THE EFFECTS OF IRON DEFICIENCY ON THE EFFICACY AND PHARMACOKINETICS OF ALBENDAZOLE IN MICE INFECTED WITH *HELIGMOSOMOIDES POLYGYRUS*

by

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ABSTRACT

The aim of this research was to determine the influence of iron deficiency on both the efficacy and metabolic patterns of albendazole in mice infected with *Heligmosomoides polygyrus*. Anthelmintic efficacy was markedly decreased in iron-deficient mice; the deficiency was also associated with a decrease in body weight, altered hematological parameters and a decreased net egg output; worm establishment in the deficient group was not affected by the deficiency. Although anthelmintic efficacy was significantly decreased by the iron deficiency, plasma concentration profiles of the main metabolites, albendazole sulphoxide and albendazole sulphone, were not changed by the deficiency. Levels of intestinal cytochrome P-450, the main metabolizing enzyme of albendazole however, was significantly depressed in iron-deficient mice. These observations suggest that although pharmacokinetic parameters are not affected by iron deficiency, nutritional status has the potential to influence anthelmintic efficacy and thus warrants further study.

RESUME

Le but de cette recherche était de déterminer les effets d'une déficience en fer sur l'efficacité et sur le profil métabolique de l'albendazole chez les souris infectées avec *Heligmosomoides polygyrus*. Les effets anti-helmintes de la drogue étaient légèrement reduits chez les souris déficientes en fer; cette déficience amenait également une reduction du poids des souris ainsi qu'un changement des parametres hematologiques, observant également une diminution de l'excretion des oeufs. Cependant, l'établissement de la population de vers n'était pas affecté par la deficience en fer. Même si l'effet anti-helminte de la drogue était diminué, la concentration du sulphoxide et du sulphone d'albendazole dans le plasma restaient inchangés. Cependant, le niveau intestinal du cytochrome P-450, principal enzyme métabolisant l'albendazole, était fortement diminué chez les souris déficientes en fer. On observe donc qu'une diète déficiente en fer n'affecte pas les paramètres pharmacokinetiques de la drogue; cependant l'efficacité de ses effets anti-helmintes peut varier nécessitant donc des études plus approfondies.

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INTRODUCTION

The relationship between parasitic infection and malnutrition is poorly understood. It is estimated that parasites infect more than one billion people each year (Pawlowski, 1984). Cases of clinical malnutrition are estimated to range from 5 to 8 million cases annually (Bundy & Golden, 1987). The impact on health is exacerbated because both conditions are chronic, are most common in growing children and, most importantly, tend to occur together in the same individuals' (Bundy and Golden, 1987). 'The available information indicates that the proportion of the world's population that bears the brunt of parasitic disease also suffers from much of the world's malnutrition and experiences the difficulties of poor socioeconomic conditions' (Crompton and Nesheim, 1982). Manifestations of iron deficiency specifically, are among the most common symptoms of undernourishment in both industrialized and developing countries (Jacobs, 1977; Kochanowski and Sherman, 1982; Cook, 1986). The interaction between iron status and parasitic infection however, is still only poorly understood (Duncombe *et al.*, 1979).

Current control of parasitic infections depends on the use of broad spectrum anthelmintics, the most predominate being the benzimidazole family of drugs (Lacey, 1988). The efficacy of these drugs is dependent on the absorption,

distribution and clearance rate of the drug, as well as the rate at which the parasite is able to take up the active metabolites; the nutritional status of an individual and the availability of nutrients are important to the regulation of these parameters. Two studies have evaluated the effects of iron deficiency on the pharmacokinetics and efficacy of benzimidazole drugs: Duncombe *et al.* (1979b) demonstrated a decreased efficacy of mebendazole, levamisole and piperazine against *Nippostrongylus brasiliensis* infections in rats maintained on an iron and protein deficient diet. Prichard *et al.* (1981), showed a decreased fenbendazole uptake by *N. brasiliensis* in protein and iron deficient rats. These studies demonstrate the potential influence of host nutritional status in determining anthelmintic effectiveness.

The **objective** of this study was to determine the impact of an iron deficiency on the efficacy and pharmacokinetics of albendazole. A moderately severe iron deficiency was induced in mice which were then exposed to *Heligmosomoides polygyrus*, a gastro-intestinal nematode common to mice. The effects of albendazole treatment in both a controlled and iron deficient state were then determined.

CHAPTER 1

LITERATURE REVIEW

1.1. EFFECTS OF IRON DEFICIENCY

Iron deficiency affects about 15 to 25% of the overall population (Briggs and Calloway, 1984). Deficiencies in iron are most widespread in certain subgroups such as infants and children since rapid growth imposes a great need for iron (Briggs and Calloway, 1984). The most common sign of iron deficiency in humans is iron deficiency anemia which is characterized by decreased muscular and tissue performance due to a reduced oxygen-carrying capacity of the blood hemoglobin (Jacobs, 1977). Iron is necessary for proper functioning of the immunological response of the host against infection (Chandra, 1975). Kuvibidila *et al.* (1983) demonstrated an impaired blastogenic response of spleen cells to both T and B cell mitogens in iron deficient mice. Other investigators have shown decreased antibody production (Nalder *et al.*, 1972), lymphoid tissue abnormalities (Rothenbacher and Sherman, 1980), impaired bactericidal activity of leukocytes (Prasad, 1979), a delayed inflammatory response (Kuvibidilia *et al.*, 1981; Krantman *et al.*, 1982) and abnormalities in neutrophil function (Chandra, 1973) in iron deficiency.

Iron has been shown to play an important role in maintaining normal DNA synthesis and cell proliferation (Robbins and Pederson, 1970; Siimes and Dallman, 1974;

Hoffbrand *et al.*, 1974 and 1976) due to the iron requirements of ribonucleotide reductase (Brockman *et al.*, 1972; Kochanowski and Sherman, 1982). Impaired cellular growth has been demonstrated in many organs (thymus, liver, spleen, heart, kidney) during chronic iron deficiency (Kochanowski, 1982; 1985). This is reflected by an overall decreased body weight that is most evident using animal models (McCall *et al.*, 1962; Kochanowski and Sherman, 1982) but that may also occur in man (Judisch *et al.*, 1966). Iron is necessary for proper digestive and absorptive function (Ghosh *et al.*, 1972; Kimber and Weintraub, 1968). Biochemical deficiencies in the intestinal mucosa include impairment of cytochrome c, cytochrome oxidase and cytochrome P-450. Iron restitution has been shown to reverse these abnormalities (Hoensch *et al.*, 1975).

Due to its influence on enzymatic activity, iron plays a role in many metabolic functions. Many investigators have shown a 20 to 50% decrease in myoglobin concentration in iron deficient rats during the growth period (Dallman and Schwartz, 1965; Siimes *et al.*, 1980; Hagler *et al.*, 1981). Other reports have described a decreased cytochrome activity (predominantly cytochrome c) in skeletal muscle of rats and mice (Dallman and Schwartz, 1965; Hagler *et al.*, 1981). In addition, severe iron deficiency causes a decrease in the activities of mitochondrial alpha-glycerophosphate

dehydrogenase which is a necessary component of the electron transport chain (Davies *et al.*, 1982; Macdonald *et al*, 1985). This enzyme is important in supporting oxygen utilization, and diminished amounts in skeletal muscle have been linked to lowered exercise capacity which is well characterized during an iron deficiency (Finch *et al.*, 1976; Davies *et al.*, 1982). Besides skeletal muscle, iron deficiency has also produced significant reduction in the activity of several respiratory enzymes in the heart, brain and liver (Blayney *et al.*, 1976) and has been implicated in angina pectoris and congestive myocarditis (Blayney *et al.*, 1976). Other studies have attributed decreases of cerebral monoamine oxidase (which results in excess serotonin in the central nervous system) and aldehyde oxidase (involved in serotonin degradation) to the irritability, apathy, lack of attention and low performance scores well characterized in iron deficient children (Viteri & Torun, 1974; Voorhess *et al.*, 1975). In addition, decreases in mitochondrial liver enzymes during iron deficiency may alter drug metabolizing pathways (Symes *et al.*, 1969).

1.1.1. Iron and Cytochrome Activity

Iron is involved in a variety of enzymatic reactions associated with mitochondria energy production (Galan *et al.*, 1984). The role of iron depletion on drug metabolism has recently begun to receive attention due to the importance of cytochrome P-450 (a heme enzyme) and NADPH cytochrome P-450

reductase in oxidation reactions necessary for drug elimination in both the liver and small intestine (Becking, 1976; Galan *et al.*, 1984; Dhur *et al.*, 1989). Studies on the interaction between iron deficiency and liver cytochrome P-450 content and activity have shown conflicting results. Becking (1972 and 1976) reported significantly increased monooxygenase activity (resulting in increased drug metabolism) after only 18 days of iron deficiency. However, several investigators (Catz *et al.*, 1970; Bailey-Wood *et al.*, 1975; Dhur *et al.*, 1988) have reported no changes in cytochrome P-450 activity or content in the liver. In contrast, Hoensch *et al.* (1975) examined drug metabolizing capacity of the intestinal villous during an iron deficiency. After only 48 hours of restricted dietary iron intake, cytochrome P450 content decreased to 42% of control values, but was restored within 24 hours by oral iron supplementation. In a later study (Hoensch *et al.*, 1976), on restriction of dietary iron intake cytochrome P-450 activity fell sharply, but was completely restored within 24 hours of oral iron repletion The study concluded that cytochrome enzyme regeneration is critically dependent on intestinal iron availability and absorption. Similarly, Dhur *et al.* (1988) demonstrated that even moderate iron deficiency can alter enzymatic systems in the small intestine thus intervening in drug metabolism.

1.1.2. Iron and Absorption

Manifestations related to the oral cavity and the gastrointestinal tract have attracted attention both because of their frequency and because of uncertainty as to their pathogenesis (Beutler and Fairbanks, 1980). Abnormalities of the gastrointestinal tract during an iron deficiency include gastric achlorhydria, gastritis and atrophy. A high incidence of blood loss and loss of plasma proteins (thus exacerbating the deficiency) have been reported in both infants and children. Impaired absorption possibly related to a decrease in iron-containing or iron-dependent enzymes in the intestinal mucosa, together with duodenitis and mucosal atrophy (resulting in diarrhea) have also been observed. Most abnormalities are corrected upon repletion with iron (Foy and Kondi, 1960; Kimber and Weintraub, 1968; Goodhart and Shils, 1980; Briggs and Calloway, 1984).

1.1.3. Evaluation of Iron Status

Severe iron deficiency is characterized by hypochromia and microcytosis of the red blood cells; clinically, this can be evaluated by measuring hemoglobin concentrations (Beutler, 1988). When observed on a blood smear the erythrocytes appear small and pale but also vary greatly in size and shape. Body weight and food intake are particularly sensitive to iron status. In most experiments both body weight gain and food intake were significantly depressed in iron deficient animals

(Siimes and Dallman, 1974; Bailey-Wood *et al.*, 1975; Kuvibidila *et al.*, 1983; Kochanowski and Sherman, 1985). Organ weights are also significantly affected by iron deficiency. Kuvibidila *et al.* (1983) showed that the heart, kidney and spleen were significantly enlarged during iron deficiency. Conversely, Siimes and Dallman (1974) showed that liver weight was significantly depressed in iron deficient animals when compared to control animals of the same age. Dallman and Reeves (1984) determined that decreased serum iron, decreased transferrin saturation and increased total iron binding capacity were particularly sensitive indices of iron deficiency anemia. However, decreased serum ferritin concentration is thought to be the most sensitive indicator of iron deficiency due to significant depletion in the early stages of iron deficiency. Other particularly sensitive indicators of iron deficiency include a decreased mean cell volume and an increased red cell distribution width in iron depleted states (Johnson, 1990), these indices however are more denotative of an anemia caused by chronic disease rather than an iron deficiency due to inadequate nutrition.

1.1.4. Iron and Parasitic Infection

Malnutrition and parasitic infection are amongst the most prevalent conditions affecting human health worldwide (Bundy and Golden, 1987). 'Synergistic interactions between nutrition and parasitism are complex. Intestinal helminths

respond to inadequate host nutrition by means of alterations in their establishment, survival and fecundity, and may also precipitate a continued decline in nutritional status by disturbing the food intake, digestion and metabolism of their hosts' (Keymer, 1986). The available information indicates that the proportion of the world's population with the highest prevalence of parasitic infection also suffers from much of the world's malnutrition (Crompton and Nesheim, 1982).

Due to the high prevalence specifically of iron deficiency anemia (it affects 15 -25% of the overall population) (Briggs and Calloway, 1984), many studies have focussed on the effects of iron deficiency on both prevalence and intensity of parasitic infection. Shield *et al.* (1981) observed a direct relationship between iron deficiency anemia and increased intensity of hookworm infection in a Papua New Guinea highland population. A study by Duncombe *et al.* (1979a) demonstrated that iron deficiency significantly reduced the development of acquired resistance to reinfection in rats infected with *N. brasiliensis*. Similarly, Bolin *et al.* (1977) observed a significant delay in the expulsion of *N. brasiliensis* from the small intestine of rats weaned on an iron deficient diet. Interestingly, an experiment by Lalonde and Holbein (1984) showed that mice infected with *Trypanosoma cruzi* exhibited a biphasic hypoferremic response to infection. The intensity of anemia correlated directly with the intensity

of the parasitemia in C57BL/6 mice; iron repletion resulted in an increased mortality rate of mice infected with *T. cruzi* whereas iron depletion protected the C_3H mouse strain by reducing mortality rates from 100 to 45%. These results indicate the potentially complex interactions occurring between the parasite and nutritional status of the host.

1.1.5. Iron and Drug Metabolism

The liver is the main organ of the body involved in drug metabolism and detoxification; both nutritional status of an individual and availability of nutrients are important regulators of drug metabolism and clearance (Bidlack *et al.*, 1986). Depletion of nutrients including protein, carbohydrates, fats and specific vitamins and minerals alter the efficiency of these reactions, both at the enzyme level and in the availability of cofactors required for completion of the reactions (Bidlack *et al.*, 1986). Studies on the effects of mineral deficiencies on xenobiotic metabolism have focussed only on hepatic drug metabolizing enzymes thus far, although the small intestine, lungs and kidneys are also known to be significantly involved in drug metabolism and clearance (Becking, 1976). This may explain in part conflicting results in the following studies in which the metabolism of various drugs known to be metabolized primarily by the cytochrome P-450 system were assessed: Becking (1976) observed a significantly increased *in vivo* rate of metabolism of both

aminopyrine and aniline in mice during an iron deficiency, *in vitro* metabolism of pentobarbital did not differ from controls; *in vivo* activity (measured by sleeping times) however, was significantly increased. Iron repletion for 7 days reversed all alterations in drug metabolism. Catz *et al.* (1970) showed that rates of metabolism of both hexobarbital and aminopyrine were significantly increased in iron deficient mice; interestingly, concentrations of microsomal cytochrome P-450 were not affected. Similarly, Becking (1972) showed a 30% increase in aniline metabolism *in vitro* and *in vivo*, with no change in the cytochrome P-450 or the reductase. These studies indicate the complex alterations that occur in cellular metabolism during an iron deficiency, thus making it very difficult to pinpoint specific malfunctions in drug metabolism.

1.2. HELIGMOSOMOIDES POLYGYRUS

Heligmosomoides polygyrus is a trichostrongylid intestinal nematode of murine rodents. It was introduced as an experimental infection in laboratory mice by Spurlock in 1943 and over the last 20 years has been used as a model for studies of pharmacology, host immunology and epidemiology of chronic gastrointestinal helminthiasis (Monroy, 1992). Its life cycle is characteristic of a direct life cycle nematode infection: eggs expelled in the feces of the host develop to infective third stage larvae within 7 days and infect a new

host via ingestion. Third stage larvae are detected in the intestinal lumen of the host within 18 hours after infection (Bryant, 1973). These larvae then migrate into the serosal musculature where they remain for 7 to 9 days undergoing maturation and differentiation processes before moving into the intestinal lumen where they further mature and begin producing eggs 3 days later. The maximum lifespan of the parasite is about 8 months and once fully mature, adult parasites produce an average of 1500 eggs/female worm/day (Kerboeuf, 1982).

Due to the many similarities in both epidemiology and immunity between *H. polygyrus* and other gastrointestinal nematodes infecting humans, this parasite has been used as a model for endemic human hookworm infection. Like human infection, exposure of mice to infection results in many lightly infected and few heavily infected individuals (Bartlett and Ball, 1972; Scott, 1987). This aggregated distribution of worm numbers has been shown to occur in many human nematodes (Schad and Anderson, 1985 & Bundy *et al.*, 1987) and in *H. polygyrus* infection (Scott, 1988a). Aggregation of parasites in certain individuals is due to many factors including behavioral differences, genetic differences and differences in development of protective immunity (Anderson and Gordon, 1982), however in the mouse-nematode interaction intrinsic features of the mice seem to play a

major role in predisposition to infection (Scott, 1988ab). The parasite causes morbidity and mortality in the host (Scott, 1987) and this is directly dependent on the intensity of infection within the mouse (ie: density dependent effects occur) (Scott, 1990). The long term survival of *H. polygyrus* during chronic primary infection has been attributed to the parasite's ability to effectively evade or depress host immune responses at the intestinal level (Behnke *et al.*, 1983; Ali & Behnke, 1984b). There is now evidence that mature worms initiate immunodepression in primary infection through release of immunomodulatory factors which effectively suppress host immunity (Behnke *et al.*, 1982; Monroy *et al.*, 1989). This finding is consistent with other studies in which immune responses to unrelated antigens such as lipopolysaccharide and sheep red blood cells were depressed during a primary infection (Ali and Behnke, 1983, 1984ab) and rejection of other nematodes such as *Trichinella spiralis* and *Nippostrongylus brasiliensis* were delayed in mice concurrently infected with *H. polygyrus* (Behnke *et al.*, 1978; Jenkins, 1975). Further support that mature parasites cause immunodepression comes from the fact that infections terminated immediately prior to parasite maturation or infections with irradiated larvae that do not develop to adults are strongly immunogenic (Prowse & Jenkins, 1981; Behnke & Hannah, 1983).

Immune responses during challenge infection have been shown to be elicited by developing larvae in the intestinal lumen (Behnke and Parish, 1979). Immune responses elicited by the developing larvae during challenge infection include release of IgG_1 and eosinophils within the mucosa granulomata (Pritchard *et al.*, 1983; Prowse *et al.*, 1978; Crandall *et al.*, 1974), an increase in B cell numbers (Parker & Inchley, 1990) and T cell proliferation (Prowse *et al.*, 1978). Immunization to the parasite can have five distinct effects: 1) 'reduction in the number of larvae surviving the tissue phase of their development, 2) arrested development of a proportion of the worms in the intestinal tissue, 3) reduction in the size & 4) fecundity of the worms which complete development and 5) expulsion of adult worms from the host intestine' (Behnke and Parish, 1981).

1.2.1. *Heligmosomoides polygyrus* and Host Nutrition

Surprisingly, little has been written on the interaction between *H. polygyrus* and nutrition. Although most studies focus on the effects of protein deficiency on the establishment, fecundity and pathogenicity of *H. polygyrus*, deficient intake of micronutrients such as zinc (Fenwick *et al.*, 1985) and iron (Duncombe *et al.*, 1979ab) have been shown to exert similar effects using other parasite systems.

In 1986, Slater and Keymer determined that mice fed on a low-protein diet and exposed to continual infection showed persistent increases in both prevalence and intensity of infection, whereas well-nourished mice showed declining prevalence and intensity of infection over time. This was attributed to the negative effect of protein malnutrition on host immunocompetence. In a later study they evaluated the influence of protein deficiency during a challenge infection with *H. polygyrus* (Slater and Keymer, 1988). They demonstrated that malnourished mice were not able to mount an effective immune response against *H. polygyrus* infection. Although protein deficient mice had slightly lower levels of circulating antibodies, they suggested that reduced resistance of the malnourished mice was due more to a delayed development of eosinophilia in the blood causing corresponding reductions in the number of granulocytes attacking the L_4 stage of infection. Brailsford and Mapes (1987) observed the effects of a primary infection of *H. polygyrus* in both protein-deficient and well-nourished mice. They suggested that worms in protein deficient mice had more severe clinical effects on the host than other similarly infected but well-nourished mice (thinner hair, decreased levels of activity, reduced weight gain, increased mortality). These studies indicate the potentially profound effects that nutrient deficiencies can have on parasite establishment and pathogenicity.

1.3. BENZIMIDAZOLES

Helminth infections pose major global concern to both the veterinarian and clinician. It is estimated that hookworms currently infect over 800 million people worldwide causing significant pathology from anaemia to stunting of growth (Horton, 1990). Similarly, prevalence of *Ascaris* infection has been estimated at over 1 billion people (Pawlowski, 1984). Only recently have the health and economic impact of human helminth infections begun to be taken seriously. Studies by Latham *et al.* (1982, 1988) showed that growth and physical fitness in African children can be improved and worker productivity increased by deworming. Another study by Nokes *et al.* (1991a) showed significantly improved cognitive function in Jamaican school children who received treatment for *Trichuris trichiura* infection as opposed to those who remained infected. These studies and others like it (Stephenson *et al.*, 1989; Nokes *et al.*, 1991b) have shown the economic benefits of treatment which has lead to mass treatment programs of entire communities in the hopes of improving both the work performance and educational attainment of individuals within the infected community.

The discovery of thiabendazole in 1961 opened the door to a new area of treatment of parasitic infection and has led to the development of a whole range of benzimidazole compounds (Lacey, 1990). 'Generally, benzimidazoles have limited

solubility and therefore absorption; they are more frequently used for intestinal parasites and particularly in veterinary practice because of their broad spectrum and low toxicity' (Horton, 1990). Treatment for human infections with benzimidazoles has been limited to the use of albendazole, flubendazole and mebendazole due to their low toxicity and high efficacy in treatment of intestinal helminth infections (Cook, 1990). In an attempt to overcome poor drug absorption and the lack of water solubility often associated with benzimidazole compounds, pro-drugs have now been developed. Netobimin is the most recently developed pro-benzimidazole, its solubility in aqueous solution offers increased intestinal absorption over the insoluble benzimidazole anthelmintics.

In general, benzimidazoles are absorbed from the gastrointestinal tract and after passage through the liver, are returned to the small intestine via the bile duct and blood (Campbell, 1990). Once taken up from the GI tract, the benzimidazole compound is rapidly distributed by the circulatory system throughout the entire body; metabolism of the drug in order to facilitate elimination commences immediately. Benard *et al.* (1985) used whole-body autoradiography and liquid scintillation counting in order to monitor the distribution of radio-labelled mebendazole in sheep treated orally with the compound. Radioactivity was observed in all organs, however both the parent drug and

metabolites were concentrated specifically in the liver and melanin containing tissues, where radioactivity residues were found for up to 30 days post-treatment. Overall, benzimidazole compounds are rapidly and extensively metabolized; usually the benzimidazole parent compound is short-lived in the host and metabolic products predominate in the plasma and tissues as well as in the parasites of treated hosts. The drug acts by selectively binding to tubulin receptors within the parasite, inhibiting polymerization of tubulin to form microtubules (Lacey, 1988). Tang and Prichard (1988) showed that benzimidazole compounds have a higher affinity for parasite tubulin as opposed to that of the mammalian host. These differences in binding affinities between the parasite and host may account for their relatively low toxicity in the mammalian host (McKellar and Scott, 1990).

1.3.1. Albendazole

Albendazole (methyl 5-[propylthio] benzimidazole-2-carbamate) is a potent broad spectrum anthelmintic (McCracken and Stillwell, 1991). It is rapidly absorbed from the small intestine resulting in high tissue concentrations when administered at relatively low dosages (Cook, 1990). McCracken and Lipkowitz (1990) postulated that its high potency was due in part to a large molecular dipole moment of albendazole sulphoxide resulting in a stronger bond at the drug-receptor binding site in comparison with other

benzimidazole drugs. Albendazole is a stable, white, odorless powder, has a melting point of 215°C, is insoluble in water and only slightly soluble in most organic solvents (Theorides *et al.*, 1975).

Metabolism of albendazole involves oxidation of the sulfur side chain of the parent compound to form albendazole sulphoxide (the principally active pharmacological agent); this reaction was originally thought to be catalyzed solely by MFMO, a flavin containing, NADPH dependent monooxygenase present in relatively high quantities in the liver (Gottschall *et al.*, 1990). However, further studies conducted by Amri *et al.* (1988) indicated that both cytochrome P-450 and MFMO contribute to the initial oxidation of albendazole. The relative involvement of P-450 and MFMO varies between species and between tissues, depending on enzyme concentrations (Gottschall *et al.*, 1990). Metabolism of albendazole sulphoxide to the inactive component, albendazole sulfone, involves another oxidation process, this time using only cytochrome P-450 in the reaction.

In order to further understand the drug action of albendazole, a number of studies have tried to characterize the pharmacokinetics of this drug. A study by Marriner (1980) using Finn-Dorset sheep showed that the two major metabolites of albendazole, sulphoxide and sulphone, were present in

plasma for up to 96 and 72 hours post-treatment respectively, at peak concentrations of 3.2 and 0.9 ug/ml respectively. Because of the rapid sulphoxidation of albendazole no parent drug was detectable in plasma at any time. A later study by Lanusse *et al.* (1990) characterized the pharmacokinetic behavior of the albendazole pro-drug netobimin after both intraruminal and subcutaneous administration. In both cases, netobimin parent drug was rapidly absorbed, distributed and cleared from the body, or metabolized into albendazole metabolites. Detectable levels of albendazole metabolites for up to 96 hours post-treatment using the intraruminal route of administration however, demonstrated a more efficient conversion of netobimin into albendazole metabolites which may account for the high clinical efficacy of netobimin observed during GI administration. Activation of netobimin is dependant on both reduction and cyclization processes carried out to a large extent by the GI flora, thus making the oral route of administration the most practical in terms of anthelmintic efficacy. Gyurik *et al.* (1981) determined quantities of urinary metabolites of radio-labelled albendazole in the rat: albendazole was present in only minor amounts (1.9% of total excreted in urine, feces and bile), albendazole sulphoxide was the principal urinary metabolite (26.6%) along with smaller quantities of albendazole sulfone (5.5%). The relatively small quantities of albendazole sulfone can be attributed to further hydroxylation of the

inactive metabolite forming 3 additional polar metabolites, thus facilitating urinary excretion. The high plasma levels of albendazole sulphoxide from 1 to 96 hours post-treatment (Marriner, 1980; Galtier *et al.*, 1991) and the low ratio of sulphone/sulphoxide (Marriner, 1980; Gyurik *et al.*, 1981) are of interest in terms of anthelmintic efficacy.

1.4. PHARMACOKINETICS

Pharmacokinetics is the study of the kinetics of absorption, distribution, metabolism and elimination of drugs and their pharmacologic, therapeutic or toxic response in man and animals (Notari, 1987). Bioavailability defines the extent to which a drug reaches the general circulation. Maximum bioavailability occurs when a drug is completely absorbed from the site of administration, or alternatively is administered via the vascular system.

1.4.1. Absorption

Many variables can influence the absorption of drugs: drugs given in an aqueous solution are more readily absorbed than those given in an oily solution, suspension or solid form because they mix more readily with the aqueous phase at the absorptive site. The dissolution rate of solid form drugs also has a direct effect on rate of absorption. In addition, the circulation to the site of absorption also effects drug absorption: increased blood flow enhancing the absorption

rate, decreased blood flow slowing absorption.

After absorption, orally administered drugs must first pass through the liver before reaching the systemic circulation. If the drug is metabolized in the liver or excreted in the bile bioavailability will be decreased; this is the so-called first-pass effect. Once in the bloodstream, most drugs can bind reversibly to plasma proteins, or alternatively, undergo distribution, metabolism and excretion (Baggot, 1978). A schematic representation of the interrelationship of the absorption, distribution, binding, biotransformation, and excretion of a drug is represented in Figure 1.1.

1.4.2. Biotransformation of Drugs

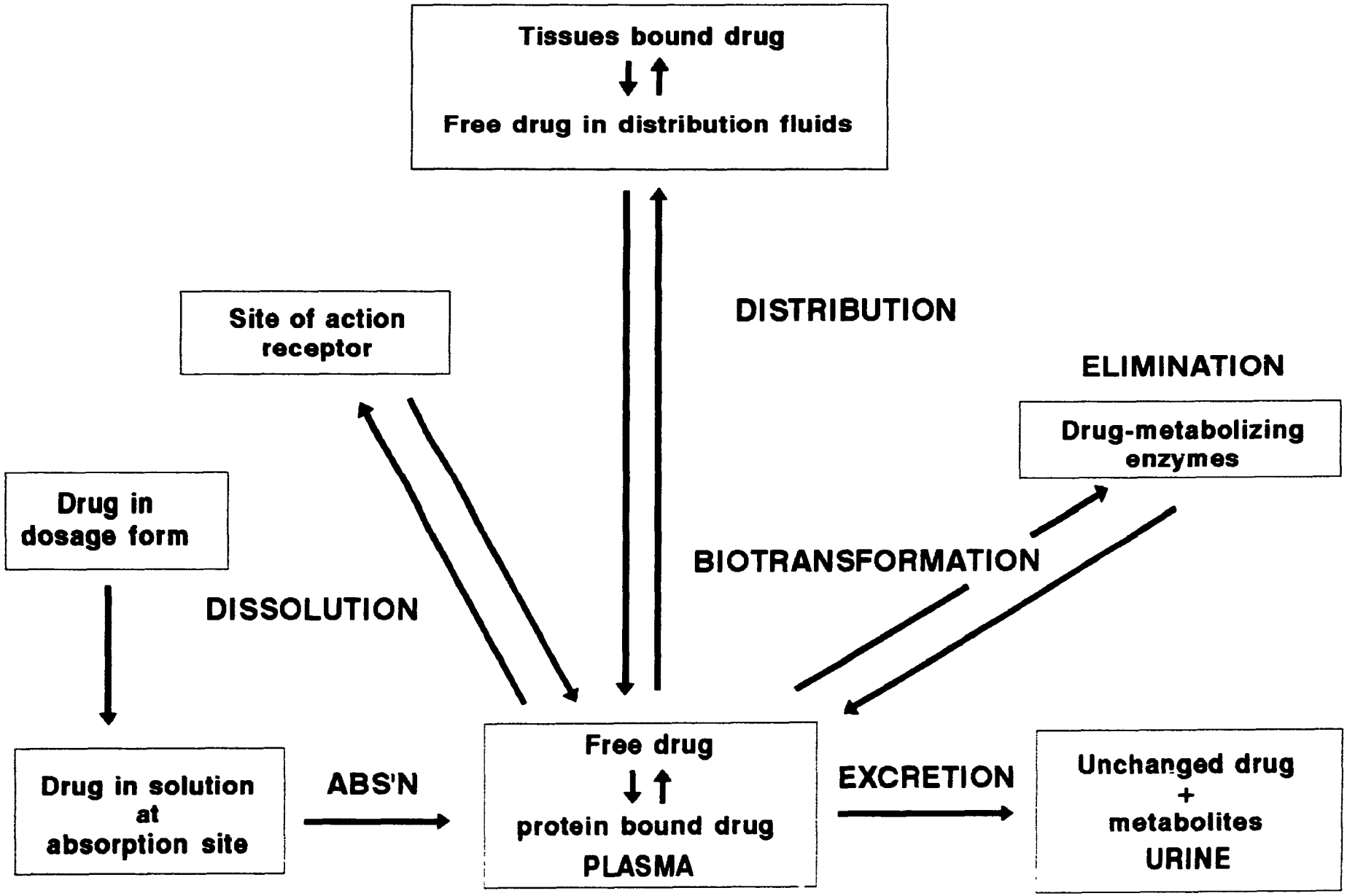
Biotransformation is the process by which the body transforms drugs into more polar and less lipid soluble metabolites thus enhancing their excretion. The metabolism of a drug determines its pharmacological activity, efficacy and toxicity in the body. Drug metabolism in mammals predominantly occurs in the liver and involves a biphasic pattern of metabolism. Phase I involves the introduction of a functional group (-OH, $-NH_2$, -SH) that converts the parent drug to a more polar metabolite. Phase I reactions often render the metabolite inactive but in some cases a more active metabolite is transformed from the parent molecule. In phase

II, the metabolites are conjugated through the functional group produced in the phase I reaction by such substances as glucuronic acid, acetate, sulphate or bile salts producing highly polar metabolites which can then be eliminated in the urine or bile. In the phase I reaction some compounds are metabolized through the flavin-monooxygenase (FMO) systems but most are metabolized through the heme-containing microsomal mixed function oxidase system involving the cytochrome P-450 enzyme complex. Cytochrome P-450 is a family of isoenzymes located in the microsomal fraction of the endoplasmic reticulum. The enzyme reaction initially involves the binding of a drug substrate to cytochrome P-450, the oxidized cytochrome P-450-substrate complex is then reduced by 2 electrons furnished by NADPH. Molecular oxygen then binds to the cytochrome P-450-substrate complex, the oxidized metabolite then dissociates and cytochrome P-450 is ready for another cycle.

1.4.3. The Relationship between Pharmacokinetics and Anthelmintic Efficacy

The broad spectrum activity of various anthelmintics combined with the large variety of tissues inhabited by internal parasites (mucosal surface of GI tract, liver, lungs, skin) makes it necessary for an anthelmintic to be absorbed so that parent drug and/or active metabolites can be transported to the site of infection. Benzimidazole

Figure 1.1. Schematic representation of the various processes that influence uptake, mechanism of action, and excretion of a drug and its metabolites from the body (Baggot, 1978).


Tissues bound drug
Free drug in distribution fluids
Site of action receptor
DISTRIBUTION
ELIMINATION
Drug-metabolizing enzymes
Drug in dosage form
BIOTRANSFORMATION
DISSOLUTION
Drug in solution at absorption site
ABS'N
Free drug
protein bound drug
PLASMA
EXCRETION
Unchanged drug
+
metabolites
URINE

anthelmintic activity is dependent on both drug affinity for parasite tubulin and a sustained effective anthelmintic concentration at the site of the parasitic infection, making the pharmacokinetic behavior of the anthelmintic an important determinant of drug efficacy (ie: parasite expulsion). It is interesting that parenteral administration of the benzimidazole oxfendazole may be equally or more effective than oral administration when treating the gastrointestinal parasite *Haemonchus contortus*. This is thought to be due to the significantly higher plasma levels achieved with parenteral administration of the drug (Hennessy and Prichard, 1981). In contrast, a study done by McKellar *et al.* (1991) using the intestinal parasite *Nematodirus battus* achieved significantly higher plasma levels of ivermectin when administered subcutaneously; however a greater expulsion of the parasite was achieved when the drug was given by the oral route. It has been demonstrated that the outcome of therapy when treating tissue inhabiting parasites (ie: parasites which infect other than the GI tract) is directly dependent on plasma concentrations achieved within the host (Elkassaby, 1991; Aziz *et al.*, 1982; Bircher *et al.*, 1984). Plasma concentrations of orally administered mebendazole and albendazole have been shown to be markedly higher when the drug is given with a fatty meal (Munst *et al.*, 1980; Lange *et al.*, 1988). Reasons for this are unknown, however one explanation may be that delayed gastric emptying may lead to

better dissolution of the drug in the stomach before it passes into the optimal absorption environment of the small intestine (Welling, 1977). These results may have practical implications with regard to the dosing regimen used during the treatment of parasitic infection. If a high systemic availability of the drug is desired then it may be practical to administer the anthelmintic together with a fatty meal.

CHAPTER 2

THE EFFECTS OF IRON DEFICIENCY ON THE EFFICACY OF ALBENDAZOLE IN BALB/C MICE INFECTED WITH HELIGMOSOMOIDES POLYGYRUS

2.1. INTRODUCTION

Nutrition and parasitism are inextricably linked. Roughly half of the world's population lives under conditions that generate both nutritional stress and parasitic disease (Crompton, 1986). Host food intake, absorption, metabolism and nutrient utilization may be disrupted by a parasitic infection; in addition, the growth, reproduction and pathogenicity of parasites may be altered in response to the specific nutrients consumed or not consumed by the host. Manifestations of iron deficiency specifically, are among the most common symptoms of undernourishment in both industrialized and developing countries (Cook, 1986), affecting up to 15 to 25% of the overall population (Briggs and Calloway, 1984).

The first broad-spectrum anthelmintic introduced into the marketplace was thiabendazole in 1961 (Brown *et al.*, 1961). Since then, many new drugs have been developed particularly of the benzimidazole family. The efficacy of these drugs is dependent on the pharmacokinetic profile of the drug, as well as the rate at which the parasite is able to take up the

active metabolites; the nutritional status of an individual and the availability of nutrients are important to the regulation of these parameters. Symptoms of iron deficiency include iron deficiency anemia (Jacobs, 1977), impaired cellular growth of many organs including the liver, thymus and spleen (Kochanowski, 1982), malabsorption of nutrients (Kimber and Weintraub, 1968) due to diarrhea, steatorrhea and gastric achlorhydria, and a decrease in the concentration of cytochrome P-450 in both liver and intestinal cells. This symptomology may in turn effect the pharmacokinetic parameters of the anthelmintic used to treat the parasitic infection, resulting in an altered uptake and metabolism of the drug by the host. This would be of major relevance to the efficacy of the drug against parasitic infection.

It has been shown that a combined iron and protein deficiency causes a significant delay in the expulsion of *Nippostrongylus brasiliensis* from the intestines of infected rats (Bolin *et al.*, 1977). Few studies however, have investigated the effects of nutrient imbalances on anthelmintic efficacy. Duncombe *et al.* (1977, 1979) described a decreased efficacy of the anthelmintics mebendazole, pyrantel and piperazine against *N. brasiliensis* in iron and protein deficient rats. Treatment of adequately nourished rats with the immunosuppressant dexamethasone prior to mebendazole treatment similarly reduced anthelmintic efficacy.

The results therefore suggested that the lowered anthelmintic efficacy was mediated by immune deficiency.

In an attempt to examine the influence of malnutrition on anthelmintic efficacy we have designed an experiment using the mouse-*Heligmosomoides polygyrus* model to determine whether altered drug efficacy occurs during nutrient deficiency and if so, whether it could be explained by other factors independant of the immune response such as alterations in the anthelmintic pharmacokinetic profile. The mouse *H. polygyrus* model was chosen because the immune system is suppressed during a primary infection, thus any differences in drug efficacy between adequately nourished and deficient mice must be due solely to alterations in the pharmacokinetics of the drug. The experiment presented here was designed to evaluate the comparative efficacy of albendazole, a commonly used anthelmintic drug, in both iron-deficient and sufficiently nourished inbred mice infected with the trichostrongylid nematode *H. polygyrus*.

2.2. MATERIALS AND METHODS

2.2.1. The Host

BALB/C female mice (Charles River, Montreal, Canada), three weeks of age were acclimatized to a 14 hours light, 10 hours dark cycle in an animal room maintained at 22-25°C. Upon arrival, mice were randomly divided into iron-deficient,

iron-sufficient and pair-fed subgroups. All mice were housed individually in Nalgene cages (Fisher Scientific, Montreal, Quebec) equipped with stainless steel grids to prevent coprophagy. All mice (excluding pair-feds) were allowed unlimited access to diets and deionized water. All diets were placed into feeders specially designed to minimize food wastage. All feeders, water bottles, cages and grids were acid-washed (10% HCl) and rinsed with deionized water before use in order to minimize iron contamination.

2.2.2. The Diet

All animals were fed a semi-purified diet using low trace element casein as the sole source of protein. The diet contained 1-3 times the requirement based on NRC guidelines (NRC, 1978) for all nutrients except iron. Iron sulphate was used to provide 51 mg/kg iron diet to the iron-supplemented diet. All diets were analyzed for iron concentration by flame atomic absorption spectrophotometry (Perkin-Elmer 360, Perkin-Elmer (Canada) Ltd., Montreal, Quebec) (see appendix II for details of diet).

2.2.3. The Parasite

The parasite is a trichostrongylid nematode, *Heligmosomoides polygyrus*. The strain was originally obtained from A.E. Keymer (Oxford University, UK) and has been maintained in stock CD-1 mice at the university.

2.2.4. Experimental Design

One-hundred-and-twenty BALB/C, female mice were used in total for the experiment. Upon arrival, mice were divided into iron-deficient, control and pair-fed subgroups; deficient and control mice were given the appropriate diet ad-libitum, pair-fed animals received the average amount of food that was consumed by the iron-deficient mice on the previous day of feeding. The pair-fed group was included in order to account for the effects of protein-calorie malnutrition that were expected in the iron deficient mice. Food consumption was recorded daily by subtracting the amount of food present in the feeder from the amount recorded on the previous day of feeding. Body weight was monitored weekly throughout the experiment.

After 5 weeks of acclimitization to the diets, mice were given an oral dose of 150 L_3 larvae of *H. polygyrus*. On day 18 of infection, the feces of all mice were collected over a 24 hour period and egg production per mouse was determined. On day 21 of infection each mouse randomly received a specific dosage of albendazole (0, 5, 10, 15, 20, 25, 30, 35, 40 or 50 mg/kg). Four days later, on day 25 of infection, all animals were bled by cardiac puncture, sacrificed by cervical dislocation and necropsied. Small intestines were removed and opened longitudinally and examined using a dissecting microscope: adult worm burden, sex and any differences in

worm distribution within the small intestine were recorded.

2.2.5. Evaluation of Iron Status

Blood samples were taken weekly from the tail vein in order to monitor the progress of the iron deficiency; total blood hemoglobin concentration was determined by the Cyanomethemoglobin method as described by Coles (1974). On the day of killing, measurements of hemoglobin and hematocrit were immediately performed in order to confirm establishment of an iron deficiency state; sodium heparin was used as the anticoagulant. Remaining whole blood was transferred to serum separator tubes in order to obtain blood serum; serum samples were stored at -20°C for later determination of serum iron concentration. At the time of sacrifice, body weight, wet spleen weight and wet liver weight of half the mice were recorded. Remaining livers were perfused in situ through the superior vena cava with a 1.15% KCL solution, carefully excised and weighed. They were then transferred to acid-washed eppendorf tubes and frozen at -70°C for future analysis of iron, copper and zinc content using the method of Schricker *et al*. (1982).

2.2.6. Statistical Analysis

Analysis of variance (ANOVA) and least significant difference (LSD) tests were used for all variables tested. Worm counts for dose response were transformed to their $\log_{10}$

values before analysis of variance in order to meet the assumption of normality. In all statistical analyses, the level of significance was set at α=0.05.

2.3. RESULTS

2.3.1. Evaluation of Iron Status

2.3.1.1. Hematological parameters

As shown in Table 2.1. and Table 2.2., the iron-restricted diet had various significant effects on both organ weights and hematological parameters. As expected hemoglobin level, hematocrit, serum iron and liver iron concentrations were significantly lower in the iron-deficient group than in the other two groups. No differences were detected in liver zinc or copper concentrations between dietary groups (Fig. 2.1.). There was significant enlargement of the spleen in the DEF group ($p< 0.001$) but no significant difference in the liver wet weight. The mean hemoglobin values (Fig. 2.2.) decreased in the DEF group from 20.5 ± 0.21 to 7.7 ± 0.32 g/dL with significant differences beginning at week 2 of the experiment. All these results lead us to consider the mice in the DEF group as severely anemic as compared to mice in the other two groups.

2.3.1.2. Effect of iron deficiency on growth

Significant differences in both food consumption (Table

2.2. & Fig. 2.3.) and body weight (Table 2.2. & Fig. 2.4.) among the anemic, pair-fed and control animals began to emerge during week 3 and 5 of the experiment, respectively. Throughout the experiment both the iron-deficient and pair-fed groups gained significantly less weight as compared to the control animals. Final body weights resulted in the pair-fed group gaining significantly less weight than the deficient group, which in turn gained significantly less than the control group. The decreased pair-fed body weight in comparison to the deficient group was not surprising. Even though mice from these two groups consumed the same quantity of food, pair-fed mice showed different eating patterns usually consuming their daily portion immediately upon receiving it. This is in contrast to a more continual pattern of consumption observed in both the iron deficient and control *ad-libitum* groups (Nielsen, unpublished observations). The implication is that deficient mice were able to use their nutrients more effectively than their pair-fed counterparts resulting in a significantly increased body weight.

2.3.2. Effect of iron deficiency on egg production and parasite establishment

Measurements of 24 h fecal egg output from infected mice were obtained on day 18 post-infection (Table 2.3). In the iron-deficient and pair-fed groups egg production (eggs/mouse/day) was significantly lower than in control group

animals ($p < 0.01$). Worm burdens were measured on day 25 post-infection (Table 2.3); no differences in worm recovery were observed between diet groups in untreated mice. In addition, there were no differences in the percentage of female worms (Table 2.3) or in the worm distribution within the GI tract between dietary groups (Nielsen, unpublished observations).

2.3.3. Effect of iron deficiency on parasite expulsion: Dose-Response Experiment

Results of the dose response in Table 2.4 and Fig. 2.5 show that at a dose rate of 25, 35, 40 and 50mg/kg, albendazole treatment was significantly less effective at parasite expulsion in deficient animals as compared with control animals ($p < 0.05$). At 50 mg/kg however, parasite expulsion in the pair-fed group was not significantly different from deficient animals indicating a possible effect due to energy restriction.

TABLE 2.1.

Effect of diet on hematological measurements in mice[1,2]

Measure	C	PF	DEF
Hemoglobin (g/dL)[3]	16.41 ± 0.17	15.94 ± 0.22	7.67 ± 0.32*
Hematocrit (%)	66.6 ± 2.1	67.7 ± 1.7	30.5 ± 1.0*
Serum Iron (μg/dL)	398.0 ± 7.1	401.4 ± 7.9	40.2 ± 2.5*
Liver Iron (μg/g)	1.35 ± 0.03	1.13 ± 0.04	1.11 ± 0.02

[1]Values are mean ± SEM, for 18-20 animals except as noted, C=control, PF=pair-fed & DEF=deficient animals.

[2]Significant difference between DEF and both C & PF groups: *$p < 0.001$.

[3]38-40 animals per group.

TABLE 2.2.

Effect of diet on mean total food intake, body weight and relative organ weights in mice[1,2,3]

Measure	C	PF	DEF
Total Food Intake/Mouse (g)	156.8 ± 1.1	141.5 ± 1.4	142.0 ± 1.5*
Initial Bwt (g)	9.55 ± 0.06	9.59 ± 0.06	9.65 ± 0.06
Final Bwt (g)	20.84 ± 0.19	16.76 ± 0.31#	17.87 ± 0.14*
Rel Spleen Wt (%)	0.65 ± 0.02	0.50 ± 0.01	1.74 ± 0.18*
Rel Liver Wt (%)[4]	6.48 ± 0.14	6.76 ± 0.21	6.01 ± 0.11

[1]Values are mean ± SEM, for 38-40 animals except as noted, C=control, PF=pair-fed & DEF=deficient animals.

[2]Significant difference between DEF and both C & PF groups: *p< 0.001.

[3]Significant difference between PF and both C & DEF groups: #p< 0.001

[4]18-20 animals per group.

Fig. 2.1. Mean liver trace iron, zinc and copper concentrations in the different diet groups. The observed decrease in iron in the deficient group is statistically significant at $p < 0.001$. Values are mean iron, zinc and copper ($\mu g/g$)/liver/diet group ± SEM, n=10-12 mice per group.

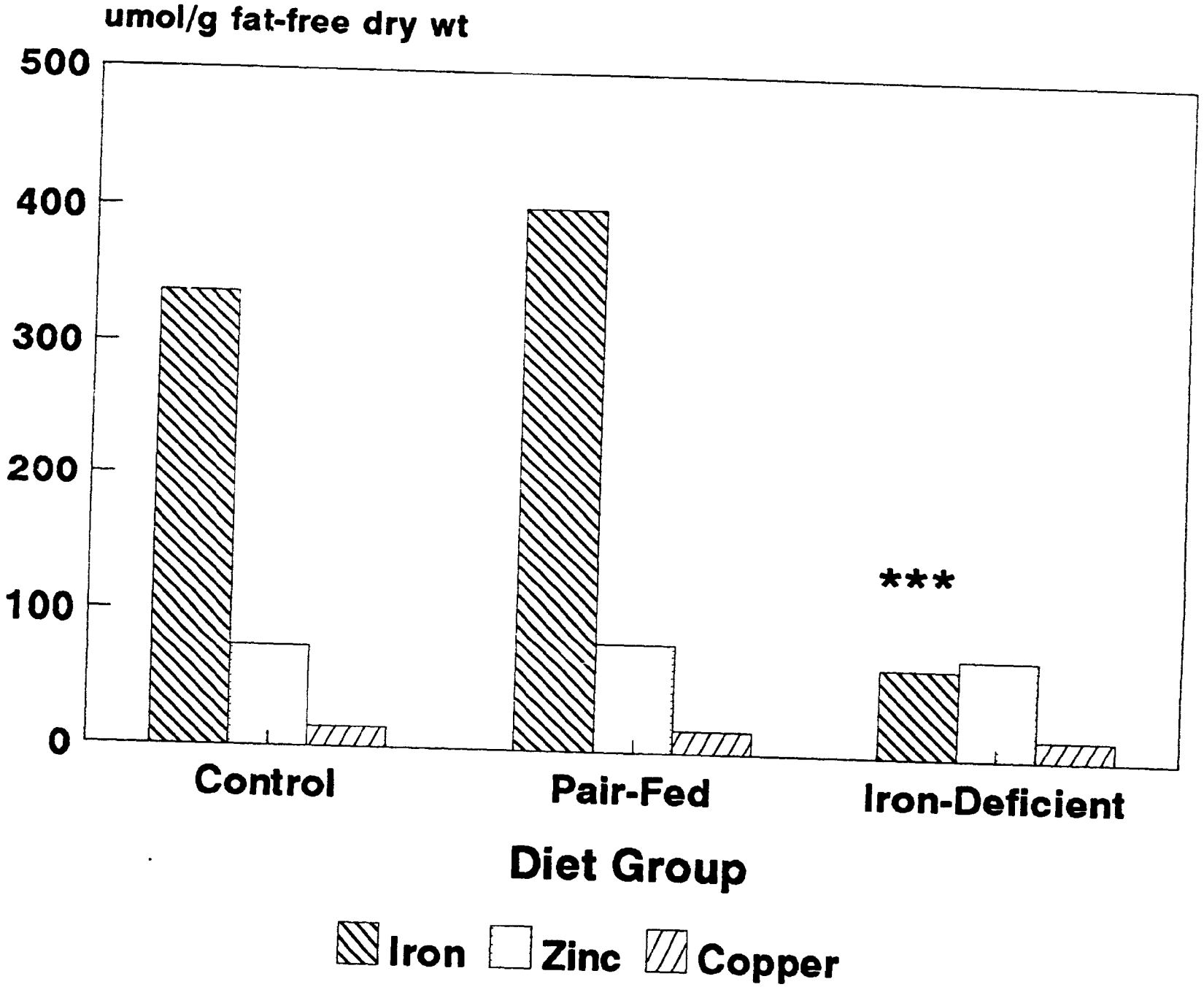

umol/g fat-free dry wt
500
400
300
200
100
0
***
Control
Pair-Fed
Iron-Deficient
Diet Group
Iron
Zinc
Copper

Fig. 2.2. Hemoglobin as a function of time on different diets. The observed decrease in hemoglobin in the deficient group is statistically significant from control & pair-fed animals at $p < 0.05$ at day 14 and $p < 0.001$ thereafter. Values are mean hemoglobin (g/dL), n=20 mice/group.

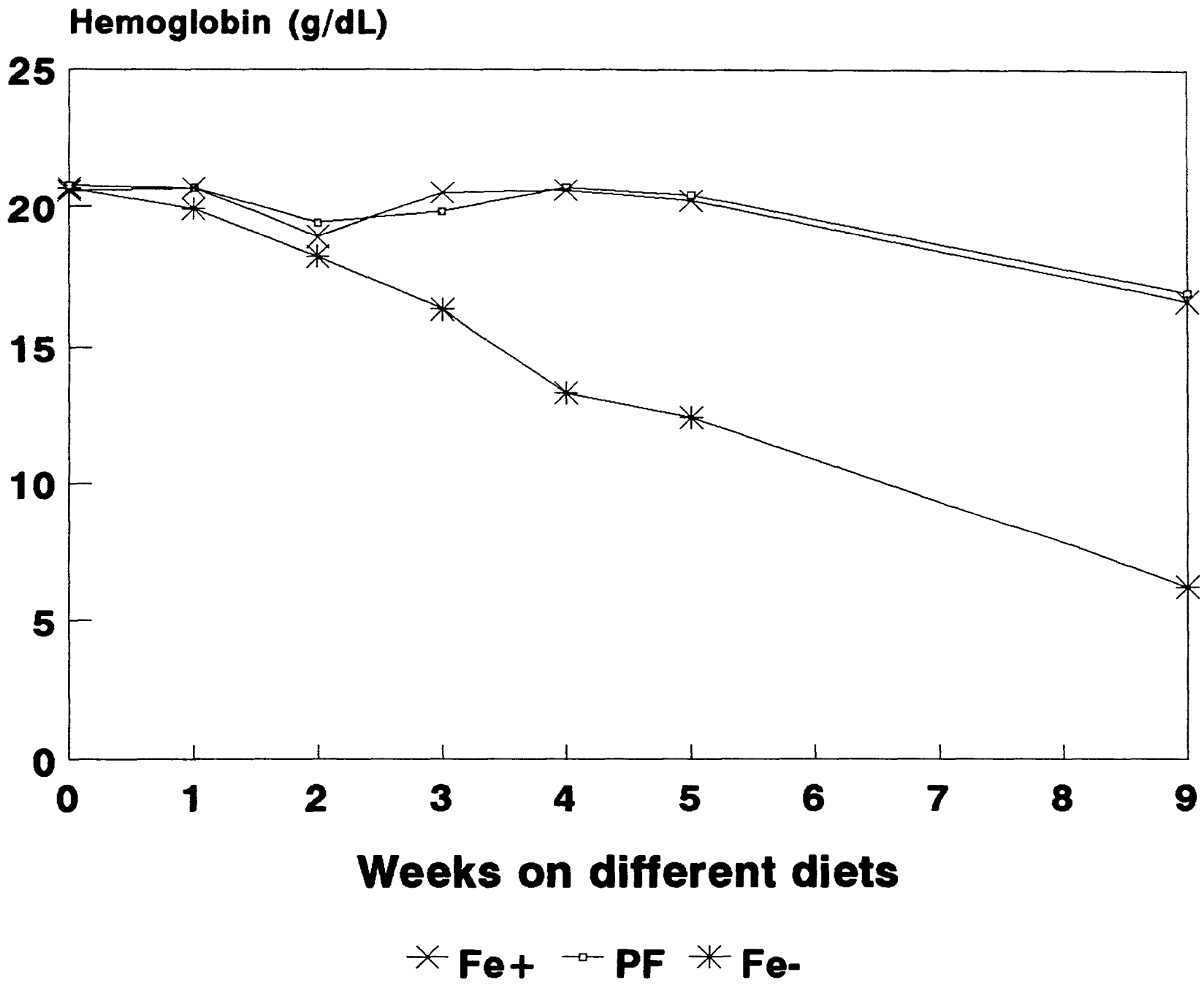

Hemoglobin (g/dL)
25
20
15
10
5
0
0
1
2
3
4
5
6
7
8
9
Weeks on different diets
Fe+
PF
Fe-

Fig. 2.3. Average weekly food intake per diet group as a function of time on the experimental diets. The observed decrease in food intake in the deficient (& PF) group is statistically significant at $p < 0.001$ from week 3 to week 8. Values are average weekly food intake/diet group (n=40).

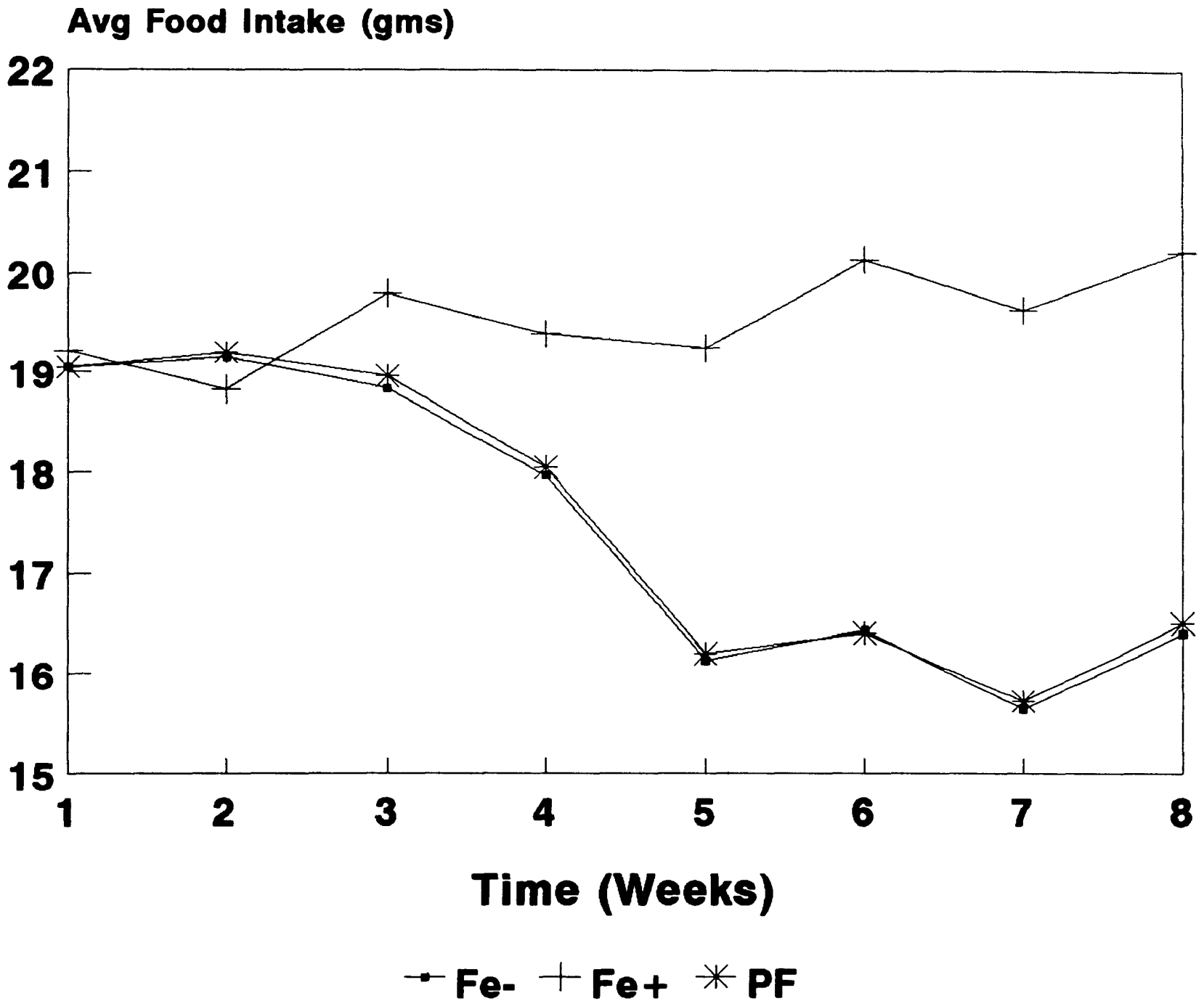

Avg Food Intake (gms)
22
21
20
19
18
17
16
15
1
2
3
4
5
6
7
8
Time (Weeks)
Fe-
Fe+
PF

Fig. 2.4. Average body weight gain as a function of time on the experimental diet. The observed decrease in body weight gain in the deficient (& PF) group is statistically significant at $p < 0.001$ from week 5 to week 8. Values are mean body weight gain, n=40 mice per group.

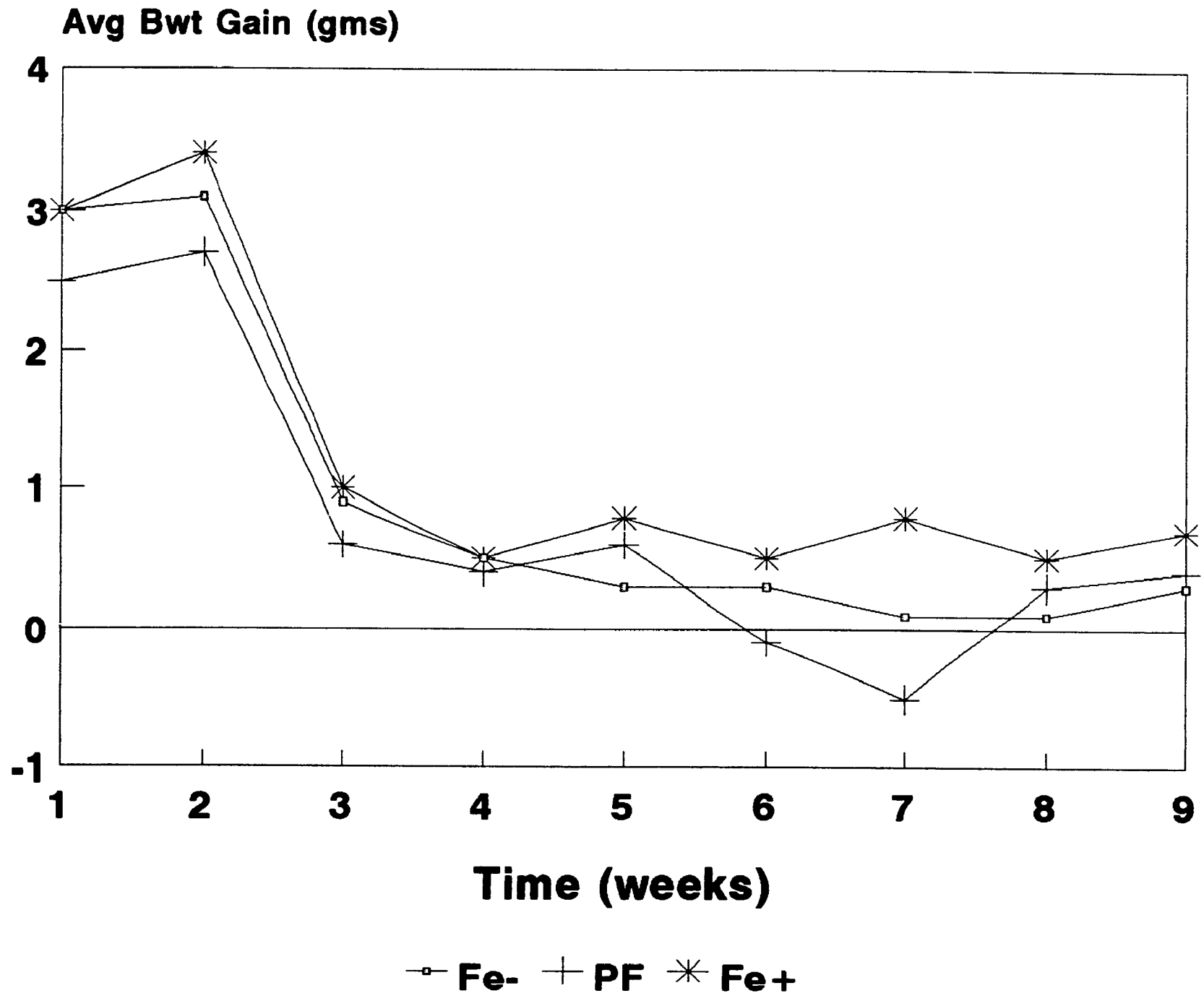

Avg Bwt Gain (gms)
4
3
2
1
0
-1
1
2
3
4
5
6
7
8
9
Time (weeks)
Fe-
PF
Fe+

TABLE 2.3

The influence of diet on egg production and worm establishment[1]

	C	PF	DEF
Eggs/Day[2]	20936 ± 1767	13781 ± 1386*	10815 ± 1316**
#Worms/mouse[3]	75 ± 15	70 ± 9	78 ± 6
#Female Worms/mouse[3]	40 ± 14	37 ± 10	46 ± 9

[1]Values are mean ± SEM, C=control, PF=pair-fed & DEF=deficient mice.

[2]Values expressed as mean eggs/day/mouse for 17-20 mice. Significant difference between C and both PF and DEF groups: *$p < 0.01$, **$p < 0.001$.

[3]4-5 mice/group.

TABLE 2.4.

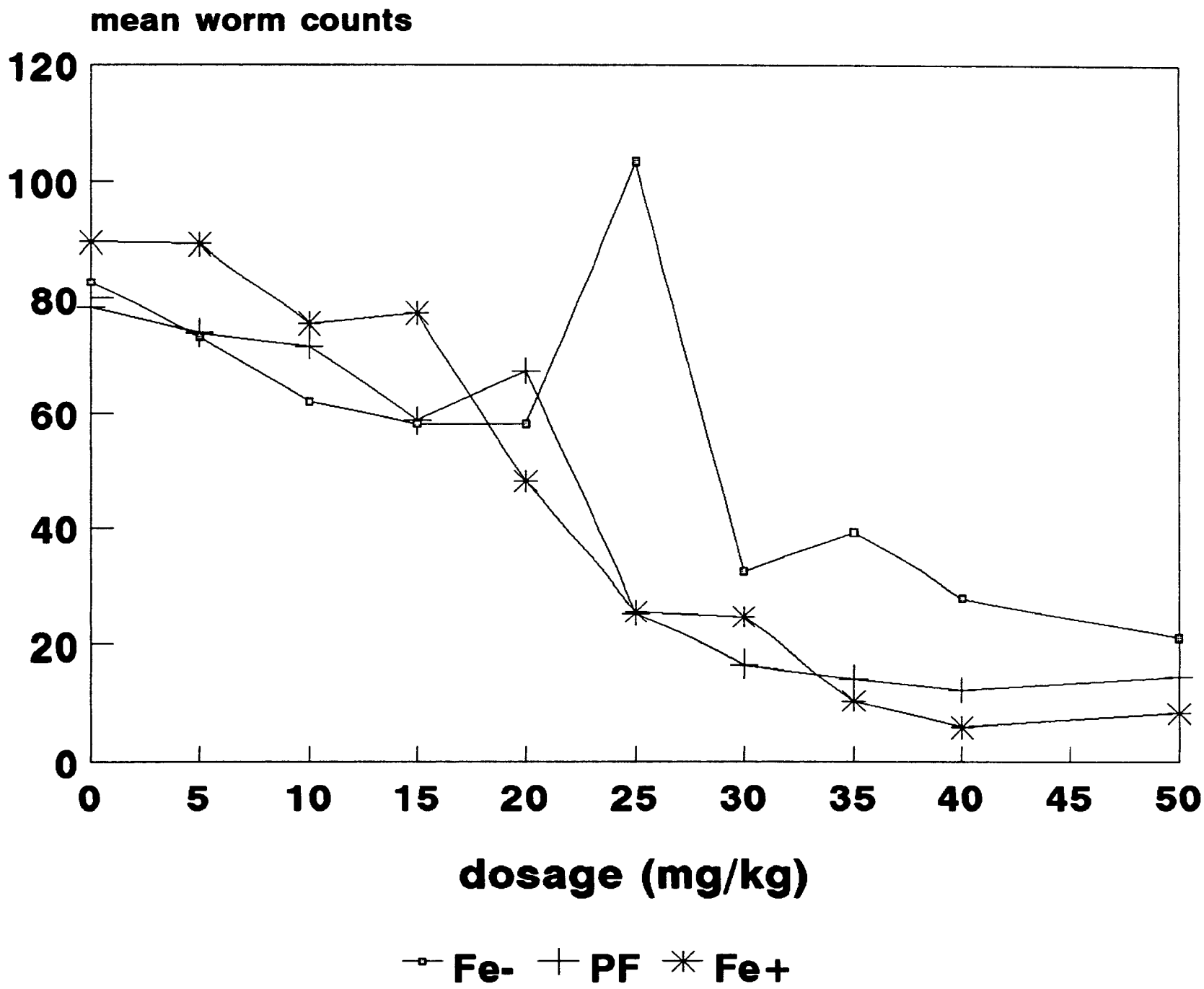
The effect of diet on the anthelmintic efficacy of albendazole[1]

Dose Rate (mg/kg)	Total Worm Counts[2] C	PF	DEF
0	75.00 ± 14.60	69.75 ± 8.59	77.50 ± 5.48
5	89.25 ± 8.10	74.25 ± 10.38	73.75 ± 4.27
10	75.75 ± 5.41	72.00 ± 7.54	62.00 ± 9.49
15	77.50 ± 7.79	58.75 ± 11.48	58.25 ± 9.14
20	57.25 ± 14.79	67.50 ± 24.77	58.25 ± 10.97
25	27.50 ± 4.77	25.50 ± 7.46	103.25 ± 17.30***
30	24.80 ± 0.66	16.75 ± 4.01	32.25 ± 13.01
35	10.20 ± 4.61	14.25 ± 4.50	39.25 ± 5.12**
40	5.00 ± 3.37	12.25 ± 4.59	28.25 ± 5.50***
50	8.00 ± 3.92	14.75 ± 4.87	21.0 ± 6.45*

[1]Values are mean ± SEM, C=control, PF=pair-fed & DEF=deficient animals.

[2]Values expressed as mean worm counts for 4-5 mice. Significant difference between DEF and both PF and C groups: *$p < 0.05$, **$p < 0.01$ & ***$p < 0.001$.

Fig. 2.5. Dose-response of albendazole in iron-deficient, pair-fed and control mice exposed to 150 L_3 of *H. polygyrus*. Values are mean worm counts ± SEM (n=4-5).


mean worm counts
120
100
80
60
40
20
0
0
5
10
15
20
25
30
35
40
45
50
dosage (mg/kg)
Fe-
PF
Fe+

2.4. DISCUSSION

In the present study, we found that the growth rate of mice fed the iron-deficient diet and consequently the pair-fed diet was significantly lower than mice fed the iron-supplemented diet. The diminished body weight of mice fed the low iron diet is consistent with observations of other investigators (Prichard *et al.*, 1981; Kuvibidila *et al.*, 1983; Kochanowski & Sherman, 1982, 1985; Dhur *et al.*, 1989). The lower hemoglobin level, hematocrit, serum iron and liver iron stores of the iron-deficient group support the results of several authors (Finch *et al.*, 1976; Kochanowski & Sherman, 1982, 1985; Dhur *et al.*, 1989). No significant effect of iron restriction on zinc or copper liver stores was observed. These results are consistent with a study by Minkus *et al.* (1992) who reported no significant differences in trace mineral liver concentrations in mice undergoing zinc deficiency. Other investigators (Kirchgessner *et al.*, 1982; Solomons, N.W., 1986) however, have reported a competitive interaction between iron, copper and zinc in the diet which is reflected in liver trace mineral stores. In this experiment it is possible that the degree of iron deficiency was not substantial enough to produce alterations in liver trace mineral stores, or alternatively, other body stores (ie: spleen, muscle) not measured in this experiment were more sensitive to these mineral interactions. The wet weight of spleens from the deficient group were consistently heavier

than controls supporting the premise of increased splenomegaly during iron deficiency (Kuvibidila *et al.*, 1981, 1983). In addition, clinical symptoms of iron deficiency such as a loss of hair, dry skin and reduced levels of activity became apparent by week 6 of the experiment (Nielsen, unpublished observations).

Daily parasite egg production was significantly lower in the iron-deficient and pair-fed groups as compared to the control group indicating an effect due to energy restriction rather than iron deficiency. This result is consistent with data from other studies (Jose & Welch, 1970; Boddington & Mettrick, 1981) in which nutrient deficiencies causing a decreased food consumption resulted in reduced parasite fecundity. Reasons for this are unclear however; it has been hypothesized that the impact of malnutrition on the parasite is modified by host genetic factors (Bundy & Golden, 1986). Observations during *Nippostrongylus brasiliensis* infection in different strains of rats support this concept (El-Hag, 1983): During the early stages of infection, egg production was independent of the dietary regime imposed on the host (zinc-deficient, pair-fed control, control *ad-libitum*). At the time of immuno-expulsion both control groups were able mount an effective expulsion of the parasite; in the deficient group worms were not expelled, however ovogenesis ceased.

In contrast, iron deficiency did not effect worm establishment thus suggesting that host iron status is not an important determinant of worm burden. These results are in contrast with several investigators (Jose and Welch, 1970; Bundy and Golden, 1987) who have observed significantly higher worm burdens in malnourished hosts. Reasons for this are unknown however, one could assume that worm establishment would vary with the species of the host, as well as the age and degree of deficiency (or deficiencies) induced in the host.

This study indicates that in the *H. polygyrus*-mouse model, iron deficiency significantly reduces the efficacy of the benzimidazole anthelmintic albendazole. This is in agreement with studies conducted by Duncombe *et al.*, (1977, 1979b) in which he demonstrated a decreased efficacy of mebendazole against *Nippostrongylus brasiliensis* infections in rats maintained on an iron and protein deficient diet. In this experiment, only at higher dosage levels (25, 35, 40 and 50 mg/kg) when parasites were being effectively expelled from the host were any differences in efficacy observed. The relatively large dosages required for effective expulsion of the parasite from the G.I. tract came as somewhat as a surprise, as other studies (Marriner *et al.*, 1980; Gyurik *et al.*, 1981) have needed much smaller dosages of albendazole (5-10 mg/kg) for effective parasite elimination.

This study indicates the complex interactions between nutrient deficiency, parasitic infection and anthelmintic metabolism. In a study conducted by *Duncombe et al.* (1979b) it was found that efficacy of mebendazole was significantly reduced by iron deficiency alone. Treatment of control rats with the immunosuppressant dexamethasone prior to anthelmintic treatment similarly reduced drug efficacy, thus leading the investigators to conclude that impairment of the immune response by malnutrition lead to decreased drug efficacy in the rats. In this study however, the immune response is suppressed during a primary infection therefore the mechanism of decreased efficacy due to malnutrition must be mediated by other factors independent of the immune system. Impairment of efficacy could be explained in terms of alterations in the pharmacokinetic profile of the anthelmintic or in the uptake of drug by the parasites. The explanation of reduced efficacy however, awaits further experimentation in order to unravel all the possibilities.

CHAPTER 3

THE EFFECTS OF IRON DEFICIENCY ON THE PHARMACOKINETICS OF ALBENDAZOLE IN MICE

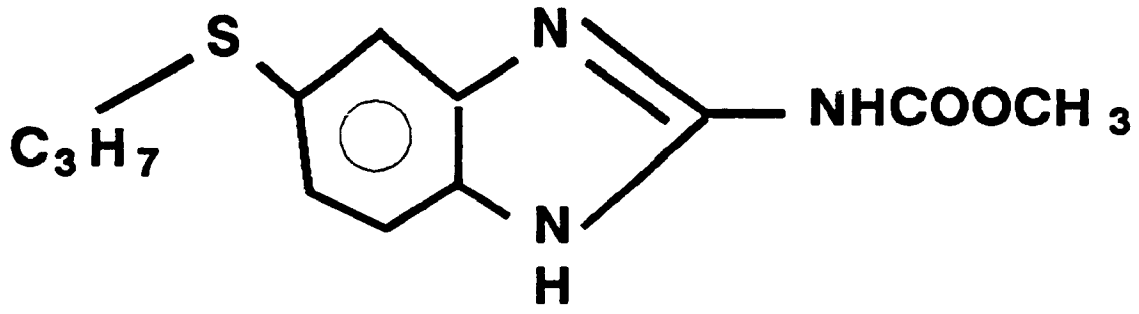
3.1. INTRODUCTION

Malnutrition and helminth infection are amongst the most prevalent chronic conditions affecting human health globally (Bundy and Golden, 1986). Albendazole is a commonly used broad-spectrum anthelmintic effective against nematodes, cestodes and trematodes. Metabolism of albendazole involves oxidation of the sulphur side chain of the parent compound to form albendazole sulphoxide, the principally active pharmacological agent; this reaction is catalyzed by both cytochrome P-450 enzymes and flavin-containing, NADPH dependent monooxygenases (MFMO). Metabolism of albendazole sulphoxide to the inactive component, albendazole sulphone, involves another oxidation process, this time using only cytochrome P-450 in the reaction (see Fig. 3.1).

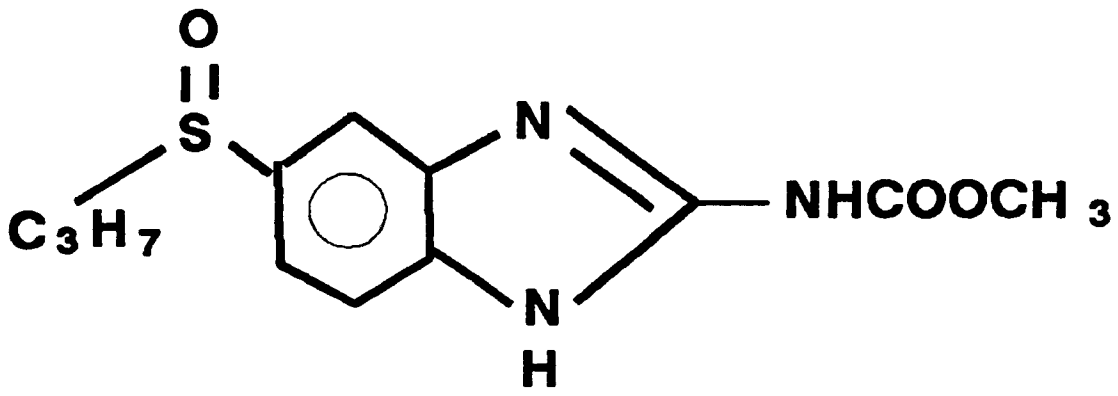
Drug efficacy is dependent on the absorption, distribution and clearance rate of the drug, as well as the rate at which the parasite is able to take up the active metabolites; the nutritional status of an individual and the availability of nutrients are important to the regulation of these parameters. The next series of experiments will attempt to characterize the plasma concentration profiles of

albendazole in both iron-deficient and sufficiently nourished mice and the effects of iron deficiency on the concentration of cytochrome P-450 in the liver and intestine. The purpose of this study was to determine whether the decreased drug efficacy observed in the previous experiment can be explained in terms of any alterations in anthelmintic pharmacokinetic behavior.

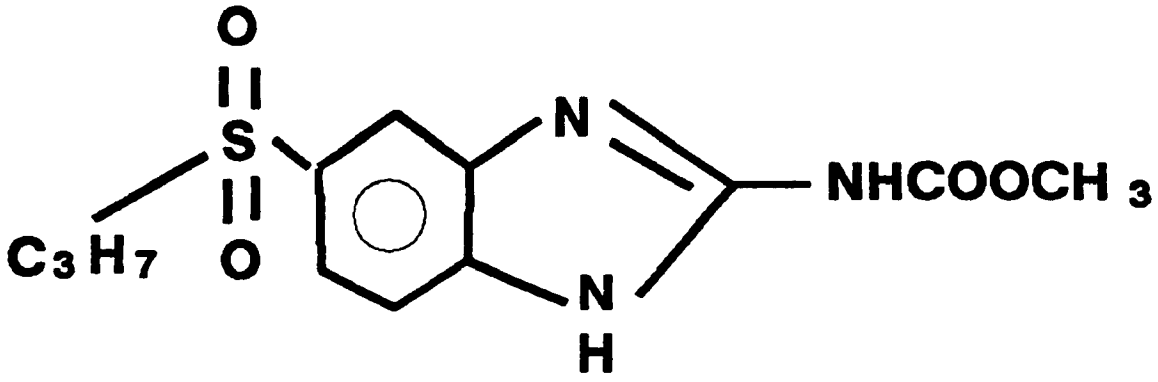
Figure 3.1. Biotransformation pathway for albendazole



ALBENDAZOLE



ALBENDAZOLE SULPHOXIDE



ALBENDAZOLE SULPHONE

3.2. MATERIALS AND METHODS

3.2.1. Experimental Animals and Diet

The study was conducted on a total of 120 BALB/C female mice, weighing from 9 to 10 grams. Mice were randomly assigned upon arrival into 3 subgroups of 40 mice each comprising the control *ad-libitum*, pair-fed and iron-deficient groups. All mice were housed in individual cages and fed *ad-libitum* with the exception of pair-fed mice which were given the amount of food consumed by the iron-deficient group on the previous day of feeding; this was done in order to account for any effects in the deficient group due to energy depletion rather than actual iron deficiency. Water was offered *ad-libitum*. Cages, feeders and water bottles were acid-washed in a 10% hydrochloric acid solution and rinsed three times in deionized water in order to eliminate any extraneous trace iron present on the equipment. The mice were fed the appropriate diet as described previously (Section 2.2.2.) for a period of 5 weeks. The diet was adequate in all nutrients except iron in the deficient group, which contained 2 mg iron/kg, as determined by atomic absorption spectrophotometry. Food consumption was recorded daily by subtracting the amount of food present in the feeder from the amount recorded on the previous day of feeding. Body weight was monitored weekly throughout the experiment. At the end of the feeding schedule, all mice were bled by cardiac puncture and sacrificed by cervical dislocation. Subsequent organ removal

was rapidly performed in order to ensure that no induction of P-450 had occurred.

3.2.2. Blood Samples

Blood samples were withdrawn from the heart and take partly for hemogram determinations (hemoglobin, serum iron concentration) and partly for analysis of drug metabolite concentrations present in the plasma. A 20 μl sample of whole blood was immediately transferred to tubes containing cyanmethemoglobin reagent for analysis of blood hemoglobin. Approximately 50 μl of whole blood was transferred to serum separator tubes in order to obtain blood serum; serum samples were stored at -20°C for later determination of serum iron concentration. Remaining whole blood was transferred to eppendorf tubes treated with sodium heparin; plasma was separated by centrifugation at 3000 *g* for 10 minutes, then frozen at -20°C until analyzed for albendazole and metabolites within two months.

3.2.3. Organ Samples

At the time of sacrifice, body weight and wet spleen weight of all mice were recorded. In order to measure cytochrome P-450 content the method of Omuro and Sato (1964) was employed. A 15-cm piece of duodenum was excised and gently washed with ice-cold 1.15% KCl solution. After a sonication the solution was first centrifuged at 11,000 x *g*

for 15 minutes to eliminate the mitochondrial fraction, and then ultacentrifuged at 100,000 x *g* for 1 hour to separate the cytosolic and microsomal fractions. The pellet (microsomal preparation) was resuspended in 3 volumes of 1.15% KCl solution and protein content immediately determined by Lowry assay (Lowry *et al.*, 1951) using bovine serum albumin as the standard protein. Microsomes were stored at -80°C for 2-3 days until used for assays. Livers were perfused in situ by the superior vena cava and rinsed for 30 seconds with cold 0.1 M KCl (to remove hemoglobin). Half of the livers were transferred to acid-washed plastic vials and stored at -80°C for future analysis of mineral content using the wet-ashing method of Schricker, *et al.* (1982). Remaining livers were weighed, homogenized and placed in four volumes of iced buffer (1.15% KCl) and the homogenate was treated like intestinal cells in solution for analysis of protein content and cytochrome P-450 concentration.

3.2.4. Microsomal enzyme analysis

The method of Omura and Sato (1964) was used for measurement of cytochrome P-450 concentration. Difference spectra of microsomal preparations were measured using a DU-7 Beckman spectrophotometer. Microsomal preparations were adjusted to 1 mg of protein per ml of 0.1 M of KCl buffer (pH 7.0), reduction of the samples was achieved by the addition of a few milligrams of solid dithionite ($Na_2S_2O_4$) to the solution.

After recording the baseline of the reduced sample cell (400-550 nm), the content of the cell was saturated with carbon monoxide (carefully bubbled through the sample for about 30 seconds) causing a reversible binding to the P-450. The carbon monoxide bound P-450 was again scanned and the difference spectrum measured.

3.2.5. Analytical procedures prior to HPLC analysis

3.2.5.1. Sample Extraction

After thawing, plasma samples (200 µl) were spiked with an internal standard (oxibendazole, 1 ug/10 µl methanol) and prepared for high performance liquid chromatography (HPLC) analysis.

The sample extraction was as described by Allan *et al.* (1980) and modified by Hennessy *et al.* (1985) using C_{18} Sep Pak cartridges (Part No. 51910, Waters Associates, Milford, Mass., USA). Each cartridge was prepared by flushing with 5 ml HPLC grade methanol (Fisher Scientific, Canada) followed by 5 ml of aqueous ammonium dihydrogen phosphate 0.017 M, pH 5.5. After passing through the spiked plasma, the cartridge was successively washed with 20 ml distilled water, 0.5 ml methanol (40%), 0.4 ml methanol (100%) and 2.5 ml methanol (100%). Albendazole and metabolites which eluted in the last 2.5 ml methanol were concentrated under a stream of nitrogen, resuspended in 0.5 ml of methanol and refrigerated until

analyzed by HPLC.

3.2.5.2. Drug/metabolite analysis

Prior to sample analysis, standard solutions of albendazole and metabolites were quantified on a LKB Bromma HPLC system (LKB, Bromma, Sweden) using an autosampler (LKB, Model 2153), Bondex 10 C_{18} reverse phase column (Phenomenex, CA, USA), LKB 2150 solvent delivery pumps, and a LKB spectral variable wavelength absorbance detector (Model 2140) reading at 292 nm for albendazole and metabolites. The mobile phase consisted of an acetonitrile/ammonium acetate (0.025 M) solution which alternated in the following proportions throughout the analysis: 28/72 (8 min), 42/58 (15 min) and 28/72 (6 min). The flow rate was 1.0 ml/min which produced a column pressure of 8.5 to 9.5 MPa. A typical chromatographic separation for albendazole and its metabolites in a spiked plasma sample is shown in Fig. 3.2.

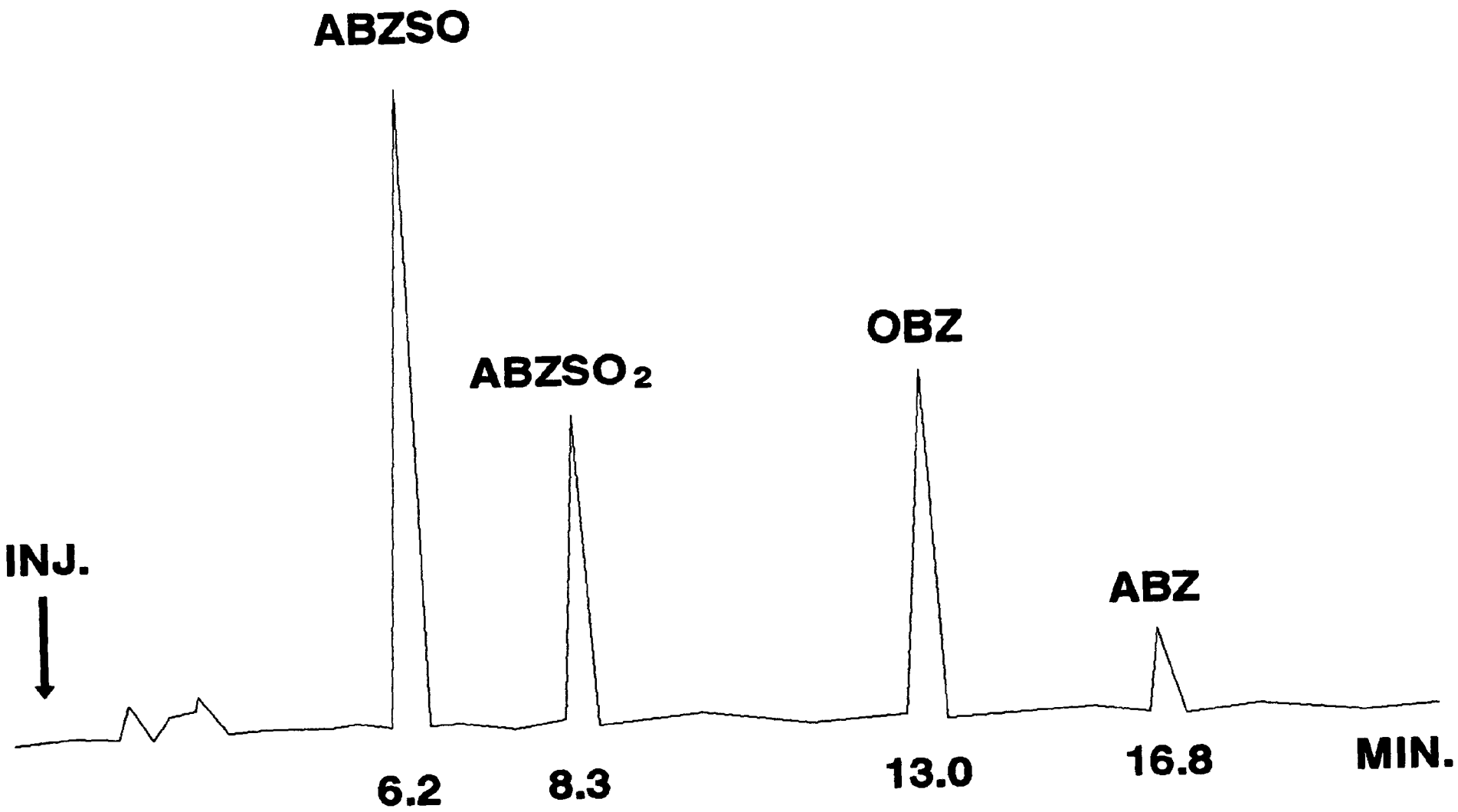
Identification of albendazole and metabolites was done by comparing retention times of samples to that of the pure reference standards. Previously, calibration curves were made up using various concentrations of the standard solutions, unknown sample concentrations of metabolites were calculated by comparison of each metabolite to the specific calibration curve. Peak area were calculated using a Nelson Analytical Software, model 2600 (Nelson Analytical, Inc., CA, USA) on an

IBM-AT computer. The limits of detection were 0.020 μg/ml for albendazole and albendazole sulphoxide and 0.025 μg/ml for albendazole sulphone.

3.2.5.3. Statistical Analysis

All variables were tested using standard one-way analysis of variance (ANOVA) followed by least-significant difference test (LSD). In all statistical analyses, the level of significance was set at α=0.05.

Fig. 3.2. Typical chromatographic separation for ABZ and its metabolites in a spiked plasma sample extracted and processed as described in 3.2.4.2. using oxibendazole as the internal standard. ABZ, although never detected in this experiment, should have eluted at 16.8 minutes post-injection.


ABZSO
ABZSO2
OBZ
ABZ
INJ.
6.2
8.3
13.0
16.8
MIN.

3.3. RESULTS

3.3.1. Evaluation of Iron Status

3.3.1.1. Hematological parameters

The effects of an iron-deficient diet on both hematological parameters and organ weights are illustrated in Table 3.1. As expected, hemoglobin levels and serum iron concentrations significantly decreased ($p < 0.001$) in the deficient group to 47% and 10% of the control values, respectively. The mean liver iron stores of the control and pair-fed mice were significantly lower than that of the deficient group ($p < 0.001$). Iron deficiency induced significant enlargement of the spleen ($p<0.001$) and a slight but not significant decrease in wet liver weight.

3.3.1.2. Effect of iron deficiency on growth

Significant differences in food consumption between control and deficient mice began to emerge during week 2 of the experiment (Fig. 3.3). Throughout the experiment both the iron-deficient and pair-fed groups gained significantly less weight as compared to the control animals. Final body weights resulted in the pair-fed group gaining significantly less weight than the deficient group ($p < 0.001$), which in turn gained significantly less than the control group ($p < 0.01$) (Table 3.1).

3.3.2. Effect of Iron Deficiency on Cytochrome P-450 Concentration in Liver and Intestinal Microsomes

As shown in Figure 3.4, no differences in P-450 concentration were observed in the liver microsomal preparations between diet groups. Intestinal P-450 however, was markedly lower ($p < 0.01$) in the iron deficient mice as compared to both control and pair-fed mice.

3.3.3. Effect of Iron Deficiency on Plasma Concentrations of Albendazole

As shown in Figures 3.5 & 3.6, no significant differences were observed in the plasma concentration profiles of albendazole sulphoxide and sulphone. Interestingly, unchanged albendazole was not detected in the plasma at any point during the analysis indicating a rapid metabolism of the parent drug to the pharmacologically active sulphoxide metabolite.

TABLE 3.1.

Body weights, organ weights and hematological measurements[1,2]

Measure	C	PF	DEF
Total Food Intake/Mouse (g)	107.7 ± 1.3	100.5 ± 0.8	100.7 ± 1.0$^{\#\#}$
Initial Bwt (g)[3]	9.64 ± 0.11	9.46 ± 0.11	9.77 ± 0.11
Final Bwt (g)[3]	19.51 ± 0.18	17.99 ± 0.15$^{\#}$	18.83 ± 0.16**
Hgb (g/dL)[3]	18.30 ± 0.16	18.54 ± 0.15	12.59 ± 0.13***
Serum Fe (ug/dL)	396.7 ± 8.1	389.4 ± 7.4	77.5 ± 16.6***
Liver iron (ug/g)	389.2 ± 41.3	357.3 ± 37.5	65.8 ± 13.2***
Rel Spleen wt (%)[3]	0.58 ± 0.02	0.52 ± 0.01	0.96 ± 0.04***
Rel Liver wt (%)	6.20 ± 0.21	6.39 ± 0.17	5.94 ± 0.18

[1]Values are mean ± SEM, for 8-10 animals except as noted, C=control, PF=pair-fed & DEF=deficient animals.

[2]Significant difference between DEF and both C & PF groups: $^{***}p< 0.001$, $^{**}p< 0.01$; significant difference between PF and both C and DEF groups: $^{\#}p< 0.001$; significant difference between DEF & C and PF & C: $^{\#\#}p< 0.01$.

[3]35-40 animals per group.

Fig. 3.3. Average weekly food intake per diet group as a function of time on the experimental diets. The observed decrease in food intake in the deficient (& PF) group is statistically significant at $p < 0.05$ at week 2 & week 3 and $p < 0.001$ at week 4 & week 5. Values are average weekly food intake/group (n=40).

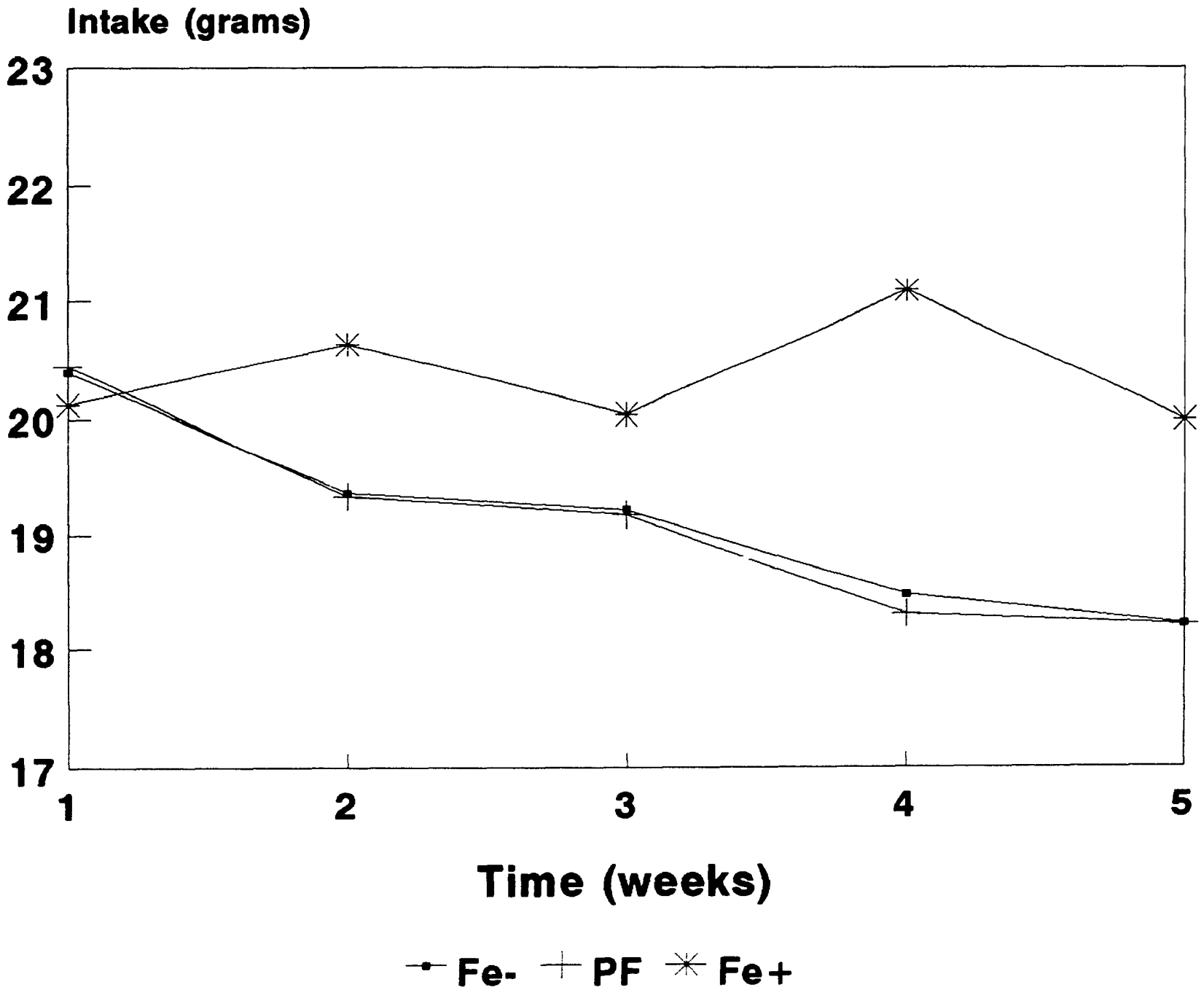

Intake (grams)
23
22
21
20
19
18
17
1
2
3
4
5
Time (weeks)
Fe-
PF
Fe+

Fig. 3.4. Cytochrome P-450 content in the livers and intestines of moderately iron-deficient mice. The observed decrease in intestinal cyt. P-450 content in the deficient group is statistically significant at ** $p < 0.01$. Values are means ± SEM for 7-8 animals.

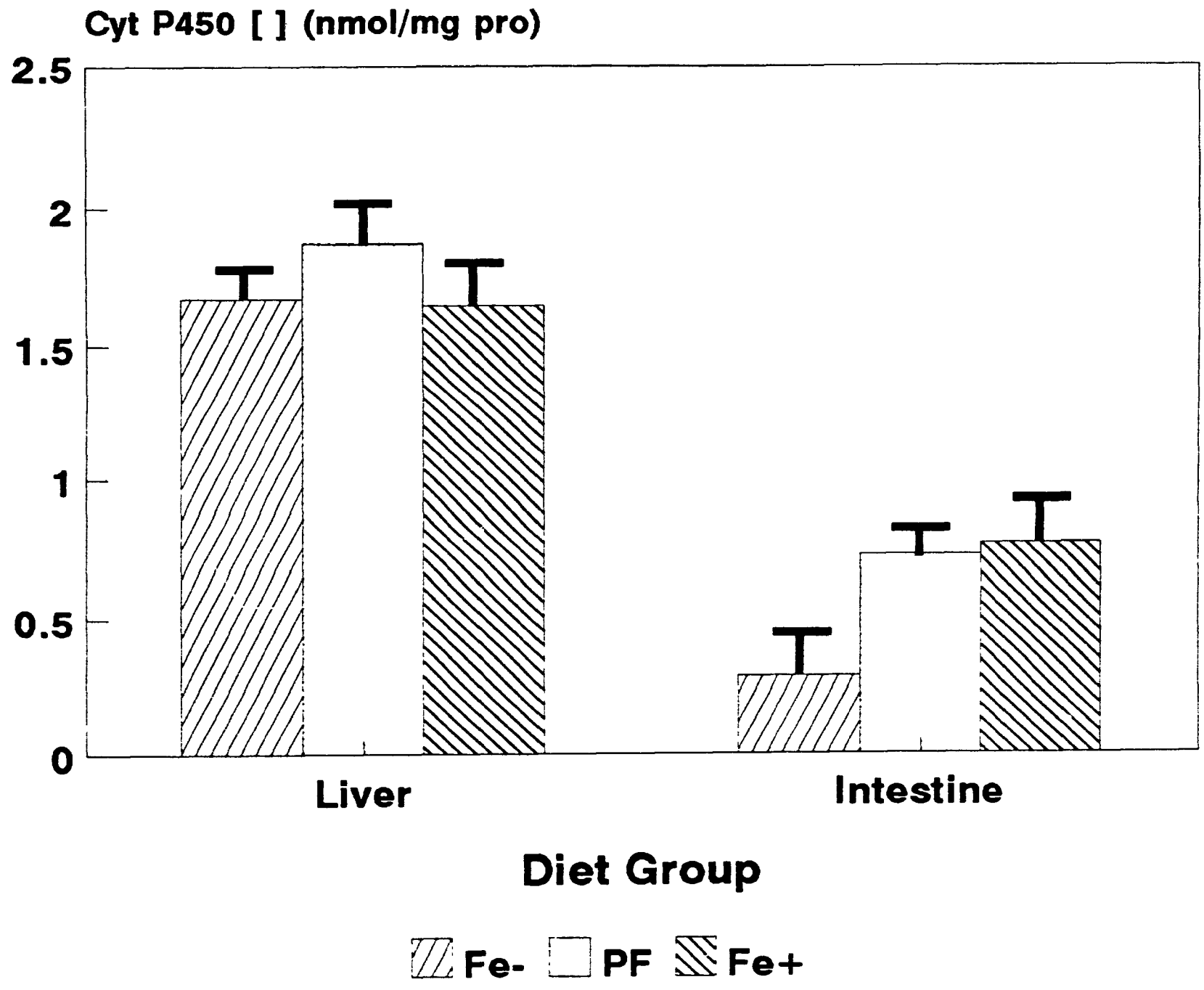

Cyt P450 [ ] (nmol/mg pro)
2.5
2
1.5
1
0.5
0
Liver
Intestine
Diet Group
Fe-
PF
Fe+

Fig. 3.5. Mean plasma concentration profiles (n=4-5) of ABZSO in different diet groups after oral administration of ABZ (40 mg/kg) in mice.

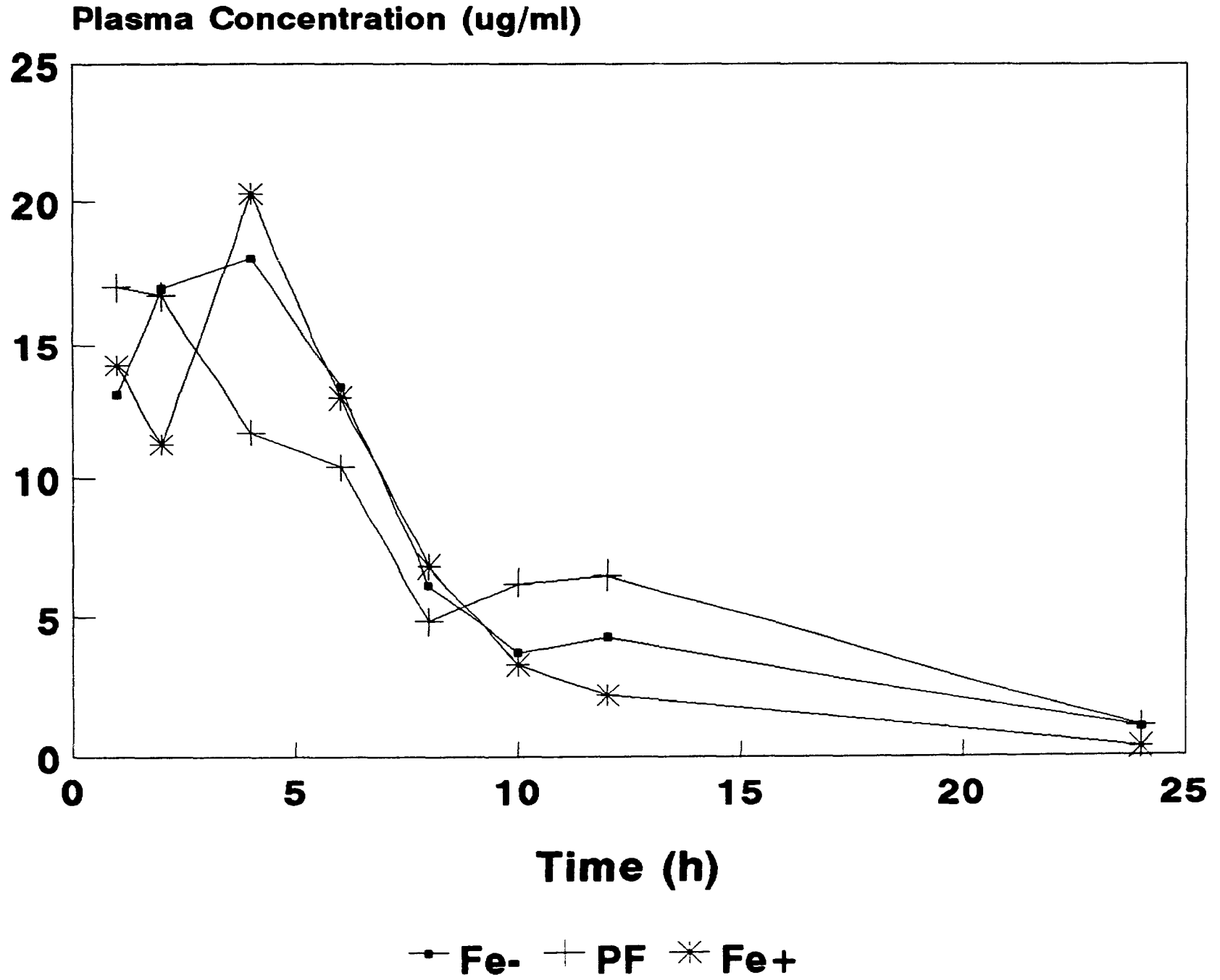
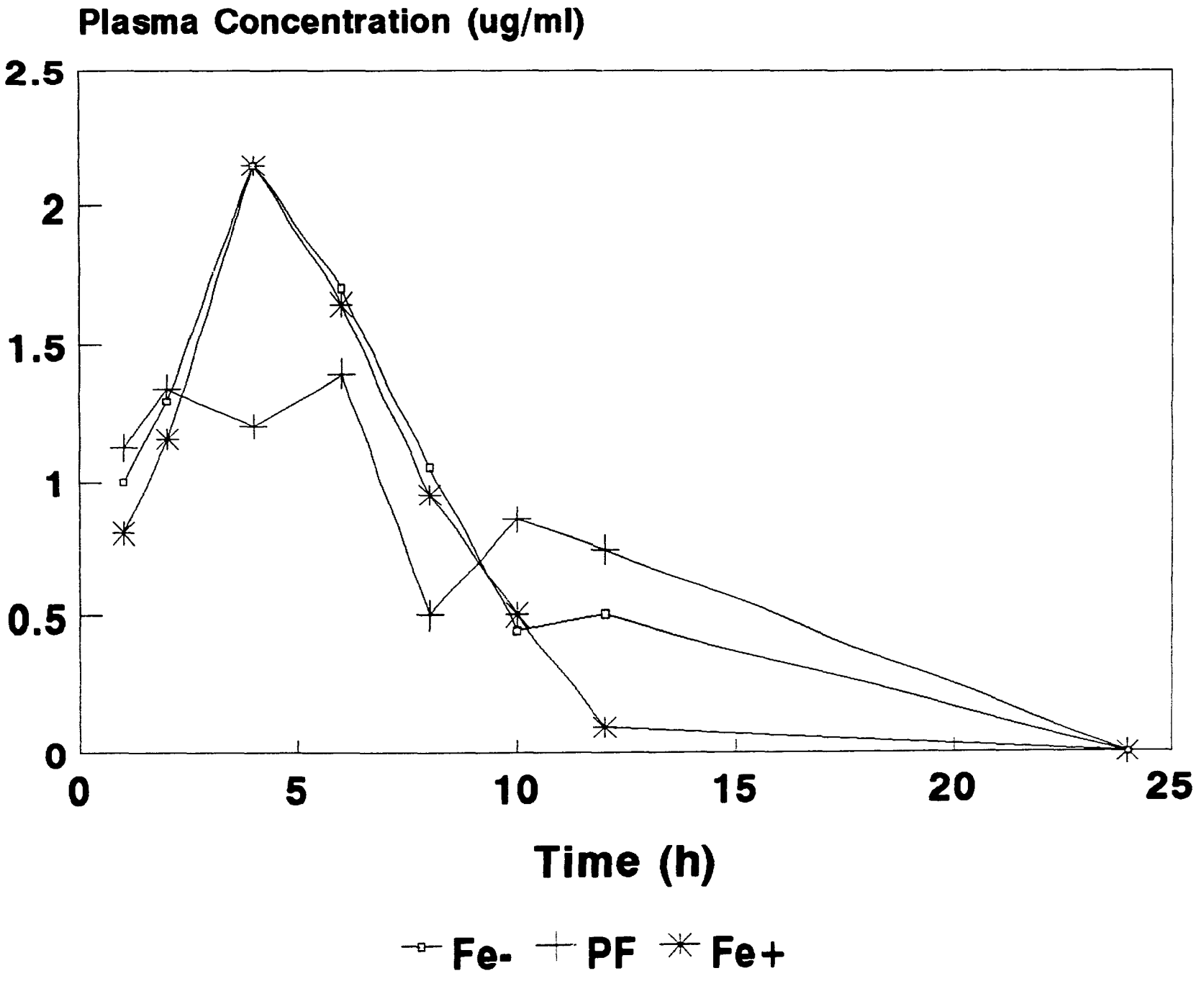

Plasma Concentration (ug/ml)
25
20
15
10
5
0
0
5
10
15
20
25
Time (h)
Fe-
PF
Fe+

Fig. 3.6. Mean plasma concentration profiles (n=4-5) of $ABZSO_2$ in different diet groups after oral administration of ABZ (40 mg/kg) in mice.


Plasma Concentration (ug/ml)
2.5
2
1.5
1
0.5
0
0
5
10
15
20
25
Time (h)
Fe-
PF
Fe+

3.4. DISCUSSION

In the present study, we found that the growth rate of mice fed the iron-deficient diet was significantly lower than that of mice fed the iron-supplemented diet. In addition, pair-fed mice had a significantly lower growth rate than both control *ad-libitum* and deficient mice. The decreased body weight of mice fed the low iron diet is consistent with observations of other investigators (Prichard *et al.*, 1981; Kuvibidila *et al.*, 1983; Kochanowski & Sherman, 1982, 1985; Dhur *et al.*, 1989). Diminished pair-fed body weight in comparison to iron-deficient mice was expected as pair-fed animals tended to consume their daily allocation immediately upon receiving it, whereas deficient mice followed a more continuous consumption pattern (Nielsen, unpublished observations). The implication is that deficient mice were able to use their nutrients more effectively than their pair-fed counterparts resulting in a significantly increased body weight.

The lower hemoglobin and serum iron values in the iron deficient group are consistent with results from several researchers (Finch *et al.*, 1976; Kochanowski & Sherman, 1982, 1985; Dhur *et al.*, 1989). Total liver iron was measured in order to assess the size of body iron stores. They were significantly diminished in the iron-deficient group as compared to both control *ad-libitum* and pair-fed animals.

This is in accordance with results of Siimes *et al.* (1974), who reported significantly decreased iron stores after only 6 days of iron deficient diet, thus indicating the dependence of liver stores on adequate dietary iron consumption. The wet weight of spleens from the deficient group were consistently heavier than controls supporting the premise of increased splenomegaly during iron deficiency (Kuvibidila *et al.*, 1981, 1983).

The primary purpose of this study was to determine whether impairment of efficacy could be explained in terms of any alteration in the anthelmintic pharmacokinetic profiles. Plasma albendazole metabolite levels were not significantly different in the deficient group as compared to both control groups, indicating that the mechanism of impaired anthelmintic efficacy during iron deficiency is not due to any differences in pharmacokinetic behavior. Results of a study conducted by Prichard *et al.* (1981) using a *Nippostrongylus brasiliensis* infection in rats showed that during a combined iron and protein deficiency, plasma fenbendazole levels were significantly higher in deficient animals as opposed to sufficiently-fed controls. The study however, did not include pair-fed controls in the experiment, thus higher plasma fenbendazole levels may have been due solely to the protein deficiency, to the combined iron and protein deficiency or perhaps an effect due to energy restriction as deficient

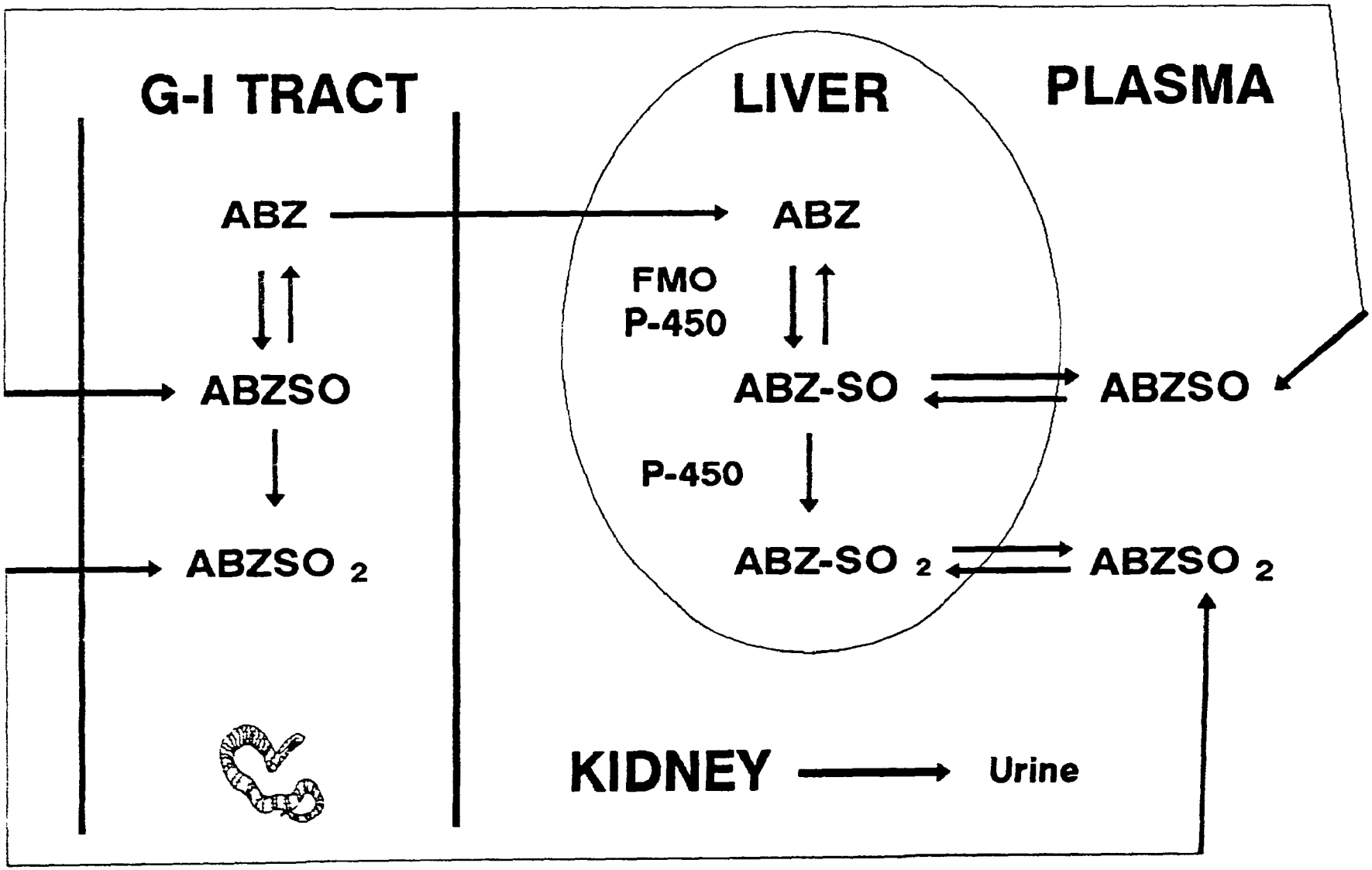
animals consumed significantly less than controls. This study and others (Catz *et al.*, 1970; Becking, 1976) suggest that different hosts may modify the influence of host nutritional status on drug metabolism.

In a further attempt to understand the mechanism of decreased anthelmintic efficacy during iron deficiency it was decided to measure cytochrome P-450 content in both liver and intestinal tissues. Although the liver is the primary organ for drug/xenobiotic biotransformation and detoxification (due mainly to cytochrome P-450), drug metabolism also takes place in other tissues such as blood, plasma, lungs, kidneys and particularly the GI tract. We did not demonstrate any modifications in the P-450 content in livers of iron-deficient mice, however intestinal P-450 was significantly depressed in deficient animals. What consequences might this have on anthelmintic efficacy? Since cytochrome P-450 plays an essential role in the biotransformation of albendazole to its pharmacologically active metabolite albendazole sulphoxide (Fig. 3.7), it would stand to reason that decreased levels of P-450 in the gut would denote a depressed rate of conversion to the sulphoxide metabolite. Clearly, this could play a significant role with regards to the quantity of active metabolite being taken up by the parasite. If less of the sulphoxide metabolite is being taken up by the parasite, anthelmintic efficacy could surely be reduced. In addition,

Prichard *et al.* (1981) found that worms in iron and protein deficient hosts took up significantly less radio-labelled fenbendazole. This observation may also partly explain the reduced efficacy of benzimidazoles in nutritionally deprived hosts. Although the reasons by which worms take up less anthelmintic is not clearly understood, it is possible that gastrointestinal effects of iron deficiency such as gastric achlorohydria, gastritis and diarrhea (Kimber and Weintraub, 1968) could adversely effect the mucosal environment, altering the homeostasis (ie: metabolic rate, absorptive capacity) of the worm. These results contribute to the understanding of drug-nutrient interactions during parasitic disease, nonetheless more research is needed in order to optimize anthelmintic efficacy during malnutrition.

Fig. 3.7. Proposed metabolic routes of albendazole after oral administration. FMO = flavin monoxygenase system; P-450 = cytochrome P-450.

Albendazole Metabolism



GENERAL DISCUSSION

The studies described above provide evidence to support the hypothesis that host nutritional status has the potential to influence anthelmintic efficacy and thus warrants further study. Improved understanding of the underlying mechanisms requires further examination of the pharmacokinetics of the anthelmintic and its interaction with host nutrition. Of particular importance are the effects of host malnutrition on the viability of the parasite (ie: does host malnutrition result in increased parasite viability or parasite malnutrition?).

In order to assess the consequences of nutrient deficiency on anthelmintic efficacy it was important in these studies to define one particular deficiency. By using the hookworm model *H. polygyrus* in murine hosts, manipulation of nutrient intake became an easy task thus providing an appropriate experimental basis for nutrition studies. The effects observed in these experiments consequently, were due only to iron deficiency thus permitting general conclusions to be drawn about the effects of iron depletion on parasite fecundity, drug efficacy and the pharmacokinetics of the anthelmintic.

If the results of these studies on anthelmintic efficacy can be extrapolated to man, they have obvious therapeutic implications. What can be done to ensure the success of drug-based control programs? In malnourished populations one option is to increase dosages of the anthelmintic. This however, may cause further side effects not previously seen in the population. A more feasible option is implementation of a nutritional supplementation program coinciding with anthelmintic treatment schedules. Finally, aggressive iron therapy may be feasible and effective in populations known to be iron-deficient. The results of these experiments however, awaits further study in order to unravel all the possibilities.

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APPENDIX I

Techniques:

(1) *Preparation of third stage infective larvae*

Faeces were collected in deionized water from infected mice and placed in Nalgene cages over a grid for a 24-hour period. After being washed through 2 sieves (150μ and 45μ) eggs were recovered and centrifuged with deionized water at 2500 rpm for 10 minutes. The sediment was retrieved and placed onto moistened filter paper within petri dishes. All dishes were covered and placed in an incubator maintained at 22°C. Larvae were recovered 7 days later from the outer edges of the filter paper. Larvae were then centrifuged and suspended in deionized water at a concentration of 200 L_3 in 0.03 ml and refrigerated until needed, all mice were given an oral dose of 150 L_3 of *H. polygyrus* accordingly.

(2) *Parasite Examination*

Small intestines of all mice were removed after sacrifice and opened longitudinally; adult worm burden, sex and parasite distribution within the GI tract was recorded using a dissecting microscope.

(3) *Measurement of Egg Production*

On day 18 of infection, feces of mice were collected over a 24 hour period. Two small sieves (150μ and 45μ) placed together were required for the procedure: feces were placed

in the 150μ sieve and rinsed thoroughly with distilled water allowing eggs to move through the top sieve into the lower sieve. Eggs were collected and placed into a graduated cylinder, equal volumes of distilled water and saturated salt solution were added to make up a volume of 28 ml. After samples were processed, the fecal specimens were placed into McMaster Egg Counting Chambers and counted using a compound microscope.

(3) *Hemoglobin determination*

Hemoglobin was determined using the Cyanmethemoglobin Method as described by Coles (1974). Whole blood (20 μl) was added to cyanmethemoglobin (HiCN) reagent. The ferricyanide present in the reagent converts the hemoglobin iron from the ferrous state to the ferric state to form methemoglobin which then combines with potassium cyanide to form cyanmethemoglobin. Color intensity was measured using a spectrophotometer and this was proportional to the concentration of hemoglobin in the blood.

(4) *Hematocrit determination*

Whole blood was placed in capillary tubes and centrifuged at high speed for 10 minutes. Packed cell volume of anemic mice was compared to controls in order to quantify severity of the anemia. Hematocrit is a combined measure of the size, capacity and number of cells present in the blood, and along

with hemoglobin, is a fairly sensitive indicator of presence and severity of anemia.

(5) *Serum Iron Concentration*

Serum iron concentration was determined using a modification of the method of Persijn *et al.* (1971). Ferrozine, a sulfonated derivative of diphenyltriazine, forms a water-soluble magenta complex with iron. At acid pH, transferrin-bound serum iron dissociates to form ferrous ions. These react with ferrozine to produce a magenta colored complex. The difference in color intensity at 560 nm before and after addition of ferrozine, is proportional to serum iron concentration.

(6) *Liver Iron Analysis*

Flame atomic absorption spectrophotometry was used to determine the iron, zinc and copper content of the livers after wet-ashing in a nitric acid solution. Absorption values were obtained in duplicate at 372 nm for iron, 214 nm for zinc and 327 nm for copper and compared against commercially prepared standards.

(7) *Blood Collection*

All mice were subjected to CO_2 treatment and once anaesthetized, were bled by cardiac puncture. After sampling, all mice were sacrificed by cervical dislocation.

(8) *Diet Composition and Preparation*

Due to the complexity in designing a trace mineral deficient diet, vitamin and mineral mixes were formulated by Teklad Inc. in order to ensure that all mice were subjected to a comparable iron deficiency. Low trace element casein was used as the sole source of protein in the diet due to the fact that it contains very low levels of iron residues. The amino acid methionine was added at a level of 0.3% to account for low levels in the casein. A level of 22% protein was chosen based on protein level recommendations in the literature that maintained adequate growth and reproduction (Knapka *et al.*, 1977 and NRC, 1978). The NRC reports on average, adequate protein levels ranging from 20 to 24% of total calories. Sucrose, cellulose and cornstarch providing approximately 60% of total macronutrients were used as the major source of carbohydrate and fibre. This was based on recommendations by the NRC (1978) in which optimal growth and reproduction occurred at levels of carbohydrate ranging from 55 to 65% of total calories. A level of 8% corn oil provided the major fat source in the diet; this again was based on recommendations by both Knapka *et al.*(1977) and the NRC (1978) in which optimal growth was observed at levels of 4 to 12%. A vitamin mix was formulated by Teklad Inc. so as to provide 1 to 3 times the recommended intake for all vitamins in order to ensure that no other deficiency was induced in the mice to compensate for reduced food intake. The mineral mix was similarly fortified

(approximately 2-3 times normal requirement) with the exception of iron content. Control diets contained approximately 51 mg/kg iron, iron-deficient diets contained approximately 2 mg/kg, a level which is supposed to induce a moderately severe deficiency state in mice (Kuvibidila *et al.*, 1983).

APPENDIX II

Diet Composition of Mouse Diets

Table A1 **Composition of basal diet for mouse**

Ingredient	Amount	
	(g/Kg)	(%)
Casein, low trace element[1]	220	22.0
DL-Methionine[2]	3	0.3
Sucrose[3]	400	40.0
Cornstarch[4]	200	20.0
Cellulose[5]	50	5.0
Corn oil[6]	80	8.0
Mineral mix[7]	35	3.5
Vitamin mix[8]	12	1.2

[1]Casein, ICN Biochemicals Canada Ltd., Montreal, Quebec.

[2]Methionine, ICN Biochemicals Canada Ltd., Montreal, Quebec.

[3]Teklad Research Diets, Madison, Wisconsin.

[4]ICN Biochemicals, Canada Ltd., Montreal, Quebec.

[5]Alphacel (hydrolyzed), ICN Biochemicals Canada Ltd., Montreal, Quebec.

[6]ICN Biochemicals, Canada Ltd., Montreal, Quebec.

[7]See Tables A3 to A7.

[8]See Table A2.

Table A2 Composition of vitamin mixture for mouse diets

Ingredient	mg/kg diet	Mouse NRC Req't mg/kg diet[1]
Niacin	42.5	10
Calcium pantothenate	30.0	10
Riboflavin	7.0	7
Pyridoxine hydrochloride	7.0	1.0
Thiamin hydrochloride	10.0	5.0
Folacin	0.9	0.5
Biotin	0.4	0.2
Cyanocobalamine (MW 1355.2)	0.05	0.02
Alpha-tocopherol acetate[2]	50 IU	20 IU
Menaquinone	5.0	3.0
Cholecalciferol[3]	1000 IU	150 IU
Retinyl palmitate[4]	4000 IU	500 IU
Choline chloride	750.0	600

[1]Nutritional Requirements of Laboratory Animals, Third Revised Edition, NRC. National Academy of Sciences, Washington, D.C., 1978.

Table A3 Composition of mineral mix[1,2] for control mouse diet

Ingredient	g/kg Salt	g/kg diet @ 3.5%
$CaHPO_4$	500.00	17.50
$KHCO_3$	220.00	7.70
K_2SO_4	52.00	1.82
NaCl	74.00	2.59
MgO	24.00	0.84
$CrK(SO_4)_2.12H_2O$	0.55	0.0192
$CuCO_3.CU(OH).H_2O$	0.30	0.0105
KIO_3	0.01	0.00003
$FeSO_4.7H_2O$	6.00	0.21
$MnCO_3$	3.50	0.1225
$ZnCO_3$	1.60	0.056
$Na_2SeO_3.5H_2O$	0.01	0.00003
Subtotal	881.97	30.868
(Sucrose)	118.03	4.132
Total	1000.00	35.00 = 3.5%

[1]See Table A5 for elemental composition of salt mixture.
[2]See Table A7 for elemental composition of microelements.

Table A4 Composition of mineral mix[1,2] for iron-restricted mouse diet

Ingredient	g/kg Salt	g/kg diet @ 3.5%
$CaHPO_4$	500.00	17.50
$KHCO_3$	220.00	7.70
K_2SO_4	52.00	1.82
NaCl	74.00	2.59
MgO	24.00	0.84
$CrK(SO_4)_2.12H_2O$	0.55	0.0192
$CuCO_3.CU(OH).H_2O$	0.30	0.0105
KIO_3	0.01	0.00035
$MnCO_3$	3.50	0.1225
$ZnCO_3$	1.60	0.056
$Na_2SeO_3.5H_2O$	0.01	0.00035
Subtotal	875.97	30.658
(Sucrose)	124.03	4.342
Total	1000.00	35.00 = 3.5%

[1]See Table A6 for elemental composition of salt mixture.
[2]See Table A7 for elemental composition of microelements.

Table A5 Elemental composition of salt mixture for control mouse diet

Ingredient (MW)	mg/kg diet	Cr	Cu	I	Fe	Mn	Zn	Se
		---------- mg/kg diet ----------						
$CrK(So_4)_2 . 12H_2O$ (499.28)	19.2	2.0						
$CuCO_3 . Cu(OH) . H_2O$ (239.19)	10.5		5.6					
KIO_3 (214.02)	0.3			0.21				
$FeSO_4 . 7H_2O$ (278.01)	210.0				42.2			
$MnCO_3$ (114.94)	122.5					58.6		
$ZnCO_3$ (125.38)	59.0						30.8	
Na_2SeO_3 (172.96)	0.3							0.16
Sucrose	578.2							
Total (a)	1000.00	2.0	5.6	0.21	42.2	58.6	30.8	0.16
NRC-Reqt. (1978) (b)[1]		2.0	4.5	0.20	25.0	45.0	30.0	---
a:b		1.0	1.2	1.1	1.7	1.3	1.0	

[1]Nutrient Requirements of Laboratory Animals, Third Revised Edition, National Academy of Sciences, Washington, D.C., 1978.

Table A6 Elemental composition of salt mixture for iron-restricted mouse diet

Ingredient (MW)	mg/kg diet	Cr	Cu	I	Fe	Mn	Zn	Se
		---------- mg/kg diet ----------						
$CrK(So_4)_2 . 12H_2O$ (499.28)	19.2	2.0						
$CuCO_3 . Cu(OH) . H_2O$ (239.19)	10.5		5.6					
KIO_3 (214.02)	0.3			0.21				
$FeSO_4 . 7H_2O$ (278.01)	0.0				0.0			
$MnCO_3$ (114.94)	122.5					58.6		
$ZnCO_3$ (125.38)	59.0						30.8	
Na_2SeO_3 (172.96)	0.3							0.16
Sucrose	788.2							
Total (a)	1000.00	2.0	5.6	0.21	0.0	58.6	30.8	0.16
NRC-Reqt. (1978) (b)[1]		2.0	4.5	0.20	25.0	45.0	30.0	---
a:b		1.0 1.0	1.2 ---	1.1	---	1.3		

[1]Nutrient Requirements of Laboratory Animals, Third Revised Edition, National Academy of Sciences, Washington, D.C., 1978.

Table A7 **Elemental composition of microelements for mouse diets**

Ingredient (MW)	mg/100 g diet	Ca	Mg	Na	K	P	Cl	S
		------- mg/100 g diet -------						
K_2SO_4 (174.27)	182				81.7		33.5	
$CaHPO_4$ (136.06)	1750	515.5				400		
$KHCO_3$ (100.11)	770				300.7			
NaCl (58.5)	259			101.8			156.9	
MgO (40.32)	84		50.7					
Sucrose	434							
	(3479)							
Microelements	20.9							
Total (a)	3499.9 3.5%	515	51	102	382	400	157	33.5
NRC-Reqt.1978 (b)[1]		400	50	50	200	400	50	--
a:b		1.3	1	2	1.9	1	3.1	--

[1]Nutrient Requirements of Laboratory Animals, Third Revised Edition, National Academy of Sciences, Washington, D.C., 1978.