries to 60% in developing nations, the population groups particularly at risk being pre-school children, pregnant
women, old individuals and adolescents (335). Even in U.S.A. some five million persons have been estimated to suffer from some degree of iron deficiency (334). The number of iron deficient persons in the world therefore surely runs into hundreds of millions (334). Iron deficiency has thus emerged as a persistent nutritional problem of worldwide concern. Furthermore, in this context it is important to realize that the consequences of iron deficiency are in fact accentuated in societies and age groups in which it overlaps the deficiency of vitamin C. In 1978 the International Anemia Consultative Group (INACG) published their "Guidlines for the eradication of iron deficiency anemia" (340).

Equally well recognized is the fact that the infant and the pregnant and the lactating women groups are uniquely vulnerable to the devastating effects of iron deficiency, the former because the rapid increase in their blood volume has to be met in the face of their poor iron stores at birth and their sole diet (milk) is naturally deficient in iron (6), and the latter because at first they have to meet the fetal requirement and then that of the infant (breast feeding) from their comparatively poorer iron stores. In this connection, it is pertinent to note that the largest sex difference that has been found to occur is in the magnitude of the female iron stores, which are only 400 mg in comparison with the 1000 mg in the average adult male (48).

The target group: The dreadful metabolic state of iron deficiency engulfs several age groups. The primary target group selected for the present study was the infant group because of its utmost importance to our country and South East Asia in particular,
and to other developing countries in general, though other vulnerable groups were also kept in view.

Iron deficiency in infants had been highlighted as early as 1928 by Mackey (341), still the tempo of research in this area of nutrition during the next 25 years (1928-53) seems to have remained in a low key. Then during the next two decades (1953-73), several reports appeared, exemplified by those in respect of iron metabolism (342-345), iron deficiency (344, 346, 347-349, 375), and iron requirements (13, 15, 341-350) in infants. Even though some earlier workers during 1954-58 (351,352) did express their doubt about the absorption of iron from the infant's main food, i.e. milk, believing that it was hindered than when given alone, yet quite a number of others in almost the same era (1958-61) (353-356, 60) came round the conclusion that iron was well absorbed from this dietary form. During the literature survey for the present programme for a period of the last ten years (1968-78), the present author came across an unexpected spurt in investigative activities in relation to the fortification of milk with iron, as a fairly large number, as many as some 35, of investigations in this area had been reported (3, 13, 15, 37, 70, 77-85, 100, 108-115, 117-120, 210, 340, 357-361). Most of these aimed at finding better and more efficient methods for the fortification of milk with iron. McMillan et al. (1976) (100) had reported on the good availability of iron from human milk in infants. These findings were confirmed by two other independent reports in 1977 (405, 406) and in WHO Report (70). Thain-Than et al. (1975) (116) reported on the inhibition of iron absorption
by coconut milk. It is quite pertinent to mention that Heinrich and coworkers, at first in 1970 in a discussion (362), and then later in 1975 (38), using a remarkably elegant and accurate methodology employing labeled iron, still cast a doubt on the absorption of iron from supplemented cow milk in infants with normal and depleted iron stores. As against this, equally elaborate and precise work of Carmichael et al. also reported in 1975 (6), and also employing labeled iron, conclusively proved good iron absorption in the presence of normal amounts of casein. As detailed elsewhere, the present investigation also proves, as conclusively as possible, good absorption of iron from both the buffalo and the cow milks when fortified with FS + AA system.

The main objective: The iron fortification of milk and other foods. In a situation like the one explained above, preventive fortification of foods with iron becomes the only rational course to adopt (357). The value of this step has been discussed (363). The main objective and the principal purpose of the present project therefore was to initiate a programme of research to design and develop a dietary form of iron which could be adequately effective as a preventive and prophylactic measure against iron deficiency in infants, our primary target group; it could also be equally effective in and beneficial for, other age groups. In this context, it is categorically imperative and cogent to bear in mind that any iron fortification programme is meant to prevent rather than to treat iron deficiency. Accordingly the form of the dietary iron and its level selected for the current investigation (1 mg of absorbed iron per day) were designed
to achieve only a limited objective - a slow but steady build up of the iron stores in the target groups, rather than to serve as a therapeutic measure against iron deficiency (70, 357, 461).

Undoubtedly, the benefit of any programme of fortification of diet with iron would depend upon the nature of the diet consumed by the concerned population; for instance, absorption of iron from a diet containing 60 mg iron given as FeCl₃ was found to be 0.3, 1.2 and 3.3 mg from maize, maize + meat, and meat diet itself (50). This naturally emphasizes the importance of the vehicle to be selected for the fortification programme. The requirements expected of a suitable and good vehicle and a fortificant have already been chalked out (pages 5-7). According to the WHO Technical Report, 1975 (70) also, the ideal vehicle should be one which is consumed by the vast majority of the target groups in adequate amounts, should be available for fortification on a large scale and at relatively few centres so that the fortification process could be adequately supervised, the resulting product should be stable under the extreme conditions likely to be encountered during storage and distribution, and the pallatability of the vehicle (or of the other foods that the vehicle might be mixed with, or on cooking) should remain unaltered. Likewise, the ideal fortificant should be readily absorbable from the vehicle, or from the vehicle mixed with diet, should not cause changes in colour and taste, and should remain stable under the conditions in which it is stored, and, above all, should be cheap. It is obvious that these are fairly rigid and quite exacting requirements, and therefore it is not surprising to find that none of the currently used vehicles or the fortificants completely
and scrupulously fulfil these criteria; all of them are known to suffer from one disadvantage or the other. In this context, the present discussion has been restricted only to a comparison of milk vs. other main vehicles currently in use. At present common salt is being tried in India on a community level as the most suitable vehicle for iron fortification. The present author is, however, convinced that an alternate approach is very much necessary and warranted; milk comes nearest to the desired vehicle. The data presented in the previous sections is being offered in defence of this thesis, and to the present author it amply testifies to the correctness of this thinking.

(b) The vehicle

The paramount importance of the vehicle in iron fortification of foods cannot be exaggerated or overrated. The main carrier foods other than milk that have been tried rather extensively include cereal foods like bread and wheat flour, and cane sugar and common salt. The minor ones include beverages and juices, drinking water, coffee, and fish sauce (pages 17-19). Several other possible vehicles found during the course of this study and considered suitable for adults, have been discussed on pages 187-196.

Cereal foods, cane sugar and common salt: The cereal foods have their own serious problems, viz., their phytate content hinders iron absorption (372), iron salts of organic acids when added to wheat flour for making bread do not withstand the baking conditions, and the availability of iron from the otherwise stable iron phosphates is rather poor and they have been found almost
useless for iron fortification. For this purpose, all iron phosphates, whether ortho-, tri- or hexa-meta-, pyro- or soluble pyro-phosphate, all behave the same way. The absorption of pyro- and ortho-phosphate is barely some 5% and 30% of ferrous sulphate or of reduced iron (50).

At present it is considered that in the wheat bran it is the phytate and not the phosphate which inhibits iron absorption (70). As regards the inhibition caused by phytate, a recent report, in January, 1979 (364), emphasized that phytate present in soybean did not hinder iron absorption in rat and in man, as iron was absorbed excellently from soybean protein. According to these investigators, all previous workers had used purified sodium phytate in their experiments. The naturally occurring phytate, being a Ca-Mg salt, was not an inhibitor. They consider that the monoiron phytate which has been found to occur in wheat (365) was a carrier of iron rather than an inhibitor of iron absorption. Several other recent reports (366-373), which discuss the availability of iron from phytate and phosphate containing foods, like bread, have been included here for reference; these are in addition to those already given in the review (pages 19 and 20). All these bring out the limitations of cereal foods as vehicle for iron fortification.

Cane sugar: During 1975-77 two groups of investigators worked out the details of the possible use of cane sugar as a vehicle for iron fortification, with and without added ascorbic acid, and with different types of meals (135, 157-159). Their results were very encouraging, as they found that cane sugar by
itself did not contain any absorption-hindering substance, and, further, that ascorbic acid was a good promoter of iron absorption in the presence of this vehicle. Among a large number of ferrous and ferric salts tried in these experiments, both ferrous sulphate and ferric-EDTA were found to give almost equally satisfactory response, whereas ferric ammonium citrate was rather poorly absorbed. Soluble iron salts like ferrous sulphate gave an undesirable blackish colour to tea and coffee, while the ferric-EDTA remained stable towards the tea tannins for some two hr. Derman et al. (159) evaluated a cereal-based meal containing cane sugar fortified with ascorbic acid and got a very encouraging response. However, inspite of these results, cane sugar as a vehicle has not found favour in several quarters, because of the storage problem in humid and hot climates, as in India, and the adverse side effects EDTA could cause on continuous use (chronic toxicity). To the present author it seems that this vehicle actually needs a different type of fortificant; saccharated oxide of iron warrants trial, and further, it is also suggested that it would be far cheaper and wiser from iron absorption angle to use raw sugar (gur) itself as is done by the rural population in India, because it contains a far higher amount of iron, already covalently bound to the sugar.

Common salt: Narasingha Rao et al. in 1972 (154) reported good bioavailability of iron from common salt fortified with ferrous sulphate + phosphoric acid; although this combination was discoloured so rapidly, on storage its bioavailability was affected adversely. In 1974 Sayers et al. (33) found good
bioavailability of iron from rice meal containing common salt fortified with ferrous sulphate and ascorbic acid; this combination was found to discolour fairly rapidly. Refind salt was found more stable than course salt, and the addition of 2.5\% starch inhibited discoloration; ascorbic acid was found to increase absorption. After evaluating the effect of a large number of chemical additives on stability and bioavailability, in 1975 Narasingha Rao et al. discovered the use of NaH\textsubscript{2}SO\textsubscript{4} as an interesting stabilizer and promoter, and, using labelled iron, found a good bioavailability from ferric phosphate + NaH\textsubscript{2}SO\textsubscript{4}, irrespective of its being ferric phosphate; they ascribed this to the effect of the promoter; the use of ascorbic acid was not found satisfactory by them. In order to reduce the cost of this combination, in 1978 Narasingha Rao et al. proposed an alternate formula based on ferrous sulphate + phosphoric acid + NaH\textsubscript{2}SO\textsubscript{4}, and found this combination quite satisfactory from the point of view of bioavailability as well as stability. It has been claimed that this formula has proved successful in community trials recently conducted in several parts of India. However, irrespective of these claims, the use of this fortificant system seems to have its own limitations. Besides questionable absorbability of iron from ferric phosphate, in spite of the use of a promoter, the use of a fairly strong acid (NaH\textsubscript{2}SO\textsubscript{4}) in itself is likely (or bound) to cause side effects on prolonged use from its accumulative effect (chronic toxicity or adverse effects). Furthermore, the use of common salt as the vehicle has its own limitations, in that it leaves certain groups totally uncovered; one is the infant group, and the other the hypertensive group,
i.e. those adults who are on restricted salt intake. Whether fortification of potassium chloride for the latter group could probably be a more feasible proposition has to be worked out. Besides, the stability of the above proposed combination in the humid and hot climate, as in our country, is another factor which has to be reckoned with. Stability and non-discolouration would surely require refind salt, as the coarse variety which is used most commonly is known to be much more susceptible to discolouration and allied defects. The cost of the FeSO₄ + H₃PO₄ + NaHSO₄ combination, particularly after including refind salt as the vehicle for reasons of stability and consumer acceptability, would warrant more detailed studies to find a better and more efficient substitute fortificant, or a de novo rethinking on the feasibility of common salt as the vehicle. It is to be pointed out that the position in respect of the "iodised common salt", i.e. fortification of common salt with KI + I₂ for use in the endemic goitre areas, was quite different. An alternate approach to the use of common salt as a vehicle for iron fortification programme there/stands warranted.

**Milk vs. other carrier foods:** The selection of milk as a vehicle for iron fortification in comparison with the above three more commonly tried vehicles cannot be ruled out as just something impracticable and inconceivable; undoubtedly, it does fulfil most of the criteria fixed for an ideal vehicle (pages 5 to 7). The good bioavailability of iron from milk stands proved (6). The preliminary estimate of cost of fortification seems comparable to that of fortification of common salt; fine details of 'costing' are awaited. But, of course, like all other vehicles,
milk too has its own limitations. For instance, most infants in our country, unlike many others in the West, are breast-fed, and so apparently they seem not within the reach to be exposed to fortified milk; but, then, in this respect too milk still scores over common salt. The organized dairy technology in India today, though still not so advanced as in U.S.A., Europe, Australia and Japan, has surely reached the stage where it can take up this problem for central fortification and distribution to bring it within the reach of even the weaker sections of our society, both in the urban and the rural population. Official assistance, as in the case of common salt, is needed to give a trial to this alternate approach as well.

Other possible vehicles: Besides the major and the minor carrier foods detailed above, the present investigation found several other solid and liquid preparations (like sprinkles, appetizers and drinks) very commonly used by all sections of Indian population, which could be used with benefit by adults as vehicles for iron fortification. These have been discussed on pages 187-196.

Ultimate choice of the vehicle: The following inferences as regards the choice of the vehicle for iron fortification should prove worthwhile to consider:

All along it has been considered that the choice of the vehicle was very important. However, Cook (1977) (50) has pointed out that, in view of the fact that an available iron salt or complex would have the same absorption as the non-heme iron of the diet regardless of the vehicle, the choice of the vehicle
should be dictated by factors other than iron availability, such as distribution within the population, and the undesirable reactions of iron with the vehicle which may alter its colour and taste. In this connection it is well recognised that in the case of milk as the vehicle the foremost factor would be the off-flavour problem, which would have to be controlled the most.

"No iron fortification at all" could be another worthwhile approach to explore. Meat and fish are well recognised as highly efficient promoters of iron absorption. It is rather strange that animal foods are seldom eaten in areas where iron deficiency is most prevalent (50); however, persuading people who are vegetarian, either from economic necessity or religious belief, to change their dietary habits is scarcely a useful public health approach to the problem of iron deficiency (357). In such a situation, fortification of diet with a substance such as ascorbic acid which is known to increase iron availability of native food iron might be a more rational approach than adding more iron to the diet (15, 50, 357). Most Indian foods are known to contain sufficient iron, which otherwise remains unavailable. In fact, the alternate approaches should actually involve finding newer and more efficient ways to add AA to cane sugar, common salt and milk as the basic vehicles. However, it is also necessary to keep in mind a balance or equilibrium, because a recent report (374) has drawn attention to the fact that excessive amount of AA destroys vitamin B12. This warrants a cautious approach to be adopted, particularly in populations with an already marginally adequate vitamin B12 intake (357).
The execution of the plan

(a) The fortificant

Rationale for selecting the present fortificant system:

In order to devise rational and more efficient ways for the fortification of the infants main diet (milk) with iron, the selection of the fortificant was based on two previous observations; one that most of the ionic organic acid ligands that had been tried for ironization of milk had given complexes which caused oxidized flavour in milk (76-84), and, second, that a chelated iron preparation, such as iron glycinate (2mg/5 ml), iron-EDTA (55 mg/10 ml), or a non-ionic polysaccharide-iron complex (100 mg/5 ml) in a syrup was well tolerated by infants (345, 356, 375); the last preparation gave the best response. Administration of iron simultaneously with milk or some cereal foods had been found to result in adequate absorption with less intestinal intolerance; ferrous sulphate was known to cause intolerance in infants (356, 375), and could also possibly result in an acute hepatotoxicity (376) and ulceration (451) in these extremely sensitive and vulnerable of the human species.

At this stage of the investigation, i.e. before selecting the actual fortificant to be tried in the ensuing studies, it was considered imperative to fix the characteristics desired and expected of a food iron fortificant, suitable for foods in general and for milk in particular. The following characteristics of iron had to be borne in mind because they would become the deciding factors: ferrous iron is oxidized to the ferric iron; iron compounds react with plant tannins (tea, coffee) to
give a blackish colouration, and with sulphur compounds to give black coloured products; they catalyse development of oxidative reactions resulting in spoilage of the food stuffs, and also activate oxidative enzymes that cause off-colour, off-taste, off-odour and off-flavour. The WHO Technical Report of 1975 (70) too had stressed these points (pages 25-30 of the said report).

In the light of the above, a line of attack based on non-ionic sugar-type ligands was preferred and initiated. More than 50 ligands, including some 21 sugars, were screened to arrive at the most suitable ligand for the ensuing studies. The work of Saltman and his group on ferric fructose proved a good guide; they had reported good absorption (377-380, 386-388) and bioavailability (6), and a rapid tissue distribution of the absorbed iron (381) using this complex. Several recent reports were also kept in view (437-439). In the first series of this programme, iron complexes from three sugars, viz. fructose, sucrose and lactose were prepared for further studies. Ferric lactose, out of the three, was preferred for detailed examination, because it was the intrinsic and characteristic sugar of milk itself, was far cheaper than the other two sugars as it was readily available in any desired amount as a by-product from an industrial 'waste' of the dairy technology, as no work had been reported on it previously, and, finally, because ferric lactose + ascorbic acid system had given somewhat encouraging results. These findings were reported last year (85, 210, 382).
These studies had afford a method for the concomitant fortification of milk with both iron and ascorbic acid.

In order to remove the shortcomings of the first series, the second series of our programme, the present investigation, was initiated using the reduced analogues of the hexose sugars, viz. hexitols, to avoid any possible adverse contribution of the enediolic species towards the structure and thence the properties of the resulting complexes. Both sorbitol and mannitol complexes were studied in detail, and on the basis of the experimental data obtained in respect of their physico-chemical characteristics (Pages 47-84), and the consideration of the following pertinent points in respect of sorbitol, ferric sorbitol was selected finally as the iron complex for the current investigation. These extra points which justified the choice of sorbitol were as follows:

Sorbitol and mannitol, though quite soluble in water (ca. 71%) are poorly absorbed sugar alcohols (383). Both sorbitol (377, 384) and fructose (378) are known to enhance iron absorption in man. Sorbitol is found uncombined in many fruits, such as apples, pears and berries, and also in several plants; mannitol is more plentiful (385). Polyols are usually employed as valuable aids in formulating a wide range of food products. As a class they are used to impart special effects to the texture or taste of a food product. 70% sorbitol syrup is normally employed (385). Recently sorbitol has been proposed as a substitute sweetener for sucrose in diabetic foods and as energy source in parenteral nutrition (389). A structural
analogue of its oxidation product, L-Sorbose, has also been proposed as a sucrose substitute because of its having \( \frac{3}{4} \) of its sweetening power (390). Metabolism of sorbitol via sorbitol pathway has been adequately worked out. Sorbitol is a well recognised sequestrant (385); in this respect it resembles other sugars like glucose (391), fructose (211), lactose (392), sucrose (70, 393, 394). It is completely nontoxic, as is evidenced by the use of ISP, iron-poly (sorbitol-gluconic acid) complex, as an oral haematinic in piglets (395), and also as parenteral haematinic in man as Ferastral (204). In this respect the latter complex resembles iron-dextran complex, which too has been used as an oral haematinic in piglets (161, 396) and as a parenteral haematinic in man as Imferron (204). Finally, sorbitol is fairly cheap and readily available in any amount, as it is an intermediate, obtained by the reduction of glucose, in the commercial synthesis of ascorbic acid. It is certainly cheaper than fructose, and not more expensive than lactose. Further points in justification of sorbitol have been summarized on pages 89-91. Certainly it is safe, cheap and a worthwhile starting material for ironization of milk.

Preparation of ferric sorbitol: It is emphasized that, because of the polymeric nature of iron-sugar complexes in which larger species exist in equilibrium with smaller chain length species, it is only the precise conditions which can give rise to complexes with reproducible properties. Any changes, howsoever minor and insignificant they might apparently look, would affect and even alter radically the nature of the
final complex, such as the size of the chain lengths, etc. The method of preparation of ferric sorbitol reported in the present study (page 140) was somewhat different from that used for ferric fructose by Saltman and his coworkers (211). They prepared their complex at pH 9.0, while the ferric sorbitol complex used in this study was precipitated at pH 11 at the time of first separation; the pH of the complex actually used in the studies at 40 ppm concentration level had a pH of 6.58, which, in the FS + AA system, was lowered still further to 3.74. The final pH of the fortified milk was almost the same as of unfortified milk, ca. 6.8, because the addition of the fortificant system (FS + AA) resulted only in a small decrease of ca. 0.2 pH unit only (page 119). The final pH of the milk after the addition of ferric fructose in the experiments of Saltman et al. (6) does not seem to have been reported.

Nature and stability of the fortificant system (FS)

Nature of ferric sorbitol complex (FS): Because of the inherent behaviour of ferric iron at physiological and higher pH, ferric complexes are not homogenous entities and exist mostly in polynuclear forms, in which larger polymeric species coexist in equilibrium with those of smaller chain lengths. Ferric fructose showed the same behaviour with a mol. weight in the range of 30-60,000 (168). Ferric hydroxide and ferric chloride (178) and even ferric citrate (397) behaved the same way. Saltman et al. (1967) (397) showed that ferric citrate at the physiological pH of 7.2 behaved like a polymer of mol. weight of 2 x 10^5. Ferric sorbitol used in the present study showed a
similar behaviour; this was quite clear from the study of its physico-chemical properties, particularly its solubility, dissociation, sedimentation and dialysis characteristics, and its behaviour on TLC, gel permeation chromatography, electrophoresis and ultra violet and visible absorption (pages 47-84).

Although these studies did afford a fair insight into the possible structure of ferric sorbitol, as given on pages 84-89, which was considered as sufficient for the present as a working hypothesis to proceed further with the ensuing work on its use as milk fortificant, it is pointed out that further more specific information regarding its precise structure, if necessary, would have to be derived from its UV and IR, and NMR, ESR, Mössbauer and mass spectra, alone and in presence of AA.

The presence of a small amount of almost free sorbitol (pages 59-72), which, so to say, was envisaged as the outermost shell (layer or covering) in ferric sorbitol complex does not seem to have been reported earlier, nor for any other ferric-sugar complex, except for ferric lactose reported by our team last year (85), which too seemed to have some free lactose associated with ferric lactose complex through a relatively weaker or loose binding force.

During the course of these studies, a newer method was evolved for the simultaneous estimation of sorbitol and iron in a complex, from the same solution by recourse to UV absorption studies on the periodate-phenylhydrazine-ferricyanide chromogen at two wavelengths 520 + 700 nm (pages 62-67). The application of this method also afforded some interesting insight into the configurational aspects of several sugars and diols (Table 18,
The other iron compounds that have been reported to give satisfactory response as milk fortificant include stabilized FeCl₃ (70; sub-references 166, 180-182 cited therein; 110), ferric glycerophosphate (selected by Layrisse et al. (1973) (112) for skim milk to prevent protein energy malnutrition in Venezuelan preschool children), ferric ammonium citrate (70, 83), ferrous gluconate (70, 79), saccharated iron oxide (70), and ferric fructose and ferric-NTA (6). But, in the hands of the present author, all of them, except ferric glycerophosphate, did cause oxidized flavour in buffalo milk. Ferric lactose (85) seemed somewhat better than most of these, and certainly better than ferric fructose and ferric sucrose, but it was found to suffer from another serious defect that on storage it settled down in milk. It is pointed out that higher mol. weight iron complexes such as ferric complexes with EDTA, poly-(sorbitol-gluconic acid)(mol. weight ca. 3000) and with dextrans and dextrins do not seem to have been tried in milk as the vehicle. This certainly warrants trial; this aspect is in progress in our research team. Their use as oral and parenteral haematirics has already been recorded (161, 204, 395, 396).

As regards the iron content of the various complexes is concerned, ferric sorbitol has an additional advantage in that it contains the highest amount of iron, as is clear from the accompanying chart compiled for the purpose.
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<thead>
<tr>
<th>Ferrous compound</th>
<th>% iron content</th>
<th>Ferric compound</th>
<th>% iron content</th>
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<td>Chloride</td>
<td>ca. 21</td>
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<td>Chloride</td>
<td>30-44</td>
<td>Amm. citrate</td>
<td>ca. 17</td>
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<td>Carbonate</td>
<td>42-48</td>
<td>Phosphates</td>
<td>10-15</td>
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<td>9-10</td>
<td>Pot. tartrate</td>
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<td>ca. 13</td>
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<td>ca. 20</td>
<td>NTA</td>
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<td>Reduced iron</td>
<td>95-100</td>
</tr>
</tbody>
</table>

Nature of FS + AA system: The reasons why a fortificant system composed of a fortificant (FS) with a co-fortificant as a promoter (AA) was selected for use in place of a fortificant molecule itself, and, further, why only AA was selected in preference to several other molecules, has all be discussed in detail (pages 34-36). Suffice it to say that this innovation did really help us immensely in all aspects of the present study, such as in the stability of the fortificant system in milk, in the consumer acceptability by removing the slight
colour imparted by the ferric form of iron to milk, in the good bioavailability of this form of iron because of its enhanced iron absorption and increased mobilization from iron stores (brought about by AA's depolymerising action and increasing the chelating power of the usual chelators, as is evidenced by an enhancement of the power of BP* (page 57-59), and in affording protection to milk against metal-induced oxidative rancidity, and oxidizability of the native or added vitamins in milk because of its antioxidant and reductant action (450, 452, 454-457). It seems now that even the sorbitol moiety, in its own right, contributes its share in protecting milk against oxidative rancidity because of its own metal-chelating capability (385). WHO Report of 1975 (70) also had recommended that all infant formulas should include both an absorbable iron salt and a known enhancer of iron absorption as AA, with an Fe:AA ratio of at least 1:10.

Stability of the fortificant system (in aqueous solution): The stability of the system in aqueous solution was gathered from studies on its dissociation characteristics. The observations detailed below reveal the complexity of the situation.

<table>
<thead>
<tr>
<th>Conc. (ppm)</th>
<th>FS</th>
<th>FS+AA</th>
<th>ΔpH</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA (500)</td>
<td>-</td>
<td>3.44</td>
<td>-</td>
</tr>
<tr>
<td>FS (10)</td>
<td>6.56</td>
<td>3.52</td>
<td>3.04</td>
</tr>
<tr>
<td>(10)</td>
<td>6.68</td>
<td>3.74</td>
<td>2.94</td>
</tr>
</tbody>
</table>

An aqueous solution of FS, at the fairly high concentration of 2000 ppm (i.e. 50 times stronger than the actually used
concentration of 40 ppm or 200 times stronger than that used for the intended level of fortification), was found to remain stable up to pH 1.5, starts dissociating around pH 1.3, and attains complete dissociation at pH 1.0 (pages 75-77). This is quite comparable to conditions in the gastric environment. In contrast, ferric fructose (concentration not known) has been reported to dissociate completely at pH 3.3 (211). This shows somewhat greater stability of ferric sorbitol in comparison to that of ferric fructose.

As regards the FS + AA system was concerned, the addition of a solution of 500 ppm of AA (pH 3.44) to a solution of 40 ppm of FS (pH 6.68), i.e. the amounts actually used in the fortification studies, resulted in a decrease of pH to 3.74. Under these conditions, FS could be considered to remain intact as a complex in the solution; i.e. the solution could be considered just a mixture of the reduced form of FS (containing Fe(II)) + excess of AA, or possibly some sort of a binary complex + excess of AA. However, because of its unique structure, AA behaves simultaneously as an acid, a reductant, a chelator and a depolymerizer. This last property in particular, which arises from its capacity to break an iron-oxygen (Fe-O) linkage, either in conjunction with or arising from its powerful reducing action converting Fe(III) to Fe(II) form, could initiate and bring about an exchange reaction resulting in the formation of ferrous ascorbate. It would be recalled that Fe(II)-oxygen linkage is far weaker than Fe(III)-oxygen linkage; Fe(II) is more stable with nitrogen as in ferrous-dipyridyl type complexes. Therefore the possibility that FS + excess AA is present in solution, or
at least in part, actually as ferrous ascorbate + excess AA + sorbitol cannot be ruled out. The methods used for preparing ferrous ascorbate (19, 20, 45, 209, 257, 286, 305, 307) as a reference standard in iron nutrition work and for use as oral haematinic (284, 285) testify to the correctness of this thinking. Whether these components exist in solution just as some sort of a stabilized mixture, or as a definite ternary complex has not been ascertained for the present. The situation might possibly be akin to that reported by Rogers et al. (1977) (399) in respect of the ternary complexes between iron-transferrin - a chelate, where the chelates tried were EDTA, NTA, phosphate or citrate; all of these could be destabilized by NaHCO₃ to give iron-transferrin-CO₃ ternary complexes. The clearance of this point awaits further work; it certainly has very deep seated significance in this fortification programme.

It is thus clear that the addition of AA to FS radically changes the characteristics of the latter (pages 47-78); the known depolymerising capability of AA (178) converts FS to lower mol. weight species (pages 77 and 78). Ferric citrate, studied by Saltman and his group (397), likewise showed that with 20 times excess AA, the original complex resulted in lower mol. weight species, tentatively identified as ferric dicitrate, which too was known to be less well absorbed.

The effect of AA on other high mol. weight iron-sugar complexes, such as iron-dextran complex (mol. weight ca. 200,000), IPSG (iron-poly(sorbitol-gluconic acid) complex, mol. weight ca. 100,000), and iron-sorbitol complex, containing citric acid, and
stabilized by dextrins (mol. weight ca. 5000), does not seem to have been reported, though, in the light of the above observations, it would not be incorrect to surmise that in their case as well lower mol. weight species would arise, which certainly should prove more advantageous in respect of their absorption characteristics when used as oral haematinics. The present author feels that it might not be impossible that this innovation, or a similar modification could, at least partly if not completely, obviate the use of these complexes by the parenteral route, and they could achieve their intended goal of being good parenteral hematinics simply and directly through the oral route.

(b) The fortified milk

The studies on the fortified milk were planned to be conducted from two angles: the in vitro and the in vivo angles. The former, which in a way were biphasic in nature, aimed at determining how the fortificant system affected the characteristics of the milk, and, on the reverse, how the milk constituents on their part affected the characteristics of the fortificant system. This required ascertaining the precise location and the nature of the fortificant in the milk, i.e. how it was distributed, where precisely it was located, in what surroundings or microenvironment and in what molecular state it existed, and, finally, how its specific existence in milk affected the physico-chemical stability of the fortificant-milk association, i.e. its set up in toto. The in vivo studies, on the other hand, were likewise planned with two major objectives in view: one aimed at determining if the fortificant could in any way affect the
availability of iron from this system, and the second was designed to assess if the iron from such a fortified system could possibly, even remotely, evoke any adverse tissue response in the consumer, or could cause even an incipient damage or injury to liver. After all, the fortified milk was meant for human consumption over life long periods, and the accumulative effects, if any, were the first to be reckoned with and deserved the utmost attention.

**The in vitro studies: Buffalo milk as the vehicle**

Most of the work on the fortification of milk with iron reported previously (pages 9-17) had been carried out using cow milk as the vehicle. The target milk for the present study, however, was buffalo milk obtained from Murrah buffalo (423), because it was the most commonly available and used milk in our region. It is specified that, as far as could be ascertained, no data previous to this work seems to have been in respect of iron fortification of buffalo milk, except the one using ferric lactose reported by our team last year (85, 210, 382). This was, actually, in continuation of our previous studies on buffalo milk (163). The fortification of buffalo milk with the currently selected FS + AA system is also being reported for the first time. In the present study cow milk was employed mainly as a reference milk for comparison, and, for that matter, fortification of even cow milk with this fortificant system had not been reported before. Some new ground in this area has thus been covered.

**Buffalo milk vs. cow milk:** The buffalo and the cow milks were found to differ radically in almost all the aspects
studied. Besides the overt and the evident differences in their gross composition (page 129), in their fat content in particular, and the relatively covert differences in the entire make up of their cream compartment (239, 407), their FGMs (408-415), their proteins (416), even their caseins (417-420), and their enzymic proteins (239, 421, 422), were all found to be quite different. As a matter of fact, these differences were found to be so vast that the present author was compelled to feel that the word "similar composition" used in connection with these two milks was in fact a total misnomer. These differences have been compiled for a separate publication.

The reference compounds: The reference compounds, Fe\(^2+\) and Fe\(^3+\) (ferrous sulphate (61, 76, 78, 108) and ferric ammonium citrate (83, 110), apart from other defects, were selected on the basis of their excellent response reported previously. The only other rational and worthwhile choice that seemed reasonable was ferrous fumarate (70, 76) or ferrous ascorbate, on the one hand, and ferric glycerophosphate (112) on the other. The lean and sparing solubility of the fumarate in buffalo milk (actually it was found to settle down in this milk on storage), and the meagre availability and non-stability of ferrous ascorbate, and the phosphate nature of the glycerophosphate came in the way of their selection as the model reference compounds. However, ferrous ascorbate was used by us as the reference standard for studying the relative bioavailability of this fortificant system in the animal experimentation. Ferrous fumarate has been reported as a promising fortificant for other food stuffs (70).
The level of fortification: The intended level of fortification, 10 ppm iron (2.5 mg of the complex per litre of milk) with 500 ppm of AA (500 mg AA per litre of milk), was meant to be a purely preventive (prophylactic) fortification level, which could provide 1 mg iron (at 10% absorption level) and 500 mg of AA per day, at the milk consumption level of one litre a day. The Fe:AA ratio certainly seems on the higher side; the reasons for selecting this amount have been explained (pages 34-36). This compares quite well with the infant's daily requirement of 0.7 mg per day (357), and also with the 5 mg per meal level suggested by Layrisse et al. (1973) (424). It is further specified that the concomitant use of AA with iron as fortificants in milk also did not seem to have been reported before, except with ferric lactose (85, 210, 382). It is admitted that in actual practice even this consumption of one litre of milk per individual infant (or adult) per day might certainly be beyond the reach of several population groups in our society. 40 ppm iron level was meant more as an experimental level to explore the limit of feasibility of fortification.

The location and the nature of the fortificants in milk: A survey of literature revealed that little information, if any, was available about the distribution pattern of exogenously added iron to buffalo milk. The previous work reported on cow milk had also not investigated how the distribution of the added iron was affected by the presence of a rather large excess of AA employed as a cofortificant. The ascorbate, unlike the extrinsic or the intrinsic phosphates and citrates, because of its redox and other unique characteristics not shared by them, was bound
to behave differently. Further, the non-ionic sorbitol ligand, as distinct from the usual ionic ligands (derived from phosphates, sulphates, citrates, gluconate, lactate, tartrate, fumarate, succinate, glycinate, aspartate, glutamate), was also bound to influence the distribution pattern in milk differently. Nevertheless, the fact remains that the distribution pattern of added iron to cow milk, even though based on ionic ligands, did serve as a good guide. In addition, it seemed curious that precious little information was available about the precise distribution of the very small amount of the intrinsic iron present in the various milks. Although it is usually considered that this 0.3 to 1.0 ppm of iron (88-99) is associated solely with the FGM as iron proteinate, yet, in reality, whether this association was shared also by other milk proteins because of their high affinity for iron remains unclear; amongst the latter, one thinks of the micellar phosphoproteins, the caseins, the skim milk lactoperoxidase (heme protein), and the iron-binding lactoferrin of whey. Why the intrinsic iron prefers the FGM and the extrinsic iron the caseins needs understanding. The iron-containing xanthine oxidase constitutes some 8-10% of the FGM proteins (224); several others are glycoproteins. Furthermore, irrespective of their gross similarities, the proteins of the buffalo and the cow milks differ widely in their molecular morphology and other characteristics, which indicates that the location of the intrinsic and the extrinsic iron in the two milks was bound to be different. The previous reports (page 15-17) showed that some 90% of the iron added to cow milk got associated with caseins, and a very small amount, some 0.1% or so, went to the
cream compartment. That even this small amount in the cream should prove sufficient to initiate the development of the oxidative defect in milk (oxidized flavour), seems due to the astonishing increase in the catalytic activity of protein-bound iron; Calvin's figures for catalase (202) are a good indication of this effect. The rest of the iron is bound by the whey proteins.

The results obtained in the present study revealed that about 95% of iron offered as ferric sorbitol was found bound to the micellar fraction, whereas this value was known to be slightly lower, in the range of 85-90% (125-127), in the case of iron derived from ionic ligands. Almost the whole of the rest of the iron was found bound to the FGM and the whey proteins. Milk lipids and the whey lactose did not seem to be able to bind iron in the presence of milk proteins. The ageing of the iron complex, whether fresh or aged, did not significantly alter the uptake of iron (125). The differences in the composition and the nature of the milk proteins definitely affected the uptake of iron, as was obvious from the present comparative studies on the buffalo and the cow milks. The studies of Basch et al. (125) showed that the procedure adopted for obtaining casein, whether isoelectric or centrifugal, or the centrifugal force and the time used, also affected the iron distribution. Prior thermal treatment of the cow milk, i.e. whether raw or pasteurized, or the sequence of pasteurization, whether before or after the addition of iron to milk, on the other hand, did not appreciably influence the iron uptake. Some of these (observed) differences in the iron uptake by proteins of the
two milks might possibly arise from the amount and the nature of their caseins or other proteins.

<table>
<thead>
<tr>
<th></th>
<th>Protein (gm %)</th>
<th>Fat (gm %)</th>
<th>Lactose (gm %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffalo milk</td>
<td>3.78</td>
<td>7.45</td>
<td>4.90</td>
</tr>
<tr>
<td>Cow milk</td>
<td>3.65</td>
<td>4.65</td>
<td>4.70</td>
</tr>
</tbody>
</table>

These differences become still more obvious in the presence of ascorbic acid, testifying to the correctness of this thinking.

Unlike the milk proteins, the uptake of iron by milk lipids seemed to be very small indeed, possibly in the range of about 0.1%, and that too presumably bound to lipoproteins rather than to the simple lipids. Mather et al. (1977) (224) have shown that quite a number of loosely bound FGM proteins are readily elutable. Besides this, the differences between the buffalo and cow milk fats also could possibly account for the observed differences in the distribution pattern of iron in the two creams. Cow milk fat is known to contain much more unsaturated fat than the buffalo milk fat and the two FGMs also significantly differ in their ultrastructural organization (page 145).

Fortification of the milks with FS + AA system was found to markedly shift the uptake of iron from the micellar protein compartment to the whey protein compartments. Ascorbic acid enhances the uptake of iron by whey proteins 5-10 fold, and this fact could prove of significance in the utilization of the waste whey of the dairy industry. The cream component in the two milks was found to be influenced quite differently in the presence of ascorbic acid; it was notable that whereas the iron
uptake by the buffalo milk cream decreased by 54%, that by the cow milk cream increased 5-fold. As the development of oxidized flavour in milk corresponds to the amount of the iron uptake by the cream compartment, this observation might be of great significance in the control of oxidized flavour in milks.

These studies further showed that milks from different species behaved differently with respect to iron distribution, and therefore milk from each species, buffalo, cow, goat, sheep, etc. would have to be examined separately. Unlike the situation in U.S.A., Europe and Australia, it is buffalo milk which is the more commonly used milk in our region.

The nature of the fortificants in milk: This aspect needed information about the state of oxidation in which the casein-bound iron, some 95% of the added one, existed in milk. It was observed that the inherent (intrinsic) reducing power of milk, arising mainly from its free-SH groups, keeps some 10% of the added iron in the ferrous form; with FS + AA combination the reduced form of iron in milk was found to increase to ca. 85% (page 164). As regards the AA itself is concerned, almost 85% of it was found to remain intact (in the reduced form) in the fortified milk; the remaining 15% was assumed to have been oxidized beyond DAA. Whether this loss was caused solely by the catalytic oxidation brought about by iron, or also by some other factor basic to milk could not be ascertained for the present. It was also notable to realize that out of this 85% of AA, only a part exists as DAA, and, further, that the oxidation to DAA stage was brought about the least by FS, in
comparison with the two reference compounds derived from the ionic ligands. It is essential to recall that both AA and DAA are equivalent biologically as regards the vitamin C activity of ascorbic acid is concerned. The ability to keep some 85% of such a labile molecule intact for its inherent biological functions, including those of enhancing the absorption of iron and its related functions in iron metabolism, even in the presence of a powerful oxidant like iron, is really very significant. That FS was found to be the least harmful in this respect is also very encouraging. Concomitantly keeping some 85% of the added ferric iron in milk in the highly absorbable ferrous iron is also exceptionally important, as it assures its high bioavailability and bioutilization in the consumer. In fact this amounts to indirectly administering ferrous iron without its undesirable side effects (adverse gastro-intestinal response) (375) and any possible hepatotoxicity (376), because the protein-bound ferrous iron would not be available for interaction with the competing sensitive protein receptor cites in the g.i.t. or in other sensitive regions.

Furthermore, as regards the precise location of the added iron on the large casein molecules is concerned, more detailed work is needed to clear the actual position. However, a few pertinent points in this regard are quite revealing. The fact that most of the added iron remains casein-bound, stresses the need to have detailed knowledge about the various caseins and their binding capacities. Demott and Dincer (1976) (127) have reported the distribution of added iron (as labelled FeCl₃) amongst the various caseins; out of the 85% of the added iron
in skim milk bound to caseins, 72%, 21% and 4% were found associated with \( \alpha_s \), \( \beta \)-, and \( \kappa \)-caseins.

In this context, the recent publication of several detailed monographs and reports is very timely (416-420). That, unlike phosvitin, iron-casein complex in which the iron is fairly strongly bound to the phosphate groups (6), still remains biologically available (6), calls for a complete understanding of the details of the mechanism involved in this binding. Saltman and his group (6) point out that iron in the presence of casein from the normally consumed amount of milk is not rendered unavailable; it would require a consumption of some 5-10 litres of milk for iron to reach the stage of unavailability. Grant (164), Grant and Taborsky (165) and Rosenstein and Taborsky (428) had proposed that, in the presence of ferrous iron and oxygen, the phosphoproteins play a different role, in that the phosphoseryl residues of casein are involved in the generation of labile high-energy phosphates. Manson and Cannon (1978) (430), on the other hand, reported that the phosphoseryl residues present in \( \alpha_s \) - and \( \beta \)-caseins rapidly catalyse the oxidation of iron from the ferrous to the ferric state with the formation of stable ferric-phosphoprotein complexes. The binding of iron by \( \alpha_s \) - and \( \beta \)-casein was found by them to be extremely firm, and was similar to that reported by Donalla et al. (1972) (429, 448) to occur with the rat cytosol phosphoproteins and with phosvitin, where the binding had been attributed to covalent rather than to electrovalent forces. According to those workers (430), the phosphoproteins acted as carriers of iron, and more specifically in the translocation of iron across the mitochondrial membranes.
(429). They did not support the thinking of Taborsky and coworkers referred to above (164, 165, 428). As far as the strong binding of iron with phosvitin is concerned, the characteristics of iron-phosvitin binding reported by Gray (180, 420) throws a good deal of light on this aspect. This phosphoglycoprotein of avian egg yolk binds $47 \pm 2$ Fe(III) ions per molecule of mol. weight 35000, to give a green derivative with a P:Fe ratio of 2.0:0.1. In this the oxygen-donor atoms are furnished by the phosphate groups of the phosphoseryl residues; other oxygen-donor ligands in this protein are aspartic and glutamic acids (only 10% in amount of serine phosphate). Nitrogen-donor histidine residues and the single oxygen-donor carbohydrate moiety are also not sufficiently abundant to be set in a prominent Fe(III) binding role (180, 420 specifically pp. 9). Many serine phosphate residues have been reported to be arranged in continuous sequences of up to 8 residues. This situation has not been found in casein, even though it is a true phosphoprotein.

In the light of the above reported excellent work on the Fe(III)-casein binding, the present author is encouraged to emphasize that the extra excess of AA present in the system of the present study, which keeps almost 85% of the added iron in the ferrous state (whose binding force with oxygen is known to be much weaker than that of the ferric form), and still keeps it protein-bound, probably as some binary or ternary complex from ferrous ascorbate, is in fact the key-stone which overcomes the difficulties expected from the Fe(III)-casein binding. And as long as this association remains ferrous-casein-ascorbate type, the bioutility of the system remains assured.
Physico-chemical stability of the fortificant and of the fortified milk:

The fortified buffalo and cow milks were found to remain as stable as the unfortified milks when stored at 4°C for 48 hr, because the fortificants, at both the concentration levels, were found to be completely miscible with the milks, and neither settling down of any sort nor decomposition of any type was observed.

The consumer acceptability of the fortified milk:

**Colour:** At the intended fortification level of 10 ppm iron, both FS as well as FS + AA caused no change in colour at all in the buffalo milk, not even after 48 hr storage. At 40 ppm concentration level, FS did impart a slight brownish tinge to milk, which disappeared in the FS + AA system-fortified milk. The concomitant fortification thus increased the consumer acceptability of milk.

**Taste:** Both AA (500 ppm) itself as well as FS and FS + AA system did not cause any perceptible change in the taste of the buffalo milk, neither at 10 ppm nor even at 40 ppm concentration level. In contrast, both the reference compounds, Fe²⁺ and Fe³⁺, imparted an acceptable taste to milk, even at 10 ppm concentration level, which became more pronounced and intense at 40 ppm level.

Thus FS and FS + AA system were found to be superior to the reference compounds (page 105).

**Flavour:** The evaluation of the effect of fortification on the flavour of milk proved a far more complex problem. The
flavour defect in milk, which is known to affect the consumer acceptability the most, originates from both the hydrolytic as well as the oxidative rancidities. The former (page 105) results in an increase in the acidity of milk, first, because of the formation of fatty acids in milk through the fortificant-induced activation of milk triglyceride lipase which leads to lipase flavour (lipolytic rancidity), and, second, because any increase in microbial activity in milk (estimated through the TTC and the DP tests) (pages 120-123) would also ultimately add to acidity, because of fermentation leading to lactic and other acids on the one hand, and the activities of the microbial proteolytic and lipolytic enzymes on the other. The estimation of the total acidity and the pH of milk could therefore afford a reasonable estimate of the combined acid defect or the hydrolytic defect (pages 116-120). In contrast, the oxidative rancidity is caused by the lipid peroxidation of the PUFAs of the FGM, and is known to be induced by the pro-oxidant action of iron with the possible involvement of the FGM xanthine oxidase and molecular oxygen (pages 124-126); it is usually measured through the TBA values of the fortified milk (page 127). Some recent methods reported in connection with the lipid peroxidation of other biological material (254-256, 258, 259, 431) have yet to be tried for milk. The overall effect on the milk flavour due to iron fortification, nevertheless, would have to be judged by considering the results obtained by the use of the above mentioned chemical methods only in conjunction with the sensory evaluation, which, irrespective of any possible limitation arising from personal factors, still remains the most sensitive organoleptic detection for consumer acceptability. The human nose, after all, cannot still be
by-passed or totally eliminated, inspite of all the electronic
gadgets that have become available for many such assessments.
So, precisely this procedure was adopted for the present study
to evaluate the flavour problem for consumer acceptability or
rejection.

Hydrolytic rancidity:

Lipase flavour: The buffalo and the cow milk lipases
were found to be two entirely different enzymic proteins; the
differences in their became more clearly demarcated when these
were exposed to AA, Fe\(^{2+}\), Fe\(^{3+}\), and to iron + AA. FS was found
to cause almost no change in the buffalo milk lipase, and the
FS + AA system rather suppressed its activity by 20\%, showing
that this fortificant system would not cause any lipase flavour
or lipolytic rancidity (page 114). Effect of AA on milk
lipase activity has been reported (223).

Microbial activity: Both the buffalo and the cow milks
differed in their behaviour towards the TTC test (page 120).
FS, which was found to be a good microbial growth promoter,
could increase the acidity.

IF test showed the formation of lesser amount of the
ferrous form of iron when FS was used, which meant lesser
extent of ultimate acidity on this account. This test thus
showed that FS would not make the milk more prone to oxidative
rancidity (page 123).

Total acidity: Unlike Fe\(^{2+}\), which was found to be
the most active acidogen, FS was/to cause the minimum of
acidity in both the milks. This behaviour was also seen after
storage, and pasteurization (page 117). The FS + AA combination likewise proved superior to the corresponding combinations derived from the reference compounds. Fortified raw milk developed more acidity on storage; in contrast, fortified pasteurized milk developed less acidity.

**pH:** The pH of the raw buffalo and the cow milks was 6.94 and 6.72; the pasteurized milks had a slightly lower pH, 6.86 and 6.63 (page 119). The 10 ppm iron concentration level was found to cause almost no change in the pH of the milks, the decrease being only some 0.01-0.02 pH unit. The 40 ppm level caused a decrease in pH of not more than 0.1 unit. Out of the three iron compounds tried, Fe$^{2+}$ was found to be the strongest acidogen, but even in this respect the buffalo milk was found to be more stable and resistant than the cow milk. AA (500 ppm) caused a decrease in the pH of buffalo milk to the extent of 0.21 units and in that of the cow milk of 0.24 units. The change in pH caused by the FS + AA system was almost the same as that caused by AA itself.

The above studies thus revealed that fortification of buffalo milk with the FS + AA system would not cause any hydrolytic defect in it, as it rather suppresses the milk lipase activity, and does not change the pH of the milk to any significant extent.

**Oxidative rancidity:** As the FGM is known to be the seat of lipid peroxidation, both whole milk and the isolated FGM were studied for getting a comparative picture. Of the three iron compounds studied, Fe$^{2+}$ was found to be the most
detrimental in causing oxidative rancidity in the whole milk; FS proved to be superior, even in comparison with Fe$^{3+}$, while FS + AA system was found to be still better in controlling the lipid peroxidation in the milk (TBA values, pages 128-130). As regards the FGMs, the use of peroxide value, hydroperoxide value, Kries test, and ketonic rancidity test failed to detect any oxidative defect, either in the FGM or in the butter fat contained therein; only the TBA test was able to reveal the extent of lipid peroxidation.

The FGMs derived from the two milks showed radically different behaviour towards the fortificants (pages 136-145). FS was found to cause a loss (40%) in the FGM proteins, which was greater in the buffalo milk FGM than in the cow milk FGM. On the contrary, FS + AA and AA itself preserved the membrane proteins from such a loss; rather they increase the protein content, up to 2-fold. As regards the membrane phospholipid, the buffalo milk FGM contained some 50% higher amount of phospholipid than the cow milk FGM; the phospholipids from the two membranes showed different behaviour towards the fortificants. FS, FS + AA and AA preserve, and increase, the membrane phospholipid in the buffalo milk FGM, but the fortificants decrease the phospholipid content in the cow milk FGM. As regards the FGM enzymes, the alk Pase was not much affected, and the values of ATPase also were not much different than those of the control. Cow milk xanthine oxidase was activated by FS + AA; buffalo milk xanthine oxidase was deactivated by both FS and AA, but their combination activated the enzymic; this latter behaviour was similar to that shown by the rat kidney aconitase towards
Fe$^{2+}$ and AA (272). 5'-Nucleotidase was found activated and preserved.

The loss of the components of FGM, phospholipid, proteins and enzymes is understandable, as it is quite feasible that the fortificant might be able to somehow destabilize and lose some constituents. Mather et al. (1977) (22+) have shown that some loosely associated proteins do get eluted. But it is rather difficult to visualize the mechanism by which the protein content of the FGM is increased; whether the fortificant brings about some rearrangement or some shifting or reshuffling of the milk proteins is not clear at present, and awaits further work. Undoubtedly, even this small amount of the fortificant taken up by the FGM, or that in the micellar casein compartment, brings about some deep seated changes in the intricately balanced physico-chemical set up of the proteins. The changes observed in the FGM enzymes of the fortified milk also reveal deep seated changes at the ultrastructural level (page 145). But one thing, amongst all these, that stands clearly marked is that the FS + AA system does successfully control the oxidative defect in milk.

The interpretation of the results obtained above could be taken as complete only when these are considered in conjunction with those obtained from the sensory evaluation of the flavour defect (pages 107-111). In the latter evaluation (page 108), AA (500 ppm) was found to cause no change in the natural flavour of the milks, neither after 48 hr storage, nor after pasteurization of the milks. Unlike the reference compounds, FS and FS + AA did not change the natural flavour of the raw and
the pasteurized buffalo milk; even at 40 ppm it was superior to the reference compounds. These experiments were conducted also at 37°C to simulate Indian summer conditions without refrigeration facilities (pages 110-111), although it is understood that in industry or in any developed country milk would never be stored without refrigeration, at least never at 37°C. These studies too confirmed the superiority of FS + AA system over the reference compounds even under the hot climatic conditions (page 111).

The overall evaluation of the flavour problem thus clearly showed the importance of the coessential composing the system; the concomitant use of the two could certainly be considered as a satisfactory method for controlling the development of oxidative defect in milk (426 and 427).

In this context it might be added that very recently two other quite interesting approaches towards the same end (i.e. the control of flavour defect in milk) have been reported. Ray et al. (1978) (425) reported a method for the control of lipolytic rancidity in milk through an effective inhibition of milk lipase brought about by a micromolar amount (8 μM) of iodine in 0.1N KI. This concentration caused some 61% inhibition of the raw cow milk lipase; it did not produce any adverse effect on body growth, body temperature and heart beat in rats. This method evidently could be safer than the use of the toxic p-chloromercuri benzoate and DFP, and better than the use of H₂O₂ which is not permitted in milk in many countries, including India. The second approach involves the use of a small amount
of trypsin. Lim and Shipe (1972) (432) proposed a mechanism for the antioxygenic action of trypsin in milk. Gregory and Shipe (1975) (433) confirmed that a small amount of trypsin was effective in inhibiting oxidative flavour development in milk. They considered that either the hydrolysis of skim milk proteins increases the chelating capacity, or that it alters the FGM arrangement. Phospholipase C, for instance, was known to inhibit lipid peroxidation by inducing a rearrangement of FGM constituents. Shipe et al. (1975) (434) reported their findings on the enzymatic modification of milk flavour. Arnold et al. (1975) (435) indicated the use of lipolytic enzymes for flavour development in dairy products. Shipe et al. (1978) (245) considered that tryptic action inhibited the oxidative development presumably by either exposing antioxidants such as the -SH groups, or by reducing the activity of the pro-oxidants.

The industrial processability of the fortified milk:
It is of practical importance for the modern dairy technology to have detailed information about the characteristics of the milk proteins and how these are affected or modified in the fortified milk, as this information is invaluable for further processings, their production and quality control. The information that has been gathered in the present study on this aspect of the fortification programme concerns the amount of the total protein that would be separated from the fortified whole milk by the use of standard techniques, the relative distribution of proteins in the skim milk derived from the fortified milk, and the effect of the fortificant system on the curdling behaviour, the electrophoretic behaviour, and on the various whole milk enzymes, particu
particularly on alk Pase, 5'-nucleotidase and xanthine oxidase. These were discussed fully on pages 146-157. However, as an overall view it could be concluded that fortification did not cause any drastic change in the characteristics of the milk proteins, except some small ordinary changes observed in respect of the rennin-induced coagulation (page 151), and the effect on some of the whole milk enzymes (page 153). In reality the buffalo and the cow milks differ so radically that each milk, and each individual process would have to be studied in detail separately to determine how it would be affected by the fortificant. Milk alk Pase inhibition by sucrose has been reported (226).

The in vivo studies

The bioavailability of iron from the fortified milk:

Promotion and inhibition of bioavailability of iron:

A considerable advance has been made in our understanding of iron nutrition (398, 401). One aspect which has really proved useful in recent times has been the unraveling of the precise molecular mechanisms by means of which the known promoters and inhibitors of iron absorption bring about their respective effects. As a matter of fact the keystone of the present study, and the one which really gave us good dividends, has been the selection of the unique promoter AA as the cofortificant. Likewise, several other promoters and inhibitors have recently proved their worth. At present the absorption of dietary iron could basically be considered as occurring from two independent pools, a heme iron pool (HI-pool), and a nonheme iron pool (NHI-pool) (357). In the former the intact heme enters the mucosal
cell, and then after its enzymatic dissociation the exchange of ligands takes place (inside the cell); the absorption from this pool is not influenced by AA, though it is known to be enhanced by nicotinomide (383). The recent suggestion of using cattle hemoglobin (86) is unique, though it would need developing proper technology for this purpose. The absorption from the NHI-pool is very much lower than from the HI-pool, only some 5-9% in comparison with ca. 38% from the HI-pool (15). The absorption from the NHI-pool is enhanced considerably by AA, which is known to enhance absorption from the iron fortified foods as well (70). This enhancing effect seems to be a dose-dependent response, probably up to a Fe:AA ratio of 1:20 or 30; the effects of higher doses is not very clear. The adverse effect of AA on vitamin B₁₂ has been reported (374). The studies on the promotion of iron absorption by red meat, liver, chick (47, 67) and fish (70) have now been concentrated on the "meat factor(s)" to which this enhancement has been ascribed (15, 70); the nature of the actual component or moiety responsible for this action and its mechanism of action remain unknown up to now. But considerable efforts are being made in this direction because of its unique significance. The meat factor is known to be some 8-9 times more powerful than AA as an enhancer of NHI-pool absorption, which simply means it could bring the latter at par with the absorption from the HI-pool. It is of further interest to learn that the effect of this meat factor is greater when phytates are present, which suggests that the meat factor was acting as an "anti-inhibitor" (unpublished observation of L. Hallberg, quoted in reference 70). The latest
work on the nature of this meat factor reported by Björn-Rasmussen and Hallberg in March, 1979 (404) is an extremely interesting account of this fascinating study. Although up till then they had not been able to pinpoint the identity of the meat factor, but they seemed to have come quite close to it. Its identification would certainly usher in a new era in the realm of iron fortification of foods.

As regards the inhibitors, Disler et al. (40, 441) reported that tea taken with rice meal decreased iron absorption from 12 to 2%, because of the tea tannins. The effect of AA on this inhibition is not known. Because of its unique structure, the phosvitin of egg (287, 288, 448) captures iron so strongly as not to permit exchange of ligands needed for iron absorption. Iron-caseins (420), in comparison, do not possess that absorption-hindering structural element(s). Amongst some recently discovered inhibitors might be mentioned laundry starch (442, 443), and gossypol (from the high protein diet made by the fortification of foods with the cotton seed cake proteins) (444). The toxicity of gossypol, due mainly because of the hinderance to iron absorption, has been reported to be eliminated by adding iron to the fortified diet (445).

Estimation of bioavailability: In justification of our selecting the relative-utilization method for ascertaining the bioavailability of iron from the fortified milk of the present study (page 172), in preference to the more popular external tag method (page 172), it is pointed out that, as clarified by Layrisse et al. (1973) (424), the external tag method cannot be used in studies where the purpose is to compare the increase in
the amount of iron absorbed when adding different amounts of iron to different types of meals. In that case (as equally valid in the present case), it becomes necessary to relate the increase in absorption observed in different series to a certain iron status, i.e. a certain reference dose. The absorption should preferably be measured from meals prepared in subjects' own homes, using the same foods and the same cooking techniques as are usually used (424). The efficiency of absorption is measured as the ratio iron absorbed : iron absorbed from the reference dose. Baker and DeMaeyer (1979) (357) also point out that the best way to measure iron absorption is by measuring the absorption of a reference dose (3.0 mg) of a standard freshly prepared labelled ferrous ascorbate given in a fasting state.

The work of Layrisse et al. (1978) (440) also proved very useful. The results obtained in the present study in respect of the bioavailability of iron from buffalo milk fortified with labelled ferric sorbitol with and without AA relative to labelled ferrous ascorbate and ferrous sulphate given to the overnight fasted animals (rats) (pages 174-176) are certainly as reliable and accurate as, in comparison, with those of the reference compounds. The use of ferrous ascorbate as the reference compound was considered more relevant, because the compound actually entering milk seems just ferrous ascorbate + excess AA + sorbitol. The additional parameters selected to evaluate adequately the response of the test compound, viz. the tissue distribution pattern of the labelled iron, the haematological response it elicited, and the tissue distribution of the heme and the nonheme ferroprotein iron (440, 458, 462-470), all relative to the reference compounds and conducted simultaneously.
(i.e. under exactly the same experimental conditions), were considered sufficient to give a fairly reliable estimate of the bioavailability of iron from the fortified buffalo milk. Most of the other studies in literature had similar screening programmes (65, 83). It is obvious (pages 176, 178-180) that FS showed almost the same behaviour as ferrous ascorbate, which in itself was almost similar to that of ferrous sulphate. The enhancing of iron absorption by AA was also quite obvious.

After ascertaining the nutritional availability of iron from the FS + AA system-fortified buffalo milk, it was considered of utmost importance also to evaluate if, on prolonged intake, this system elicited any adverse tissue response in the consumer. This in reality is a fairly complex problem if the parameters to be fixed are to be purely biochemical in nature, apart from the histochemical and the cytochemical techniques. The usual practice of just checking the rate of body growth was considered insufficient, and, therefore, besides studying the effect of the fortificants on the rate of both the body and the organ growth, in the present study, particularly because of the powerful oxidizing action of iron, the known still more powerful pro-oxidant action of ferrous iron, of AA, and of their combinations (449, 459, 460), some adverse effects of AA (449, 453, 454), particularly on lipid membranes, and of the products of lipid peroxidation in the FGM (248-251, 436, 459, 460), it was decided to assess any possible hepatic damage, even in an incipient stage, by using a combination of two sensitive indicators, viz. the status of the hepatic redox state, as well as that of certain hepatic enzymes, known to be affected
by such damage or cell injury (402, 403, 472).

The status of the redox state was evaluated by studying the hepatic thiol redox state (the GSH levels), the reduced ascorbic acid level (318-320), the xanthine oxidase level (471), and the lipid peroxidation (452, 453, 459, 460). The hepatic enzymes selected were the GOT, GPT, the alkaline and the acid Pases. The redox state of the blood was studied likewise. It was considered that evaluation of these parameters would be sufficient for this stage of the study to get a reliable and reasonable estimate of even an incipient damage caused to liver by the month long intake of the fortified milk. The results showed that the system under study did not cause any significant change in any of these parameters, thus assuring the safety of the system.

(c) Fortification of other foods and feeds

During the course of this investigation, it became clear that, besides milk and the other major carrier foods, discussed above, several other preparations could also serve as vehicles, because they too fulfill almost all the criteria fixed for an ideal vehicle. These are in common use by all types of population groups, both rich as well as the weaker sections and both urban as well as rural populations. They too need evaluation as common vehicles. For the present these have been examined only from the point of view of sensory acceptability (pages 187-196), but warrant the normal detailed studies with respect to their bioutilization. These are: the 'sprinkles', the 'drinks', and the 'spreadings'; the former could be a good
substitute for common salt as the vehicle. It is suggested that these might also be found useful and acceptable in other countries as well.

1. **The drinks:** Mineral waters; fruit and vegetable juices, sugarcane juice; soft drinks like citrated waters (lemon water, ginger water, panna), squashes, syrups; whey drinks, plain or carbonated, vitaminized or with soluble high-protein products. Appetizers like jal jeera, rasam (based on tamarind water). Mild alcoholic drinks like the lohasav and the drakshasav (containing ferric acetate in syrup and fermented to a minor extent, and containing also some other herbal extracts and essences).

It would be useful to recall that high mol. weight iron complexes like IPSG have been given in drinking water to piglets (395). Ferric oxyhydroxide microparticles in water have also been reported (447).

2. **The sprinkles:** These are based mostly on common salt mixed with condiments and/or sour and sharp powders: they may include Kalanamak (black rock salt, containing several other trace metals), condiments (like black pepper, cumin seeds, cardamom, cinnamon, clove, dried ginger, magh, peppermint powder, coriander powder, anisi seeds, dried onion or garlic powder, glycyrrhiza powder), ground mango or pomegranate powder, or a little ammonium chloride.

3. **The spreaders:** Jams, jellies, marmalades, molasses. These may include (though not strictly spreaders) also pickles, catchups (chutneys), vinegars, etc.
Another group that was examined was fruit salads, desserts, sweets (Indian style), and biscuits, pastries, etc. These can mask the taste, if any, of any iron preparation.

One aspect that deserves mention in this respect is the need to educate the public to procure iron only from a central distribution centre, so that the required amount could be properly mixed with the other ingredients, and controlled.

Besides, the FS and FS + AA also proved useful as poultry and cattle feed. The preliminary experiments in this direction (page 196) indicate that it might prove as useful or even better than the existing haematinics, particular as a veterinary and poultry haematinic.

(iii) The conclusions drawn from the present study

The findings of the present study when considered in their entirety indicate that the following points stand out as significantly evident:

1. Iron deficiency anemia is a persistent nutritional problem of worldwide concern which engulfs several age groups. It is disturbingly common in the infants, and the damage caused to this group is dangerously threatening. Iron nutrition of infants, the main target group of the present study, necessitated the selection of milk as the vehicle; the use of common salt as the vehicle, presently being tried in India, leaves this group almost wholly uncovered. Availability of iron from fortified milk stands fully established, because its phosphoprotein casein differs radically from the egg phosphitin. 10 ppm iron concentration level for fortification
of milk, which could provide 1 mg iron per day per one litre of milk consumed, could be considered sufficient and a safe preventive measure to slowly build up the iron stores.

2. The selection of the non-ionic fortificant ferric sorbitol, as opposed to the other commonly tried fortificants derived from ionic ligands, used particularly in combination with a known safe enhancer of iron absorption ascorbic acid as the cofortificant, provided both the missing factors of milk, conferred stability on the system, and solved the additional problems of the consumer acceptability (based on colour, taste and flavour of milk), and of the bioavailability of iron from the fortified milk. It completely suppressed the oxidized flavour in milk which otherwise could have been caused because of the strong oxidizing power of iron. About 85% of both the fortificants (iron in the absorbable ferrous state and AA in the reduced form) bound to casein assured the nutritional assimilation and utilization. This also overcame any possible adverse tissue response as assessed by the use of a battery of fairly sensitive tests. The industrial processability of the fortified milk remained almost unaltered.

3. This combination could prove equally beneficial for other age groups, as well as for the fortification of poultry and cattle feeds. The bioavailability data in the case of the latter (the animals) would have to be obtained to substantiate the assimilation/utilization.

4. Several other dietary forms, which are in common use in India, could also be tried as vehicles for iron
fortification for the adult group using this fortificant system. These are the drinks, the sprinkles and the spreadings. Bioavailability from them needs detail studies.

5. The fortificant system under study was found to be fairly cheap, as it could be easily prepared from abundantly available starting materials. It is stable with a fairly long shelf life.

6. The use of this system in milk could serve as an alternate approach to common salt as the vehicle for iron fortification programmes.

(iv) Suggestions for future work in this area

At the conclusion of the above studies, the present author feels that further work is warranted on the following lines:

A. Re: the presently investigated system (FS + AA)

(i) The structural aspects: It is necessary to ascertain the precise structure of ferric sorbitol and of the iron-casein complex, especially as it exists in the presence of a large excess of ascorbic acid. The precise nature of the polymeric chains, the depolymerizing effect of ascorbic acid, the presence of free sorbitol as the so-called "outermost covering", the distribution of the non-ionic ferric sorbitol amongst the various caseins (in comparison with iron complexes derived from ionic ligands) and the exact location and the nature of the iron-casein binding, as it is affected by the
excess ascorbic acid, all need elaboration.

Likewise more work is needed to clarify the association between iron and the FGM proteins, to help us evolve more rational approaches towards the control of oxidized flavour in milk caused by the powerful catalytic effect of iron-protein complexes. Further, still more sensitive methods are needed to detect this defect, right in incipient state for ready control.

(ii) Bioavailability of iron: The presently obtained data should be compared with that obtained from the use of external tag method, to confirm these findings. More work is needed to unravel the precise distribution and deposition of iron in the tissues when it is taken up from FS + AA system in comparison with that from other systems, in order to evaluate the precise utilization, and also assess any adverse tissue response at the incipient stage to check even the remotest possible chance of any untoward consequence in the consumer, because the fortificant system is to be used for life-long periods.

(iii) Extension of the present work: The present fortificant system should be tried for the preparation of fortified milk products, like cheeses, yoghurt, cream, lassi (butter milk), ice creams, and powdered and condensed milks; further it should also be tried for the fortification of other vehicles such as cane sugar, common salt, and the cereal products (may be the cooked ones).

B. Re: Iron fortification in general

(i) Increasing the iron content of milk: There is a
need to evolve a system by which the iron content of human and
cattle milk could be increased adequately enough to avoid iron
deficiency in the infant in particular, and in other age groups
in general. None exists at present.

(ii) Development of newer more effective vehicles:
Vehicles other than those being tried at present need to be
developed, as all of those in current use suffer from one defect
or the other. The easiest and the cheapest could be drinking
water itself, which, like chlorination and fluoridation could be
ironized at the central distribution centre. The drinking water
supply is quite separate than the water supply for non-drinking
purposes, in most centres. Newer fortificants would have to be
developed for this purpose. The use of IPSG in drinking water
against piglet anemia is known (395); similarly water from a
blacksmith's shop in which hot iron used to be quenched was
known as a good hematinic (162). Ferric oxyhydroxide as
microparticles in water has been reported (447). Further, in
this context it might be indicated that different types of
mineral waters (plain, citrated, carbonated, flavoured, sweetened,
or as high-protein waters) could be developed. Likewise,
whey drinks, or lactose drinks, or mild alcoholic drinks on
the lines of lohasava or drakshasavas could be developed.

(iii) Development of newer fortificants: There is need
to develop newer, more effective, and cheaper fortificants.
The present author sees innumerable possibilities in this field,
as the number of possible ligands is countless. Protein and
polysaccharide hydrolysates and agricultural wastes, on the one
hand, and low mol. weight oligosaccharides obtainable from
monomers with epichlorhydrin, and polyaminoacids like poly glu, 
ply asp, etc. as oligo-peptides, on the other, offer immense 
possibilities.

Even the known fortificants like IPSG could be tried in 
other vehicles as water, cane sugar and common salt etc.

(iv) Development of newer promoters: Modified ascorbic 
acids might give still better results. Newer promoters as the 
meat factor would greatly enhance this scope.

(v) Development of newer fortificant systems: Newer 
cofortificants and newer systems need development for iron 
f fortification.

So, at the end of this investigation, it might be 
emphasized that the field is endless indeed! The present author 
feels very optimistic that the iron problem would certainly be 
solved, much sooner than it was expected, even only a short 
time back, and, like many a scourge of yesteryears, iron 
deficiency anemia too would be conquered and would stand 
vansished. We all have to work hard in this direction.