INFLUENCE OF TWO FORMS OF IRON ON ASCORBIC ACID IN INFANT FOODS

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ABSTRACT

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Iron fortification in infant foods is an issue of contention among nutritionists, industry and consumers. Two forms commonly added are ferrous sulfate and electrolytic iron. Ascorbic acid enhances iron absorption, but iron is catalytic in oxidation of reduced ascorbic acid, and may decrease the utility of iron-fortified infant foods as source of vitamin C nutriture.

Methodology for analysis of reduced vitamin C and dehydroascorbic acid was evaluated in reference to standards. Observations lead the researcher to question some methods reporting concentration of ascorbic acid in literature. Correction equations were obtained for use with experimental data.

The experiment evaluated mixed cereal (dry cereal with apple juice) with electrolytic iron, and wet cereal with ferrous sulfate, over 10 days of refrigerated storage. Only mixed cereal had significantly greater rate of oxidation with iron present than the non-fortified product. Considering a normal storage period, neither product showed substantially increased reaction rate in presence of iron to warrant concern.

I DEDICATE THIS WORK TO THE ONES
WHO HAVE OFFERED ME SUSTENANCE
AND SUPPORT WITH THEIR LOVE THROUGH
THE MANY HOURS OF ITS PREPARATION --

to my parents, whom I shall always love,
to my brother, Chuck,
and to a friend, Nancy.

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In gratitude to those who have touched the author's life, to

her parents and to her friends:

Be his
My special thanks, whose even-balanced soul,
From first youth tested up to extreme age,
Business could not make dull, nor passions wild:
Who saw life steadily and saw it whole.

Matthew Arnold

1822-1888

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INTRODUCTION

Nutrient fortification of several foods is considered commonplace by consumers today, and it is intended to provide an adequate supply of nutrients that may be consumed at low levels in the diet of some individuals. The fortification of table salt with iodine has been credited with eliminating the widespread prevalence of goiter in parts of the United States. The state of Michigan, in 1921, reported 47 percent of school children as having goiter. The use of iodized salt was extensively promoted, and in 1951, only 1 percent of the population was reported to have simple goiter (Gutherie, 1975).

Iron is an essential nutrient that has been accepted in fortification of white bread and flour since 1941, as result of an executive order enacted during World War II. The effort in fortification of foods with iron was intended to combat the incidence of iron deficiency anemia. Limited diets, such as those consumed by infants, may not include all essential nutrients and in the quantities required for optimal growth and physiological development. Some foods that traditionally comprise a major portion of an infant diet have been fortified and enriched to contain significant amounts of many needed nutrients -- iron and vitamin C are two nutrients that are commonly added.

Two forms of iron are currently added to dry infant cereal and wet infant cereal packed in jars. The two forms, electrolytic iron and ferrous sulfate respectively, differ in their bioavailability with ferrous sulfate as the more readily absorbable form. The two forms also differ in respect to their potential food applications. Reduced ferrous sulfate is not

appropriate for use in dry cereal since the particles precipitate from an even distribution in a package; and with time, the cereal becomes grey in appearance from a chemical reaction with the reduced iron.

Presence of vitamin C with iron has been shown to enhance iron absorption (Bjorn-Rasmussen and Hallberg, 1974), yet iron is also recognized as a catalytic agent in promoting oxidation reactions that degrade the quality of reduced forms of nutrients. The objective in fortification is to promote health, yet if the presence of iron with vitamin C leads to an increased rate of degradation for the latter nutrient relative to when iron is not fortified, then its addition in baby foods would not be advised.

Therefore, a project was initiated to evaluate the influence of two forms of iron on ascorbic acid in two products where both nutrients are supplied in substantial amounts. No research had previously demonstrated change over time for concentration of various forms of ascorbic acid in the opened product.

Vitamin C is declared on the nutrient label as the amount of reduced vitamin present after a specified period of shelf storage. Yet, nutrient content of foods as consumed may be substantially different in product once opened and stored for a period of time. Also, the interaction with other food components may be active in altering nutrient composition. The nutrient profile of baby foods, as consumed, was evaluated to reflect the influence with presence of the two forms of iron in their respective products.

It was of primary interest to establish the rate of change for ascorbic acid contrasting the two products in respect to iron presence or its absence. By evaluating the reaction rate as influenced by iron, the utility of iron-fortification and vitamin-C enrichment of baby foods

could be established and reported.

LITERATURE REVIEW

Dietary Requirement of Infants

Individuals are more vulnerable to nutritional inadequacies during particular stages of the life cycle. The Surgeon General reports that the health of the American people has never been better (U.S.H.E.W., 1979). The present situation is attributable in part to advances in technology which have provided Americans with a nutritious, consistent and available food supply. However, nutrient needs are high during periods of physical growth and development, or emotional stress. During these times when change is occurring, the demand that the diet supply required amounts of all nutrients is challenged, and nutritional supplements sometimes play a role in meeting needs.

Some nutritionists advocate that nutrient requirements be fulfilled by including a wide variety of foods from the four basic food catagories without reliance on the use of vitamin/mineral supplements (N.A.S., 1973). Therefore, when nutrient needs are high, more careful consideration should be given to the diet to ensure that requirements are met. Infancy is a time when development of all physiological systems are in a critical state and the nutritional needs of the infant are of particular concern. Further, some researchers suggest that certain adult characteristics, including mental retardation, obesity, hypertension, and atherosclerosis have their origins in early feeding patterns (Dairy Council, 1979).

Nutritional research, as directed by the recommendation of the Food and Nutrition Board of the National Research Council (Food and Nutrition Board, N.R.C., 1979) has focused on prevention of degenerative diseases that may be incurred naturally during the aging process. Many of the major health problems in the U.S. are known to be of multiple etiology. Attainment of optimal nutrition during infancy with its continuation throughout the life cycle may contribute to a reduced incidence, or delayed onset, of some of these illnesses.

The controversy regarding breast-feeding has been reviewed (Jelliffe, 1975; Gerrand, 1974; Oseid, 1975, Fomon, 1974c). It is generally agreed that a distict advantage of breast-feeding is that human milk is best suited to the infant's nutrient needs during the earliest stage of growth. Additional advantages include promotion of an harmonious mother-child relationship and, in some situations, the economic advantage of breast-feeding relative to artificial feeding. Onsted and Sleigh (1975) and the American Academy of Pediatrics (1976) have recently recommended that infants be breast-fed for the first four to six months of life. It is acknowledged, however, that there is a certain segment of the population that cannot breast-feed for anatomic, physiologic, or toxicologic reasons.

While breast-feeding may provide protein, calories, most minerals, vitamins, and fluids in proportions best suited to infants' needs, it is not intended to provide sufficient nourishment for growth during the entire first year or two of life. Human milk does not contain sufficient amounts of iron to meet infants' requirement (Brown, 1973). The concentration of iron in breast milk is only slightly higher than that of cow milk or unfortified cow milk formula (Underwood, 1971;

Murthy, 1971), yet iron in breast milk is uniquely bioavailable (McMillan, 1976; Saarinen, 1977). The need for supplemental iron in infants on prolonged breast-feeding is, therefore, likely to be less critical than in those fed unfortified cow milk formula. The newborn infant is well supplied with iron, both in terms of iron needed for metabolic and enzymatic functions. Eighty percent of iron stored is in hemoglobin (Sjolin, 1968; Smith, 1974) and the remainder is in such storage forms as ferritin and hemosiderin which function in iron homeostasis. Iron requirements of pregnancy are high (Recommended Dietary Allowances, 1974), yet iron deficiency at this stage has greater consequence on the developing fetus, since nutrients available in only limited amounts are preferentially transferred to the fetus (Widdowson, 1951). Within the first year of life the infant must double its body iron as it triples its body weight, and transplacental delivery of iron that guaranteed iron nutrition for the fetus is replaced with a smaller and less consistent dietary supply (Dallman, 1980). Iron stores are depleted during early development, but it is not until the fourth month of life for the term infant that the increased dependence on dietary iron is apparent.

Prevalence of Iron Deficiency and Anemia

Iron deficiency anemia (hypochromic and microcytic) in infancy has been defined as the state in which hemoglobin concentration is less than 11 mg/dl and that which responds to the administration of iron (Fomon, 1974a). Iron deficiency anemia has been recognized as the most prevalent nutritional disorder among infants and children in the U.S., particularly those between the ages of six and 24 months (Fomon, 1970). A low iron

content of either breast milk or unsupplemented cow milk formula (about 0.5 mg/liter) (Fomon, 1974b) predisposes the infant to depleted iron reserves. The total iron store in preterm and low-birth-weight babies is less than normal at birth and a rapid rate of postnatal growth quickly exhausts reserves before four months. Lundstrom et al. (1977) observed iron deficiency in low-birth-weight infants between the ages of two and three months. Thus, it is clear that an exogenous source of iron is even more critical for the preterm than for term infants (Lundstrom, 1974; Schulman, 1954; Committee on Nutrition, American Academy of Pediatrics, 1976).

Filer (1969) reviewed the significance of iron-deficiency anemia as a public health problem and concluded that its existence is less prevalent in more favorable socioeconomic circumstances and in large metropolitan centers (Haughton, 1963; Andelman, 1966; Danneker, 1966). Haddy and co-workers (1974) evaluated iron status in 109 infants and children ages four months to two years from low-income families. Incidence of iron deficiency (hemoglobin concentration less than 11 mg/dl and hematocrit values lower than 33%) was slightly more than 50 percent. While incidence of iron deficiency is lower in more affluent socioeconomic strati, Sturgeon (1959) found approximately seven percent of term infants, not consisdered underpriviliged, with hemoglobin levels less than 10 mg/dl by one year of age. Further, Hutcheson (1968) reported that 10 percent of Tennessee's rural poor children under six years of age were anemic.

Recommendations Made

The high risk of iron deficiency, with or without clinical

symptoms of anemia, prompted the Committee on Nutrition of the American Academy of Pediatrics to issue several recommendations over recent years for iron supplementation of infants and children (Committee on Nutrition, 1969; Committee on Nutrition, 1971; Committee on Nutrition, 1976). The first recommendation was made in 1969 when the Committee published a review of iron requirements for infants. The Committee urged that pediatricians recommend inclusion of iron-fortified foods in the infant's diet for at least 18 months. In 1971, a statement was issued recommending the use of iron-fortified formula until at least 12 months of age. Additionally, it was suggested that iron-fortified whole milk or iron-fortified evaporated milk be made available for infant feeding. Iron-supplemenation in breast-fed infants was not specifically advised due to the enhanced bioavailability of iron in breast milk.

The Committee faced both praise and demonishment by numerous professionals for thses recommendations (Schafer et al., 1971; Haddock and Harvey, 1971; Diamond, 1971; Melhorn, 1971; Filer, 1971a; Fisher and Allen, 1971; Filer, 1971b; Pearson, 1971; Oski, 1972; Pearson, 1972; Lopez, 1972; Feurth, 1972). Opponents argued that infants from middle-class families were not as likely to incur iron-deficiency anemia as would those from low-income groups (Owen et al., 1970; Burks et al., 1976) and would thus not benefit from iron supplementation. Kripke and Sanders (1970) reported prevalence of iron-deficiency anemia among children ages six months to three years seen at ambulatory clinics in Iowa. Anemia was identified in only 4.1 percent of a sample whose socioeconomic status was different than previous groups that had been studied in low-income areas of large metropolitan centers.

It is argued that iron additives given to infants with an already adequate iron status could result in an iron overload (Danneker, 1966). Infants with hemolytic diseases such as sickle cell anemia or a form of hereditary hemochromatosis would be particularly susceptible to dangers of excessive iron intake. In light of the rare occurence of such diseases, more concern was voiced with regard to the ultimate outcome of eating additional iron-fortified cereal and possibly iron-fortified bread over an extended period of time. Some physicians continued to be concerned with possible increased incidence of feeding problems or gastrointestinal disturbances although no documented evidence has been demonstrated that this was a significant problem (Committee on Nutrition, 1971).

Reevaluations Offered by Opponents

Owen et al. (1970) reported that anemia was not as prevalent in American preschool children as might have been anticipated from earlier reports. However, Owen and co-workers (1971) reported that, while anemia predominated among preschool children from lower income families, iron-deficiency (based on levels of plasma iron) was relatively common in all segments of the population. In a recent communication from Ahn and Maclean (1980), data was presented indicating that growth of infants receiving human milk as a sole source of protein and energy was comparable to that of the National Center for Health Statistics (NCHS) population up through the ninth month of life. Consequently, it was implied that human milk alone provides adequate nutrition for growth of an infant during this period of time. In closer comparison of the NCHS growth curves, it was demonstrated that the curve tended to successively cross lower percentile lines towards the end of the 10-month observation period.

This was particularly true for male children. This observation denotes a declining ability of human milk to adequately serve as sole source of nutrients for the infant towards the end of this 10-month period (Morley, 1976). Values did, nonetheless, stabilize between the 25th and 75th percentiles before the end of 10 months and did not indicate imminent malnutrition.

Evaluation of the Prevalence of Breast-Feeding

The nutritional and psychological merits of breast-feeding warrant its continued support as a method of feeding the newborn infant. Prevalence of breast-feeding in the U.S. has fallen 100 percent over the past 75 years and less than one percent of one-year-old infants in previous years were breast-fed (Bain, 1948; Hill, 1967). Recently, there has been renewed interest in breast-feeding, particularly among more educated mothers and the declining trend is reversing (Jelliffe, 1976). Reports have surfaced that morbidity and mortality rates for breast-fed infants are generally lower than for artificially-fed infants in underdeveloped countries (Mata et al., 1971; Mellader, 1959; Adebonojo, 1972; Jackson et al., 1974). Similar rates have been reported comparing infants in developed countries. Reasons for lower rates of mortality and morbidity existing for breast-fed infants appear complex, but a major contributing factor appears that supplemental solids or cow milk and formula derived thereof may easily become contaminated since bottles and nipples may not be adequately sterilized. Further, water supplies may not be sanitary, and may thus bottle-feeding may become a vehicle for infection and disease. A newborn infant has not developed the

capacity to combat even low-level insult from bacterial contamination that predominates in some underdeveloped communities and an increase in morbidity and mortality rates of infants results (Mata et al., 1971; Monckeberg, 1973; Plank and Milanesi, 1973).

Economic implications of breast-feeding are salient in developing countries and in areas of the U.S. where a choice to bottle-feed an infant may have serious economic consequences. The purchase of formula and manufactured weaning foods may divert scarce monetary resources for families in developing countries who earn an average of 4 U.S. cents per day (Latham, 1979). Such purchases may jeopardize the health and nutritional status of other family members.

Effect of Iron Deficiency Exclusive of Anemia

Among other advantages, human milk gives the infant immunologic protection against respiratory and gastrointestinal infection as well as allergic reactions (Dairy Council, 1979). While some of the cellular components considered beneficial in human milk are not secreted after 5 days post partum (Lawrence, 1980), there are both cellular and humoral factors associated with breast milk that may have long-term advatages for the infant. Cellular lymphocytes synthesize the antibody IgA, which is the most important immunoglobin in milk -- not only in concentration, but also in biological activity. The IgA content of human milk is very high in colostrum and drops precipitously after 4 to 6 days, but remains constant thereafter for as long as 27 months.

The "bifidus factor" in human milk facilitates the growth of Lactobacillus bifidus which appears to have an 'intestinal guardian'

function in checking the proliferation of undesirable and possibly harmful organisms, such as pathogenic E. coli (Jelliffe and Jelliffe, 1978). Colonization of the alimentary canal for breast-fed infants is primarily with L. bifidus, whereas the intestinal flora of babies fed cow milk is primarily gram-negative bacteria, especially coliforms and bacterioides.

However, prolonged duration of breast-feeding does little to replete exhausting iron stores, and if deficiencies are allowed to persist will result in nutritional anemia. Other symptoms of iron-deficiency can exist without clinical signs of anemia and may easily go undiagnosed and untreated. While it is difficult to separate the physiological consequences of deficiencies in tissue iron from those of anemia, there has recently been some success in making this distinction. This has been by the demonstration of impaired exercise tolerance in the rat and defects with the immune response in man. Both conditions may occur independent of anemia (Dallman et al., 1978). The symptoms of iron-deficiency are due partly to the comprimised delivery of oxygen to tissues resulting from a decrease in concentration of hemoglobin (Anderson and Barkue, 1970; Viteri and Torun, 1974; Gardener et al., 1975). Iron-deficiency may also result from depletion of iron-containing compounds in tissues (Jacobs, 1969; Fairbanks et al., 1971; Dallman, 1974), and may thus give rise to clinical manifestations of anemia.

Tissue iron may be classified in several catagories. The first, heme iron compounds, exist in cytochromes and myoglobin and are responsible for the oxidative production of cellular energy in the form of adenosine triphosphate (ATP). Myoglobin is essential for storage of

oxygen and functions during muscle contraction. Second, the non-heme related iron-containing compounds are the iron-sulfur proteins and metalloflavoproteins. These compounds account for more iron in the mitochondria than with the cytochromes, and include the enzymes NADH dehydrogenase and succinic dehydrogenase. These are essential in the functioning of cellular respiration. A third classification of compounds do not contain iron but rather require iron as a cofactor. Iron-deficiency that has progressed beyond simple depletion of iron stores will affect the tissue iron compounds in a non-symmetric manner that cannot fully be predicted from the degree of anemia (Dallman, 1974).

Biochemical and morphological abnormalities result from depleted iron reserves and may be related to aberrant physiological functioning. Researchers attribute the anemia of iron-deficiency in striated muscle for an observed decreased work capacity (Anderson and Barkue, 1970; Viteri and Torun, 1974; Gardener et al., 1975). Finch (1976) showed in rats that striated muscle dysfunction is not immediately reversed by transfusion to correct the anemia, but may be alleviated with four days of iron therapy. Iron-deficiency has been implicated in adult patients with altered behavior including apathy, irritability, and inability to concentrate (Dallman et al., 1978). Dallman (1975) reported that iron-deficiency initiated early in life for the rat results in deficient cerebral iron which persists even after iron repletion of other tissues. Mackler et al. (1978) corroborated earlier reports of a casual relationship with iron-deficiency in his observation of decreased activity for aldehyde oxidase, a key enzyme in the pathway of serotonin degradation, and observed an elevated concentration of serotonin in brain tissue of iron-deficient rats.

Further abnormalities resulting from iron-deficiency that involve the central nervous system are associated with elevated urine catecholamine levels. Webb and Oski (1974) proposed that elevated levels were the consequnce of the decreased activity for monoamine oxidase, an enzyme involved in the catabolism of neural mediators. In addition to mental changes, alteration of catecholamine concentration can explain the increased irritability and abnormalities in appetite sometimes described in association with iron-deficiency.

A condition frequently associated with iron-deficiency is an increased susceptibility to infection (Pearson and Robinson, 1976). The infant, with its yet undeveloped immune system is particularly vulnerable to disease. Several research teams have provided evidence of impaired lymphocyte and neutrophil function as a basis for such a relationship (Arbeter et al., 1971; Johnson et al., 1972; Chandra, 1973; Macdougall et al., 1975; Srikantia et al., 1976). The lymphocyte abnormalities may be related to a lower incidence of positive skin tests for iron-deficient than for iron-replete groups when tested with a purified protein derivative and Candida antigen (Joynson et al., 1972; Macdougall et al., 1975). Neutrophils are defective in the oxidation-reduction of a nitro blue tetrazolium dye, suggesting that the iron-containing enzyme for this reaction is present in diminished amounts.

Gastrointestinal functioning may also be impaired when iron reserves are depleted. Ghosh et al. (1972) reported a reduction in acid secretion by the stomach with iron-deficiency. Other researchers have found that intestinal absorption (Yeoman and St. John, 1975) including iron absorption (Kimber and Weintraub, 1968) is impaired without an

adequate supply of tissue iron. Biochemical deficiencies of the intestinal mucosa of the rat that affect cytochrome enzymes were thought to be the cause for decreased absorption.

Thus, knowledge that iron stores are depleted by four to six months of age in the term infant suggest that some type of iron supplementation should be considered. Saarinen (1978) found that iron supplemented infants rarely had laboratory evidence of exhausted iron stores. Clinical symptoms of anemia are not always apparent with suboptimal iron status, yet the long-term consequence of inadequate iron nutrition has been documented.

Transitional Foods

In view of the 1976 recommendation by the Committee on Nutrition of the American Academy of Pediatrics (1976) for iron fortification of infant foods, Saarinen (1978) evaluated iron status of infants on prolonged breast-feeding relative to others receiving a cow milk formula and infants receiving a proprietary infant formula. Results indicated that supplemental iron should be considered for breast-fed infants after six months of age. In contrast, infants who were weaned to cow milk needed iron supplementation earlier -- even at four months of age or before. It was speculated that increased bioavailability of breast milk accounted for the observed enhanced iron status for breast-fed infants relative to those receiving the cow milk formula.

Further, Siimes et al. (1979) reported that iron concentration in breast milk declines during the course of lactation and infants on prolonged breast-feeeding are forced to depend on a depleting iron reserve. Transitional solid foods may be readily introduced to the

infant's diet at four months of age and may supplement breast- or bottle-feeding (Cook et al., 1972). Saarinen (1978) reported that non-fortified solid foods do not provide a sufficient source of iron for infants on prolonged breast-feeding. It was suggested that among solid foods consumed by infants, iron-fortified cereals could provide a major source of iron and would be expected to prevent iron-deficiency during the latter portion of infancy.

Some nutritionists have attempted to discourage dependence on vitamin/mineral supplements and instead suggest that individuals select a well-balanced diet to attain optimal nutritional status (N.A.S., 1973). It is important to consider dietary recommendations aimed at improving iron nutrition from an economic as well as nutritional perspective, and additionally to develop habits that will be beneficial in later life. Opportunity is limited for an infant to consume a wide variety of foods, since the ability to digest some foods or physically handle foods is not yet developed. In the U.S., the inclusion of iron-fortified dry cereal with introduction of solid foods in the infant's diet is a convenient and practical mode to attain adequate iron nutrition.

Iron Bioavailability

It is recognized that the availability of iron from foods depends greatly on several factors including body iron stores, form and amount of iron in foods, and the combination of foods in the diet (Monsen et al., 1978). Cook and co-workers (1972) in their report implied that the unusually high bioavailbility of breast milk-iron is modified by the introduction of various solid foods. The inclusion of meat in the diet has been found to profoundly enhance absorption of all forms of food

iron (Layrisse and Martinez-Torres, 1971). Further, the form of iron in meat that exists in heme protein is more readily absorbed than is non-heme iron in vegetable and grain products. In one study, using human subjects, 37 percent of the heme iron was absorbed from a test meal in contrast to only five percent of the non-heme iron (Bjorn-Rasmussen et al., 1974). There is almost no heme iron in the infant's diet when milk is the major source of calories. The relatively small amount of iron present exists primarily in the non-heme form. Emphasis on meat in an infant's diet may facilitate formation of adequate iron reserves, but such focus may not be economically practical and may be excessive in respect to the infant's capacity to digest high protein foods. Cereal products may be effectively utilized in preventing iron-deficiency within practical constraints, providing that such foods are iron-fortified at adequate levels with a form of iron that is both available and the application technique technologically feasable.

Recommendation Made For Feeding Dry Cereals to Infants

The Committee on Nutrition of the American Academy of Pediatrics (1976) made the recommendation that current nutrition counseling should emphasize the use of dry infant cereals to provide an adequate source of iron in the infant's diet. The recommendation included that dry cereals be used during the first and second year of life since requirements for iron continue to be large during this extended period.

Iron Enrichment

The concept of iron enrichment for cereals has found forum for contention with legislators, nutritionists, and with the milling and

baking industry. The alarming prevalence of low iron intakes reported in the Ten-State Nutrition Survey (1971) led the FDA and several nutritionists to propose a 300 percent increase in the present mandate for the amount of iron required to be added to enriched white bread in the United States (Abraham et al., 1974; Finch and Monsen, 1972; Goldsmith, 1973). Inadequacies of this report have been widely discussed (Crosby, 1974). However, the enrichment of flour and bread with vitamins and iron has been practiced since 1941, when it was initiated as one of the measures taken to handle the food emergency encountered as result of World War II. The levels of enrichment currently in practice have been in effect since 1943.

However, evidence that persistence relating to the prevalence and severity of iron-deficiency anemia even with the current iron enrichment program of cereal products lends justification to doubt the efficacy of such a practice. Nutrition surveys of adult U.S. Army personnel in basic training from the period 1970 to 1971 revealed surprisingly high prevalence of anemia among both young males and females (Col, 1972). The infant and young child require relatively greater amounts of dietary iron in order to maintain stores. When iron-deficiency anemia is observed in adolescents and adults, it is generally accepted that this is a reflection of iron stores never having attained adequate levels in infancy (Council on Foods and Nutrition, 1972); and thus, the patent importance of establishing adequate iron nutrition in the infant is indicated.

Medical nutritionists, pediatric and hematological groups concerned with iron-deficiency anemia have been expressing their views for many years, yet the controversy of cereal enrichment with

iron intended for infant feeding continues to provide forum for debate. Primary contention concerns selection of the particular source of iron used in enrichment of cereal-based foods. Stipulation of the proposal in the Federal Register (1971) is that added iron be in a form which is harmless and assimilable. All chemical forms of iron used in foods must meet the criterion of safety, as do all food additives, but experimental animal data indicate that bioavailability varies widely depending on (a) the specific chemical form of the iron, (b) the food to which the iron is added, (c) the overall diet of which the enriched food is a part, (d) the particular type of processing for the food product, and (e) nutritional status of the individual. Research results of bioavailability in animals is abundant with respect to most forms of iron currently added to foods, yet there is a paucity of data regarding availability of iron from food in the human (Council on Foods and Nutrition, 1972).

Enrichment of Dry Infant Cereals

Most of the dry infant cereals produced in the late 1940's and 1950's contained sodium iron pyrophosphate or other sources of iron which were characterized with probably less than one percent of the iron actually being absorbed (Committee on Nutrition, 1976). A report by Rios et al. (1975) discredited the value of iron-fortified cereals in infants' diet claiming that sodium iron pyrophosphate and ferric orthophosphate were so poorly absorbed from infant cereal that iron supplemented cereals failed to provide an effective or predicatable source of dietary iron. In a comment regarding the statements made by Rios and associates (1975), Stewart (1975) maintained that Gerber

Products initiated substitution of reduced iron for sodium iron pyrophosphate in its cereals in 1970. Specification of iron particle size requires that 95 percent of the iron passes through a 325 NBS mesh screen, or particles must be 44 microns or less in diameter. Fritz et al. (1975) reported that bioavailability of such iron was estimated to be 34 percent that of ferrous sulfate (as standard) compared with 14 percent for sodium iron pyrophosphate.

In 1972, electrolytic iron was introduced as the form for fortification in cereals manufactured in the U.S. and remains as the form currently in use. Specifications include that 80 percent of iron is less than 20 microns in particle size. In addition, it is reported that a higher solubility index for iron is indicative of increased bioavailability (Rios et al., 1975: Waddell, 1974), and electrolytic iron with a large porous surface enhances solubility relative to other forms of reduced iron powder.

In 1970, electrolytic iron was selected for use in cereal products because it is finely divided in cereal with the largest surface area of all iron products available at the time. It is still in use since no compound with greater bioavailability that is equally appropriate for use in cereals has been found.

Anderson et al. (1974) experimented using miniature pigs and compared bioavailability of various forms of iron in rapidly growing animals. Experimental forms of iron were compared in a cereal-milk mixture similar to that commonly fed to infants. It was determined that the effect of iron-deficiency anemia on growth performance of animals was statistically significant by the fourth week of the study. At that time, gain in body weight was highly correlated with

concentration of hemoglobin in blood. Iron status of pigs fed a diet fortified with sodium iron pyrophosphate did not differ statistically from that of pigs whose diet was not iron supplemented. However, elctrolytic iron of small particle size was 70 percent as available as the reference iron source, ferrous sulfate. Therefore, efficacy of supplementing deficient diets with sodium iron pyrophosphate is questionable, but research results supported supplementation with electrolytic iron of small particle size.

Further Evidence for the Effect of Small Particle Size and Greater Solubility in Enhancing Bioavailability of Iron

Shah and Belonje (1973) had claimed that bioavailability of iron is dependent on particle size. A research team of Fritz and Pla, along with numerous co-workers, have examined bioavailability of several forms of fortification iron (Fritz et al., 1974; Pla and Fritz, 1971). Pla and Fritz (1971) used the hemoglobin repletion method for measuring bioavailability whereby anemic animals respond to supplemental iron feeding and clinical symptoms of anemia are no longer present. Researchers consistently found that electrolytically reduced iron of small particle size was more assimilable than were other forms of iron previously used for enrichment purposes. Electrolytically reduced iron 7 to 10 microns in particle diameter had relative biological value (RBV) of 63.5 $\pm$ 10.9 when ferrous sulfate ($FeSO_4 \cdot 7H_2O$) was used as the reference standard ($FeSO_4$=100). Other forms of iron were less available. Similarly prepared electrolytic iron of larger size (27 to 40 microns) was also less available with a RBV of only 37.9 $\pm$ 12.2.

It has been established that bioavailability of elemental iron

powders depend to a large extent on particle size distribution (Hoglund and Reizenstein, 1969; Pla et al., 1973; Motzok et al., 1975), and also on the manufacturing process (Pennell et al., 1975; Pla et al., 1976). The method of preparation for iron powders influences solubility and porosity of surface area. Pla et al. (1976) and Motzok et al. (1978) demonstrated that solubility rate shows good promise as predictor of the relative bioavailability for some types of elemental iron powders. Motzok et al. (1978) compared several methods for manufacture of food grade elemental iron powders in regard to particle size, RBV and solubility. Electrolytic and carbonyl iron powders were well utilized by rats, while iron powders produced by reduction at high temperatures with gases such as CO and H_2 were poorly utilized by rats. Respective RBV (mean of 12 to 15) for these samples was only one-third or less of the value obtained with feeding electrolytic iron. Particle size may account for some variability in RBV between types of iron powders, and the high proportion of very fine particles (69 percent less than 7 microns in diameter) in a carbonyl iron sample may have accounted for its relatively high solubility in 0.2 percent HCl. Electrolytic iron samples contained particles about 37 percent of which were less than 27 microns in size, while iron powders produced by reduction with H_2 and CO gases contained high proportion of particles whose size was greater than 19 microns. Pennell and co-workers (1975) also reported that the high proportion of annealed surface structure of elemental iron produced by reduction at high temperatures contributed to the low solubility and boiavailability of this type of iron powder. When using this discrete fraction of various iron powders, all of similar particle size, effect on RBV

could be assessed for the different methods of manufacture. Small particle size fraction (7 to 10 microns) of electrolytic iron had RBV only 5 to 10 percent higher than those comparable fractions of H_2 and CO reduced iron powders, whereas RBV and *in vitro* solubility of this fraction of electrolytic iron was two times that of the H_2 and CO reduced iron. Thus, fortification with electrolytic iron appears to be the most efficacious mode for prevention of iron-deficiency via iron-fortification programs with iron powders.

An important consideration remains that research reported herein are *in vitro* studies. Application to human infant feeding situations is speculative — or presumptive at best. Animal research with depletion-repletion feeding trials is not a realistic physiologic iron status, but conclusions may serve as indication for human iron nutriture.

Technological Restrictions in Iron-Fortification

Iron-fortification and enrichment of food products in the United States has focused on the use of four iron compounds. These compounds include ferrous sulfate, powdered elemental iron, and two insoluble iron salts: ferric orthophosphate and sodium ferric pyrophosphate. Other iron compounds account for less than 5 percent of the total number of forms used (Waddell, 1974). The technical problems encountered with iron enrichment are greater than the difficulties overcome for the addition of vitamins. The soluble forms of iron undergo notable changes in cereal products during storage including the development of rancidity with changes in color, odor, and baking performance. Thus, insoluble phosphate salts were commonly added to cereal foods (Council on Foods and Nutrition, 1941) even though biological efficacy of such

practice was questionable (Rios et al., 1975; Fritz et al., 1975). Reduced iron reportedly imparts a grayish appearance in white or lightly colored foods. The two insoluble phosphate salts are light colored which makes their addition particularly applicable to cereal products. While it is accepted that ferrous sulfate is the most bioavailable form from the standpoint of iron nutrition (Pla and Fritz, 1971; Amine et al., 1972; Fritz and Pla, 1972; Ranhotra et al., 1971), use of this form in cereal products presents problems that cannot be tolerated in foods that face extended shelf-storage. The soluble ferrous salt can be used in a strained food system that is heat processed and is thus more shelf-stable.

Recommendation of the Committee on Nutrition of the American Academy of Pediatrics (1976) includes that ready-to-feed cereals with fruit in jars be consistent in iron content and form, and that the form supplied be well-absorbed. Waddell (1974) reported the superior bioavailability of ferrous sulfate as compared to less soluble sources whether the iron was added directly to the diet or was consumed in the form of enriched bread. Fritz et al. (1970) also determined that ferrous iron, whether it be in the chloride form or sulfate salt, was somewhat better utilized in both rats and chicks than were comparable ferric salts.

Influence of Specific Foods on Absorption of Enriched Iron

It has already been noted that the amount of iron available to improve iron nutrition via fortification depends on factors including form and level of iron-fortification, iron status of the individual, and composition of the diet with foods consumed concurrent with supple-

mental iron (Monsen et al., 1978). It is well recognized that the critical relationship of dietary iron to establish iron nutrition is accentuated for the infant relative to the adult. Specifically, about 95 percent of the iron required for the production of red blood cells in the adult is recycled from the breakdown of senescent red cells and only 5 percent is derived from dietary sources (Hillman and Finch, 1974). It is estimated that the one-year-old infant derives less than 70 percent of red blood cell iron from senescent red cells and therefore requires about 30 percent of iron needed for the production of red blood cells from the diet. In order to avoid the need for costly prophylactic supplementation with iron, it becomes increasingly desirable to enhance iron absorption from foods consumed. The relevance of type of fortification iron used and bioavailability has already been discussed. The role of other dietary components in influencing absorption is worthy of further consideration.

Role of Ascorbic Acid in Enhancing Iron Absorption

The enhancing affect with the inclusion of meat in the diet, referred to as the "meat-factor" (Cook and Monsen, 1976), has been mentioned. That presence of ascorbic acid has an enhancing influence has been widely reported (Apte and Venkatachalam, 1965); Conrad and Schade, 1968; Herbert et al., 1966; Layrisse et al., 1968) and is particularly beneficial in respect to absorption of the less available non-heme iron compounds (Cook and Monsen, 1977). Non-heme iron in foods exists primarily in an inorganic ferric (III) iron complex. These complexes are broken down during digestion and the iron is partly reduced to the more readily absorbed ferrous (II) iron form. This conversion process is facilitated by the presence of endogenous

hydrochloric acid in gastric secretions and by ascorbic acid (Dallman, 1980). That the conversion takes place prior to digestion in the stomach has been reported. Hodson (1970) investigated a liquid weight-control dietary fortified with iron as ferrous sulfate or ferric orthophosphate. The beverage contained an excess of ascorbic acid. After two to five months storage,the iron added in the trivalent ferric form had been solubilized, ionized and reduced to the bivalent form while the ferrous salt remained in its original soluble form. Lee and Clydsdale (1979) found 85 percent conversion from aqueous ferric chloride to the ferrous ion in canned beans and a rehydrated breakfast beverage. Lee and Clydsdale (1980) further highlighted the potential for changes to occur in iron valence during processing and storage with an investigation studying the effects of various forms of iron in an ascorbate-fortified beverage over three days of storage. Iron, as ferrous sulfate, remained 100 percent soluble but a slight increase was observed for amount of soluble complexed iron. When iron was added to the beverage containing ascorbic acid as ferric orthophosphate, 21 percent of the iron became solubilized with 17 percent being converted to the more biologically available ferrous form. Iron added in the elemental form became solubilized rapidly and no iron remained as elemental iron after four days of storage. The loss of elemental iron occured geometrically with 90 percent disappearing the first day after hydration. After four days, the iron profile of the beverage containing ascorbate and elemental iron was practically identical to that with ferrous iron added.

In a later report, Nojeim and Clydsdale (1981) reported that at pH 4.2, over 90 percent of elemental iron present in a phthalate/HCl/NaOH buffer system with ascorbic acid was ionized to the ferrous form

regardless if ascorbic acid was added or not. Gradual oxidation to the trivalent state was observed with time. After four weeks, approximately 25 percent of the ionic iron was present in the ferric state.

Physiological amounts of ascorbic acid in adults can result in a two- to five-fold enhancement of absorption for intrinsic iron or iron added in fortification of cereal products (Sayers et al., 1973; Bjorn-Rasmussen and Hallberg, 1974; Sayers et al., 1974a; Sayers et al., 1974b). It was suggested in a report of the International Anemia Consultative Group (1977) that a ratio by weight of at least 1:5 iron to ascorbic acid be used in infant cereal and milk products that are fortified with iron.

Some studies have indicated that ascorbic acid increased absorption of food iron and iron in the ferric form, but had little advantage in iron therapy with ferrous iron (Bothwell et al., 1958). Reports by Brise and Hallberg contradicted earlier reports and found that ascorbic acid, when given in sufficient amounts (200 mg or more ascorbic acid with 30 mg of iron), increased the absorption of ferrous iron and that the absorption-promoting effect increased with increasing amounts of ascorbic acid. It was noted that individual variation was great between subjects and that such variation could have led to previous conclusions where no significant effect of added ascorbic acid was detected.

It is thought that the reducing affect of ascorbic acid may help keep the complex in the ferrous state and thus prevent -- or at least delay, the formation of insoluble or undissociated ferric compounds. Nojeim and Clydsdale (1981) have described the influence of pH in this phenomena.

It has been indicated that ascorbic acid promotes absorption of iron. Mechanism for increased absorption with vitamin C present may be an action via internal iron transfer systems. Mazur and co-workers (1960; 1961) have shown a mutual relationship of ascorbic acid with ATP for the incorporation reaction of transferrin bound plasma iron into ferritin for storage. Lockhead and Goldberg (1959) reported that ascorbic acid increases the transfer of iron to heme biosynthesis as protoporphyrin. However, Brise and Hallberg (1962) concluded from their observations that the main effect of ascorbic acid under conditions examined (30 mg iron and 50-500 mg ascorbic acid administered orally) is intraluminal and probably due only to its reducing potential.

Prospective for Combining Ascorbic Acid with Fortification Iron in Infant Foods

The concern regarding the persisting condition in the United States and in less developed countries with nutritional anemia has prompted attempts to fortify accessory food items with iron (Zoller et al., 1980). Development of iron-fortified salt, sugar, coffee, tea, oils and MSG have been accomplished. In view of the significant contribution for ascorbic acid in promoting iron absorption, attempts have been made in the past to ensure that both of these nutrients are generously supplied in a variety of foods. Sayers et al. (1974b) found that common salt could be fortified with both iron and ascorbic acid provided that starch was added to prevent development of discoloration. Sayers indicated that such fortification would significantly improve the iron nutrition in countries where the staple food is rice or maize. Such efforts would prove helpful in diets of adults

when iron nutrition is low and consumption of iron-rich foods or of traditionally iron-fortified foods is less than desirable.

However, a proposal for the use of iron-fortified salt or sugar in baby food is not consistent with current practice (Dallman, 1980) that includes recommendation for reduced salt and suagar in infants' diets. A more practical approach to improving infant nutrition is through enhancement of iron absorption from foods already providing significant calories and iron in babies' diet.

The ability of ascorbic acid to enhance iron absorption from iron-fortified cereals, provided that it is added after cooking or taken during the meal as a solution, as in orange juice, has been demonstrated (Bjorn-Rasmussen and Hallberg, 1974; Callender and Warner, 1968; Elwood et al., 1978 ; Kuhn et al., 1968; Steinkamp et al., 1955). Cereal foods are promoted as standard dietary ingredients and thus may be an excellent vehicle for improving iron status in infants. The efficacy with iron-fortification in the presence of ascorbic acid in infant cereal is clear; but the combination of the two nutrients in a single food item is feasible only if the appearance, taste and nutrient content is maintained until the time of serving.

Additionally, the package distributed to infants as part of the national WIC (Women, Infants and Children Supplemental Food) program includes both an iron-fortified cereal and a fruit juice with ascorbic acid. The opportunity for the two nutrients to be served in a combined product is provided to a population who may be marginal for iron nutrition. Interest is therefore generated for investigating the chemical interaction of the combined nutrients.

Ascorbic Acid -- Structure and Properties

The Three Forms Idendified

Reduced ascorbic acid (R-AA) (Figure 1) is a highly soluble, acid compound with strongly reducing properties. It is a white crystalline solid melting at 192°C. It is the γ -lactone of an hexonic acid with an enediol structure of carbon atoms 2 and 3 (Tannenbaum, 1976). The vitamin is readily labile to heat, low-acid pH, presence of oxygen, and metal catalysts.

Dehydroascorbic acid (DHA) (Figure 1) is formed with the removal of two equivalents of hydrogen from the reduced form. The oxidized form retains the basic ring structure but no longer contains a conjugated system. The acidity associated with the enediol structure of R-AA is lost on oxidation to DHA (Tannenbaum, 1976).

Further oxidation results eventually in formation of diketo-gulonic acid (DKGA) (Figure 1) with the primary hydroxyl group being oxidized to a carbonyl group. Additionally, the completely oxidized form loses the lactone ring structure and subsequently all vitamin C activity (Tannenbaum, 1976).

Influence of Acid Conditions on Reactivity

R-AA possess two acidic hydrogens with pKa's in water of 4.25 and 11.79. The influence of pH on oxidation rate is marked and has been reported by numerous researchers (Khan and Martell, 1967; Lee et al., 1977; Joslyn and Miller, 1949). Below pH 6.0, the undissociated and monovalent forms of ascorbic acid are the main species in solution (Khan and Martell, 1967). In this range, only the monoionic species was found to be reactive toward molecular oxygen and

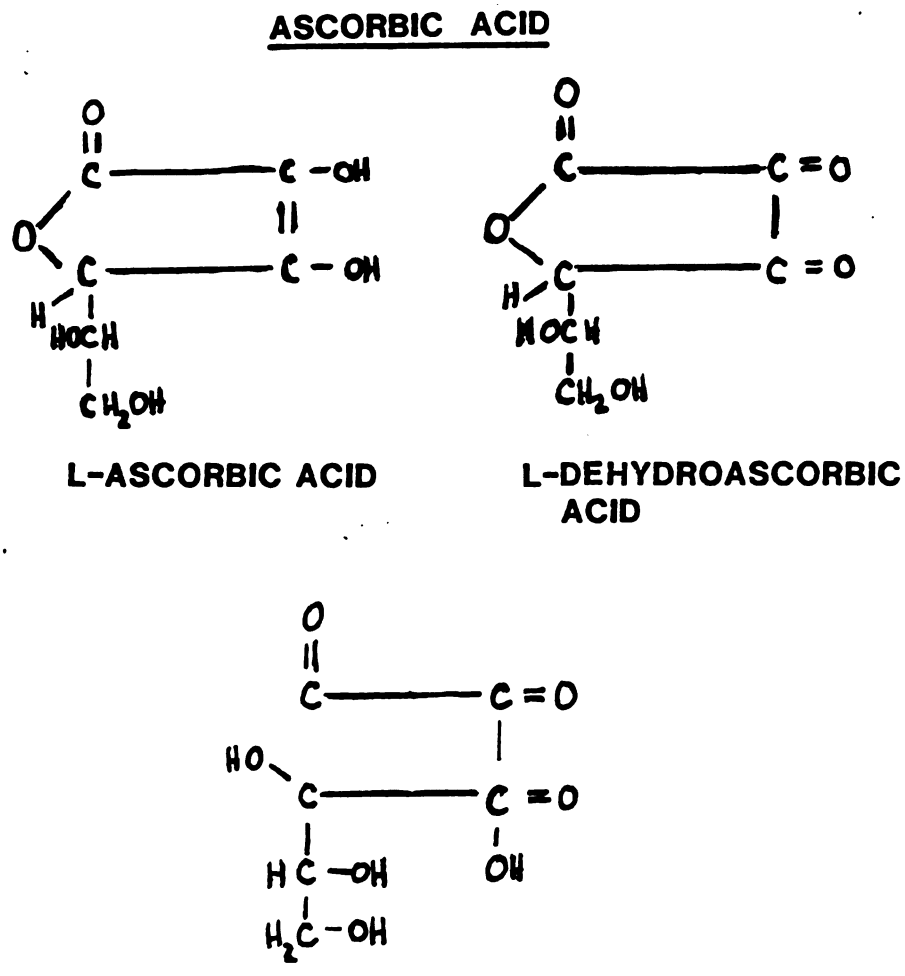


Figure 1. Three chemical forms of ascorbic acid produced during oxidation.

the spontaneous rate of oxidation varied linearly with the concentration of ascorbic acid. The contribution of the neutral species was essentially zero. Finholt et al. (1963), and Blaug and Hajratwala (1972) found the rate of oxidation to reach a maximum near pKa_1 of ascorbic acid. Joslyn and Miller (1949) determined that autoxidation of ascorbic acid in the presence of sufficient cyanide and thiocyanate to supress the catalytic effect of metals at pH 4.7 to 9.2 in phenolsulfonate buffer (pH 7.8 to 9.2) and phosphate buffer (pH 4.7 to 7.6) proceeded with the divalent ion reacting 10^5 times faster than the monovalent ion with atmospheric oxygen. Under partial pressure, the reaction rate for the divalent ion is reduced one-fifth but is not reduced for the monovalent species.

Influence with Presence of Metal Catalyst

Joslyn and Miller (1949) reported that in the absence of thiocyanate and in the presence of copper, the reaction rate for the monovalent ion was proportional to the oxygen concentration, yet the effect was observed only when the metal was present in high concentration. At low copper concentration, rate of oxidation increased more rapidly than did concentration of the metal. It is reported that a complex formation occurred between the ascorbate ion and the metal. Khan and Martell (1967) supported their results and found linear variation in rate with concentration of ferric (III) ions in the range of pH 1.5 to 3.85. In both cupric and ferric catalyzed reactions, the rate showed an inverse dependence with the hydrogen ion concentration, but only at pH below pKa_1 of ascorbic acid (Figure 2). At pH values greater than pKa_1, there is an

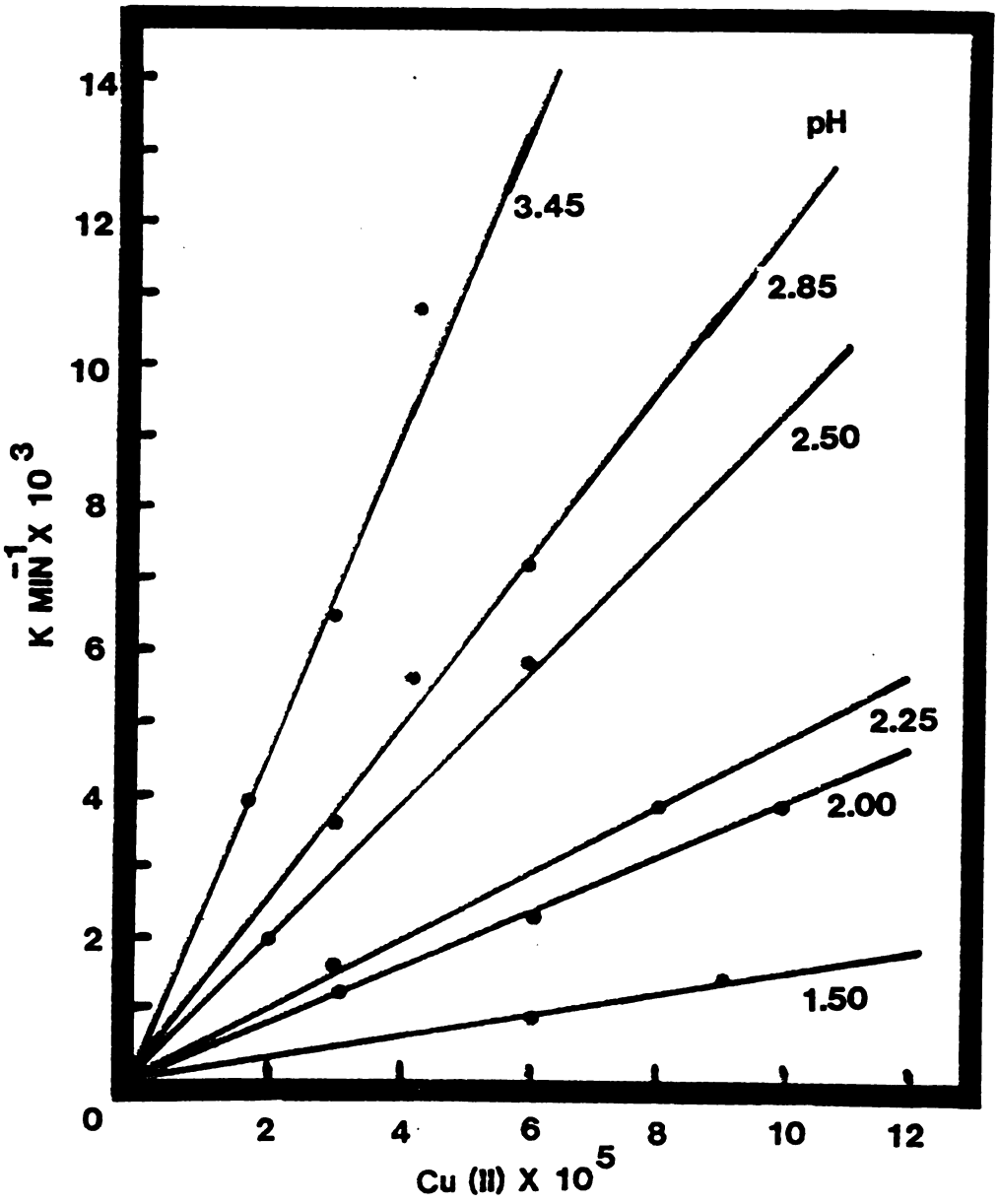


Figure 2. Catalytic effect for the oxidation of ascorbic acid in the presence of Cu(II) ion at 25 C, as function of pH.
K = difference between the first-order rate constants in the presence and in the absence of the metal ion.

increasing proportion of divalent ions in solution. Joslyn and Miller (1949) reported that the rate of oxidation increased to maxima between pH 6 and 7. This finding may reflect some spontaneous oxidation with the increased rate observed for the divalent ion in reaction with atmospheric oxygen.

The relationship with pH and metal catalyst is complex and is influenced by a variety of factors including buffers in the system and amount of oxidation already taken place. During the course of oxidation, pH value changes somewhat as the less acidic DHA accumulates. Joslyn and Miller (1949) observed an increase in pH given pH values of 6.5 and below; but at pH above 6.5, a decline in final pH occurred, indicating that products of oxidation other than DHA must accumulate.

Despite varying rates of ascorbic acid degradation reported over time, researchers including Lee et al. (1977) have shown a first-order reaction rate for ascorbic acid concentration versus storage time. This has been reported for anaerobic conditions (Lee et al., 1977; Finholt et al., 1963) and for aerobic conditions (Khan and Martell, 1967).

Influence on Stability by Water Activity

Much interest has been generated by investigators for the relationship between loss of food nutrients and moisture content. The moisture content is most appropriately represented as water activity (a_w) defined as;

$$a_w = \frac{P}{P_o} = \frac{\% \text{ ERH}}{100} \qquad (1)$$

where P_o = vapor pressure of pure water, P = vapor pressure of water in the food product and % ERH = percent relative humidity of the system in equilibrium, whereby there is neither gain nor loss of water. The majority of interest with water activity is focused in dehydrated foods or intermediate-moisture (IMF) technology. Nutrient stability of a system with low water activity is quite different from one in a more aqueous state. Karel and Nickerson (1964), and Vojnovich and Pfeifer (1970) have reported that in foods of water activity up to a_w = 0.5, there is an increased rate of ascorbic acid destruction with increasing moisture content. Lee and Labuza (1975) reported the influence of a_w on destruction of vitamin C in systems with a_w up to 0.84 and found that the same phenomenon existed whereby a_w of a food system corresponded with increased rate of ascorbic acid degradation (Figure 3).

Three mechanisms were proposed by which water may act to control reaction rate. First, the mechanism for oxidation may differ at various a_w's; second, that water may act to dilute the concentration of ascorbic acid which could reduce the rate of reaction. Alternatively, an increased water content may facilitate the reaction since higher moisture corresponds with a less viscous solution and enables more diffusion to take place. Analysis by Lee and Labuza (1975) concluded that oxidation rate does not change as a_w increases and a dilution effect may be occurring. However, in their studies, it appears that the dilution effect may be masked by some other mechanism that caused the reaction rate to increase. The increased mobility of a reactive species in the more aqueous solution was considered as a most likely agent for enhancing oxidation rate. The increased

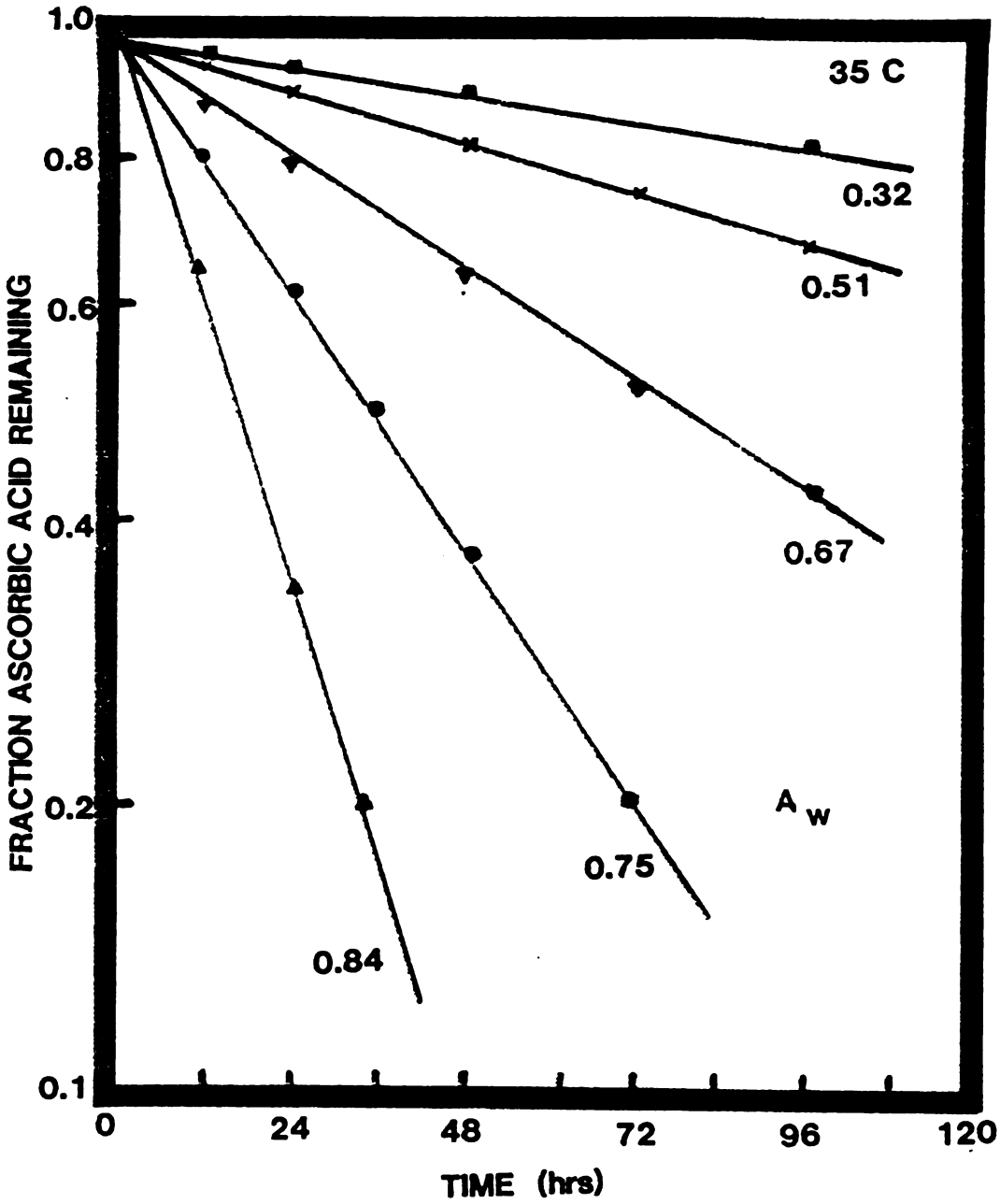


Figure 3. Retention of ascorbic acid as function of a_w at 35 C.

rate could be due to either increased mobility for the metal catalyst or for ascorbate itself. It was further postulated, however, that above a certain moisture content, the aqueous phase viscosity was not greatly altered and the rate of oxidation should approach a constant value.

Methods of Analysis for Ascorbic Acid

Numerous analytical procedures exist for ascorbic acid in foods, yet none are completely satisfactory since foodstuffs contain interfering agents and some processing techniques characteristic for a food item contribute interfering agents (Freed, 1966). Analysis is generally by means of a procedure based on an oxidation-reduction reaction, or by means of treatment for a chromagen formed by coupling oxidized ascorbic acid with 2, 4-dinitrophenylhydrazine dye (DNPH). Classic analysis follows oxidation by a redox dye, such as 2, 6-dichloroindophenol (DCIP). The DNPH technique may be used to determine only the reduced form of ascorbic acid, or may yield "total" vitamin C which includes both active forms: reduced form, R-AA and DHA. DHA is reduced to R-AA, and DKGA is quantified by the amount of dye required to form a highly colored hydrazone. The amount of total ascorbic acid present is indicated by difference.

The three forms of ascorbic acid may be quantitatively determined using a step-wise reaction with the compound DNPH in a method first outlined by by Roe et al. (1948). The analysis is based on the coupled DHA and DKGA derivative. The reaction is known to be quite sensitive (Penney and Zilva, 1945). The technique discussed by Roe et al. (1948) is time-consuming and laborious, however,

methods have been outlined for various particular foodstuffs. The coupling reaction is delicate and temperature is a critical factor. At temperatures in excess of 37°C, the reaction is affected by glucose, fructose, and glucuronic acid (Roe, 1961). At low temperatures, the reaction is quite slow.

Extraction of the three compounds from tissues must be conducted so as to prevent the oxidation of R-AA to DHA, and to keep at a minimum the change of DHA to DKGA. Roe and Oesterling (1944) prevented such oxidation in plant tissue by using a metaphosphoric acid solution containing 1 percent thiourea. In animal tissue, a more powerful antioxidant agent is required due to the strength of the oxidizing affect of oxyhemoglobin (Roe et al., 1948). The problem was solved by adding stannous chloride in 10 percent concentration to the metaphosphoric solution, then diluting the sample to contain 0.5 percent $SnCl_2$.

Cereal and fruit foodstuffs may not require presence of $SnCl_2$ as antioxidant, but iron added in fortification may become an interfering agent and catalyze the oxidation of ascorbic acid. Thus, the use of $SnCl_2$ may be recommended for analysis of iron-fortified products. Furthermore, Mattews and Hall (1978) compared ascorbic acid in fresh and frozen green peppers using thiourea and $SnCl_2$. Researchers determined that R-AA was protected from oxidation to a greater degree with $SnCl_2$ than when thiourea was used. It is therefore apparent that recommendation should follow for use of $SnCl_2$ as antioxidant in ascorbic acid analysis of cereal and fruit infant foods when an antioxidant is required in the procedure.

In light of difficulties inherent with the method proposed by

Roe et al. (1948) which includes use of several toxic reagents, alternative means of quantifying the three forms of vitamin C have been investigated. The classic method for analysis of reduced ascorbic acid includes oxidation by DCIP. Oxidation of the two loosely-bonded hydrogen atoms of the dienolic group of ascorbic acid is generally measured by visual titrametric techniques using the indophenol reagent, yet these techniques are limited by interfering agents and by difficulty in determining the precise endpoint of titration. Photometric analyses have aided precision, but no satisfactory adaptation of the technique has been developed that avoids interference of compounds such as thiosulfate, sulfite, reductones; and ferrous, cuprous, or stannous salts which react rapidly with indophenol.

DHA and total ascorbic acid (TAA) may be determined by a fluorometric procedure involving the reaction of DHA with orthophenylenediamine (OPDA) to yield a fluorescent quinoxaline, 3- (1, 2-dihydroxyethyl) furo [3, 4-b] quinoxaline-1-one (Deutsch and Weeks, 1965). Specificity of the procedure is enhanced through use of boric acid. Boric acid is added to the oxidized aliquot prior to the addition of OPDA, causing formation of a boric acid-DHA complex. DHA may not condense with OPDA and any resulting fluorescence is then due to extraneous material. Background interference may be subtracted from the fluorescence of the complex formed without addition of boric acid and true DHA is indicated. Use of Norit as oxidizing agent for oxidation of all three forms of vitamin C present allows for the determination of TAA, and R-AA is attained by difference.

A simple, rapid, quantitative method for continuous flow analysis for DHA and for TAA has been developed by Kirk and Ting (1975). The method, using the Technicon Autoanalyzer II, relies on

a modification of the manual method of Deutsch and Weeks (1965). Oxidation of R-AA is by DCIP-thiourea oxidation in substitution for Norit, since Norit oxidation is incompatable with the continuous flow system. Use of the automated system decreases analytical time without adversely affecting either the accuracy nor precision of the OPDA-based assay.

EXPERIMENTAL PROCEDURES

Preparation of Model Systems

Two types of model systems were prepared. The intent was to simulate experimental products of (a) a mixed cereal system of dry cereal and apple juice and (b) a prepared wet cereal product. The mixed product, referred to as mixed cereal, was simulated with a 10^{o} Brix sucrose solution, and the wet cereal was emulated with a 5 percent gelatinized rice starch solution. Rice flour was heated to gelation temperature and cooled thoroughly before addition of ascorbic acid. Both products were freshly prepared for use each day and were stored at room temperature.

Ascorbic Acid Determination

R-AA and DHA were determined by the continuous flow ortho-phenylene-diamine micro-fluorometric procedure described by Kirk and Ting (1975). Ascorbic acid was extracted with 0.5 percent oxalic acid and samples were filtered before introduction to the Autoanalyzer (Technicon Autoanalyzer II, Tarrytown, N.Y.). DHA condenses with the OPDA dye forming a fluorophor which may be detected fluorometrically with an excitation wavelength of 360 nm and emission wavelength of 436 nm. DHA blanks are determined with addition of boric acid to prevent condensation of OPDA with DHA.

Total ascorbic acid is measured as DHA following oxidation of R-AA with 2,6-dichloroindophenol (DCIP) dye. R-AA may be determined by difference of DHA from TAA once respective blanks have been subtracted. A set of R-AA standards (Eastman Kodak) were run daily and R-AA was determined by application of a linear regression equation. DHA standards (ICN Pharmaceuticals)

were run simultaneously when DHA was to be determined and quantity of DHA was calculated similarly by application of linear regression.

Reduced Ascorbic Acid

Analysis for R-AA followed the classical titrametric technique described in AOAC (1975; Freed, 1966). Samples were prepared by extraction in 3 percent metaphosphoric acid ($mHPO_3$) and 0.005 M EDTA solution. Extracts were titrated with DCIP and concentration was determined visually by the amount of dye required to oxidize the ascorbic acid and produce a faint pink coloration.

Dehydroascorbic Acid

DHA was determined by the automated micro-fluorometric procedure described by Kirk and Ting (1975) and also by adaptation of the colorimetric method outlined by Roe and associates (1948; Freed, 1966). The method for automated analysis has been described above for R-AA, but DHA concentration was determined by application of linear regression formulas for DHA standards.

The procedure outlined by Roe et al. (1948) permits identification of R-AA, DHA, and DKGA; but involves the use of hazardous reagents and is difficult to manage. Both hydrogen sulfide and bromine are required in the assay, which are corrosive and toxic; subsequently, a modification of the method outlined by Roe et al. (1948) was adopted. The analysis used is based on the coupling reaction of both DHA and DKGA with 2,4-dinitrophenylhydrazine (DNPH) under carefully controlled conditions to give red-colored osazones. DHA content in model systems and in samples is determined after comparison of color produced in samples and a set of DHA standards. Analysis for baby food samples would indicate both DHA and DKGA

concentration present. Preparation for each sample is by grinding in a mortar with 1/20th of the final volume af 5 percent $mHPO_3$ and sufficient dry $SnCl_2$ to make a 10 percent $SnCl_2$ solution in the volume of 5 percent $mHPO_3$ added. A 10 percent concentration for the $SnCl_2$ solution during grinding protects ascorbic acid from oxidation and leaves DHA and DKGA unaltered. A 20-fold dilution of samples follows grinding to yield the final extract. Specifically, to each sample 0.5 g $SnCl_2$ was added with 5 ml $mHPO_3$, samples ground and diluted to 100 ml. Extracts were subsequently filtered; 4 ml aliquots pipeted into each of three test tubes. One set of tubes served as blank, while other tubes were treated with 1.0 ml of 2 percent DNPH. Tubes were placed in a 37°C water bath for exactly six hours to allow the coupling reaction to proceed. Samples were immersed in an ice bath to cool, and 5 ml of 85 percent sulfuric acid was added dropwise to each tube with continuous shaking. To the blank tubes, 1.0 ml of 2 percent DNPH was added and all tubes were permitted to stand at room temperature for exactly 30 minutes. Since color intensity is critically dependent on time following addition of H_2SO_4, a definite schedule was followed for spectrophotometric measurement of color. Tubes were allowed to stand 30 minutes and read in a Bausch and Lomb Spectrophotometer (Spectronic 70, Bausch and Lomb, Rochester, N.Y.) at 520 nm. Evaluation at 520 and 540 nm revealed that maximum absorbance occurred at 520 nm. Concentration of DHA was evaluated by comparison with a set of DHA standards and application of a linear regression model.

Diketogulonic Acid

DKGA in samples was determined by the method described by Roe et al. (1948). The colored osazone is the coupled complex of DKGA with DHA. The completely oxidized component DKGA was determined by difference,

subtracting DHA determined by the automated analysis. By using the automated analysis for determination of DHA, the use of corrosive and toxic reagents was avoided.

Pilot Experiments

Comparison of Methodology for R-AA

Recovery of R-AA from both sucrose and starch model systems was evaluated simultaneously by the automated analysis and by the titrametric procedure. Samples were prepared with R-AA standard at concentration of 100 ug/ml. Samples prepared for automated analysis were further diluted with 0.5 percent oxalic acid to contain 4, 6, and 8 ug/ml R-AA. Starch solutions were filtered before introduction to the Autoanalyzer; sucrose solutions did not require filtration. Blanks for model systems were also evaluated by preparing samples with no ascorbic acid added. Samples were prepared for analysis for the titrametric procedure by diluting with 3 percent $mHPO_3$-0.005M EDTA solution to contain 200, 400, and 600 ug/ml R-AA. Differences in sample preparation between the two methods corresponded with sensitivity for each of the two methods of assay. Blanks of both model systems were prepared as for the automated analysis to evaluate background interference.

Comparison of Methodology for DHA

Recovery of DHA from both sucrose and starch model systems was evaluated by the automated analysis and by the spectrophotometric procedure. Samples for both systems were prepared to contain 100 ug/ml DHA. Samples for analysis using the Autoanalyzer were diluted with 0.5 percent oxalic acid to contain 4, 6, and 8 ug/ml DHA. Only starch samples were filtered before introduction to the system. Blanks for both sucrose and

starch solutions were run simultaneously with samples, as were DHA standards. Samples prepared for evaluation by the spectrophotometric assay with DNPH required addition of 10 percent $SnCl_2$ as antioxidant, grinding with mortar and pestle, and dilution with 5 percent $mHPO_3$ to contain 4, 6, and 8 ug/ml DHA. Concentration was determined by comparison with a standard curve. Standards were prepared in either oxalic acid or $mHPO_3$ respectively. Blanks were run for both sucrose and starch solution in both automated and spectrophotometric analyses.

Preparation of Infant Food Samples

Two products were evaluated. These were a mixed cereal: dry rice cereal with bananas prepared in a 1:5 dilution with apple juice, and a wet cereal: rice cereal with applesauce and bananas. The wet cereal is a thermally processed product packed in glass jars with a net weight of 100g. All samples including the dry rice cereal, apple juice, and wet cereal were prepared by Gerber Products (Fremont, Michigan). Rice cereal is commercially prepared with electrolytic iron added in fortification to make label declaration of 47.5 mg/100g. Considering a serving size of 14.2 g (0.5 oz), cereal may provide 45 percent the U.S.R.D.A.(Code of Federal Regulations, 1981) infants. Apple juice is prepared to enable label declaration of 32.0 mg reduced ascorbic acid per 100g (3.2 fluid oz), a level that represents 120 percent the U.S.R.D.A. for infants and 100 percent the U.S.R.D.A. for a child one to four years of age. Complete nutritional information for the three products as prepared by Gerber is given in Table 1.

Rice cereal was also prepared with no iron added in fortification. Unfortified cereal was prepared with apple juice identically as was product containing electrolytic iron. Three batches of each mixed product with and without iron were prepared in sufficient quantity to enable

Table 1. Average nutrient values for baby food products per 100 g.

nutrient	Product: Rice cereal with bananas	Product: Apple juice	Product: Rice cereal with applesauce and bananas
Calories	396	49	81
Protein (g)	9.1	0.1	1.3
CHO (g)	75.6	11.9	18.1
fat (g)	6.4	0.1	0.4
crude fiber (g)	1.0	0.1	0.2
moisture (g)	5.1	87.6	79.7
Calcium (mg)	634	4	10
Phosphorus (mg)	352	5	23
Iron (mg)	47.5	0.4	5.0
Sodium (mg)	100	2	5
Potassium (mg)	660	93	49
Vit A (IU)	29	-	1
thiamin (mg)	1.58	-	0.17
riboflavin (mg)	1.90	0.01	0.20
niacin (mg)	14.08	0.08	2.67
Vit B_6 (mg)	0.63	0.03	0.13
Vit C (mg)	2.1	32.0	11.7

sampling over a 10 day period. Samples were stored at 4.4°C in covered plastic containers and were held in the dark between sampling. Two batches of vitamin C-enriched juice and two batches of iron-fortified and unfortified cereal were provided by Gerber. Three different combinations of the mixed product, both with and without iron, were prepared as diagramed in Figure 4.

Wet cereal, rice cereal with applesauce and bananas, was prepared as for commercial distribution fortified to a level adequate to make label declaration per 100g of 5.0 mg iron as ferrous sulfate, and enriched to declare 11.7 mg reduced ascorbic acid. Based on serving size of 135 g (contained in a single serving jar), this corresponds with 45 percent of the the U.S.R.D.A. for infants for both iron and ascorbic acid. Products were prepared by Gerber both with and without iron-fortification, but with vitamin C-enrichment included in all products evaluated in the experiment. Three batches of each product, both with and without iron added, were prepared in sufficient quantities to permit sampling over the experimental period. Sampling from each replicate was conducted from a single jar. All jars were stored with refrigeration between sampling at 4.4°C and in the dark. Schemmatic diagram of all samples as prepared for analysis is given in Figure 5.

Both mixed cereal and wet cereal samples were prepared for introduction to the Autoanalyzer for determination of R-AA and DHA, and for spectrophotometric analysis for DHA and DKGA. Dilution to the appropriate range of assay required that 1.0 g of mixed cereal or 3.0 g of wet cereal be diluted to 100 ml with 0.5 percent oxalic acid. Extracts were filtered before introduction to the system. Identical samples were prepared for spectrophotometric analysis. Range of the spectrophotometric assay necessitated that 2.0 g of mixed cereal be diluted to 200 ml with 5

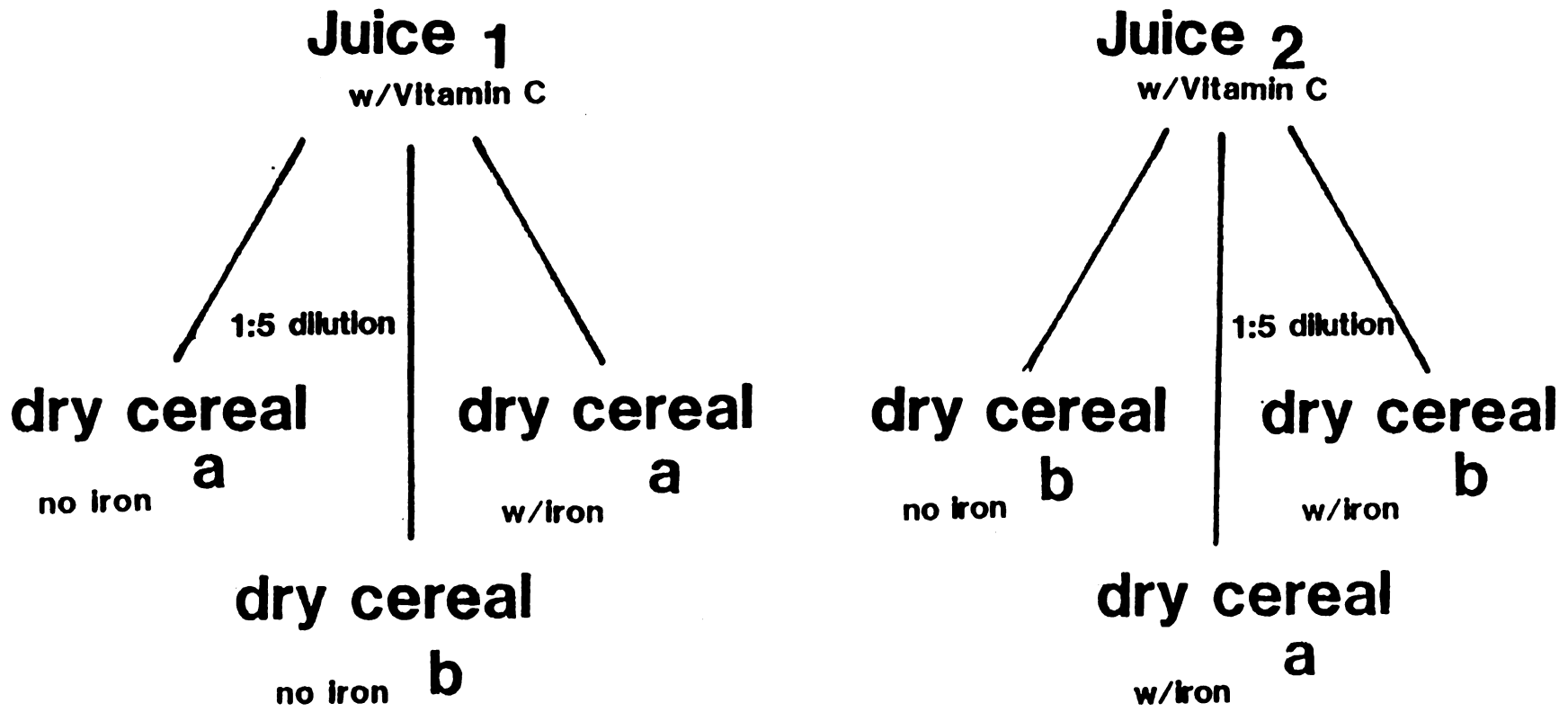


Figure 4. Schematic diagram of mixed cereal preparation.

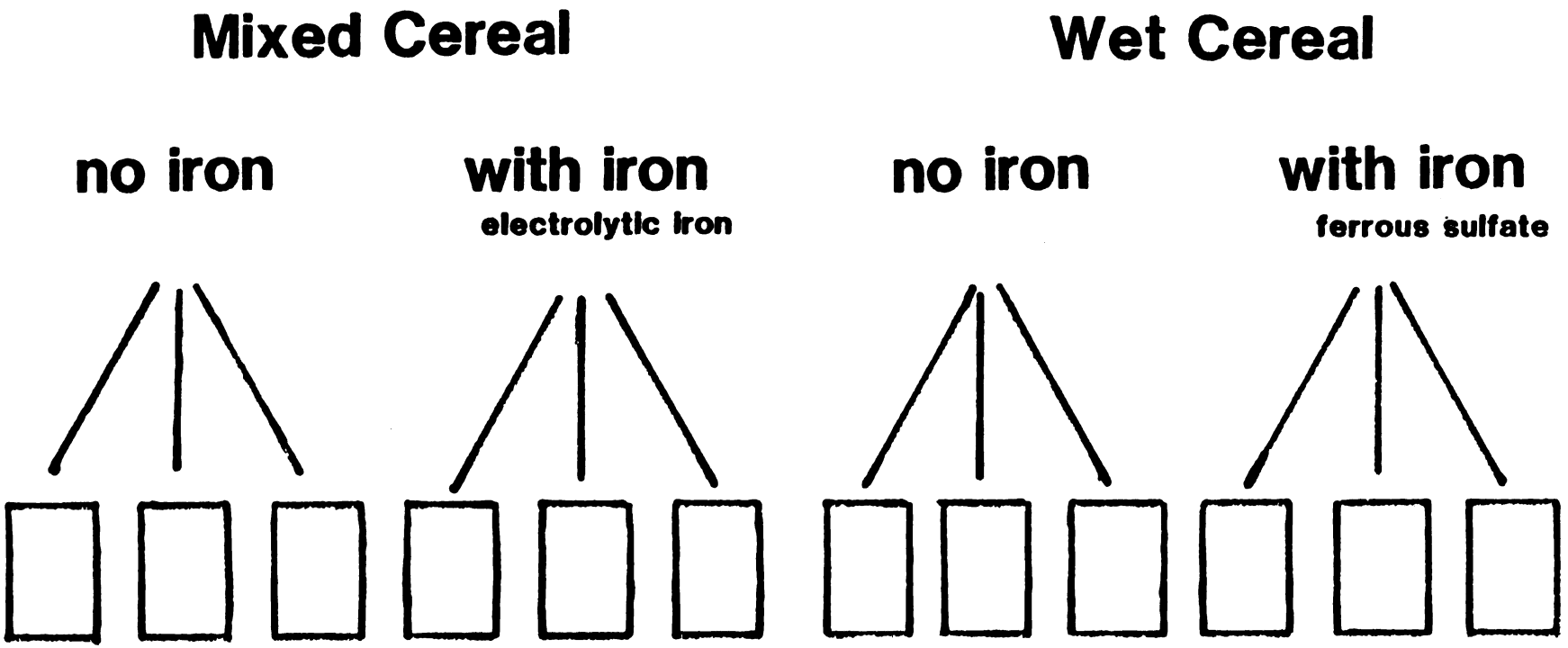


Figure 5. Samples prepared for analysis.

percent $mHPO_3$ and that 2.0 g samples of wet cereal be diluted to 100 ml.

Iron Analysis

Iron assay was conducted for all samples at the beginning and end of the 10 day experimental period. Analysis was by atomic absorption spectrophotometry. Sample preparation was conducted using a wet ashing technique and digestion with perchloric and nitric acids. Samples for day 0 of the experiment were stored until the conclusion of the time period with the addition of 10 ml nitric acid before storing. Digestion was completed upon termination of the experiment and all samples were evaluated simultaneously.

An atomic absorption spectrophotometer (Model IL 951 - Instrumentation Laboratory, Inc., Wilmington, Ma.) was used. The model uses a premixed gas burner. Fuel used was acetylene with air as oxidant. Light source was a hollow cathode lamp (8 ma). Absorption of radiation was detected at a wavelength characteristic for iron, 248.3 nm, with a band width of 0.3 nm. Sample concentration was determined by integration with a set of iron standards within the predicted range of samples being evaluated.

Data Analysis

Results of the pilot study comparing methods relative to standards were compared for statistical differences by means of a three-way analysis of variance (ANOVA). Analysis was run with the SPSS computer program (Statistical Package for the Social Sciences).

The loss of R-AA and TAA, and the accumulation of DHA and DKGA was evaluated in terms of actual concentration change by means of the SPSS computer program. Differences were first described based on three-way ANOVA. Evaluation followed according to the repeat measure split-plot

design for the experiment (Appendix I-III, V).

A first-order reaction rate was assumed for loss and accumulation of the respective forms of ascorbic acid:

$$\frac{d(C)}{dt} = k\,C \qquad (2)$$

where (C) = concentration of ascorbic acid; t = any time during storage (days); and k = first-order reaction rate constant ($days^{-1}$). The k constant was calculated for each system, both mixed and wet cereal products, and for each form of ascorbic acid: TAA, R-AA, DHA and DKGA in terms of concentration ratio:

$$\text{concentration ratio} = c / c_o \qquad (3)$$

In cases where negative values would have resulted in calulation for equation 3, another form of this expression was applied:

$$\text{concentration ratio} = c - c_o / c_o \qquad (4)$$

The k constant is useful in that it can be applied to calculate concentration (c) at any time (t) given knowledge of initial concentration (c_o) by means of the first-order reaction equation:

$$c = c_o \exp(-kt) \qquad (5)$$

The k constant was also used in calculation of one-half life, or doubling time, for each form of ascorbic acid. This would indicate the time required to reduce by one-half the amount of TAA or R-AA present,

or to double the quantity of DHA or DKGA in the mixed and wet cereal products.

Experimental Design

Based on the considerable interest expressed regarding the persisting condition in the U.S. and in less developed countries with nutritional anemia in infants (Fomon, 1970; Filer, 1969), and the increasing interest in iron-fortification with highly available forms of iron, along with the potential for interaction of nutritional components enhancing bioavailability (Sayers et al., 1974), a project was designed that would assess the effects of the two forms of iron commonly added in infant foods. The foods selected have been serving as vehicles for both iron-fortification and vitamin C-enrichment. That ascorbic acid enhances iron bioavailability has been established (Apte and Venkatachalam, 1965; Layrisse et al., 1968); yet, if iron in combination with ascorbic acid acts to degrade the vitamin then its addition may not be acceptable in infant foods that contribute substantially to the vitamin C nutrition of infants.

Electrolytic, or elemental iron is currently added in fortification of dry infant cereal which may be prepared with apple juice for infant feeding. Reduced iron, ferrous sulfate, is added to wet cereals that are simultaneously enriched with vitamin C so that they may provide substantial quantities of both nutrients in infants' diets. Reduced iron may possess greater catalytic capacity to oxidize R-AA since it is chemically a more reactive species of iron than that in the elemental form.

Pilot Projects for Comparison of Methodology

A study was conducted preliminary to the project designed to evaluate accuracy in each model system relative to standards for the methods used in the experiment. The combination of methods with the automated analysis for determination of TAA, R-AA and DHA, and the spectrophotometric analysis for quantification of the coupled DHA and DKGA complex was intended to permit evaluation of DKGA by difference from the DHA component. Since methods were not quantitative, the DKGA component could not be identified. Correction factors were required that would permit comparison of results obtained to standards in model systems.

Reduced ascorbic acid standards were run for the automated analysis and the classical titrametric technique. DHA standards were run for both the automated analysis and for the adapted method from Roe et al. (1948). Sample size for the two comparative studies was predetermined to obtain statistical power greater than 0.95 for an f-test; analysis of variance of fixed effects.

Ten-Day Storage Study for the Cereal Infant Foods

The project was then initiated in order to enable comparison for catalytic reactivity in destruction of vitamin C for mixed cereal with electrolytic iron, and wet cereal with ferrous sulfate. Since sampling was to be conducted daily from each sample over a 10 day period, a non-random, split-plot, repeat measure, experimental design was applied. Each sample may be treated as its own block, thereby reducing need for excessive replication of product. The design obtained adequate statistical power with three replicates of each product, both with and without iron. Experimental error (mean square error) was reduced without sacrificing power of significance. The split-plot, repeat measure design is summarized

in Figure 6. The design is useful for indicating trends over time as response to a given treatment (Gill, 1978). Bonferroni-t analysis and a Student's t-test were also applied to make pair-wise comparisons between combinations of cereal products.

Indication of Background Interference with Ascorbic Acid

Samples were prepared by Gerber identically with experimental product but with no vitamin C added in enrichment. Analysis was conducted similarly for the unenriched product as for the enriched samples to indicate the amount of natural vitamin C in each product.

Iron Evaluation

Each product was evaluated for iron content at the beginning and end of the 10 day experiment. This was conducted to verify that iron was present at levels declared by Gerber and that iron content was not changing dramatically during the course of the evaluation.

Data evaluation was conducted according to the Student's t distribution for pair-wise comparison. Change in samples without iron added was compared over time, as was change in samples with iron added in fortification. Results are expressed in terms of significance with a t-test statistic (Appendix VII).

SPLIT - PLOT DESIGN (repeat measure)		
trt (3)		d.f.
	CT	1
	IRON	1
	CT X IRON	1
	batches/CT X IRON	8 E_1
	DAYS	9
	CT X DAYS	9
	IRON X DAYS	9
	batches X DAYS	72 E_2

Figure 6. Split-plot design of the experiment.

RESULTS AND DISCUSSION

The study of nutrient interaction within a food product is particularly significant when considering the infant's diet, since the consequence of suboptimal nutritional status during development may influence health in adulthood. The effect of two forms of iron added to infant food on ascorbic acid was evaluated. The two products evaluated represent vehicles with substantial nutrient contribution in infants' diet, and are currently significant sources of iron and ascorbic acid for infants fed commercial cereal foods. The systems were chosen to represent a real-life feeding situation and thus, were not manipulated to equate iron level nor ascorbic acid content. Nutritional implication for feeding each product could be assessed.

Comparison of Methodology for R-AA

Results obtained from the automated method were compared with that of the classical titrametric vitamin C analysis. Identical readings were not obtained for each method. Comparison for the 10^{o} Brix model solution in relation to R-AA standards is illustrated in Figure 7. Both methods indicated less ascorbic acid than was present by addition of amounts of R-AA standard. Results for the titrametric method were significantly below that obtained for the automated analysis.

Comparison for methods with the starch model system indicated a similar but reversed situation. Both methods indicated less R-AA than was present by addition of the standard; but in starch, the titrametric assay indicated a greater amount present than was indicated with the automated analysis. This difference was significant ($p < .001$) and is

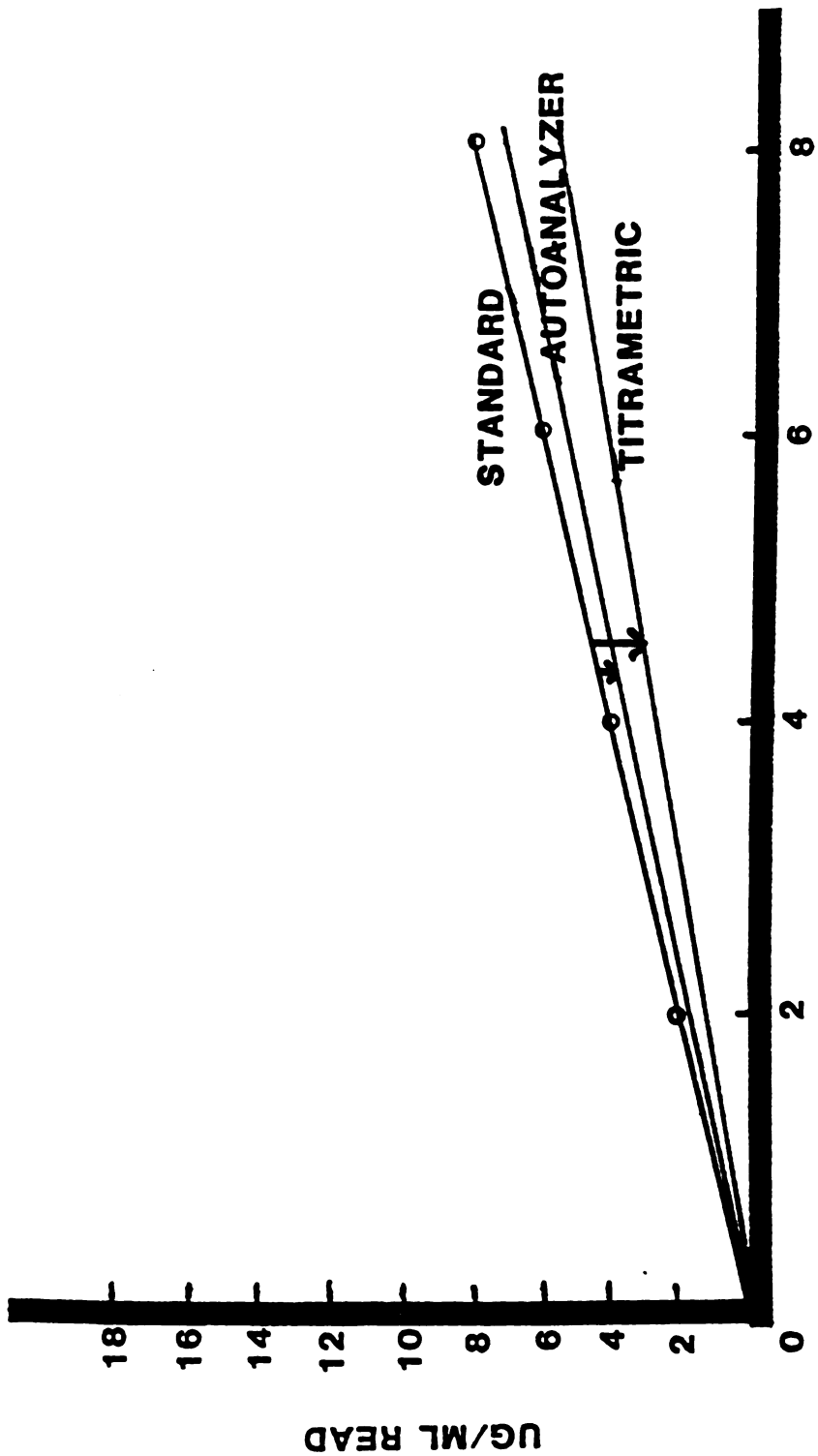


Figure 7. Comparison for R-AA in 10^{o} Brix model solution with R-AA standards by autoanalyzer and titrametric methods.

illustrated in Figure 8.

The contributing factor in the sugar model system that caused more R-AA to be reported in samples relative to the DCIP titrametric analysis may be attributed to interaction from interfering agents. No method for vitamin C analysis has proven to be free of interference from all chemical substances.

Roe (1961a) reported that the method based on DCIP oxidation has highest specificity of any oxidation-reduction method proposed. Bessey (1938) showed highest specificity when the reaction was carried out at pH 3.5. At pH values above 4, interference with phenolic or sulfhydral groups was pronounced. Essentially, any substance with a reduction potential lower than that of the indicator is a possible source of interference (Bessey and King, 1933). In addition, the visual technique is limited by difficulty in evaluating a precise endpoint. For the sucrose model, the closer estimation with R-AA to standards obtained with the automated fluorometric method could reflect an increased degree of precision.

It may be that sucrose, which is not a reducing sugar, is hydrolyzed by phosphoric acid used in the titrametric analysis, thereby creating a reducing sugar which could react with indophenol dye.

The automated fluorometric analysis has as principle rate-controlling factors for the formation of quinoxalines of carbohydrates: molar ratios of the reactants, acidity, and temperature of the medium (Towne and Spikner, 1963). The formation of a fluorescent compound is illustrated in Figure 9. Deutsch and Weeks (1965) reported that pyruvic acid (α-ketopropionic acid) reacts with OPDA to form a fluorescent 2-hydroxy-3-methyl-quinoxaline. Fluorescence is an additive phenomenon and it is necessary to differentiate between that representing dye interaction

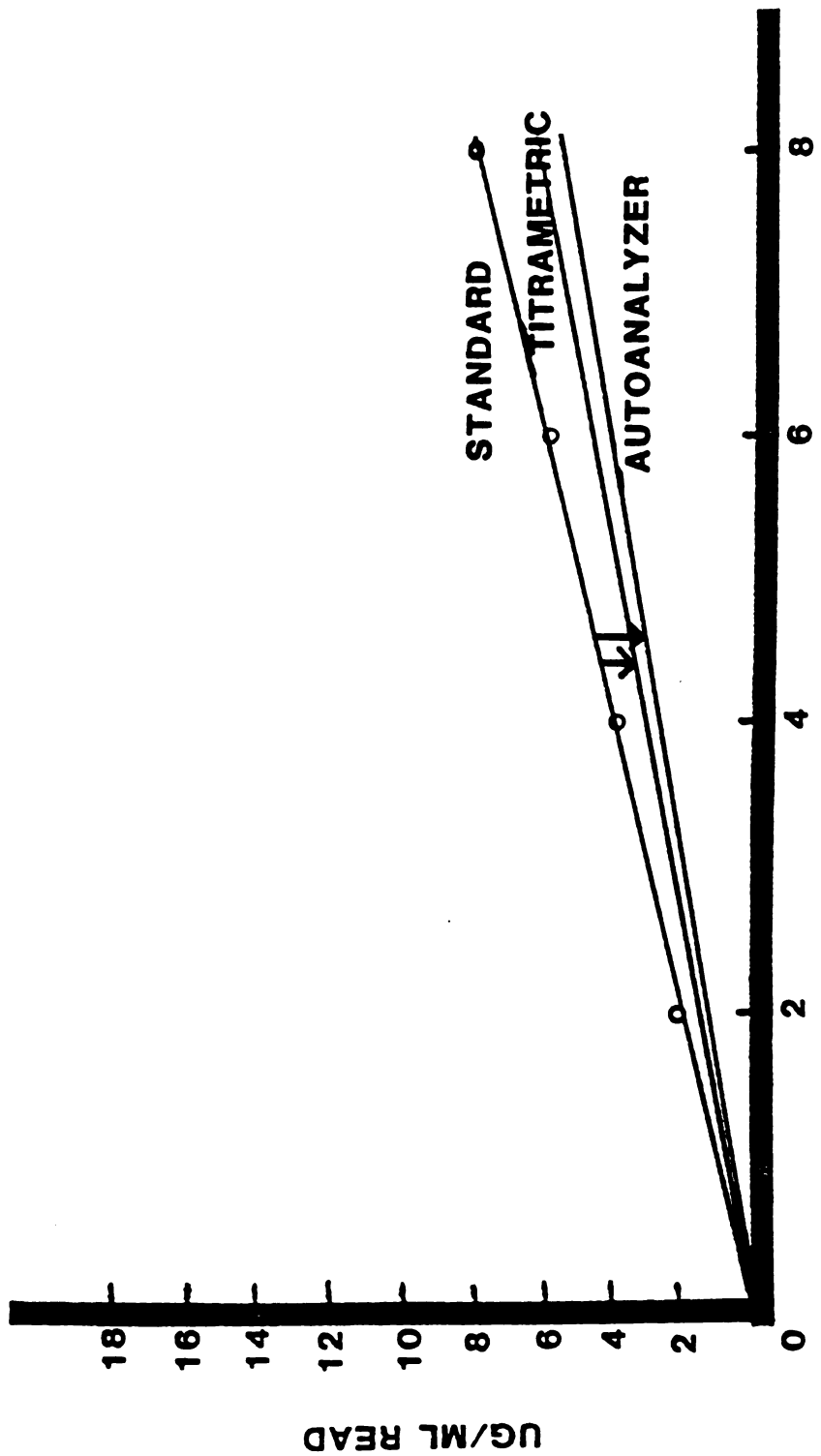


Figure 8. Comparison for R-AA in 5% rice starch model solution with R-AA standards by autoanalyzer and titrametric methods.

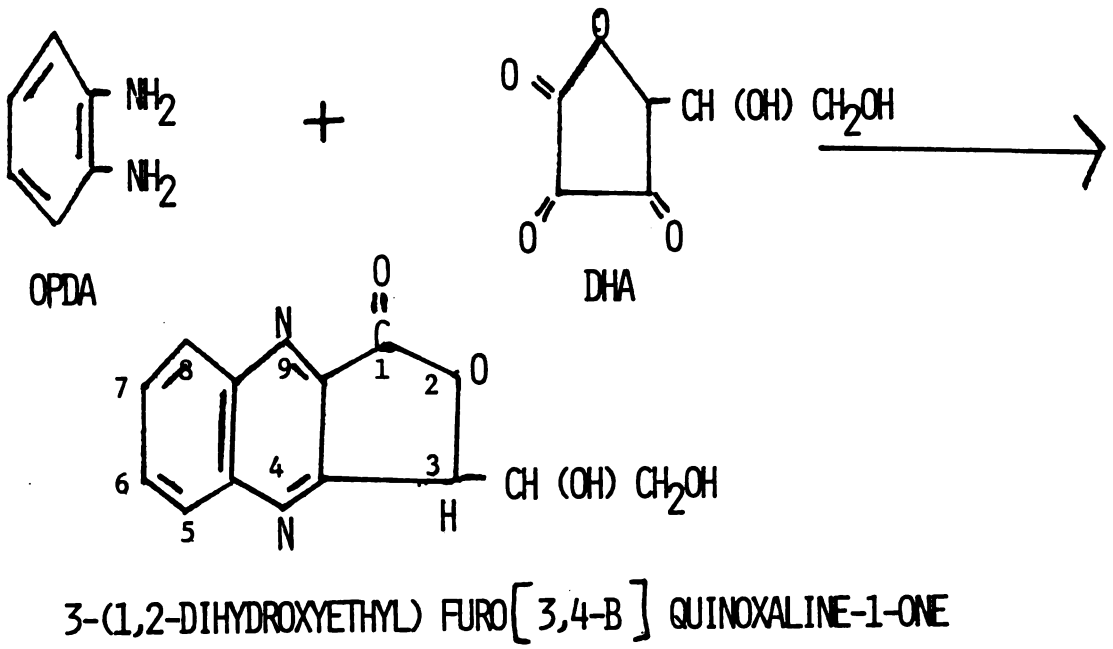


Figure 9. Formation of a fluorescent compound.

with DHA that resulting from interfering compounds. Boric acid, used in the analysis to account for interference from compounds other than DHA, complexes with adjacent cis hydroxyl groups of carbohydrate (Malcolm et al., 1964). The diol groups of the furanose structure of DHA bind with boric acid to form mono-, neutral and bisdiol complexes (Figure 10) (Pigman and Wolfrom, 1949). Once complexed prior to addition of OPDA, DHA does not condense with indicator dye. Therefore, any resulting fluorescence should result from extraneous material. However, Deutsch and Weeks (1965) indicated that a compound posessing all the following qualities could interfere with the accuracy of the fluorometric method:

(a) an α-diketo group which reacts with the diamine dye
(b) fluorescence wavelength of the formed quinoxaline within the

region of the assay
(c) contain cis hydroxyl groups which react with boric acid solution to form a complex.

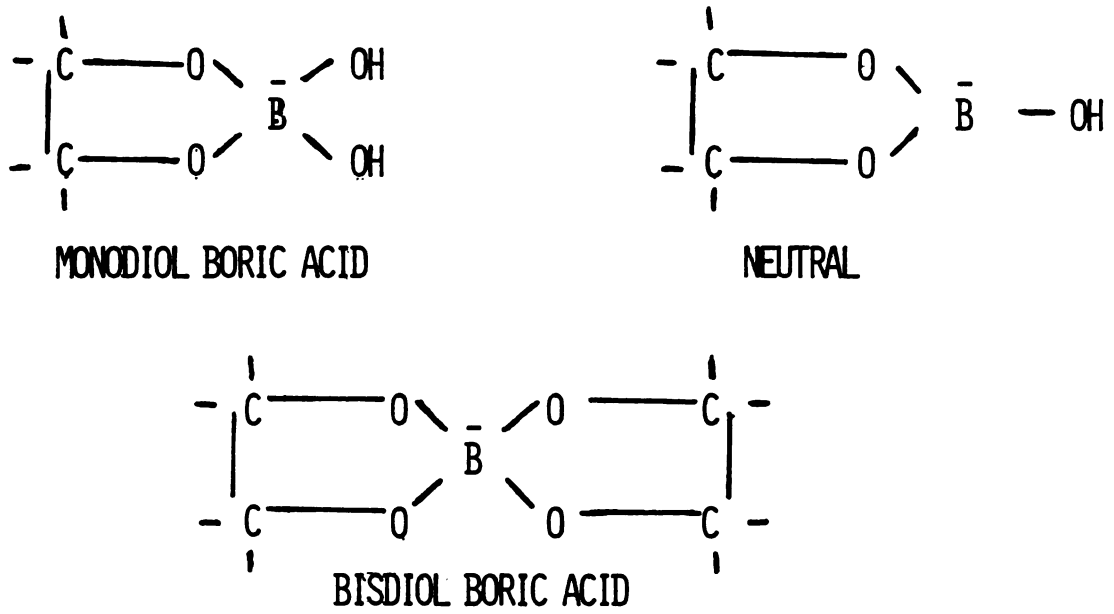


Figure 10. Borate-carbohydrate complexes.

It is not likely that the observed result stems from a binding of boric acid since this would result in detection of fewer extraneous substances and would increase rather than decrease the observed amount of R-AA. Should any agent be active in inhibiting the borate-complex formation, then a larger factor would be subtracted from the total fluorophor perceived as oxidized DHA. This could account for the low results obtained in this comparison study.

An additional contributing factor to the low observed result could be incomplete oxidation of reduced ascorbic acid to DHA by DCIP. Therefore, the amount of available DHA bound as the fluorescent complex would not represent all R-AA present.

Although result of the comparative study in starch was reversed relative to the result obtained for the sugar model solution, both methods

indicated a lower concentration of R-AA than was added to each solution as a standard. This observation reveals a similar phenomenon as that which occurs in sugar, that is, there is interference of some nature. Natural starch is comprised of glycosidic sidechains and yields only glucose on hydrolysis. Glucose is a reducing sugar and upon hydrolysis, would interfere with accurate determination of R-AA present. It may follow that the fluorometric method is less affected in starch than sucrose, or that the influence of the model system affects the titrametric method disproportionately.

Comparison of Methodology for DHA

Results obtained from the automated analysis were compared with those of the spectrophotometric assay for a set of DHA standards. Again, readings obtained by the two methods were not identical, and neither method yielded results as would be expected by known amounts of DHA standard which was added to the sugar and starch model solutions. Results for the spectrophotometric analysis for the 10^{o} Brix model system recorded amounts that were significantly higher than known amounts of standard added ($p < .001$), and were significantly higher than were indicated by the automated method ($p < .001$) (Figure 11).

Roe (1961a) reported that the coupling reaction of DNPH method is not sensitive to reducing compounds which interfere with the oxidation-reduction techniques. Only at temperatures that exceed those advised for the coupling reaction would there be significant interference from sugars. Sugars resulting from possible hydrolysis in the sucrose model solution may have appeared as interfering agents for the titrametric analysis for R-AA that was reportedly sensitive to such compounds. Without the interference present in the DNPH assay for DHA, observed

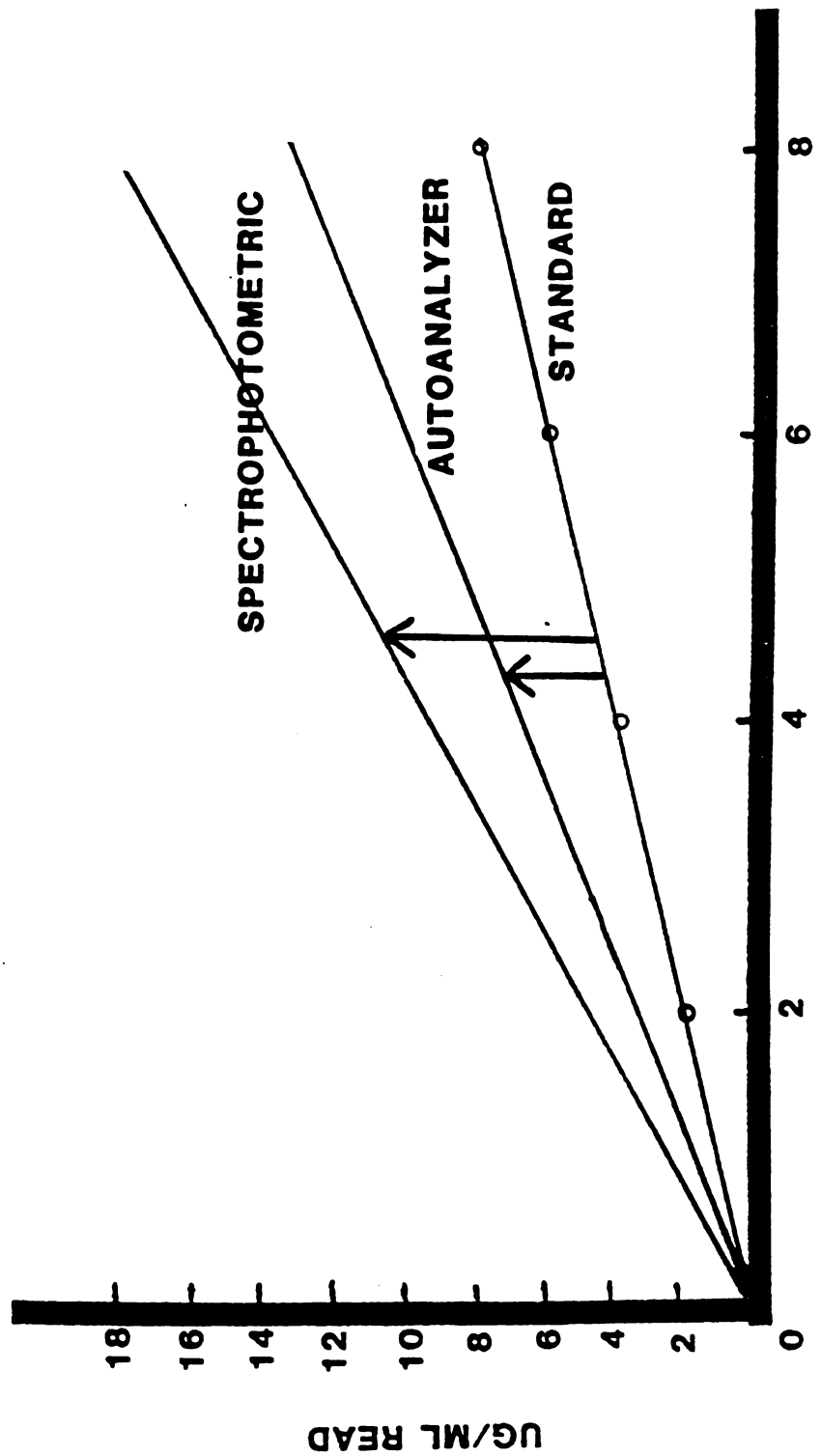


Figure 11. Comparison for DHA in 10^{o} Brix model solution with DHA standards by autoanalyzer and spectrophotometric methods.

results appeared quite high.

Results obtained by the fluorometric analysis were elevated relative to DHA standards in the sugar model system. This observation corresponds with that obtained for the R-AA standards, but the degree of the relative difference with respect to standards for DHA was more extreme. Since tha analysis was conducted identically with that for R-AA, which recorded slightly less ascorbic acid than standard, it may be concluded that the source for the distinction is with the form of ascorbic acid being evaluated. Norit oxidation has been traditionally used in oxidation of R-AA to DHA before analysis. Kirk and Ting (1975) found that DCIP served as an adequate substitute since Norit oxidation was not suited for continuous analysis. It may be indicated that DCIP oxidation is not complete and not all R-AA was converted to DHA. Since only DHA is indicated by the analysis, less R-AA may have been detected than was actually present. It may then follow that result for DHA would read higher than standard since completeness of oxidation for DHA is not of consequence when only DHA standard is present.

In starch, differences were not as dramatic as for the sucrose model. However, the automated analysis recorded more of the DHA standard in the starch solution than for similar concentration solutions evaluated by spectrophotometric method ($p < .001$) (Figure 12).

Roe (1961a) indicated that reductones and reductic acid interfere in both the DNPH method and in the visual oxidation-reduction procedures. The starch model system was prepared by heating starch in water to gelation temperature with subsequent cooling. Reductones are formed from sugars heated in a mildly alkaline medium. Reductones produced in the gelation process for the starch model may interfere with analysis for DHA in excess of known amount of DHA standard added to the starch

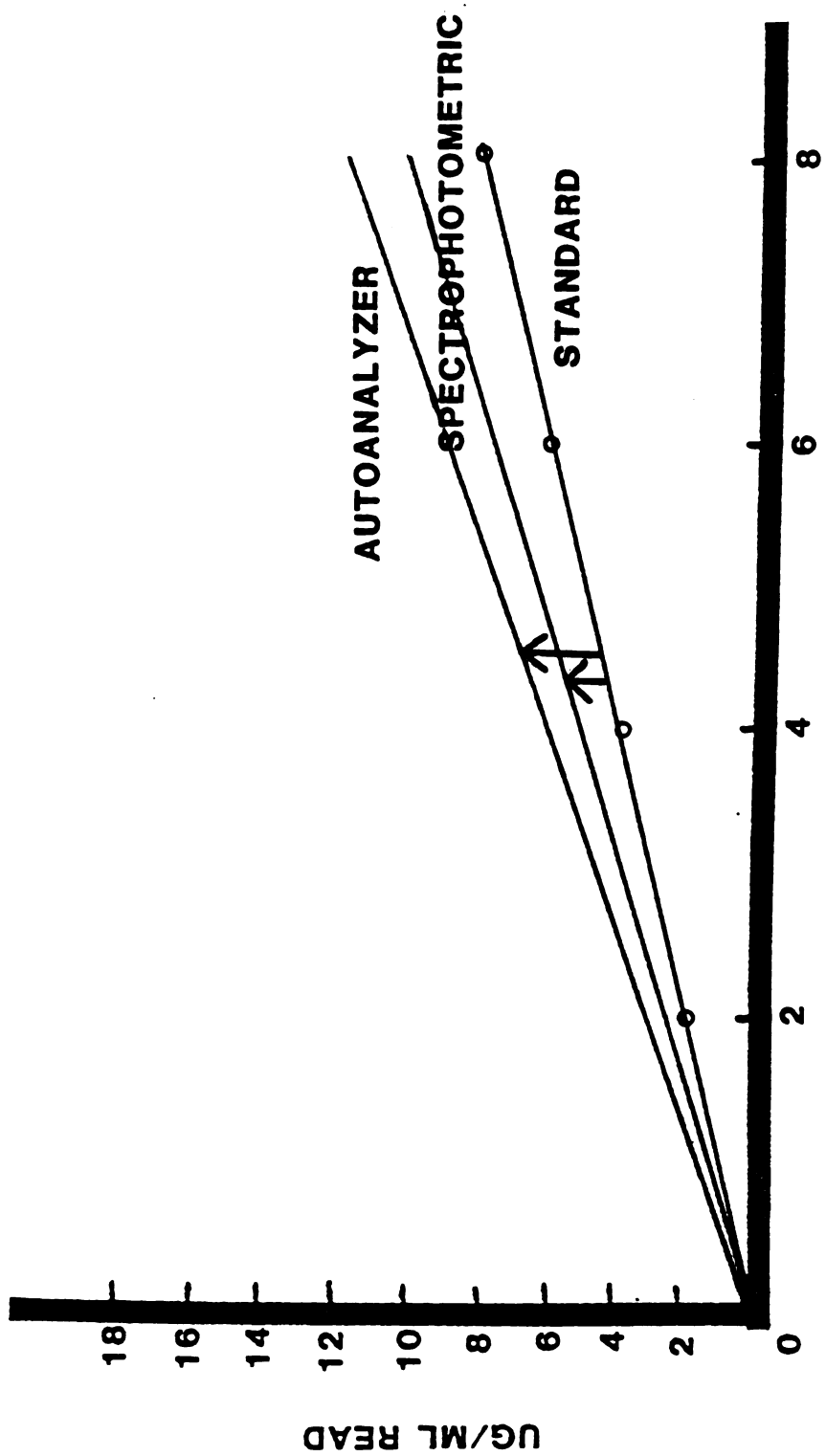


Figure 12. Comparison for DHA in 5% rice starch model solution with DHA standards by autoanalyzer and spectrophotometric methods.

model solution.

Purity for the DHA standard was determined since R-AA was not obtained by the automated OPDA analysis. It was concluded that results could be used to determine correction equations for experimental data. Raw data obtained for DHA would reflect observed aberations from standards due to the respective form of analysis.

Correction equations for change in DHA concentration over time were based on linear regression models for each model system and standards with each method of analysis. Equations are presented in Table 2.

Elevated results indicated by the spectrophotometric method relative to standards may be indicative of contamination from fully-oxidized DKGA within the DHA standard. The DNPH dye couples with both DHA and DKGA, and would indicate both the vitamin-active DHA and the completely oxidized DKGA forms of ascorbic acid. The fluorometric method includes a fluorophor-producing reaction of DHA with OPDA (Figure 9). Review of Figure 1 indicates that DKGA could also contribute two keto groups that could react with OPDA and may, at least in part, be additive to the amount of DHA being recorded.

The Experiment

In overview, observations indicated that rate of loss during the 10 day experiment for TAA and R-AA, and rate of accumulation for DHA were significantly different between the two cereal types. The rate of increase for DKGA was not significantly different for the mixed cereal compared with the wet cereal product.

Total Ascorbic Acid

Experimental data indicate that TAA concentration was diminished during the course of the experiment ($p < .05$), but the influence of iron

Table 2. Correction equations for concentration based on linear regression for each model system and set of standards.

R-AA, DHA and DKGA
sugar solution model:
AA vs standard → y = 1.583 X + 0.2100
spectro vs standard → y = 1.771 X + 3.538
starch solution model:
AA vs standard → y = 1.1344 X + 0.5990
spectro vs standard → y = 1.0146 X + 2.9546

in promoting loss of TAA was not significantly different for electrolytic iron or ferrous sulfate. Results indicate that conditions within the particular cereal product evaluated were more influential on TAA than was the form of iron added in fortification. Statistical evaluation for TAA is presented in Appendix I. There was a significantly different amount of TAA loss between mixed cereal and wet cereal throughout the 10 day period of analysis ($p < .001$). Change in TAA concentration ratio during the trial period is illustrated in Figure 13. Concentration at the end of 10 days appeared similar for both iron-fortified and non-fortified wet cereal product, but the mixed cereal product without iron present had greater loss of TAA than the non-fortified product. This result stands out as an anolmoly in the experimental data since it was anticipated that the product with iron present would have a greater amount of ascorbic acid loss.

Figures 14 and 15 indicate the influence with presence of iron in mixed cereal (Figure 14) and in wet cereal (Figure 15). Results are shown for all ascorbic acid metabolites; TAA, R-AA, DHA and DKGA as a concentration ratio comparing non-fortified to fortified product. The product with no iron present has been assigned a concentration ratio of 1.0, and the iron-fortified product's average concentration is expressed in relation to the average concentration for the product when no iron was added. It would be expected that a ratio of less than 1.0 would be indicated for TAA in the iron-fortified product; however, the anomoly appears and the average concentration in iron-fortified mixed cereal exceeded that for the non-fortified mixed cereal product. This apparent difference was significant ($p < .001$).

Figure 15 illustrates the contrast for average concentration between the non-fortified and fortified wet cereal products. For wet cereal, the

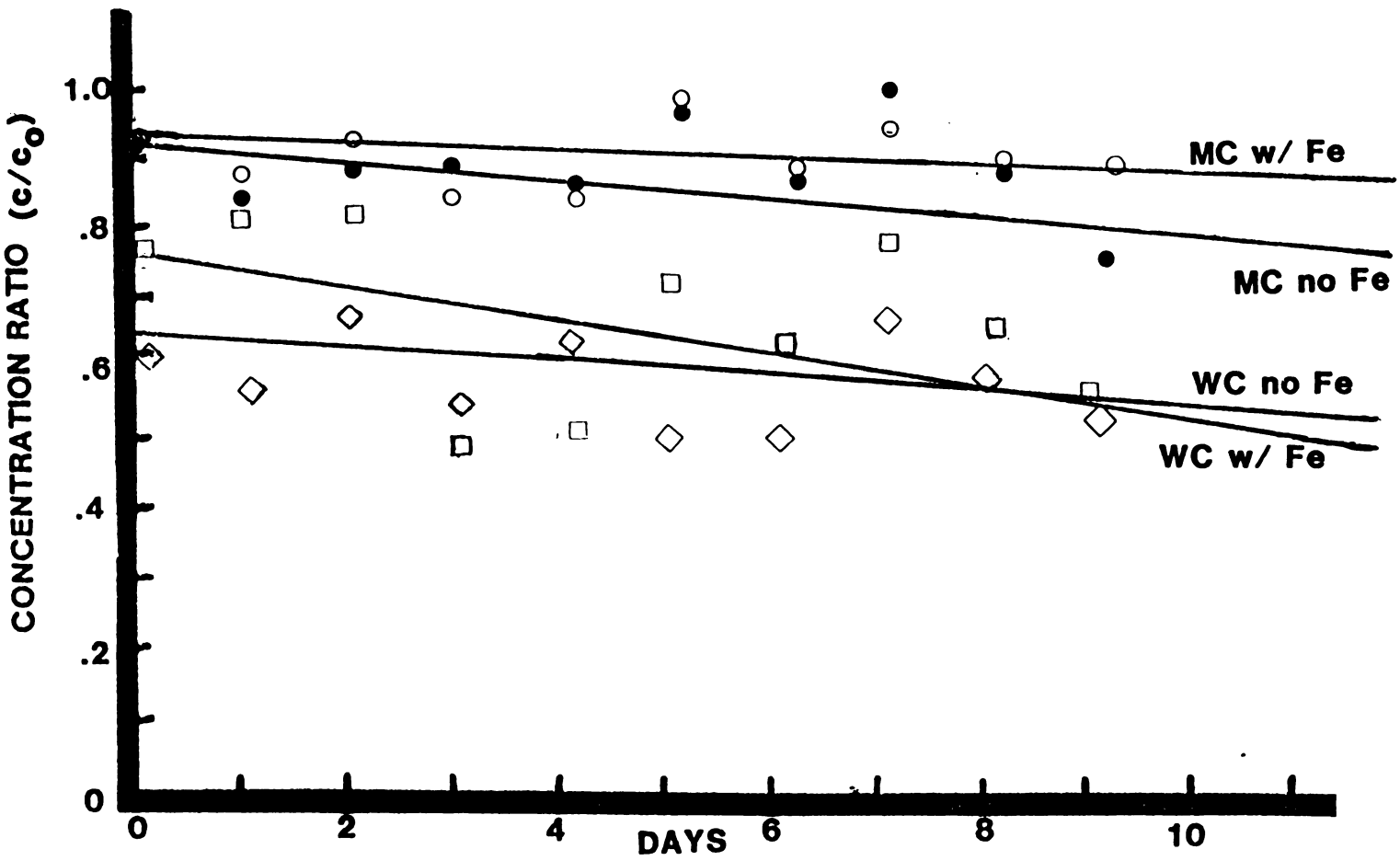


Figure 13. Change in TAA concentration ratio for non-fortified and iron-fortified cereal products during 10 days of refrigerated storage.

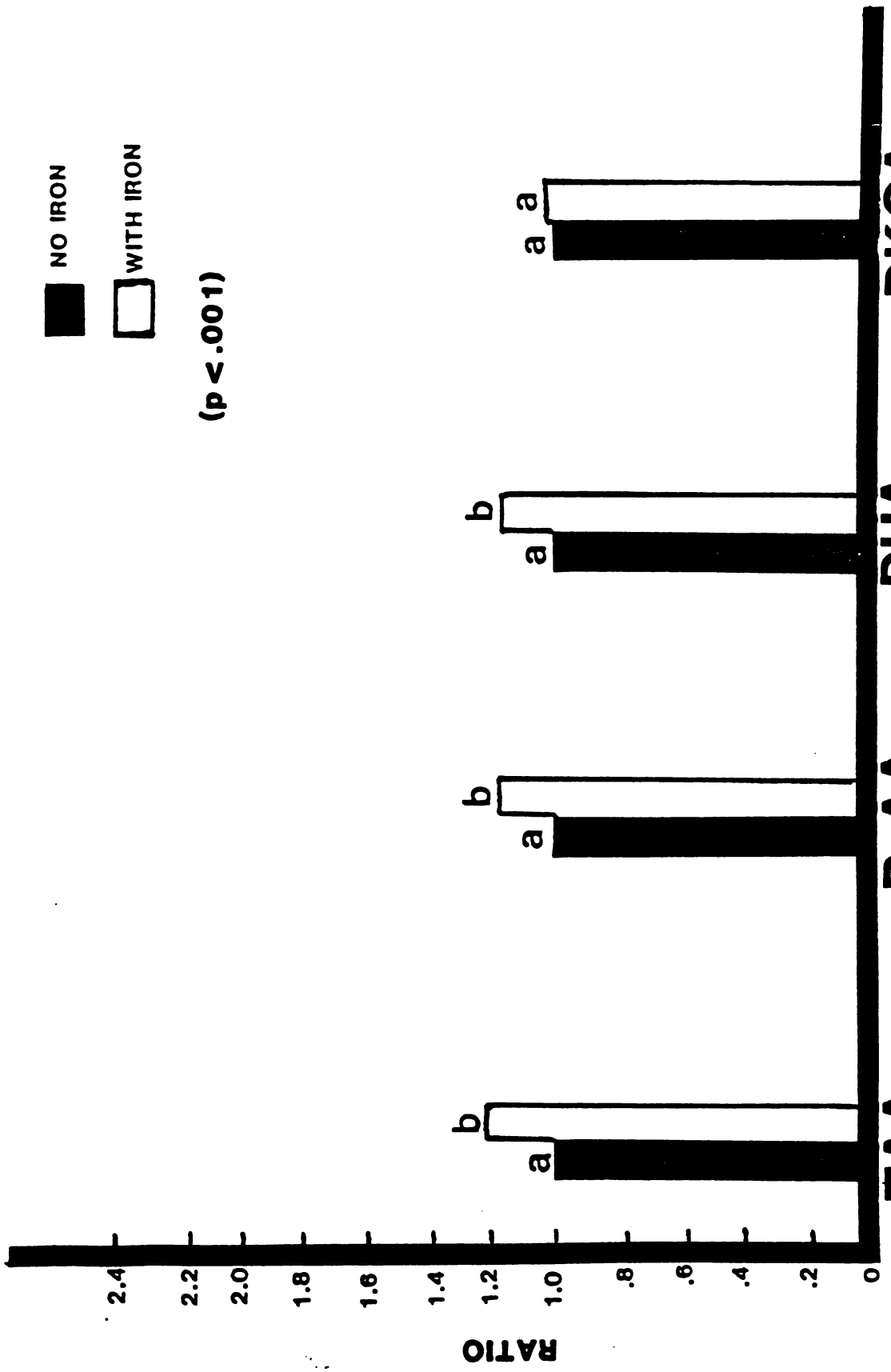


Figure 14. Ratio for difference in ascorbic acid metabolites for mixed cereal (expressed as ratio of iron-fortified: non-fortified product).

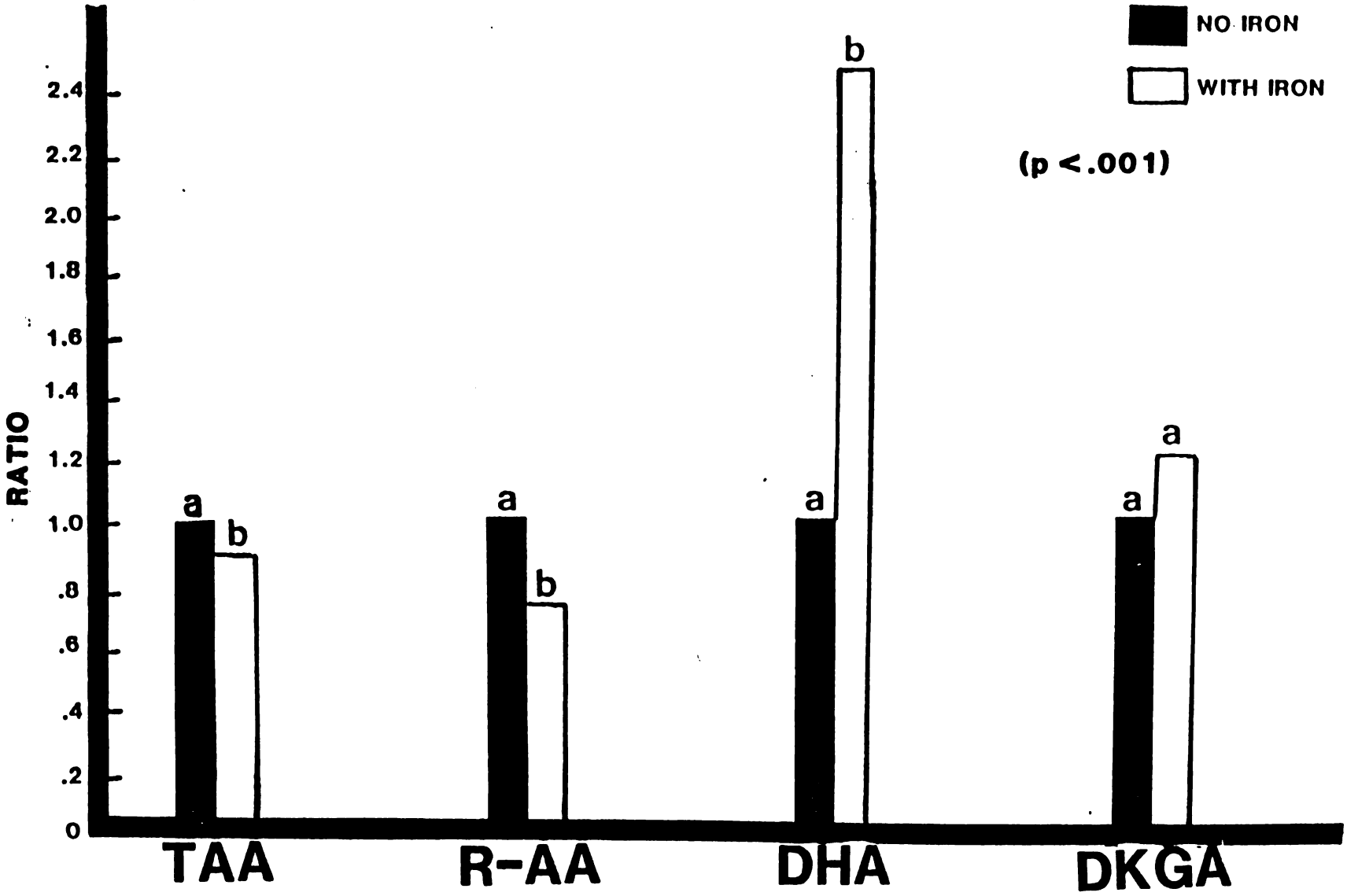


Figure 15. Ratio for difference in ascorbic acid metabolites in wet cereal (expressed as ratio of iron-fortified: non-fortified product).

average TAA for iron-fortified product is significantly less than the average TAA in the wet cereal product with no iron present. It appears that the influence of ferrous sulfate in wet cereal, averaged over the 10 day period, is more catalytic than is electrolytic iron in mixed cereal.

Reduced Ascorbic Acid

Results for the decline in R-AA over the 10 day period corresponded with loss of TAA. Experimental data revealed that R-AA was diminished over the storage period ($p < .001$); and that wet cereal had a significantly lower concentration than did mixed cereal ($p < .001$). Statistical evaluation is included in Appendix II. The declining R-AA in all products is presented in Figure 16. A similar situation as that which occurred for TAA is evident for R-AA and average concentration change for the iron-fortified products was not significantly different from the non-fortified products. Again, it appears that the influence of the cereal type itself was predominant in promoting loss of ascorbic acid, and that iron was of secondary consideration.

Figure 14 illustrates the contrast for the mixed cereal between iron-fortified and non-fortified product. For mixed cereal, the anomoly that appeared with respect to TAA is apparent for R-AA and the iron-fortified mixed cereal product has a greater average concentration of R-AA during the experiment than does the non-fortified product. Figure 15 illustrates the influence of ferrous sulfate in wet cereal, and the average concentration of R-AA is significantly less for the iron-fotified rice cereal than for the non-fortified product.

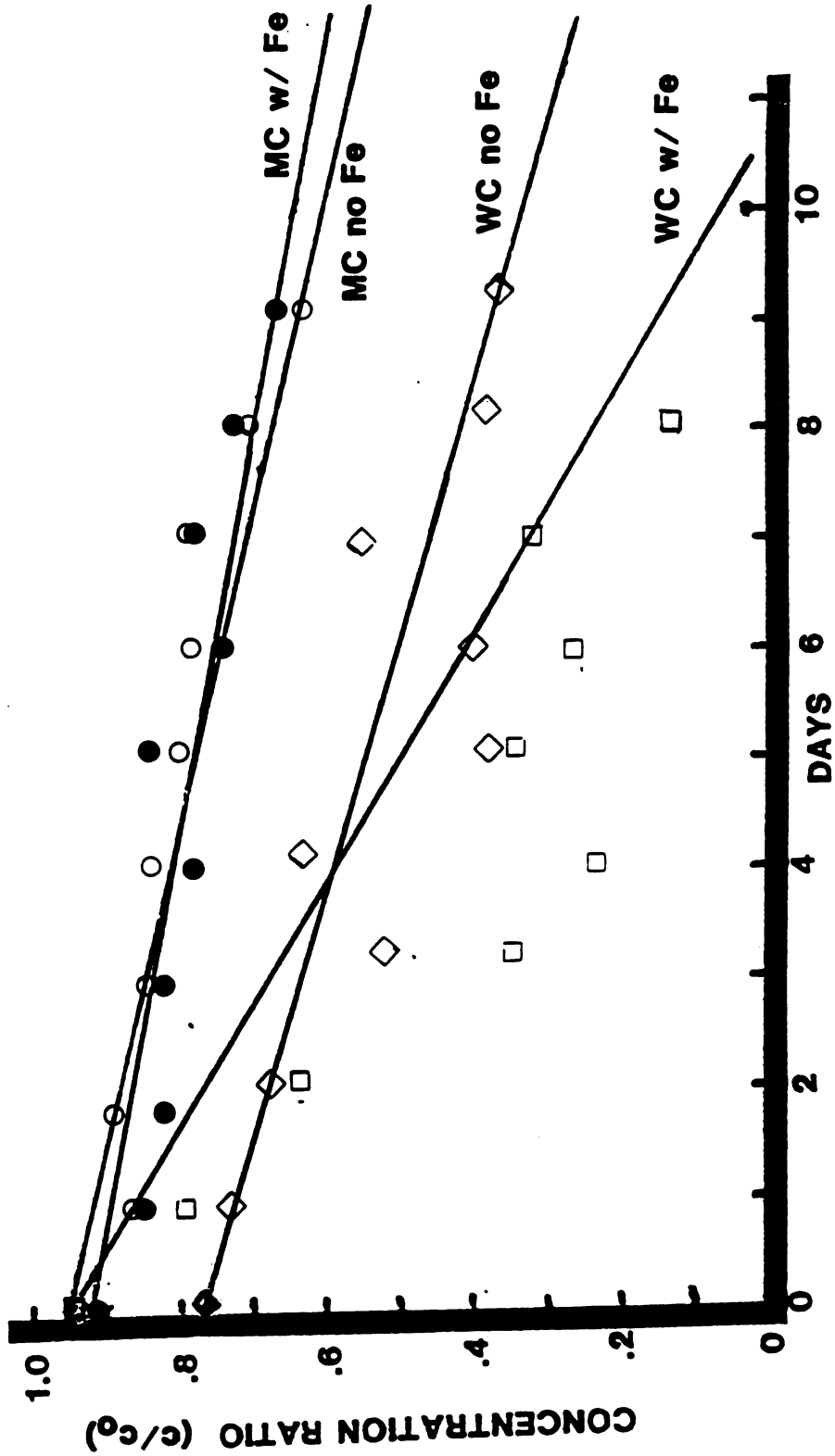


Figure 16. Change in R-AA concentration ratio for non-fortified and iron-fortified cereal products during 10 days of refrigerated storage.

Dehydroascorbic Acid

Experimental results for DHA were adjusted in relation to DHA standards in order to reflect the influence of the analytical method used. Results revealed significant differences for DHA between iron-fortified and non-fortified products. Statistical evaluation is presented in Appendix III. Significant interaction with time shows that the products had different rates of DHA accumulation, and the iron-fortified mixed cereal and iron-fortified wet cereal had a greater rate of DHA increase than the non-fortified mixed cereal and wet cereal respectively.

It appears that not only is the environment within each cereal type influencing a change in ascorbic acid, , as was apparent for both TAA and R-AA, but that the presence of iron may have added to the degradative process with oxidation of reduced ascorbic acid.

The influence of iron in each cereal product is presented in Figure 17. Results given as k first-order reaction rate constants are indicated in Table 3. Rate of DHA increase in iron-fortified mixed cereal is significantly greater than the rate of accumulation in non-fortified mixed cereal product ($p < .05$). This distinction is not significant with respect to iron-fortification in wet cereal product. However, rate of increase in DHA concentration ratio in cereal types averaged for iron content was significantly greater in wet cereal than the rate of increase in mixed cereal ($p < .005$) (Appendix III). Thus, it appears that the experimental results are consistent and the type of cereal product itself had considerable influence on the rate of ascorbic acid degradation.

The significant effect in mixed cereal on DHA is shown in Figure 18. The product with iron present had a greater k value during the course of the 10 day storage period, and the concentration at the end of the

Figure 17. The influence of iron on DHA in mixed cereal and wet cereal (expressed as k first-order reaction rate).

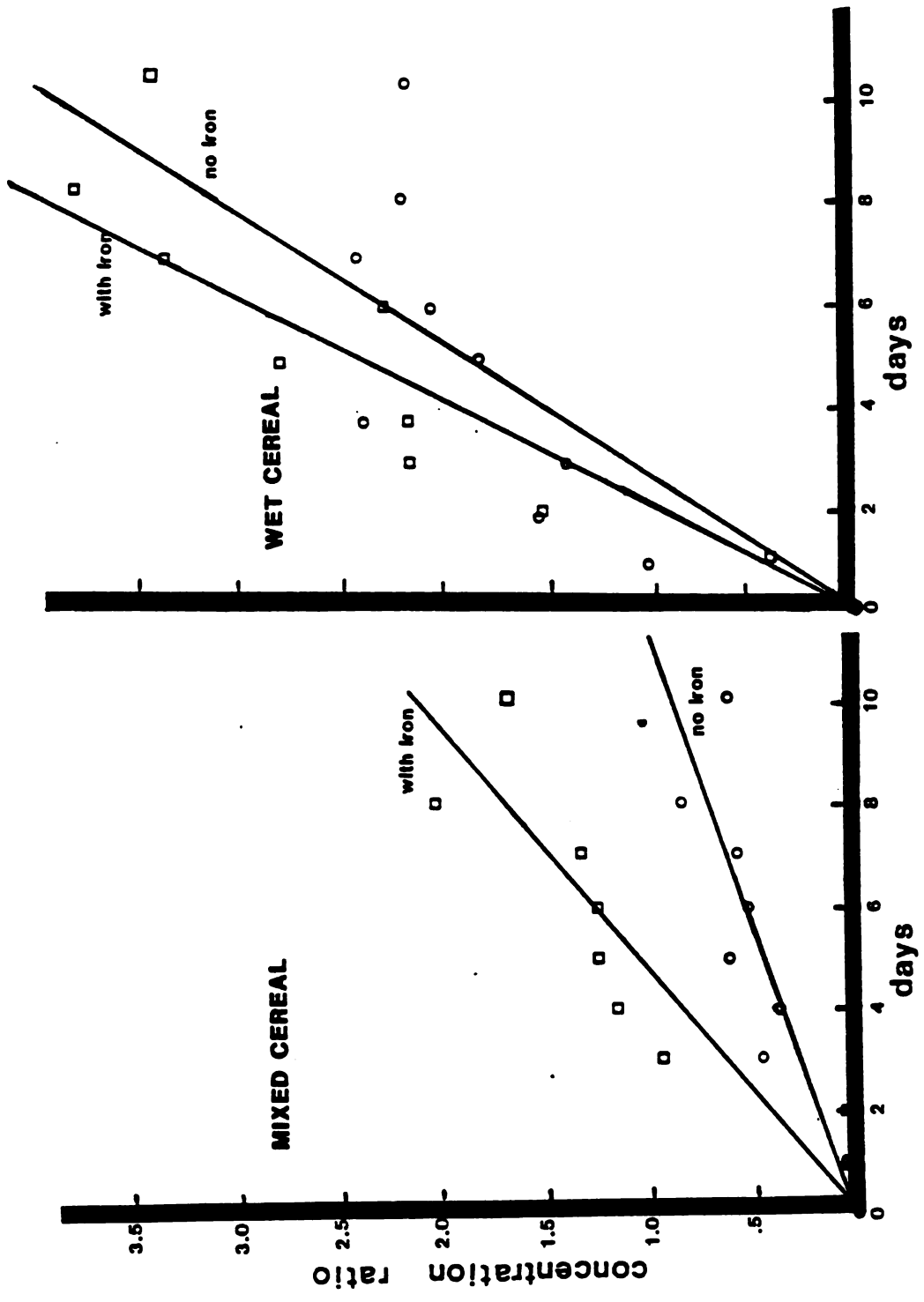

concentration ratio
3.5
3.0
2.5
2.0
1.5
1.0
.5
0
2
4
6
8
10
days
MIXED CEREAL
with iron
no iron
WET CEREAL
with iron
no iron
days

Table 3. k-reaction rate constants for mixed and wet cereal products.

ascorbic acid metabolite	Product: mixed cereal (no iron)	mixed cereal (w/iron)	wet cereal (no iron)	wet cereal (w/iron)
TAA	-.0104[a]	-.0039[a]	-.0007[a]	-.0158[a]
R-AA	-.0228[a]	-.0274[a]	-.0418[a]	-.1087[a]
DHA	.0611[a]	.2239[b]	.2257[a]	.3562[a]
DKGA	.2722[a]	.4775[a]	.8438[a]	3.2560[b]

*Contrasts indicating significance apply only for each ascorbic acid metabolite and not between metabolites. Comparisons not made for (a)mixed cereal (no iron) vs. wet cereal (w/iron), or (b)wet cereal (no iron) vs. mixed cereal (w/iron).

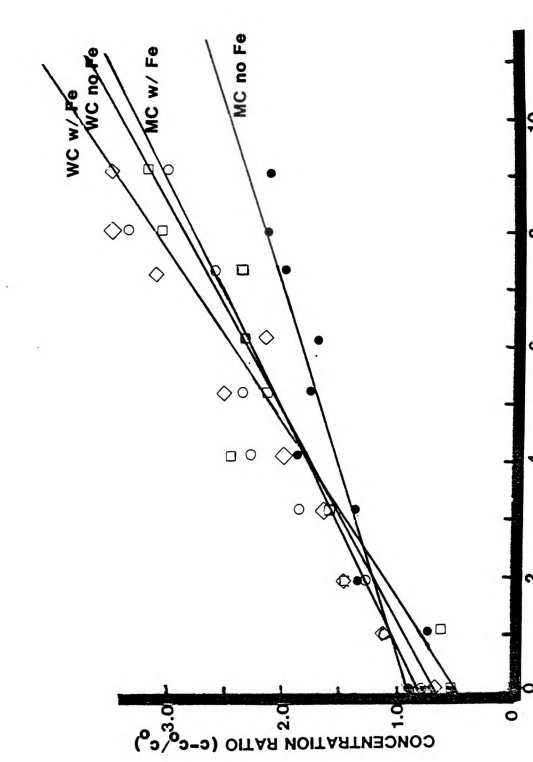


Figure 18. Change in DHA concentration ratio for non-fortified and iron-fortified cereal products during 10 days of refrigerated storage.

period was significantly greater than for the same product without iron-fortification. DHA for wet cereal with iron was only slightly greater than for the wet cereal product without iron. This distinction may be the result of a definently enhanced oxidative ability of electrolytic iron compared to that of ferrous sulfate; or may be the result of conditions imposed on both nutrients, iron and ascorbic acid, within the two cereal products.

Figures 14 and 15 indicate that the difference in DHA when iron was present was more dramatic in wet cereal (Figure 15) than in mixed cereal (Figure 14). The observed result appears to support the concept that cereal type had a pronounced influence on the catalytic effect of iron added to the product in fortification.

Figures 19 and 20 more clearly show the distinction between non-fortified and fortified products. Concentration for DHA in fortified product is presented as a ratio of DHA in non-fortified cereal. Figure 19 illustrates for mixed cereal that an increase in DHA was evident after day 4 of storage. The increased ratio for fortified product continued until the conclusion of the 10 day period. No trend was observed that could indicate a gradual increase in DHA, and the difference between non-fortified and fortified product was not altered after the single substantial increase on day 4. Figure 20 presents the parallel situation for wet cereal product. No trend was observed and results do not reveal any significant differences. There was a greater concentration for DHA achieved in the iron-fortified product on day 6, and a ratio exceeding unity in favor of the fortified product continued until the end of the 10 day period. The difference between the wet cereal products was not significant, whereas the mixed cereal did show a significant effect for

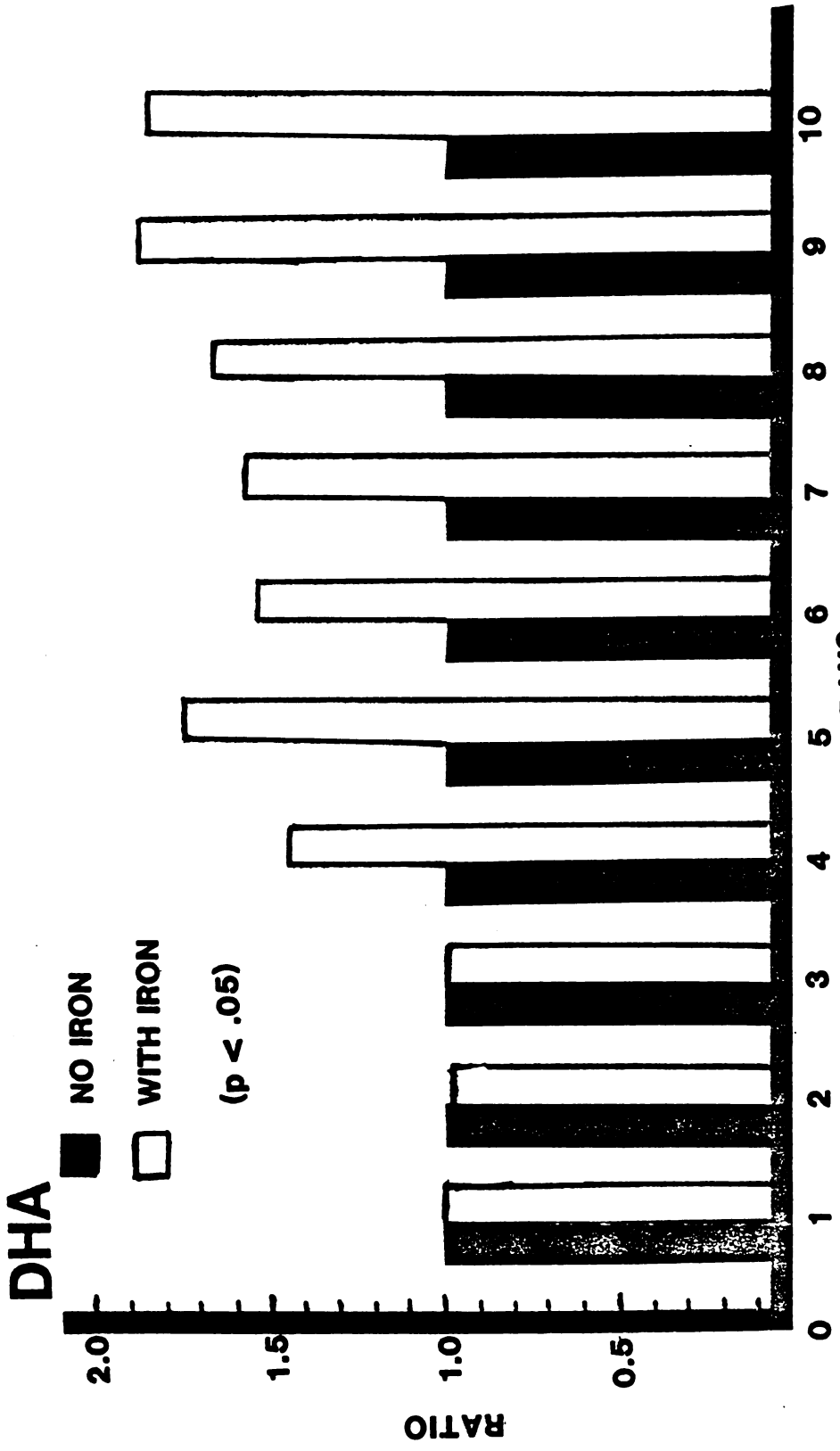


Figure 19. Change in DHA concentration ratio for non-fortified and iron-fortified mixed cereal (expressed as ratio of iron-fortified: non-fortified product).

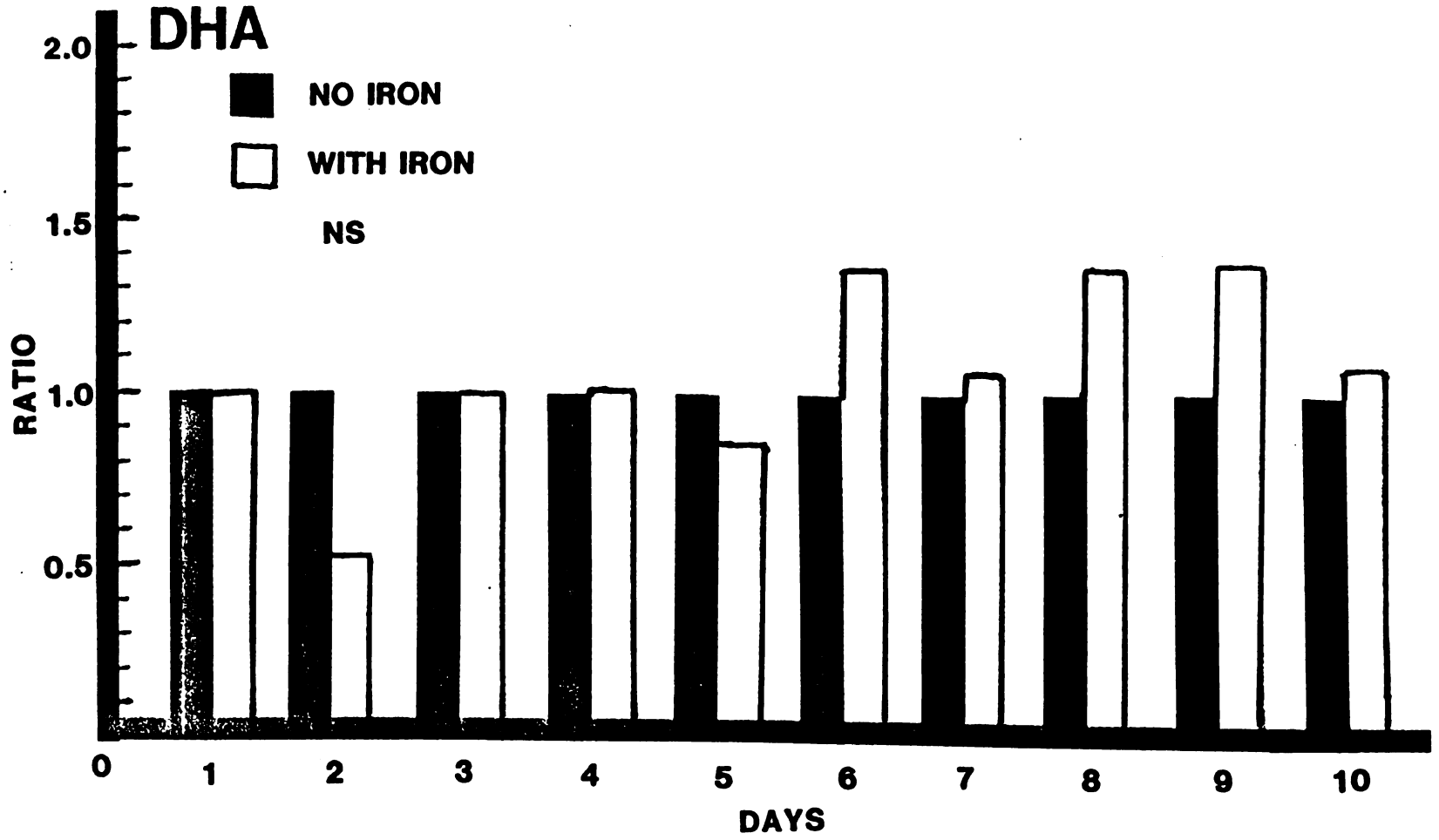


Figure 20. Change in DHA concentration ratio for non-fortified and iron-fortified wet cereal (expressed as ratio of iron-fortified: non-fortified product).

DHA when iron was present.

Diketogulonic Acid

Results for the completely oxidized component, DKGA, were more erratic than for the other forms of ascorbic acid. All factors influenced DKGA, but it appears that they do so independently. There was an overall increase in DKGA during the 10 day storage period ($p < .001$). Also, the average concentration of DKGA was significantly greater in wet cereal than in mixed cereal ($p < .05$). These results support the notion that the cereal product itself may predominate in influencing ascorbic acid. The influence of iron was significant for DKGA ($p < .001$), but the iron factor failed to interact with either cereal type, i.e. the influence of iron was similar for both cereal types. Statistical evaluation for DKGA is presented in Appendix V and Appendix VI.

k-Reaction Rate Constants

First-order reaction rate values, given in Table 3, are graphically illustrated in Figure 21 for mixed cereal and Figure 22 for wet cereal. Negative reaction rates for TAA and R-AA reflect loss of these components over time. Alternatively, positive values for DHA and DKGA reflect the accumulation for these components over the course of the experiment. Although the concentration ratios determined for TAA and R-AA begin at 1.0, results have been normalized so that values could be described on the same axis with DHA and DKGA, with all values originating at zero. The significantly greater rate of DHA accumulation in iron-fortified cereal relative to the non-fortified product is apparent in Figure 21 ($p < .05$). Iron-fortification with electrolytic iron does not cause

Figure 21. k First-order reaction rate for ascorbic acid metabolites in mixed cereal.

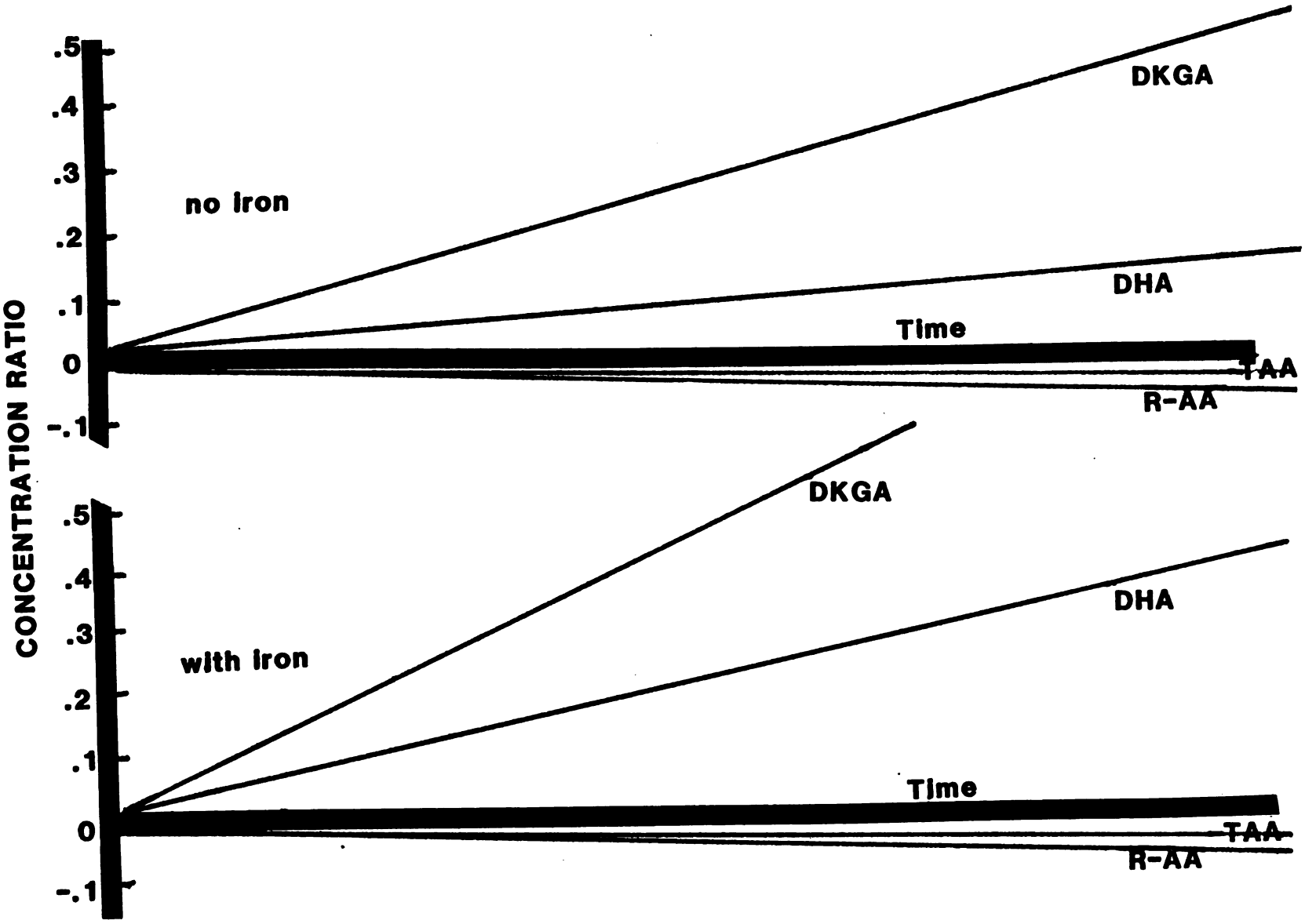
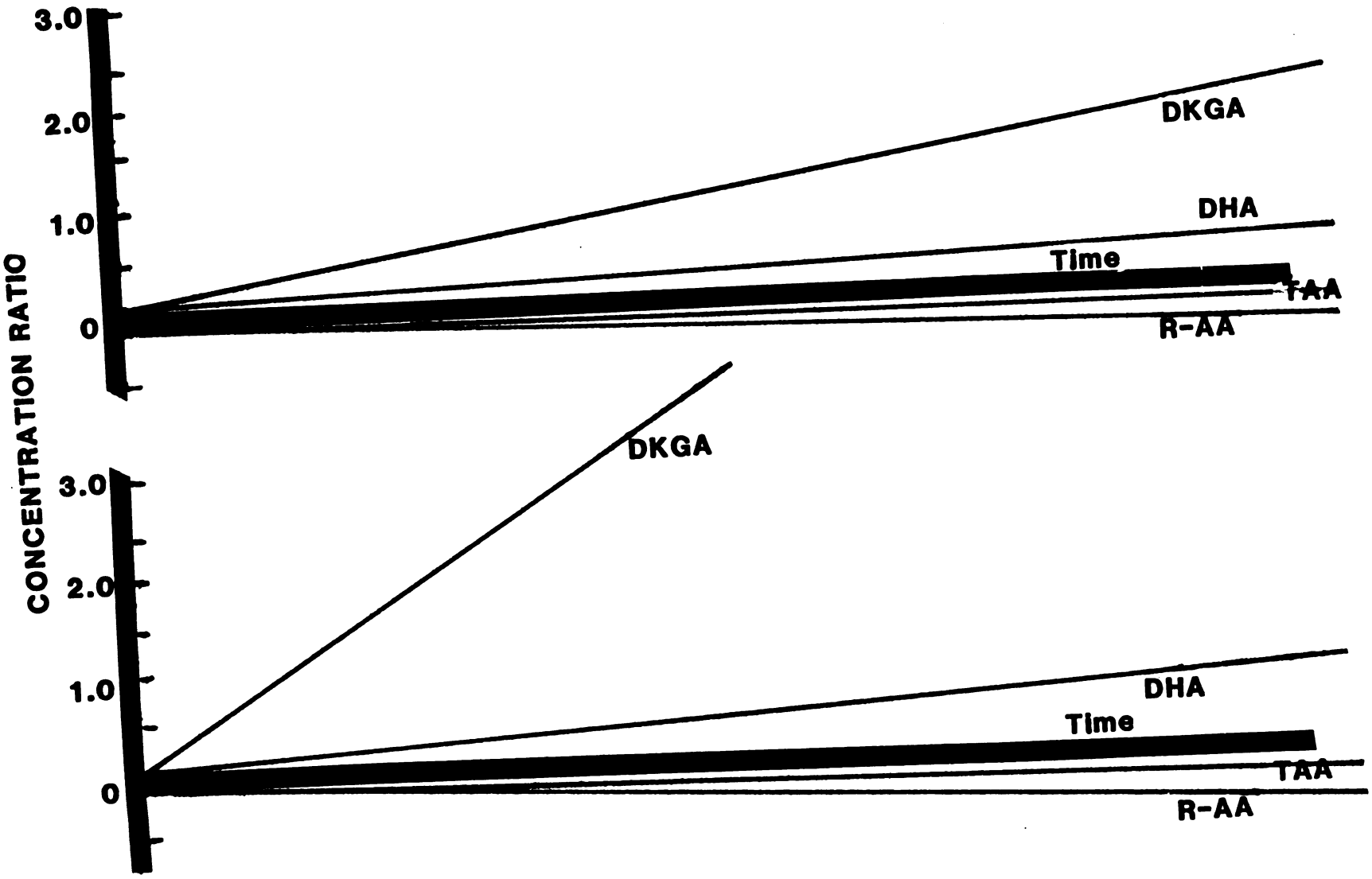

CONCENTRATION RATIO
.5
.4
.3
.2
.1
0
-.1
no iron
DKGA
DHA
Time
TAA
R-AA
with iron
DKGA
DHA
Time
TAA
R-AA

Figure 22. k First-order reaction rate for ascorbic acid metabolites in wet cereal.


CONCENTRATION RATIO
3.0
2.0
1.0
0
DKGA
DHA
Time
TAA
R-AA
DKGA
3.0
2.0
1.0
0
DHA
Time
TAA
R-AA

a greater rate of loss for TAA or R-AA, nor a greater rate of increase for DKGA compared to the non-fortified mixed cereal. Figure 22 illustrates the similar situation for wet cereal; however, there is no significant difference between reaction rates obtained for ascorbic acid metabolites with the addition of ferrous sulfate in the wet cereal product. When k values are contrasted for cereal products, there is a significantly greater rate of DKGA accumulation in fortified wet cereal than in iron-fortified mixed cereal (Appendix VI). No other ascorbic acid metabolite shows significantly different reaction rate between the two non-fortified cereal types or the two iron-fortified cereal products.

Differences in k are further illustrated in Figure 23 for loss of TAA, Figure 24 for loss of R-AA and Figure 25 for DHA increase. The concentration ratio for each cereal type has been averaged for both non-fortified and iron-fortified product, and is presented to indicate change in concentration ratio over the 10 day storage period. The DKGA component failed to show significant differences in the averaged concentration ratio over time for the two cereal products, and is thus not indicated. The most dramatic difference is for DHA, with wet cereal having a markedly greater rate of increase in concentration ratio over time than for mixed cereal (Figure 25).

An additional means of comparison for each metabolite of ascorbic acid evaluated in cereal products is expression of one-half life. In terms of R-AA and TAA, one-half life represents time (days) required to reduce by one-half the concentration present on day 1; and for DHA and DKGA, a parallelism of the expression amounts to the time necessary to achieve a two-fold increase from the initial concentration. Half-life, or doubling time (in days) for all products is presented in Table 4. Ratio for change in one-half concentration, or two-fold concentration, is

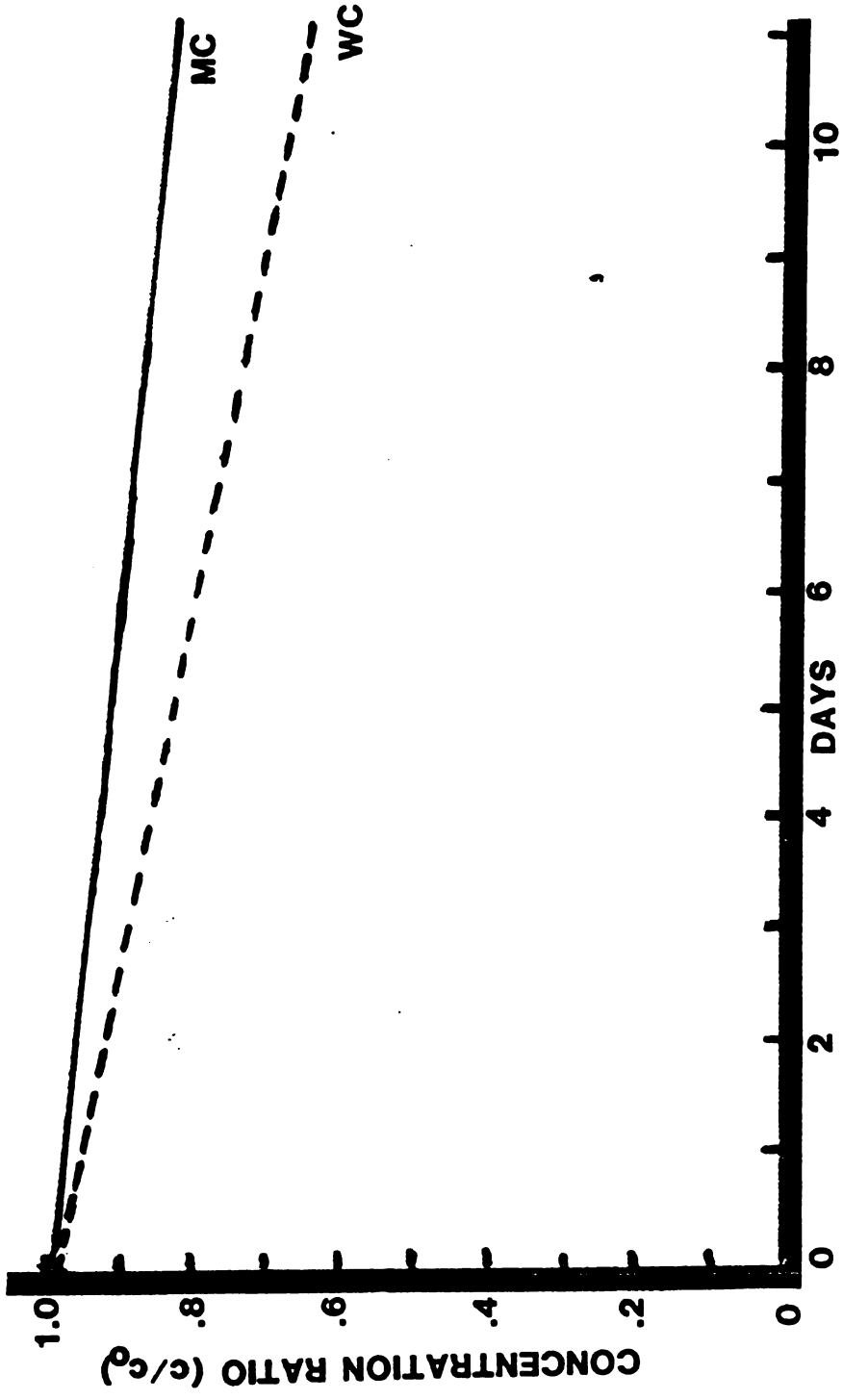


Figure 23. k-Rate constants for TAA in mixed and wet cereal products (averaged for non-fortified and iron-fortified products).

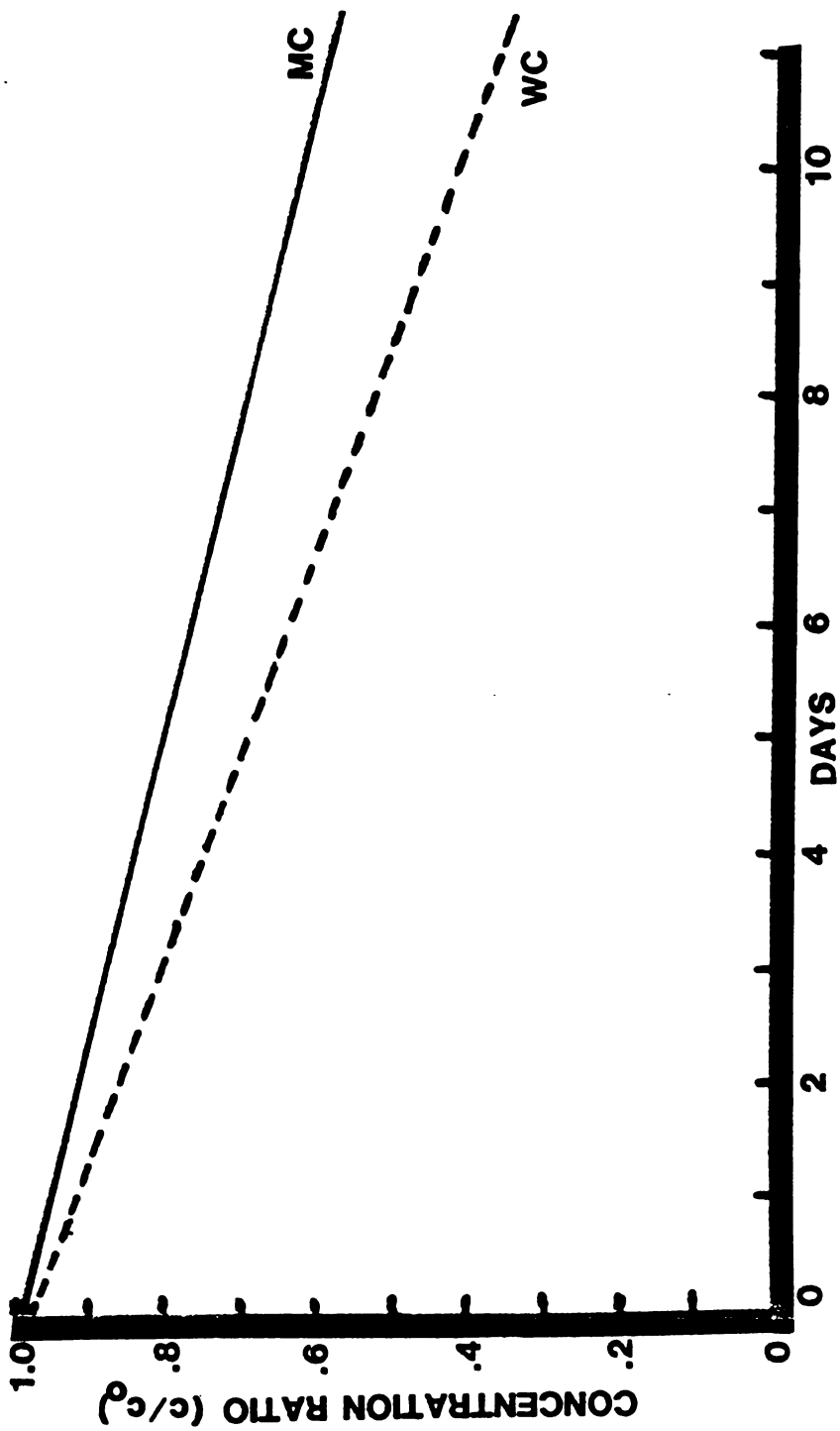


Figure 24. k-Rate contants for R-AA in mixed and wet cereal products (averaged for non-fortified and iron-fortified products).

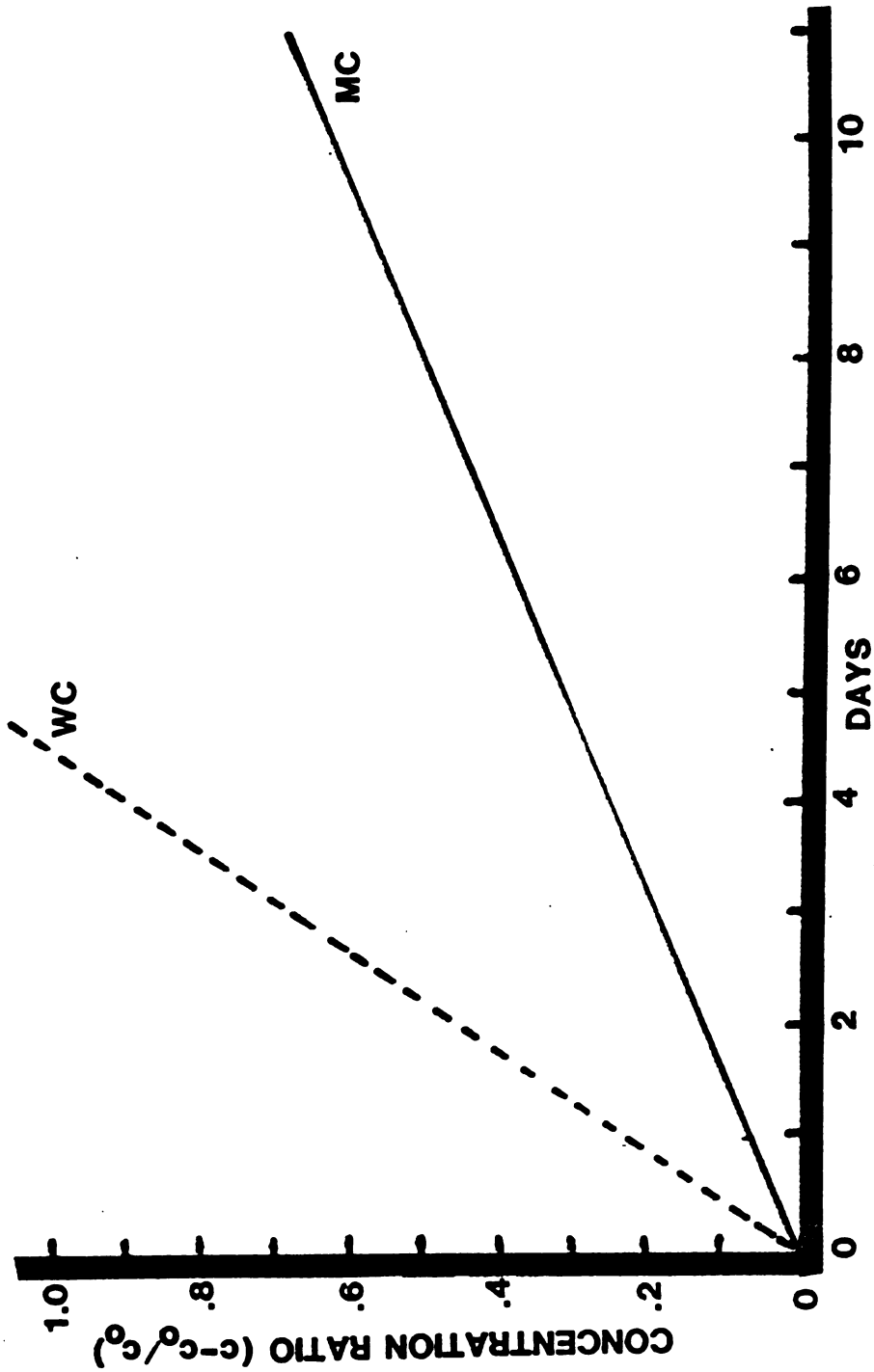


Figure 25. k-Rate constants for DHA in mixed and wet cereal products (averaged for non-fortified and iron-fortified products).

given in each case so that the influence of iron may be assessed. The greater the one-half life, or shorter the doubling time, the more stable the system is to change in amount of each respective metabolite of ascorbic acid. The anomoly again appears for mixed cereal with TAA, and samples with iron added seem to be sparing for vitamin C.

Since the ratio is calculated for the product with no iron relative to that with iron added, the ratio is anticipated to be greater than 1.0. The larger the value is above 1.0 indicated the greater catalytic ability for iron to induce chemical reactions that degrade ascorbic acid. The greatest ratio occurs for DHA, reinforcing the observation that presence of iron may have influenced a change in DHA to a greater degree than for any other metabolite. Furthermore, the ratio is larger for mixed cereal than for wet cereal which supports the observation that the influence of electrolytic iron in mixed cereal induced a more rapid degradation of ascorbic acid than did presence of ferrous sulfate in the wet cereal product.

As mentioned previously, no attempt was made to evaluate the two iron forms in products of identical iron or ascorbic acid concentrations. Rationale was that the study was intended to enable reporting of the nutritional value in the two products, as consumed, given their particular form of iron added in fortification and interaction of nutrients in their own particular food environment. Results reported herein have been related in terms of concentration ratio, in order that the reaction rate for each product may be compared. Conclusions obtained for experimental data indicate that the reaction leading to destruction of the reduced form of ascorbic acid is greatly influenced by factors present in each of the cereal systems, and to a lesser degree by the presence of iron either as electrolytic iron or ferrous sulfate. The two cereal

products are characteristically different in respect to their potential for concentration of dissolved oxygen, moisture content or a_w level, and pH. Also, wet cereal has been thermally processed with ascorbic acid in the presence of iron, whereas mixed cereal is not heat treated after ascorbic acid and iron are combined.

Influence of Oxygen Concentration

Due to the two products' different method of preparation, there is a difference in the potential for dissolved oxygen to exist in each product. Although juice is a thermally processed product, the final mixed cereal product is not heat treated.

All samples were opened and exposed to air during sampling. Samples were stored in an aerobic environment. The effect of dissolved oxygen in the system could influence oxidation rate, and Mack et al. (1976) reported a direct proportionality between rate of oxygen uptake and the initial concentration of oxygen. Spontaneous oxidation rate for ascorbic acid was proportional to the initial concentration of dissolved oxygen.

Khan and Martell (1967) observed that the rate-determining factor in oxidation of the ascorbate anion is the semiquinone formation by the interaction of the ascorbate anion with DHA. In mixed cereal, the rate for DHA accumulation showed a substantial increase at day 4 (Figure 19), and the wet cereal showed an increase in DHA only after day 6 (Figure 20). The time lapse from the moment the product was first prepared or opened may reflect the time required to effect semiquinone formation from molecular oxygen oxidation.

Weissberger et al. (1943) described that DHA may also take part in the oxidation of the ascorbate anion as a side reaction. This side reaction may contribute to the effect observed in Figure 17, where DHA accumulation

in wet cereal was more rapid than in mixed cereal. However, k for the reaction with no iron was not significantly different from that when iron was present (k = .2257 for wet cereal/no iron, and k = .3562 for wet cereal/with iron). Spontaneous oxidation appears to be more prevalent in wet cereal. Increasing concentration of DHA with oxidation of the ascorbate anion may have enhanced the rate that oxidation proceeded in the product, even when no iron was added.

That the potential may be greater for dissolved oxygen to exist in mixed cereal, where the final product was not thermally exhausted, could have influenced the marked distinction observed between iron-fortified and non-fortified mixed cereal products. The potential for the greater concentration of dissolved oxygen to exist in the mixed cereal products, along with the catalytic ability of electrolytic iron, could partially account for the significant difference between k values for the iron-fortified product in relation to the product with no iron present (k = .0611 for mixed cereal/no iron, and k = .2239 for mixed cereal/with iron). Interaction of the metal catalyst and air may be different for the two products when dissolved oxygen concentration is not identical for the mixed cereal and wet cereal products.

Influence of Water Activity

That moisture content influences ascorbic acid stability has been established (Lee and Labuza, 1975; Karel and Nickerson, 1964; Vojnovich and Pfiefer, 1970), and destruction rate of ascorbic acid increases with increasing water activity. Although it is likely that water activity was a minor influence for the two products evaluated, since both the viscous wet cereal and the more aqueous mixed cereal are probably quite close to a_w = 1.0, moisture content was considered as a potential factor

attributing to the differences observed in k between products. Values for k (Table 3) reveal accelerated ascorbic acid degradation in wet cereal relative to mixed cereal (with the exception of loss for TAA in wet cereal without iron). Results for ascorbic acid one-half life, or doubling time, (Table 4) indicate that wet cereal is the more reactive environment than mixed cereal with generally shorter half-life or doubling time, for all ascorbic acid metabolites. Lee and Labuza (1975), in an attempt to account for the accelerated rate observed with increasing moisture content, present a paradox; a dilution effect with increasing water content would reduce the rate of degradation, while diffusion of ascorbic acid in a more aqueous environment would allow for increased mobility for reactants and would facilitate the oxidative reaction. In a more diffuse system, degradation rate for ascorbic acid would be accelerated.

Results for this experiment indicate that the more diffuse system is the wet cereal since it seemed to support a more rapid rate of spontaneous oxidation; but the more aqueous mixed cereal system allowed for the greater reactivity of iron. Lee and Labuza (1975) found that the dilution effect was masked by some mechanism that allowed a rate increase.

Investigations by Lee and Labuza (1975) involved use of systems with a wide spectrum of a_w's considerably lower than that for either of the two infant food products. Table 5 relates data from Gerber for moisture content of the three products used in the investigation. Calculation for the combined mixed cereal product revealed a moisture content of 73.9%. The difference between water content for the two cereal products evaluated is slight. Therefore, it should follow that the observed results may not concur with results of earlier research wherein investigation with a wide spectrum of a_w's reported more dramatic differences. The research of Lee and Labuza (1975) reported an increased reaction rate for products

Table 4. One-half life, in days, for ascorbic acid degradation (TAA & R-AA) or doubling time (DHA & DKGA);and ratio between non-fortified and iron-fortified product.

ascorbic acid metabolite	Product: mixed cereal (no iron)	mixed cereal (w/iron)	wet cereal (no iron)	wet cereal (w/iron)
	ratio (no iron:iron)		ratio (no iron:iron)	
TAA	41.15	113.30	1157.10	56.07
	0.36		20.64	
R-AA	17.98	16.42	21.17	13.06
	1.10		1.62	
DHA	16.76	5.52	6.32	2.34
	3.03		2.70	
DKGA	4.31	3.22	0.49	0.28
	1.34		1.75	

Table 5. Moisture content for infant food products.

product	moisture content (%)
wet cereal -- Rice cereal with apple-sauce and bananas	79.7
mixed cereal -- Apple juice	87.6
dry Rice cereal with bananas	5.1

from Gerber (1977)

with a_w = 0.84 relative to that with a_w = 0.32. Figure 26 illustrates the relative increase in a_w for increasing percent moisture for an adsorption isotherm. As indicated in the graph, products with a high moisture content as infant food samples would not have an a_w appreciably different than 1.0.

Furthermore, experimental data supports the concept that oxidation is more rapid in products prepared on the desorption branch of the hysteresis loop which contain a higher moisture content than for adsorption systems at the same water activity (Labuza, 1971; Labuza et al., 1972). The wet cereal is produced by desorption with heat treatment from a more liquid product. The mixed cereal is prepared from dry cereal with the addition of liquid, and would therefore have attained its equilibrium moisture by adsorption. Although already indicated that the influence of water activity was probably minimal in this experiment, it would be anticipated that the wet cereal would be the environment that supports a more rapid rate of spontaneous oxidation.

Interaction of Water Activity with Metal Catalyst

The influence of iron-fortification on ascorbic acid stability was

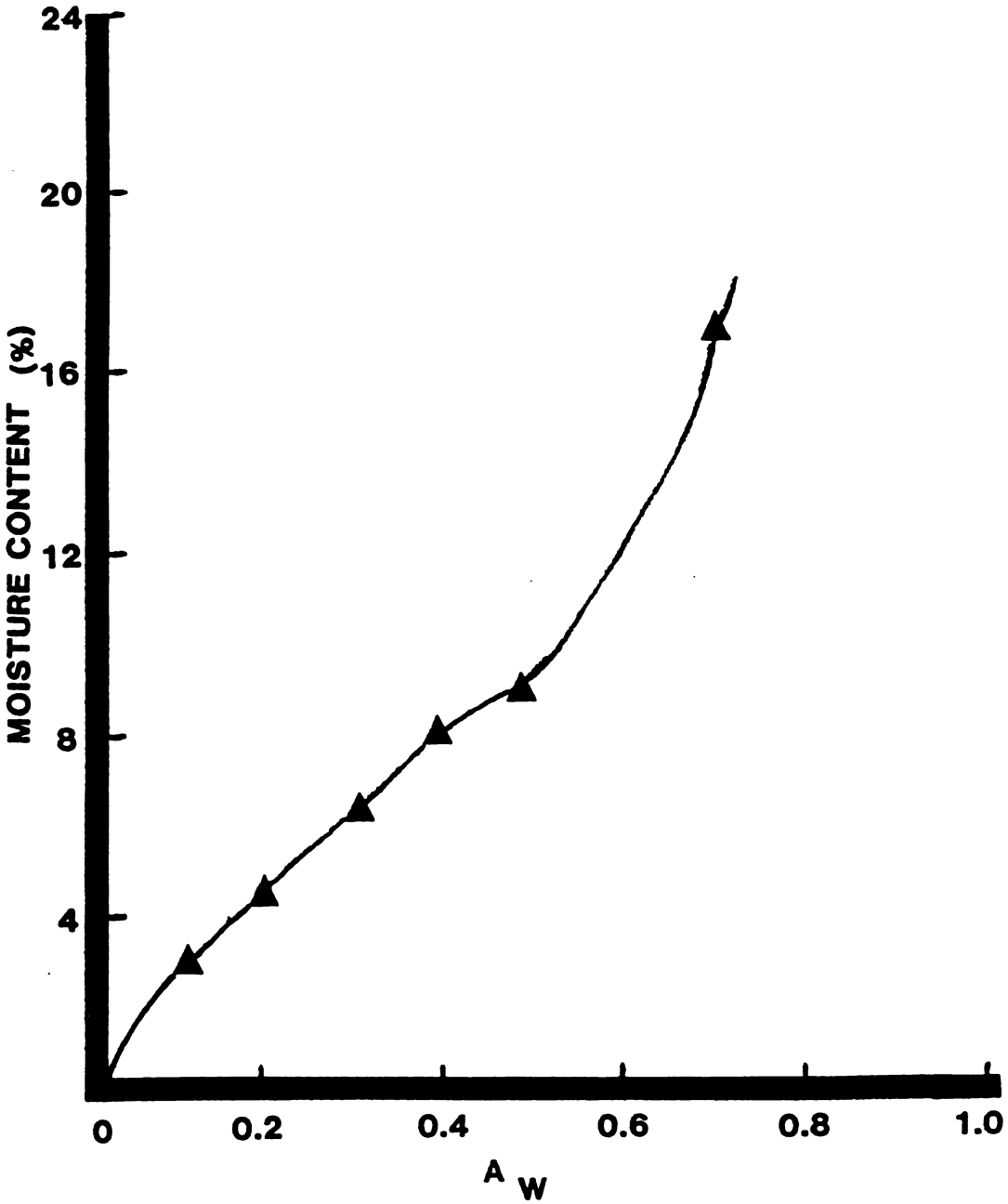


Figure 26. Adsorption isotherm for the dehydrated model food system at 20°C. (From Dennison, 1978)

minimal for TAA and for R-AA, since k constants showed little increase for any iron-fortified product evaluated relative to the non-fortified cereal product (Table 3). The more pronounced influence due to metal ion catalysis is for DHA accumulation and is illustrated in Figure 17. Mixed cereal showed a significantly greater rate of increase in DHA with the presence of electrolytic iron, relative to the non-fortified product, than did the wet cereal in the presence of ferrous sulfate. Ratio for k between fortified and non-fortified product was greater for mixed cereal relative to wet cereal, with ratios of 3.66 and 1.58 respectively.

Dennison (1978) observed no rate increase due to metal ion catalysis for TAA nor for R-AA degradation at a_w = 0.40. At or below 0.40 a_w, a lack of iron catalysis reflects a reduced ability of the aqueous phase to solubilize and/or mobilize the metal ions in the system. At a_w of 0.65, which is within the capillary region of the adsorption isotherm, a two- to three-fold increase in the degradation rate was noted for the model system with added trace minerals as compared to the sysytem with no mineral fortification. Reaction rate data indicate that free mobility of ferrous iron (Fe II) may require the complete hydration of the metal ion in its octahedral configuration, which may only be possible in the free water of the capillary region. Below this region, the metal ions may have one or more charged species from the product matrix as substituted liquid, effectively immobilizing or limiting metal ion migration.

Solubility Factor for the Iron Catalyst

Even though products evaluated in this research were of greater moisture content than products used by Dennison in his report (1978), enhanced solubility for the reduced ferrous sulfate salt relative to

the elemental species may be proposed as a factor contributing to the observed differences in the reaction rate between products. Greater mobility for the metal ion within the wet cereal product than for electrolytic iron in mixed cereal may allow for the increased rate of catalysis by iron in wet cereal.

Influence of pH on Valence State of Iron

There is a somewhat similar pattern for both cereal types with respect to the time elapsed before the initiation of increased oxidation rate in the iron-fortified product relative to non-fortified product (Figures 19 and 20). Such similar behavior may indicate a change of the iron form in one product to that which more closely approximates the iron form in the other product. Nojeim and Clydsdale (1981) have established that iron valence in model systems reflects pH of the environment. A 4-week study involving elemental iron showed the greatest amount of ionization and conversion to the ferrous valence occurring at pH 2.7, the least at pH 6.2. Ascorbic acid aided the ionization of iron and favored the ferrous valence at low pH of 2.7.

Results of the experiment indicate that there was no significant difference for TAA, R-AA or DHA between k values for iron-fortified mixed cereal and wet cereal; that is, that the rate of loss in TAA and R-AA, or the rate of increase in DHA, was similar for the two products regardless of the form of iron originally present in the products. Nojeim and Clydsdale (1981) showed over 80 percent conversion for elemental iron to ferrous iron at pH 5.0 or below. The phenomenon of conversion for the elemental compound to the soluble form is illustrated in Figure 27. After 48 hours, percent concentrations of elemental iron and ferrous sulfate were similar, but less than 1 percent of either

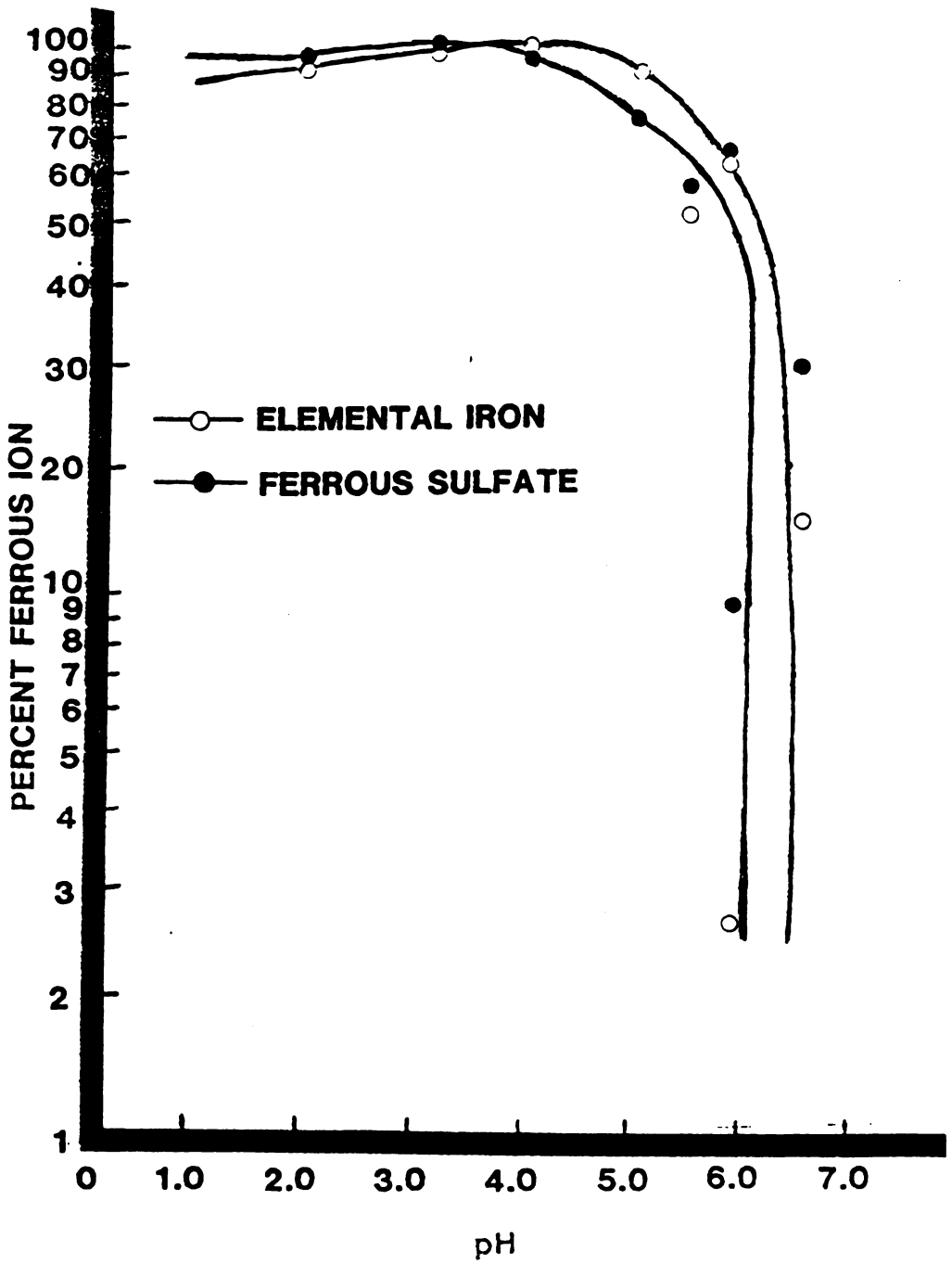


Figure 27 . Semilog plot of pH vs. the percentage of added iron converted to ferrous ion after 48 hrs. Less than 1% of either ferric ortho phosphate or sodium ferric EDTA was ionized in this time and are therefore not shown. (From Nojeim and Clydsdale, 1981)

ferric orthophosphate or sodium ferric EDTA was ionized. These two latter compounds were subsequently not included on this chart.

The two cereal products investigated both have pH values below pH 5.0: mixed cereal has pH 4.15 and wet cereal has a slightly lower concentration of hydrogen ions and pH 4.85. At pH 4.2, Nojeim and Clydsdale (1981) had over 90 percent ionization of elemental iron to ferrous iron within 48 hours, regardless of the presence or absence of ascorbic acid. Gradual oxidation to the trivalent state occurred with time. After 4 weeks, approximately 25 percent of the ionic iron was in the ferric state. Concentration of ascorbate failed to influence ionization of iron.

Pattern of DHA accumulation in Figure 19 for mixed cereal, and Figure 20 for wet cereal indicate a similar type phenomenon with a rapid increase in DHA occurring for the iron-fortified product at day 4 and day 6, respectively, for cereal types. Increase in each product is followed by a plateau in concentration ratio to the conclusion of the experimental period. Based on the report of Nojeim and Clydsdale (1981), electrolytic iron in mixed cereal would ionize to the ferrous form within 48 hours. In this research, the ascorbic acid present in the wet cereal product maintains iron in the ferrous valence and the two products appear to act in harmony with respect to DHA during the remainder of the study.

Differences observed between Figures 19 and 20 reflect the variant conditions within the two respective products. It has been mentioned previously that composition including iron and ascorbic acid concentration was not consistent between products. Experimental data reveal that average concentration over the 10 day period for iron in mixed cereal was 3.13 mg/100 g. Average concentration in wet cereal was 6.33 mg/100 g.

This represents a two-fold difference in concentration of iron available to affect ascorbic acid, with ascorbic acid present in an inverse two-fold concentration differential. At the onset of the experiment, mixed cereal contained an average of 102.6 mg/ 100 g reduced ascorbic acid, and wet cereal had an average of 59.0 mg/ 100 g of R-AA. The greater rate of degradation for ascorbic acid in wet cereal, illustrated by k in Figure 21 for mixed cereal and in Figure 22 for wet cereal, reflect the disproportionate concentration of reactants between products.

Influence of pH on Oxidation Rate

Reaction rate constants presented in Table 3 are for each type of cereal product evaluated. Given that the food environment differs substantially between products warrants further analysis of observed results. Again, pH may be recognized as a factor in predicting the difference in oxidation reaction rate for the two products. Khan and Martell (1967) reported that reaction rate increased with increasing concentration of the the metal (as ferric ion) for pH range 1.50 to 3.85. Earlier researchers showed that the rate increased proportionally to pH 6 and 7 (Joslyn and Miller, 1949).

Mechanism for influence of hydrogen ion on oxidation rate appears to involve the particular chemical species of ascorbic acid present. Maximum rate for ascorbic acid degradation in acidic conditions occurs near pKa_1 of R-AA (Finholt et al., 1963; Blaug and Hajratwala, 1972). Lee et al. (1977) reported pKa_1 of 4.09. At pKa_1, ascorbic acid exists primarily as the monovalent ion. At pH values below pKa_1, the neutral ion predominates; and as pH values approach 6 and 7, the divalent ion is more abundant.

Lee et al.(1977) proposed a mechanism whereby a complex forms between iron and the monovalent ascorbate. The complex requires less activation energy for its destruction than for the dissociated and undissociated forms of ascorbic acid. The amount of complex formed may reach maxima near pKa_1 of ascorbic acid.

Khan and Martell (1967) reported that in the pH range below 6, the undissociated and monoionic forms of ascorbic acid are the main species in solution. The concentration of divalent ion is negligable within this range, but increases as pH increases beyond pKa_1. Khan and Martell (1967) described that metal ion-catalyzed reactions showed an inverse dependence on hydrogen ion concentration. That is, as pH increased within the range below pH 6, reaction rate also increased. Experimental results confer with the conclusions of Khan and Martell (1967), whereby conditions with a pH 4.85 would promote a more rapid rate of oxidation than at pH 4.15. Complex formation may be maximum at pHa_1 for ascorbic acid, but contribution of the divalent species from spontaneous oxidation described in the literature review may be sufficient to account for the observed enhanced rate of oxidation in wet cereal relative to mixed cereal when iron is not considered as a factor in the experiment.

Effect of Heat Processing on Iron Form

Valence or form of iron present in fortified foods is altered by the food environment and by the method of preparation for the product. It has been mentioned that a distinction between products evaluated herein is that wet cereal is a thermally processed product and the final mixed cereal product is not heat treated. The wet cereal was the only product processed with both iron and ascorbate present.

Research reports have assessed the changes during heat processing

in a variety of food products (Lee and Clydsdale, 1980a; Lee and Clydsdale, 1981; Hegenauer et al., 1979). Processing of ferrous sulfate-fortified spinach samples resulted in formation of significant amounts of insoluble iron (Lee and Clydsdale, 1981). Insoluble iron forms are not as bioavailable as the reduced ferrous sulfate form. Processing with ascorbate did not prevent the formation of insoluble iron. The effect of baking on the iron in enriched flour has also been investigated (Lee and Clydsdale, 1980b). Ferrous sulfate added to flour and baked as biscuits generated large amounts of insoluble iron. Iron profile of the iron source originally present in each product had vanished in the final product after heat processing. Hegenauer (1979) found that prolonged batch heating of iron-fortified raw milk decreased oxidation by ferrous salts. Formation of insoluble compounds of iron during heating could have attributed to the diminished catalytic ability observed for the reduced iron added.

The above research results may pertain to the phenomenon observed with experimental data. The addition of iron as ferrous sulfate in wet cereal did not yield significant differences between k for DHA in non-fortified and iron-fortified product. It is likely that the formation of substantial amounts of insoluble, and therefore less reactive, iron would have resulted in a diminished rate of oxidation relative to that if iron was present in 100 percent of its original reduced state.

Iron Content in Samples

For both cereal products, initial iron concentration exceeded that required by label declaration. Mixed cereal contained, on the average, 3.62 mg/100 g iron and wet cereal contained 6.72 mg/100 g iron. Actual values correspond with required amounts to be in accordance with label declaration of 3.33 mg/100 g and 5.0 mg/100 g respectively.

Results for iron evaluation on day 1 and day 10 are presented in Appendix VII. It appears that iron content did change over time for both mixed cereal and wet cereal products, and a diminished iron concentration was observed after 10 days of storage. A significant loss in iron occurred for both iron-fortified mixed cereal and wet cereal products, with the more dramatic change observed for mixed cereal ($p < .001$). Change in iron concentration for wet cereal was slightly less pronounced, but was significant with $p < .01$. Results revealed that a small amount of iron was present in both cereal products when no iron was added in fortification, thus indicating that iron was naturally present in the product. The significant decline in iron concentration during the 10 day storage period was unexpected.

Significance of the Research

The design of the experiment was intended to permit a comparison of the influence for reduced ferrous sulfate and electrolytic elemental iron in the oxidation of ascorbic acid. Experimental results indicated that the cereal type itself was of considerable influence on reaction rate for ascorbic acid metabolites. Therefore, the effect on ascorbic acid is presented in context of each cereal product evaluated. The observed difference between mixed cereal and wet cereal appeared to be integrally embodied in the influence of oxygen concentration, a_w, pH, and the effect of heat processing on ferrous sulfate.

Observed results indicate that there was a loss of ascorbic acid during the 10 days of refrigerated storage. Destruction of the vitamin in a wet cereal, rice cereal with applesauce and bananas, is only minimally accelerated by the practice of iron-fortification with ferrous sulfate. A caretaker who prepares a mixed cereal of dry rice cereal with

apple juice may expect the presence of iron in dry cereal to enhance the rate of oxidation for ascorbic acid in apple juice. Oxidation would proceed more rapidly in the mixed cereal product than if the juice was stored separately.

Conversion of R-AA to DHA is not of major nutritional concern, since DHA reportedly has 75 to 80 percent the vitamin C activity of the reduced form. Furthermore, it is emphasized that significant loss of ascorbic acid due to the presence of iron, occurred only in the mixed cereal product, and only after a substantially extended storage period. The result pertains to a situation where a product may be prepared in the home and stored for a period greater than 4 days. Since ascorbic acid is a labile nutrient, storage recommendations generally suggest that vitamin C-rich foods not be kept exposed to air for periods longer than a few days (Tannenbaum, 1976).

The recommendation as result of this research to cease iron-fortification in dry cereals in order to prevent acceleration of ascorbic acid degradation in a mixed product is not warranted. The prevailing incidence of iron-deficiency anemia in infants and children underscores the critical contribution of iron-fortified cereals in efforts to alleviate the problem. An appropriate interpretation of this research is to affirm and encourage the widespread use of iron-fortified cereal products for infants introduced to solid foods.

Relevance of the research also pertains to the national policy, wherein WIC (Women, Infant and Children Supplemental Food Program) infant packages provide both iron-fortified dry cereal and ascorbic acid-enriched fruit juice. The two products may be fed in combination without substantial loss of ascorbic acid, provided the mixed cereal product is not stored for a period longer than 4 days.

The observed experimental results may be of interest considering any policy decision regarding nutritional labeling requirements. Although no recommendation is made suggesting that product be stored for long periods of time after opening, and label declaration only pertains to the sealed product as purchased, a conscientious food company would be interested in the nutrition provided by the product as consumed. Additionally, it is important that the consumer be cognizant that changes in nutritional quality occur for a food product over time, and be knowledgable of the food ingredients that may influence the rate of nutrient loss.

It is also interesting that the reduced form of iron, ferrous sulfate, is generally regarded as catalytic in promoting a reduction-oxidation reaction. However, in this experiment it is observed that the pronounced influence on oxidation from iron is in mixed cereal where electrolytic iron is the iron source. This result supports the notion that many influences come to bear in alteration of the nutritional quality of a food product, and that consideration must include the complex associations of the food environment, and the method of food processing and preparation.

SUMMARY AND CONCLUSIONS

The rate of ascorbic acid degradation was evaluated in two infant food products. The two systems investigated were a mixed cereal with dry rice cereal and apple juice, and a wet cereal with applesauce and bananas. Mixed cereal is prepared containing electrolytic iron and wet cereal is fortified with ferrous sulfate. The influence of the two forms of iron in their respective products on ascorbic acid is described as changes occur during a 10 day period of refrigerated storage.

A preliminary project was undertaken to enable comparison for methodology used to assay R-AA and DHA. No method precisely measured amount of standard added to model systems, and the particular model solution used showed predictable influence on results obtained for each method of analysis. Table 6 summarizes relative concentrations for both R-AA and DHA according to each method of analysis.

Table 6. Relative concentrations according to method of analysis.

R-AA == Sucrose: standard > OPDA > titrametric
Starch: standard > titrametric > OPDA

DHA == Sucrose: spectrophotometric $\gg$ OPDA $\gg$ standard
Starch: OPDA > spectrophotometric > standard

For R-AA in the sucrose model solution, the automated OPDA procedure yielded results that more closely agreed with known amounts of standard added than did the titrametric procedure. Results in starch were inverted and the titrametric assay produced more precise recordings relative to a

set of standards.

For DHA, the pattern of results was similar with the automated OPDA analysis indicating closer agreement with standards relative to the spectrophotometric method in sucrose; but in starch the spectrophotometric method was more accurate.

Results for DKGA, obtained by difference, were not quantitative between methods. A correction factor, relative to a set of standards, was required to allow comparison between products in the experiment.

The three inconsistencies in methodology described leave the researcher in question regarding the precision with some techniques used for ascorbic acid assay. There appears to be considerable differences for ascorbic acid concentration determined by some methods of analysis. Magnitude and direction of the inaccuracy depends largely on characteristics of the food system evaluated. Further comparative research may validate the observations reported herein that reveal inconsistency between methods currently used to determine ascorbic acid concentration, and are commonly reported in literature.

It was postulated that iron in the reduced ferrous form, present in wet cereal, would enhance the rate of oxidation relative to that for elemental iron in mixed cereal. This assumption was not supported by experimental data. Significant differences were obtained for DHA between non-fortified and iron-fortified mixed cereal during the course of the storage period; however, there was not a corresponding significant distinction between wet cereal products.

The pattern was apparent in both cereal products with a delay in the initiation of the oxidative process in iron-fortified product until day 4 or day 6 of the experiment. The longer delay was observed for the wet cereal product. This similarity between cereal types is supported by

work of Nojeim and Clydsdale (1981) who observed a conversion of elemental iron to ferrous form in 48 hours. With such conversion, there is minimal nutritional significance given the product is stored for a period longer than 2 days, since iron in both products would be present in the optimal form for bioavailability. Loss of vitamin C in iron-fortified cereals does not continue to increase following the initial rate increase relative to the non-fortified product.

Characteristic differences between products, aside from the form of iron present, may have contributed to the distinction regarding the rates of ascorbic acid oxidation. The two products differed in initial concentration of iron and ascorbic acid, in potential for dissolved oxygen, in moisture content, and in pH. Additionally, the wet cereal product was thermally processed with both iron and ascorbate present, whereas mixed cereal did not receive similar heat treatment. Previous researchers have shown that the iron profile is altered by heat processing, and ferrous sulfate originally present in wet cereal may have been substantially converted to an insoluble, and less reactive, form.

The presence of ascorbic acid with iron is reported to enhance the absorption of iron. Results reported herein indicate that loss of ascorbic acid, promoted by iron, did not occur for several days of refrigerated storage. The catalytic effect of iron is only significant in the mixed cereal product that received no heat treatment. Since it is is most likely that infant foods would be fed with only a day or two of storage, it seems that the imapct on vitamin C nutriture of infants fed vitamin C-enriched and iron-fortified foods is of minimal concern. Both mixed cereal and wet cereal products may appropriately serve as vehicle for both vitamin C-enrichment and for iron-fortification.

APPENDICES

APPENDIX I

Split-plot Analysis

	Total Ascorbic Acid	
source of variation	F	sign. of F
day	3.272	.05
cereal type (CT)	43.620	.001
iron	1.161	NS
day X CT	9.539	.001
day X iron	0.536	NS
CT X iron	8.490	.001
day X CT X iron	0.730	NS

$SS_{E_1} = 54.986$

$SS_{E_2} = 37.298$

$MS_{E_1} = SS_{E_1}/8 = 6.873$ (used to test all not involving days)

$MS_{E_2} = SS_{E_2}/72 = 0.518$ (used to test all involving days)

APPENDIX II

Split-plot analysis

source of variation	Reduced ascorbic acid F	sign. of F
day	12.311	.001
cereal type (CT)	43.259	.001
iron	0.028	NS
day X CT	12.762	.001
day X iron	2.938	NS
CT X iron	8.867	.001
day X CT X iron	1.119	NS

$SS_{E_1} = 63.264$

$SS_{E_2} = 27.792$

$MS_{E_1} = SS_{E_1}/8 = 7.908$ (used to test all not involving days)

$MS_{E_2} = SS_{E_2}/72 = 0.386$ (used to test all involving days)

APPENDIX III

Split-plot Analysis

source of variation	Dehydroascorbic acid F	sign. of F
day	80.640	.001
cereal type (CT)	67.058	.001
iron	17.777	.001
day X CT	7.058	.005
day X iron	15.511	.001
CT X iron	1.728	NS
day X CT X iron	3.122	.05

SS_{E_1} = 501.056

SS_{E_2} = 10.041

$MS_{E_1} = SS_{E_1}/8 = 0.923$ (used to test all not involving days)

$MS_{E_2} = SS_{E_2}/72 = 0.139$ (used to test all involving days)

APPENDIX IV

Bonferroni-t Analysis and Student's t Test -- k constants

	Dehydroascorbic Acid			
contrast*	t_B	sign. of t_B	t	sign. of t
A. mixed cereal/ no iron vs mixed cereal w/iron	-1.9474	NS	-1.9474	.05
B. wet cereal/ no iron vs wet cereal w/iron	-.3836	NS	-.3836	NS
C. mixed cereal vs wet cereal (no iron)	.8926	NS	--	--
D. mixed cereal vs we cereal (w/iron)	.4441	NS	—	--

*Contrast A denotes the effect of iron in mixed cereal.
Contrast B denotes the effect of iron in wet cereal.
Contrast C denotes the difference between cereal products without added iron.
Contrast D denotes the difference between cereal products with iron present.

APPENDIX V

Split-plot Analysis

	Diketogulonic acid	
source of variation	F	sign. of F
day	72.00	.001
cereal type (CT)	3.66	.05
iron	205.89	.001
day X CT	1.29	NS
day X iron	1.39	NS
CT X iron	0.54	NS
day X CT X iron	1.06	NS

$SS_{E_1} = 14.19$

$SS_{E_2} = 38.64$

$MS_{E_1} = SS_{E_1}/8 = 1.77$ (used to test all not involving days)

$MS_{E_2} = SS_{E_2}/72 = 0.54$ (used to test all involving days)

APPENDIX VI

Bonferroni-t Analysis and Student's t Test -- k constants

	Diketogulonic Acid			
contrast*	t_B	sign. of t_B	t	sign. of t
A. mixed cereal/ no iron vs mixed cereal w/iron	.3722	NS	.3722	NS
B. wet cereal/ no iron vs wet cereal w/iron	-.9626	NS	-.9626	NS
C. mixed cereal vs wet cereal (no iron)	.7033	NS	--	--
D. mixed cereal vs wet cereal (w/iron)	6.1430	.01	--	--

*Contrast A denotes the effect of iron in mixed cereal.
Contrast B denotes the effect of iron in wet cereal.
Contrast C denotes the difference between cereal products without added iron.
Contrast D denotes the difference between cereal products with iron present.

APPENDIX VII

Student's t-test distribution — evaluation of change over time

Product	Iron Concentration Day 0 (mg/100g)	Day 10 (mg/100g)	t		s	sign. of t
Mixed cereal (no iron)	1.03	0.67	2.99	2	.210	.05
Mixed cereal (w/iron)	3.62	2.64	58.98	2	.029	.001
Wet cereal (no iron)	0.97	1.22	-.93	2	.465	NS
Wet cereal (w/iron)	6.72	5.94	7.08	2	.190	.01

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