

AN ABSTRACT OF THE THESIS OF

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Title: The Effect of Type and Level of Dietary Protein  
on the Bioavailability of Dietary Fluoride in the Rat.

Abstract approved: \_\_\_\_\_  
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This study was designed to determine whether dietary protein is a nutritional factor influencing the bioavailability of dietary fluoride. To accomplish this, a factorial experiment was conducted with weanling rats fed a purified diet to determine the influence of dietary protein type (casein or lactalbumin) and level (12% or 36%) on fluoride bioavailability. Dietary fluoride in each case was 2 or 10 ppm supplied as sodium fluoride. After four weeks, fluoride retention was significantly reduced ( $P < 0.001$ ) in rats fed high protein diets at 2 ppm fluoride, which was reflected in decreased femur and tibia fluoride concentration. A significant reduction in total femur fluoride content was observed only for rats fed diets containing 36% casein and 10 ppm

fluoride. Results for molar fluoride content were less reliable than either femur or tibia in this regard. This protein-induced reduction of fluoride retention was observed despite the fact that apparent fluoride absorption was enhanced in all groups fed high protein diets ( $P < 0.001$ ). Urinary excretion of fluoride was significantly increased in all rats fed high protein diets, thus accounting for the observed reduction in skeletal fluoride uptake. Significantly greater ( $P < 0.001$ ) body weights were observed among rats fed high levels of protein than among rats fed normal protein diets, despite the fact that food intake for all treatment groups was adequate and similar. No consistent effect on skeletal or molar fluoride uptake due to protein type was evident under the conditions of this study. Although this study was not designed to investigate the mechanism involved in decreased fluoride retention with high protein diets, increased urinary fluoride excretion may be due to increased glomerular filtration rate coupled with decreased renal tubular fluoride reabsorption. Therefore, the results of this study demonstrate that, in the rat, a threefold increase in dietary protein level negatively influences fluoride bioavailability by promoting increased urinary fluoride excretion thus reducing fluoride available for incorporation into bones and teeth. These results

suggest that excess dietary protein consumption common in the U.S. combined with marginal fluoride intake may adversely affect fluoride bioavailability in humans. This could reduce the fluoride content of teeth and bones, which may decrease resistance to dental caries and compromise skeletal integrity.

The Effect of Type and Level  
of Dietary Protein on the Bioavailability  
of Dietary Fluoride in the Rat.

by

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THE EFFECT OF TYPE AND LEVEL OF  
DIETARY PROTEIN ON THE BIOAVAILABILITY  
OF DIETARY FLUORIDE IN THE RAT

INTRODUCTION

The trace element fluoride is considered to be an essential nutrient for humans due to its beneficial effects on tooth development (1). Fluoride is incorporated into the structure of teeth and is necessary for maximal resistance to dental caries (2, 3). Optimal fluoride intake may also be beneficial in reducing the prevalence of osteoporosis (4, 5, 6). Information on total daily intake of fluoride is increasing, and it is now apparent that foods can contribute a significant portion of total daily fluoride intake when foods and beverages are prepared with fluoridated water (7, 8, 9). This is particularly true for infants and small children. In order to ensure optimal benefits and safety of fluoride, it is important to determine not only dietary intake, but the actual amount of fluoride available for incorporation into the growing skeleton and teeth. Although the absorption of fluoride in water is nearly complete (8, 10, 11), only 50-80% is absorbed from foods (8, 12). A

better understanding of the effect of specific food components on fluoride bioavailability, therefore, is needed.

At present, factors known to influence fluoride bioavailability, defined as absorption and utilization (13), include fluoride concentration and chemical form, physiological state, frequency of ingestion and interactions with certain minerals (8, 13, 15). For example, high levels of calcium, magnesium and aluminum reduce fluoride absorption due to the formation of insoluble complexes in the gastrointestinal tract (16, 17, 18). Several investigators have observed that milk and milk products reduce fluoride bioavailability in rats (20, 21) and in humans (11, 22) by as much as 50 per cent.

Recent evidence indicates that high levels of dietary protein influence the metabolism of certain trace elements. For example, excessive levels of protein have been reported to alter the metabolism of zinc (23, 24, 25), copper (24) and selenium (26). On the other hand, the effect of high levels of dietary protein on fluoride bioavailability is not known. In addition, it is not known if the source of dietary protein is important. It is possible that either type or level of protein in the diet may reduce fluoride

bioavailability by interfering with gastrointestinal absorption or by increasing renal excretion. A variety of physical and chemical properties associated with proteins such as amino acid composition, sulfur amino acid content, and structural characteristics, may be factors which could influence fluoride metabolism. In addition, the small size and extreme electronegativity of the fluoride ion may increase the chances of a protein-fluoride interaction. For example, the proteins in milk may contribute to the reduction of fluoride bioavailability observed when fluoride is administered with milk. Because protein consumption in the United States often exceeds the recommended dietary allowance (RDA) by two to three times (27, 28, 29), and dietary fluoride intake is highly variable (1), the relationship between dietary protein and fluoride bioavailability could be important.

In order to assess the adequacy of fluoride intake, accurate estimates of bioavailability are required. Further investigations are necessary to identify nutritional factors influencing fluoride bioavailability. This information may be particularly relevant to infants and young children, who have large amounts of milk in their diets, and when the need for optimal fluoride intake is greatest. In addition, the existence of a protein-fluoride interaction may help to

identify possible mechanisms of the observed reduction of fluoride bioavailability when ingested with foods. Because foods prepared with fluoridated water can contribute significantly to total daily fluoride intake, especially for younger age groups, it has become increasingly important that total daily fluoride intake be considered when prescribing fluoride supplements. Additional information on fluoride bioavailability would facilitate the evaluation of fluoride status of individuals, and therefore the formulation of supplementation regimens, which are currently based on the concentration of fluoride in water alone.

#### Research Hypothesis and Objectives

The purpose of this study was to investigate the effect of both type and level of dietary protein on fluoride absorption, fluoride retention and skeletal uptake of fluoride under normal dietary conditions using the growing male albino rat as an animal model. It was hypothesized that high levels of dietary protein would reduce the bioavailability of dietary fluoride. A corollary hypothesis that certain milk proteins influence fluoride bioavailability was also tested. The specific objectives of this study were to:

1. Determine the effect of two different dietary proteins on fluoride bioavailability at two levels of dietary fluoride.
2. Determine the effect of two different levels of dietary protein on fluoride bioavailability at two levels of dietary fluoride.
3. Determine the bioavailability of dietary fluoride in terms of fluoride absorption, fluoride retention and skeletal and molar uptake of fluoride.

The following section reviews fluoride intake and metabolism, and develops the rationale for the research hypothesis in detail.

## REVIEW OF LITERATURE

Fluoride as an Essential Nutrient

The essentiality of the trace element fluoride has been reaffirmed with the recent recommendation of a safe and adequate intake range (1). The status of fluoride as an essential nutrient is based on its unique physiologic role in promoting optimal dental health. Optimal ingestion of fluoride has been proven to provide increased resistance to dental caries. When the fluoride concentration in public water supplies is approximately 1 part per million (ppm), either naturally or by artificial adjustment, a 50-60% reduction in the caries rate is observed (2, 3, 30). This benefit is not confined to children, since it persists throughout adulthood (3, 31).

Although the mechanism by which fluoride prevents dental caries is not yet completely understood, it is generally agreed that the incorporation of fluoride into tooth structure reduces enamel solubility in acids that are produced by oral microflora (2, 3, 31). The fluoride ion enters the mineralized tissues either by ionic exchange or by direct incorporation into the crystal structure during the phase of crystal growth (2, 31). Fluoride increases internal bond strength within the apatite lattice of both teeth and bone by

replacing hydroxyl and carbonate groups to form fluorapatite, thus producing a more ordered crystal structure (2, 3, 32). This ionic replacement and the larger, more perfect crystal it produces, causes teeth to be more resistant to acid demineralization. The outer surface of enamel continues to acquire fluoride throughout life by ionic exchange with fluoride present in oral fluids (33). Additional proposed cariostatic mechanisms of fluoride include the development of smaller teeth with more shallow fissures, which reduces contact points and other food-trapping areas, promotion of remineralization and possible inhibition of bacterial enzymes (2, 32).

In addition to protecting teeth from decay, fluoride may also play a role in the maintenance of a normal skeleton. Increased bone density and skeletal mass promoted by fluoride ingestion may exert a general protective effect against degenerative bone changes in later life (5, 6, 34, 35, 36). The skeleton continues to accumulate fluoride with age by ionic exchange despite the gradual decrease in remodeling activity, although a plateau is reached around age 55 (33, 37). Epidemiologic evidence suggests that there may be a decreased incidence of osteoporosis in fluoridated areas as indicated by reduced incidence and severity of bone fractures and collapsed vertebrae (4, 5, 6).



Beneficial effects of pharmacologic doses of fluoride have been demonstrated in post-menopausal osteoporosis and have been used for years in the treatment of osteoporosis (38, 39). Thus, the benefits of optimal fluoride ingestion extend into adult life with the continued deposition of fluoride in the outer layers of enamel (2, 31) and for improved bone health.

Fluoride has a relatively narrow range of effectiveness. However, the only known adverse effect associated with the chronic ingestion of relatively small amounts of fluoride is dental fluorosis, also known as enamel mottling (2, 31, 35). This disorder, characterized by opaque whitish or yellowish spots of varying size in the enamel, is a permanent feature of affected teeth (2). It occurs only when excessive amounts of fluoride are ingested during the pre-eruptive development of the teeth, from birth to about 16 years of age (2, 32). Mottling may become evident when drinking water fluoride concentration exceeds 1.4-1.6 ppm. This phenomenon, however, is usually noticeable only to trained dentists at this level. Higher fluoride intake during enamel formation increases both the incidence and severity of the defects. The cause of mottled enamel is thought to be disturbances in ameloblastic activity, since these

enamel-forming cells appear to be the most sensitive cells in the body to fluoride (2, 31).

#### Dietary Intake of Fluoride

Because the fluoride ion is distributed widely throughout nature in soils and water supplies, almost all foods and beverages contain at least trace amounts of fluoride (8, 40, 41). The quantity ingested by different individuals, however, may vary considerably. Factors contributing to individual variation in total daily fluoride intake include concentration of fluoride in the local drinking water, the volume of water consumed, processing and preparation of foods in fluoridated water and the food habits of individual consumers. For example, because water intake is generally greater in warmer environmental temperatures than in cooler climates, the amount of fluoride added to community water supplies varies from 0.7 to 1.2 ppm, depending on the local climate (1, 2).

In general, the fluoride content of most foods is low, and it is difficult to obtain the recommended intake of fluoride from the diet alone (1). Exceptions are marine fish, especially if the bones are eaten (the mean fluoride concentration of seawater is 1.3 ppm) and black or green leaf teas (41). Certain wines, beer and bottled mineral water may contain significant amounts

of fluoride, which reflect the fluoride concentration of water used in the preparation of these beverages (8, 41). Although the fluoride concentration in drinking water is considered the most important source of dietary fluoride, the primary source of fluoride is the diet where water fluoride concentration is low (42). When foods are processed with the use of fluoridated water, the food fluoride content rises significantly (8, 43). For example, fruit juices, vegetables, soups and cereals processed with fluoridated water were found to contain 5 to 20 times more fluoride (table 1) than when non-fluoridated water was used (43). If foods are then prepared in the home by diluting or cooking with fluoridated water, the fluoride content is further increased. The fluoride concentration of local water supplies greatly influences the total daily fluoride intake of infants and toddlers, particularly if water-diluted formulas are used (43). Although manufacturers have discontinued the use of fluoridated water in the processing of infant foods (8, 9), there appears to be a wide range in total daily fluoride intake of infants and toddlers due to variations in consumption of ready-to-feed formulas, cereal products, vegetables and certain dairy products (44). Thus, the fluoride content of foods takes on practical importance when foods such as fruit juices, vegetables, and cereals are

Table 1. Fluoride content of foods (ppm).<sup>1</sup>

Food	Non-fluoridated water	Fluoridated water
Tomato soup	0.04	0.38
Beer	0.30	0.68
Green beans	0.20	0.89
Whole potatoes	0.38	0.76
Diced carrots	0.19	0.61
Ginger ale	0.02	0.77
Oatmeal	0.98	4.87
Rice cereal	2.11	6.35
Fruit juice (mixed)	0.014	0.38
Apple-cherry juice	0.14	1.48

<sup>1</sup>Source: Reference (43) and (52).

processed with or prepared with fluoridated water, and may greatly enhance total daily fluoride intake in these age groups.

Based on the analysis of composite "market basket" food collections in four geographic regions of the U.S., Singer et al. (45) estimated the total daily fluoride intake of young adult males from foods, including water, to range from 0.9 mg/d in a non-fluoridated community to 1.72 mg/d in a fluoridated community. In similar studies, estimates of 0.315 to 0.610 mg/d were calculated for two-year olds (46). From a comparison based on the analysis of collections of commercial infant foods obtained from fluoridated and non-fluoridated areas, Singer and Ophaug (43) calculated the total daily fluoride intake for 6-month old infants to range from 0.153 to 0.763 mg/d. Assuming a body weight of 8.1 kg for the average 6-month old infant, this fluoride intake expressed on a body weight basis ranges from 0.026 to 0.067 mg/kg body weight. Recommended fluoride intake for this age group is 0.05 to 0.07 mg/kg expressed on a body weight basis (8). Thus, these estimates for total daily fluoride intake appear to fall within the range of intake regarded as optimum for these age groups, although intake for toddlers was slightly low.

In a recent study of more extensive food collections, Ophaug et al. (9) found a minimum intake of 0.42 mg/d in infants and a maximum intake of 0.62 mg/d for toddlers, representing levels of fluoride intake which generally fall within or slightly below optimum intake. Variations due to quantity of water ingested and level of water fluoride concentration were observed, but the daily intake from foods was similar for both age groups. The authors stressed the need for accurate bioavailability data, especially for these age groups. Singer et al. (47) estimated an average daily fluoride intake of 1.85 mg/d in fluoridated cities, and 0.86 mg/d in non-fluoridated cities for young adult males. However, it is not clear if fluoridated water from the communities was included in food preparation as it would be in the home, so these estimates may be low.

In a fluoridated community, average fluoride intake from foods is currently estimated to be 0.4 mg/d, accounting for approximately one-quarter of total daily fluoride intake (7). However, there is little agreement on the fluoride content of individual foods and beverages. Improved analytical techniques, such as the use of the fluoride ion-selective electrode (48) and the separation of fluoride by diffusion from biological materials (49, 50, 51), have demonstrated

that early estimates of food fluoride were too high (52, 53, 54). Earlier data were based on a colorimetric method not specific for fluoride. When current analytical methods were compared to earlier methods, a consistent overestimation of fluoride content of fruits, vegetables and infant foods was found (table 2), with values differing by as much as a factor of 100.

Because recent investigations have indicated that there has been a decline in caries prevalence, along with an increase in the occurrence of mild fluorosis in fluoridated as well as non-fluoridated communities (9, 55), concern has been expressed that the total daily fluoride intake is increasing. Leverett (55) has suggested that there is an increasing amount of fluoride present in the food chain as a result of extensive water fluoridation, and that fluoride concentration of drinking water should be adjusted downward. Others (56) disagree, pointing out that community water fluoridation remains the safest, most effective and most economical way to provide optimal caries protection for all residents regardless of age, socioeconomic status, education or access to dental care. Horowitz and Horowitz (56) suggest that any efforts to reduce total fluoride intake should be

Table 2. Comparison of analytical techniques for determining fluoride content of foods, after diffusion at 60° C.<sup>1,2</sup>

Food	Method	
	Colorimetric μg/g	Fluoride electrode μg/g
Applesauce, cherries	3.25 ± 0.17	0.028 ± 0.002
Creamed spinach	2.00 ± 0.08	0.70 ± 0.03
Chicken, chicken broth	5.65 ± 0.17	5.10 ± 0.32
Fresh apple	4.12 ± 0.45	0.023 ± 0.01
Fresh cherries	2.60 ± 0.60	0.011 ± 0.01

<sup>1</sup>Mean + SD; n = 4 samples

<sup>2</sup>Source: Reference (57).



directed at non-dietary sources of fluoride, such as toothpastes and fluoride supplements. In addition, Taves (57) concluded that evidence of an increase in daily fluoride intake was based on inaccurate data. Analyses with the fluoride ion-selective electrode for fluoride in foods, bone and urine failed to confirm any significant increase in intake (7, 9, 57). Furthermore, data suggesting an increase in fluoride intake as a result of years of fluoridation were based on unreliable colorimetric methods (52, 53, 54). A more likely explanation for the observed reduction in caries rate and increased mild fluorosis is the increased ingestion of non-dietary fluoride sources, such as fluoride dentrifrises (usually 1000 ppm fluoride), mouthrinses, and the use of topical fluoride applications (9, 35, 58, 59). The bioavailability of fluoride in toothpastes is high (85 - 100%) and should be considered in total daily fluoride intake estimations for children (59). For example, if a child swallows 0.5 g of a 0.1% fluoride toothpaste, 0.5 mg fluoride is obtained thus contributing to increased total daily fluoride intake (59).

## Fluoride Bioavailability

This section reviews the absorption, distribution, utilization and excretion of fluoride. Emphasis is placed upon factors influencing fluoride bioavailability with special reference to skeletal and dental tissues.

### Bioavailability of Dietary Fluoride

Bioavailability can be defined as the proportion of a nutrient present in food which is absorbed and utilized (13). Absorption may be defined as either true absorption, which takes endogenous secretion of a nutrient into account, or as apparent absorption, which does not. Utilization includes the processes of transport, cellular assimilation and conversion to biologically active forms, as well as routes of internal excretion (13). Bioavailability of dietary fluoride is receiving increasing attention and has recently been reviewed by Rao (8). In general, only 50 to 80% of the fluoride in foods is absorbed compared to essentially 100% in water in the fasted state (8, 12). When given to rats (21) and humans (10, 11) sodium fluoride (NaF) is almost completely absorbed. For this reason, NaF is often used as a bioavailability standard in fluoride metabolism studies (10, 11, 21). Any factor which can alter either gastrointestinal

absorption of fluoride or renal excretion of fluoride, may influence fluoride bioavailability by altering the amount that is available for incorporation into bones and teeth.

The most useful indicator of fluoride bioavailability in animals is skeletal fluoride content (8, 41). The femur is often used in animal studies, due to the fact that it has an adequate fluoride content for analytical purposes, it is conveniently removed and it has lower variation in fluoride content than other bone samples (21, 60, 61, 62). In human subjects, estimates of fluoride bioavailability are based on fluoride balance data calculated from total fluoride intake and fecal and urinary fluoride excretion (8, 63, 64). More recently, plasma fluoride concentration has been suggested as an index of bioavailability using a pharmacokinetic technique (10, 11, 65). However, plasma fluoride levels are maintained at such low levels ( $< 0.01$  ppm) that accurate and reliable determinations using this method are consequently difficult to obtain (66).

While knowledge of the fluoride content of foods and daily total fluoride intake is improving, more accurate information on fluoride bioavailability is needed because foods with identical fluoride content

may exhibit large differences in bioavailability (8). Although there is evidence that foods can make a significant contribution to total daily fluoride intake, it is not known how much of this fluoride is absorbed and utilized. Further investigations are therefore necessary to identify dietary factors influencing fluoride bioavailability in order to accurately determine adequacy of intake.

#### Fluoride Absorption

Soluble salts of fluoride are rapidly absorbed beginning in the stomach and continuing throughout the entire gastrointestinal tract (14). Free ionic fluoride is absorbed by passive diffusion across the gastric and intestinal mucosa (14, 15). Recently, it has been proposed that the passive diffusion of non-ionic fluoride as hydrogen fluoride (HF,  $pK_a$  3.45) may also be important under the acidic conditions of the stomach (67), and that gastric fluoride absorption is a pH-dependent process. Although fluoride sources may be inorganic or organic, only the fluoride ion is of importance in human nutrition (14, 15), and is the major form of fluoride found in foods and water supplies.

The rate and extent to which fluoride is absorbed is dependent on several factors, including the

solubility of the compound, chemical form, age of the individual, frequency of ingestion, quantity ingested, and both inorganic and organic constituents of the diet (14, 15). In general, any substance capable of complexing with fluoride or rendering it insoluble can reduce fluoride absorption from the gastrointestinal tract. For example, the presence of food in the gastrointestinal tract of rats has been shown to decrease fluoride absorption by 32% (17). When NaF was added to a dry diet to supply 5 ppm fluoride, there was a 36% reduction in femur fluoride storage in rats compared to identical amounts of NaF administered in water (60). Similar findings were reported by Wagner and Muhler (16) based on whole carcass analysis. Conversely, when rats were fed high levels of dietary fat, which delays gastric emptying, fluoride absorption and retention were increased (68). Fluoride absorption can be reduced by the presence of calcium, magnesium or aluminum, which form relatively insoluble fluoride complexes. Early investigators studying fluoride absorption frequently used gastric intubation techniques, involving simultaneous infusion of fluoride and other ions. For example, Weddle and Muhler (60) observed a significant decrease in femur fluoride in rats when various salts of magnesium, aluminum and calcium were administered together with a NaF solution

by stomach tube. Because fecal fluoride has been shown to increase with increased calcium content of the diet, it is apparent that absorption is decreased, presumably due to the formation of insoluble complexes which are less efficiently absorbed (14). Similar findings have been reported in human subjects. Jowsey and Riggs (19) observed that serum fluoride was reduced by 22% when orally administered NaF and calcium carbonate were given concurrently to human subjects. On the other hand, Spencer et al. (69), using fluoride balance methods, found no effect of calcium given as the gluconate in human subjects. This may be attributable to the form of calcium used. However, when aluminum hydroxide, an active ingredient in some antacids, was administered with NaF, a significant decrease in fluoride absorption was observed, resulting in increased fecal excretion and negative fluoride balances in human subjects (18). Of current interest is the fact that a variety of antacids, many of which are essentially all calcium carbonate, are widely marketed as a source of calcium for women. It is possible that this practice could contribute to decreased fluoride bioavailability.

Sodium chloride (350 mM), administered with a 4 ppm fluoride solution by stomach tube to rats, significantly reduced femur uptake of fluoride when

given with either water or flour (61). Ruzicka et al. (70) reported that NaCl ingestion reduced skeletal uptake of fluoride in mice by approximately 20%. These authors hypothesized that increased sodium consumption caused increased urinary excretion of fluoride. In contrast to the gastric intubation study of Ericsson (61), Cerklewski et al. (71) recently found slightly greater fluoride absorption in rats fed normal or high chloride diets, compared to rats fed a low chloride diet. However, urinary excretion of fluoride was decreased by approximately 30% in the chloride-deficient rats. This resulted in greater fluoride retention as indicated by increased femur fluoride content. The results of this dietary study suggest that the influence of chloride on fluoride bioavailability depends on the level of chloride as well as the route of administration.

Milk and milk products have been reported to reduce fluoride bioavailability by several investigators. Early studies explored this because milk had been suggested as an alternative vehicle for fluoridation for portions of the population without access to fluoridated water (3, 21). When 2 ppm fluoride was given to rats in milk, 45% less fluoride was stored in the carcass compared to when an identical amount was

given in water (20). Ericsson (21) reported slower intestinal absorption of radioactive fluoride ( $^{18}\text{F}$ ) and reduced femur uptake when  $^{18}\text{F}$  was given in milk, compared to  $^{18}\text{F}$  given in water. He hypothesized that coagulation of milk and the formation of calcium fluoride ( $\text{CaF}_2$ ) under the acidic conditions of the stomach may impair fluoride absorption.

Data from human studies generally confirm the findings of animal experiments. In experiments using a pharmacokinetic approach, Ekstrand and Ehrnebo (11) observed a 30-50% reduction of fluoride bioavailability when subjects were given NaF tablets with a variety of dairy products, as compared to 100% bioavailability from water during fasting. They speculated that the observed decrease in bioavailability was partially due to the formation of  $\text{CaF}_2$ , which has poor aqueous solubility. These authors suggested that the interaction between milk products and fluoride be taken into account when establishing dosage regimens for fluoride supplementation for children, and that NaF tablets should not be taken with milk products. Also using pharmacokinetic techniques, Spak et al. (22) provided further evidence that less fluoride is retained when given with milk. Fluoride given in milk or infant formula to young adult males produced delayed and reduced plasma fluoride peaks, indicating a slower



absorption rate. A significant decrease in urinary fluoride excretion was also observed.

Because milk is a complex food, no single hypothesis adequately explains the mechanism involved in the observed reduction of fluoride bioavailability when taken with milk. Some of the alternative hypotheses proposed include:

1. Milk, as other foods, acts as a physical barrier preventing fluoride access to mucosal surface of gastrointestinal tract (22).
2. When milk is coagulated in the stomach, poorly soluble  $\text{CaF}$  is formed, thus reducing fluoride absorption (11, 21).
3. Milk may cause an increase in gastric pH, reducing the formation of  $\text{HF}$  (11).
4. Gastric acidity reduces the pH of milk, causing an increase in unbound calcium which promotes  $\text{CaF}_2$  formation (11).
5. Fluoride is incompletely soluble in milk (22).

There have also been suggestions that protein may reduce the absorption of fluoride (41, 62, 72, 73).

However, studies that have specifically investigated the effects of either type or level of dietary protein on fluoride bioavailability when fluoride is presented in the diet appear to be unavailable.

#### Physiological Distribution of Fluoride

Fluoride has a strong affinity for calcified tissues, and therefore is concentrated in bones and teeth. The skeleton has a great capacity to accumulate fluoride, containing approximately 96% of the fluoride in the body (74). Up to half of an ingested quantity of fluoride may be retained by the hard tissues and the remainder is rapidly excreted by the kidneys at most levels of intake (5, 33). Fluoride is incorporated into the skeleton during the stages of bone formation and maintenance during normal bone remodeling. Uptake is maximal during the active mineralization phase of bone growth or tooth development when a large proportion of crystals are still forming (37). Fluoride ions present at crystal surfaces may also migrate into vacant spaces in the crystal interior during recrystallization (33). The level of fluoride in all calcified tissues is directly related to the quantity ingested. The fluoride content of bone and teeth increases linearly with the concentration of fluoride in the water supply up to approximately 8 ppm, and increases steadily with age (33, 74).

Plasma fluoride is maintained at extremely low levels ( $< 0.01$  ppm) due to the rapid processes of skeletal uptake and urinary excretion (33). These regulatory mechanisms are so efficient that minimal fluctuation of plasma values occurs even with variable fluoride intake (74). Fluoride is therefore not allowed to accumulate in the soft tissues. Although a transient rise in plasma fluoride may be seen following fluoride ingestion, none remains for any length of time due to the rapidity of skeletal uptake and urinary excretion. In addition, the large volume of extracellular fluid in which the absorbed fluoride is diluted contributes to maintaining low plasma and soft tissue levels (33). This indicates that the body maintains an effective fluoride homeostasis even at widely varying levels of intake.

The amount of fluoride taken up by different parts of the skeleton is determined by growth rate, vascularity of the tissue and by the duration of fluoride exposure (15, 33). Formative activity of bone is the main factor controlling the extent of fluoride uptake (33). For this reason, a greater proportion of fluoride is retained by the growing skeleton than by the adult skeleton (33, 75). The greater hydration of young mineralized tissue ensures a high permeability to

ions which provides better access of tissue fluids to crystal surfaces (33). In addition, a greater proportion of the skeleton is available to the circulation in individuals who are actively laying down bone mineral (76).

At all ages, some regions of bone are more active than others, resulting in uneven distribution of fluoride within bones (33). Due to site variability and irregular fluoride deposition, there is great variability in individual bone fluoride levels, as well as between different areas and types of mineralized structures within the individual. Based upon chemical analyses and histologic examination, cancellous (spongy) bone appears to store more fluoride than compact bone, epiphyses more than diaphyses, and biologically active surfaces of bone more than interior regions (33, 77, 78). Once deposited, fluoride may be removed from skeletal tissues by the bone remodeling process (33). Likens et al. (78), however, concluded that fluoride which is released during remodeling does not necessarily enter the circulation, but may be rapidly redeposited in adjacent regions of active bone formation.

Dental tissues differ from bone in that once formed, there is no remodeling. This is particularly true for formed enamel, which has no cellular activity

(33). Low permeability also restricts ionic mobility as opposed to bone tissue. Therefore, once fluoride is deposited in the teeth, it is not subject to resorption (3, 33). Maximal uptake occurs during the pre-eruptive period of tooth formation and calcification. However, fluoride uptake continues at the tooth surface during the post-eruptive maturation phase because the teeth are incompletely calcified at this time (3, 32). Thus, the full benefit of fluoride for caries prevention depends on optimal fluoride ingestion from birth to the age of 16 years. The concentration of human dentin fluoride is much higher than enamel (33), possibly because of its physiological similarity to bone. As with bone, fluoride is not homogeneously distributed in dentin. The highest concentration of fluoride is found closest to the pulp chamber, where the systemic blood supply is greatest, and where the formation of secondary dentin occurs (31, 32).

#### Fluoride Excretion

The principle route of fluoride excretion is by way of the urine (10, 76). Small amounts of fluoride are also excreted through sweat under hot and moist environmental conditions (79). A small portion of fluoride excretion is probably endogenous, absorbed and reexcreted via the gastric and intestinal fluids (76).

Because fluoride is a bone- and teeth-seeking element, and because excessive intake during the period of tooth formation and calcification may result in fluorosis, a thorough understanding of fluoride elimination is important.

Excretion generally reflects daily intake and it has been shown that there is a linear relationship between the concentration of dietary fluoride and the concentration of fluoride in the urine at fluoride levels in diet and water up to 8 ppm (64, 76). For this reason, urinary fluoride level is a useful indicator of fluoride intake (8, 76). Although trace amounts of fluoride appear in the blood within minutes, up to 30% of an ingested quantity of soluble fluoride is found in the urine within three to four hours, and between 80 and 90% is absorbed in eight hours after administration in rats and dogs (17, 80). These results have been confirmed in the rat (21, 81) and in humans (82) using the radioactive isotope  $^{18}\text{F}$ .

Glomerular filtration removes fluoride from the circulation and normal kidney mechanisms appear to account for urinary excretion of fluoride (15, 76). Fluoride excretion increases with the rate of urine flow and the glomerular filtration rate (10, 82). A steady state, where fluoride excretion approximately

equals fluoride intake, is established following ingestion of fluoride at constant levels of intake (76, 83). In contrast, individuals who have an irregular exposure to fluoride show higher excretion of fluoride when normal intake is exceeded (76), due to the rapid process of urinary excretion. Individual variation can be extremely large. Some of the factors influencing renal excretion of fluoride include the amount of fluoride ingested, water intake, previous exposure to fluoride, age and health status of the individual. For example, urinary fluoride excretion is generally lower in children because a larger proportion of fluoride intake is deposited in the growing skeleton, as compared to adults consuming the same amount of fluoride (76).

Recently, Whitford et al. (84) suggested that urine pH may also play a role in regulating fluoride excretion. In drug-induced diuretic studies with rats, increased fluoride excretion and decreased fluoride reabsorption was observed when an alkaline urine was produced. In contrast, when a slightly acid diuresis was induced, much lower fluoride excretion and greater fluoride reabsorption was observed. They proposed that the mechanism of renal fluoride reabsorption is non-ionic diffusion of HF from the tubule and the subsequent dissociation and trapping of ionic fluoride

in the interstitial fluids. From these data, Whitford and Pashley (85) concluded that fluoride reabsorption is greatest when the hydrogen ion concentration, and therefore the HF concentration is highest. More recently, Ekstrand et al. (65) demonstrated an increased fluoride excretion in human subjects with sodium bicarbonate-induced alkaline urine, and decreased fluoride excretion with ammonium chloride-induced acid urine, concluding that increased reabsorption of non-ionic HF was responsible for decreased fluoride excretion during these imposed acidic conditions. No differences in urine flow rate under either acidic or alkaline conditions were observed, which suggested that a concentration gradient within the distal tubule in favor of HF diffusion may be a major force for HF diffusion. However, the significance of these findings to normal individuals was disputed by Ekstrand et al. (10) who pointed out that even at a slightly acid urine pH, such as 5.2, less than 1% of fluoride would be present in the form of HF. Further investigations are therefore needed to determine the possible influence of urinary pH on fluoride reabsorption under normal dietary conditions.



### Rationale for a Protein-Fluoride Interaction

Dietary protein is known to influence the bioavailability of several trace elements. Of the trace elements which have been studied, effects of dietary protein on zinc, copper, iron and selenium have recently been reported (23, 24, 25, 26, 86). For example, a threefold increase in dietary protein, fed as lactalbumin, increased the apparent absorption of zinc as indicated by increased liver zinc and increased tibia zinc in rats (24). The addition of cysteine to low protein diets also had the effect of enhancing zinc bioavailability in this study. Similar findings have been reported in human subjects in balance studies. Greger and Snedeker (23) reported increased zinc absorption, increased serum zinc, and increased urinary zinc excretion when adult males consumed high protein diets. Higher copper absorption and retention were also observed with high protein diets. Although increased copper absorption could not be demonstrated in rats fed high protein diets, total liver and kidney copper was increased (24). In a recent study, Sherman et al. (86) observed that tissue concentrations of zinc, copper and iron were increased as a result of increasing dietary protein threefold. In addition, an age-protein interaction was observed. For example, a high protein diet resulted in increased iron in the spleens of young

rats, but lower iron content in the spleens of aged rats, compared to normal protein diets. A threefold increase in dietary protein level resulted in improved apparent absorption of selenium in adult males, but also increased urinary selenium excretion rates significantly (26). Because high levels of dietary protein influence the utilization of other trace elements, it is possible that excess dietary protein may influence some aspect of fluoride metabolism.

The source, as well as the level, of dietary protein is well-known to be a factor in trace mineral bioavailability. For example, phytic acid and various types of fiber and other chelators associated with plant proteins bind zinc and inhibit its absorption (87). Recently, higher tibia zinc concentration was observed in rats fed 30% lactalbumin compared to 30% soy protein diets, and zinc absorption was improved by the addition of sulfur amino acids to these diets (88). More efficient absorption of iron was also observed in the lactalbumin-fed rats. Soy protein may also reduce the absorption of non-heme iron (89). Adair and Wei (90) have suggested that the phytate or tricalcium phosphate present in soy-based infant formulas could decrease fluoride bioavailability by providing molecular-binding sites for fluoride.

It is possible that type or level of dietary protein may influence fluoride bioavailability either by altering gastrointestinal absorption or renal excretion. Proteins vary considerably in physical and chemical properties, and a number of factors associated with certain proteins may be important determinants of fluoride bioavailability. These factors include amino acid composition, secondary structure, and the state of hydration and net charge of proteins. Casein, for example, has a relatively higher proportion of basic amino acid residues, especially lysine, than lactalbumin (91). The significance of this observation is that lysine has been reported to reduce the skeletal uptake of  $^{18}\text{F}$  in rats (61). In a more recent investigation, excess dietary lysine (2.1%) fed to rats reduced liver and plasma copper concentrations (92). Mitchell and Jenkins (92) hypothesized that proteins or their breakdown products in the gastrointestinal tract affected copper bioavailability either by reducing tissue utilization or by promoting increased urinary copper excretion. Under the acidic conditions of the stomach, where fluoride absorption begins, casein may complex fluoride through the formation of salt linkages between fluoride and protonated amino groups (93), rendering it less available for absorption. In addition, casein has been shown to be associated with

approximately one quarter of the fluoride present in milk, whereas only traces of fluoride are associated with fats or the albumin-globulin fraction (94).

Major differences between casein and lactalbumin include the sulfur amino acid content, the ratio of cysteine to methionine and the fact that casein is a phosphoprotein (91, 95). These differences between casein and lactalbumin may influence either glomerular filtration rate or pH of the glomerular filtrate. As previously discussed, fluoride homeostasis is maintained by skeletal uptake and renal excretion. Of the factors influencing renal excretion, the most important are the plasma concentration of fluoride and urine flow rate (10, 82). Fluoride excretion increases proportionately with increased urine flow rate (76). Elevated levels of dietary protein are known to increase urine flow rate (96, 97), which may decrease the bioavailability of dietary fluoride by increasing urinary output and thus fluoride excretion.

Finally, the fluoride ion itself exhibits several unique properties which may favor an interaction with proteins in the gastrointestinal tract. Fluorine is the most electronegative element known, and reacts with almost every element in the periodic table (98, 99). Fluoride compounds thus tend to be either extremely

reactive, such as HF and F<sub>2</sub>, or extremely stable, such as organic fluorides. The fluoride ion is also the smallest negatively charged ion. Because of its very small size, fluoride may adsorb onto the surface of proteins, or interact with surface electrochemical or stereochemical properties of proteins (93). For example, fluoride has been reported to cause enzyme inhibition by adsorption onto active sites required for the formation of the enzyme-substrate complex (41). Surface binding of this nature is influenced by a variety of factors, including molecular conformation, state of hydration, the properties of the colloidal matrix and the properties of the anions (93).

In conclusion, although it is generally agreed that foods decrease fluoride bioavailability by 30-50% (8, 12, 15), the significance of specific dietary factors and their influence on fluoride bioavailability is not well understood. In the present report, the major hypothesis advanced is that dietary fluoride bioavailability may be particularly influenced by either the type or level of protein in the diet. Studies on the effects of protein on fluoride bioavailability may help to define which constituents in milk and other foods contribute to a decrease in fluoride utilization. This information would improve our understanding of the mechanisms, as well as the

practical implications of variable utilization of fluoride. Because protein is one nutrient which is commonly consumed in excess of the RDA (27, 28, 29), and fluoride intake in this country is highly variable, a protein-fluoride interaction could be important. Accurate bioavailability data would permit more accurate, precise assessment of fluoride status, and thus provide better guidelines for fluoride supplementation practices. This information may have particular implications for infants and children, especially during the critical period of tooth development when the need for optimal fluoride intake is greatest.

## MATERIALS AND METHODS

### Experimental Design

The experimental design was a factorial experiment involving three factors: fluoride, protein level, and protein type with two levels of each factor (table 3). There were eight treatments with six replicates per treatment.

### Diet Formulation

Two different basal diets were formulated (table 4). The normal protein diet provided 12% protein, a level considered to be adequate for the laboratory rat (100). The high protein diet provided three times this amount (36%) and was prepared with less dextrose in order to accomodate the larger amount of protein. Diets were composed of purified ingredients and met the known nutritional requirements for the growth and development of the laboratory rat (100). The selection of casein and lactalbumin as test proteins was based on the fact that they are both major milk proteins. The significance of this is that the proteins in milk may contribute to the observed reduction in fluoride bioavailability when given with milk (11, 22). Basal diets, without supplemental addition of fluoride, provided < 0.5 ppm fluoride. Components were mixed with a commercial food mixer (Model A-200, Hobart Mfg. Co.,

Table 3. Experimental design<sup>1</sup>

Fluoride	Protein type	Protein level	
		12%	36%
2 ppm	Casein	1	2
	Lactalbumin	3	4
10 ppm	Casein	5	6
	Lactalbumin	7	8

<sup>1</sup>Numbers 1-8 refer to dietary treatments. Replicates per treatment = 6.



Table 4. Basal diet composition

Component	Normal Protein	High Protein
	g/kg	
Protein source <sup>1</sup>	120	360
DL-methionine <sup>2</sup>	3	-
Vitamin mix <sup>3</sup>	50	50
Mineral mix <sup>4</sup>	30	30
Cellulose powder <sup>1</sup>	40	40
Cornstarch <sup>5</sup>	150	150
Corn oil <sup>6</sup>	50	50
Dextrose <sup>7</sup>	557	320

<sup>1</sup>U.S. Biochemical Corp., Cleveland, OH. 92.5% crude protein. Rats were fed diets containing either casein or lactalbumin (120 g/kg or 360 g/kg) as the sole source of protein.

<sup>2</sup>J.T. Baker Chemical Co., Phillipsburg, NJ.

<sup>3</sup>Ref. 108, except that choline was supplied as the bitartrate (40 g/kg mixture).

<sup>4</sup>See Table 5.

<sup>5</sup>Best Foods CPC International, Englewood Cliffs, NJ.

<sup>6</sup>Gregg Food Division of A.E. Staley Co., Portland, OR.

<sup>7</sup>Staleydex 333, A.E. Staley Co., Decatur, IL.

Troy, OH) equipped with a stainless steel bowl, mixing paddle and splash guard. Diets were stored in a refrigerator at 4°C.

It was possible to keep diet content of calcium, phosphorus, sodium, potassium, magnesium and zinc constant for all diets despite contribution of minerals from variation in protein type and level by formulating the mineral mixtures shown in table 5. Equilibration of these minerals eliminated possible confounding effects and limited the study to three experimental variables, thus allowing conclusions to be drawn based on the physical and chemical characteristics of the proteins alone.

Sodium fluoride (NaF) was used as the source of fluoride in this study because it is a soluble fluoride compound known to be virtually 100% absorbed by rats (21). The levels of fluoride chosen were 2 ppm and 10 ppm. Two ppm fluoride is within the range of the suggested requirement of this nutrient for the rat (100). The higher level was tested in order to improve the possibility of detecting differences in skeletal storage of fluoride, and also because the level of dietary fluoride may be an important factor in a possible protein-fluoride interaction. All experimental diets were formulated to supply 2 ppm

Table 5. Mineral mix composition<sup>1-3</sup>

Component	Diet			
	1,5	2,6	3,7	4,8
CaHPO <sub>4</sub>	13.62	5.71	15.81	12.30
CaCO <sub>3</sub>	3.00	8.78	1.35	3.94
NaCl	2.54	2.54	2.54	2.54
K <sub>2</sub> CO <sub>3</sub>	1.78	1.78	1.48	0.92
K <sub>2</sub> SO <sub>4</sub>	1.82	1.82	1.82	1.82
K <sub>3</sub> C <sub>6</sub> H <sub>5</sub> O <sub>7</sub> ·H <sub>2</sub> O	4.75	4.42	4.93	4.94
MgCO <sub>3</sub> -n-hydrate (26% Mg)	1.54	1.51	1.46	1.31
MnCO <sub>3</sub>	0.122	0.122	0.122	0.122
CuCO <sub>3</sub>	0.009	0.009	0.009	0.009
Ferric citrate (18.4% Fe)	0.190	0.190	0.190	0.190
ZnCO <sub>3</sub>	0.0483	0.0299	0.0522	0.0414
KIO <sub>3</sub>	0.0003	0.0003	0.0003	0.0003
Na <sub>2</sub> SeO <sub>3</sub> ·5H <sub>2</sub> O	0.0003	0.0003	0.0003	0.0003
CrK(SO <sub>4</sub> ) <sub>2</sub> ·12H <sub>2</sub> O	0.0192	0.0192	0.0192	0.0192
NaF	0.0044	0.0044	0.0044	0.0044

<sup>1</sup>Provides in g/kg diet.

<sup>2</sup>Made to 1 kg with dextrose.

<sup>3</sup>Final mineral content of diets, adjusted minerals only: Ca (0.52%), P (0.40%), K (0.36%), Na (0.10%), Mg (400 ppm), Zn (30 ppm). Mineral levels include the amounts provided by basal diet components.

fluoride by the addition of NaF. In order to provide the 10 ppm level, diets 5, 6, 7 and 8 were supplemented with further additions of NaF, using dextrose as a carrier to facilitate incorporation into diets. Thus, the variation in protein type, protein level and fluoride level resulted in eight different treatment diets, where each protein type and level was represented at each level of fluoride.

### Animals

Male, weanling, outbred Sprague-Dawley albino rats (Cr1:CD (SD) BR, Charles River Laboratories, Wilmington, MA) were used in this experiment. Rats were individually housed in suspended stainless steel screen-bottomed cages (Hazelton Systems, Aberdeen, MD) which were maintained in a temperature- and humidity-controlled animal room with a 12-hour light-dark cycle. Six rats of initial age 27 days and initial weight of 82 g (73-91 g range) were assigned to each of eight different treatments. Rats used in this research were kept in accordance with established regulations (101).

Individually formulated powder diets were provided in glass jars. Although diet intake was not restricted, daily intake was monitored in order to limit excess to a few grams over what was eaten the previous day as a management practice to minimize

waste, especially during the metabolic collection period. Fresh diet was given every other day, except during the collection period when it was provided on a daily basis. Distilled-deionized water was available ad libitum in glass bottles fitted with stainless steel sippers and silicon rubber stoppers.

### Conduct of Study

The experimental period was 28 days. This represents a substantial portion of the young, growing rat's life (102), and is commonly used in growth and metabolism studies (23, 68). Body weights were recorded weekly. During the fourth week, all rats were placed in stainless steel metabolic collection units (Hazelton Systems, Aberdeen, MD) in order to collect urine and feces for a five-day period. Urine was collected into polyethylene bottles. Feces were collected into unit funnels, then stored in plastic bags.

### Sample Collection and Preparation

At the end of the 28-day experimental period, all rats were euthanized by decapitation under carbon dioxide anesthesia. Tibias and femurs were excised and stored frozen at -20° C. Heads were collected and stored frozen in 0.9% saline, prior to extraction of molars. Tibias and femurs were steam autoclaved at 15 pounds pressure for 8 minutes to permit defleshing,

then extracted with 95% ethyl alcohol in a Soxhlet apparatus for 24 hours. The dried bones were ashed in a muffle furnace at 590° C for 24 hours (Thermolyne Corp., Dubuque, IO). Ashed bone samples were stored in a desiccator after dry ash weights were recorded.

The pH of collected urine samples was adjusted to approximately 5 by addition of 3 N HNO<sub>3</sub> and stored frozen at -20° C in polyethylene bottles. Upon thawing and immediately prior to analysis, urine samples were centrifuged at 420 x g for 10 minutes (Model PR-2, International Equipment Co., Boston, MA). Feces were frozen with liquid nitrogen and then ground to a uniform powder using an electric sample grinder (Braun Model KSM 2, Schawbel Corp., Cambridge, MA).

Heads were steam autoclaved at 15 pounds pressure for 12 minutes in order to soften connective tissues and allow extraction of all maxillary and mandibular second and third molars. Second and third molars for individual rats were pooled to ensure adequate sample size for fluoride analysis, and dried in an oven at 100° C for three days. The dried molars were stored in a desiccator and dry weights recorded.

#### Diet Analysis

Samples of each individual diet (5 g) were weighed out in duplicate and wet ashed on a temperature-

controlled hot plate using reagent grade  $\text{HNO}_3$ , followed by 30% hydrogen peroxide. Ashed samples were then dissolved in 3 N hydrochloric acid ( $\text{HCl}$ ) with gentle heating, and diluted with distilled water to appropriate volume for analysis. Calcium, magnesium and zinc were determined by atomic absorption spectrophotometry (Perkin-Elmer Model 2380, Norwalk, CT). For calcium determination, the final dilution was made with 0.1% lanthanum chloride solution to prevent interference by phosphorus (Analytical Methods for AAS, Perkin-Elmer Corp., Norwalk, CT). Phosphorus was measured by the colorimetric method of Fiske and Subbarow (103). Procedures for diet fluoride analysis are discussed below.

#### Fluoride Determination

All fluoride determinations were made using the fluoride ion-selective electrode (Model 96-09 combination electrode and Model 901 Ionalyzer, Orion Research Inc., Cambridge, MA). Sample pH was adjusted to 5.0 - 5.5, for all fluoride determinations with the electrode, by adding equivalent volumes of total ionic strength adjustment buffer (TISAB II), as described by Sekerka and Lechner (104). Use of this buffer is critical in fluoride determinations because for fluoride ion activity to approximate fluoride

concentration it is necessary to optimize and control factors such as pH, ionic strength, interference by other ionic species and temperature (48).

To minimize fluoride contamination, all laboratory ware used in this research was washed carefully, rinsed with distilled water, soaked in 10%  $\text{HNO}_3$  for three hours, and rinsed again with distilled water, as suggested by Singer and Armstrong (105). All fluoride solutions were kept in plastic bottles. Redistilled water was used in the preparation of all reagents, sample dilutions and standards used for fluoride analysis (105).

#### Bone and Urine Fluoride Determination

Bones were analyzed for fluoride content using the method of Singer and Armstrong (106). Ashed tibias and femurs were dissolved in 3 N  $\text{HCl}$  in 20 ml polystyrene beaker cups (VWR Scientific, Seattle, WA). When completely dissolved, the beaker cup contents were transferred to polymethylpentene flasks (50 ml, Nalge Co., Rochester, NY) containing 25 ml of TISAB IJ using several rinses of redistilled water. Final pH adjustment was made with the addition of 1 ml of 5 M sodium hydroxide ( $\text{NaOH}$ ), and contents were made to volume with redistilled water. The fluoride content of the bones was then directly determined with the



fluoride ion-selective electrode against similarly prepared fluoride standards (1 and 10 ppm). Aliquots of urine were diluted 1:1 with TISAB II in polystyrene beaker cups (10 ml) and urine fluoride was directly determined using the same instrumentation (107).

#### Diet, Molar and Fecal Fluoride Determination

Fluoride content of unashed diet samples, feces and molars were based on the perchloric acid diffusion of hydrogen fluoride (HF) according to the method of Osis et al. (50), prior to fluoride analysis with the electrode. In this procedure, fluoride in biologic materials is separated from interfering ions as HF, which is then collected in a trapping solution (NaOH) as NaF. Polypropylene Conway diffusion plates were used (83 mm outer diameter, Bel-Art Products, Pequannock, NJ), fitted with polystyrene petri dish lids (No. 4061, Lab-Tek, Naperville, IL). For all diffusion procedures, the center well was used as the collecting area and samples were placed in the ring surrounding the center well. For diet analysis, 0.5 g of unashed diet was used. For fecal analysis, 0.5 g of fecal sample for rats fed diets containing 2 ppm fluoride, and 0.3 g for rats fed diets containing 10 ppm fluoride diets were used. Entire molar samples, for both second and third molars were pulverized to a fine powder using an amalgamator apparatus (Model VM-C,

Caulk Co., Milford, DL) equipped with plastic capsules and stainless steel balls (108) before loading into plates. Silicon high-vacuum grease (Dow-Corning, Midland, MI) was generously applied to the outer rim of the sample ring to achieve tight seals with petri dish covers. Samples introduced into the sample ring were wetted with 2 ml redistilled water. To the center well 0.2 ml of 1.25 M NaOH was added, followed by 2 drops of absolute ethanol to facilitate spreading of the NaOH collecting solution over the well surface. To the sample ring 2 ml cold 50% perchloric acid were added and a petri dish cover immediately applied. Plates were stacked on boards, swirled gently to mix plate contents, and placed in an oven at 50-55° C for 22 hours.

Following the diffusion period, lids were removed and 0.2 ml of 1 N HCl and 2.2 ml redistilled water were added to the center well. The contents of the center well were mixed and transferred with disposable plastic transfer pipettes (Samco, San Fernando, CA) to beaker cups (10 ml) containing 2.5 ml TISAB II. The fluoride content of the resulting aqueous solution was then determined with the fluoride electrode. Duplicate fluoride standards and blanks were diffused with each set of analyses to check the recovery of fluoride, as

compared to non-diffused fluoride standards. For fecal analysis, duplicates were accepted if the variation about the mean was  $\leq 5\%$ .

#### Calculation of Fluoride Absorption and Retention

Urine, feces and diet fluoride content values were used to estimate apparent absorption and retention of dietary fluoride, as defined by the following equations:

$$\% \text{ Absorption} = \frac{\text{Intake} - \text{Feces}}{\text{Intake}} \times 100$$

$$\% \text{ Retention} = \frac{\text{Intake} - (\text{Feces} + \text{Urine})}{\text{Intake}} \times 100$$

The extent of utilization of absorbed fluoride was expressed as the ratio of retained fluoride to absorbed fluoride according to the following formula:

$$\frac{\text{micrograms fluoride retained}}{\text{micrograms fluoride absorbed}} \times 100$$

#### Statistical Analysis

The experimental design was a 2 x 2 x 2 factorial experiment with eight treatments and six replicates per treatment. Data were analyzed by factorial analysis of variance. Significant treatment effects were partitioned into effects due to fluoride, protein level, protein type and the interactions of these three factors (109). Individual means were compared using

the least significant differences method (LSD), only where the F test for treatments was shown to be significant (110). Differences between individual treatments were considered significant at  $P < 0.05$ .

## RESULTS

Food Intake and Growth

Food intake and growth for rats fed the eight treatment diets are presented in table 6. Rats fed diets containing 36% protein gained significantly more weight ( $P < 0.001$ ) than rats fed diets containing 12% protein. Final body weight was 7-13% higher in high casein groups compared to low casein groups, and 11-17% higher in high lactalbumin groups compared to low lactalbumin groups. The effect of high protein diets on growth observed in this study cannot be attributed to differences in food intake, since there were no significant differences in the four week total food intake between groups. Similar effects of high protein feeding on body weight have been reported by others (24, 111). Rats fed high protein diets tended to have greater femur ash weights than rats fed normal protein diets (table 6). However, when the ash weight of the bones was expressed as mg/100 g of body weight, no significant differences were found between treatment groups.

Food intake was within the expected range for rats of this age, and compares with or exceeds that of similar studies (24, 88, 112). However, individual rats in three of the four lactalbumin groups (12%

Table 6. Effect of protein level, protein type and dietary fluoride on food intake, weight gain and femur ash.<sup>1,2</sup>

Measures	2ppm F				10 ppm F				LSD (P < 0.05)
	12% protein		36% protein		12% protein		36% protein		
	cas	lac	cas	lac	cas	lac	cas	lac	
Total food intake (g)	449 ± 13	428 ± 28	453 ± 4	447 ± 6	454 ± 3	431 ± 25	439 ± 27	421 ± 22	—
4 week weight gain (g)	155 ± 9	138 ± 11	189 ± 9	183 ± 8	158 ± 8	136 ± 13	177 ± 14	166 ± 11	12.6
Left femur ash wt.(mg)	153 ± 10	153 ± 10	165 ± 20	179 ± 10	162 ± 10	147 ± 10	170 ± 20	166 ± 20	14.6
mg ash/100 g BW	65 ± 2	70 ± 5	63 ± 6	67 ± 2	68 ± 3	67 ± 7	66 ± 5	67 ± 4	—

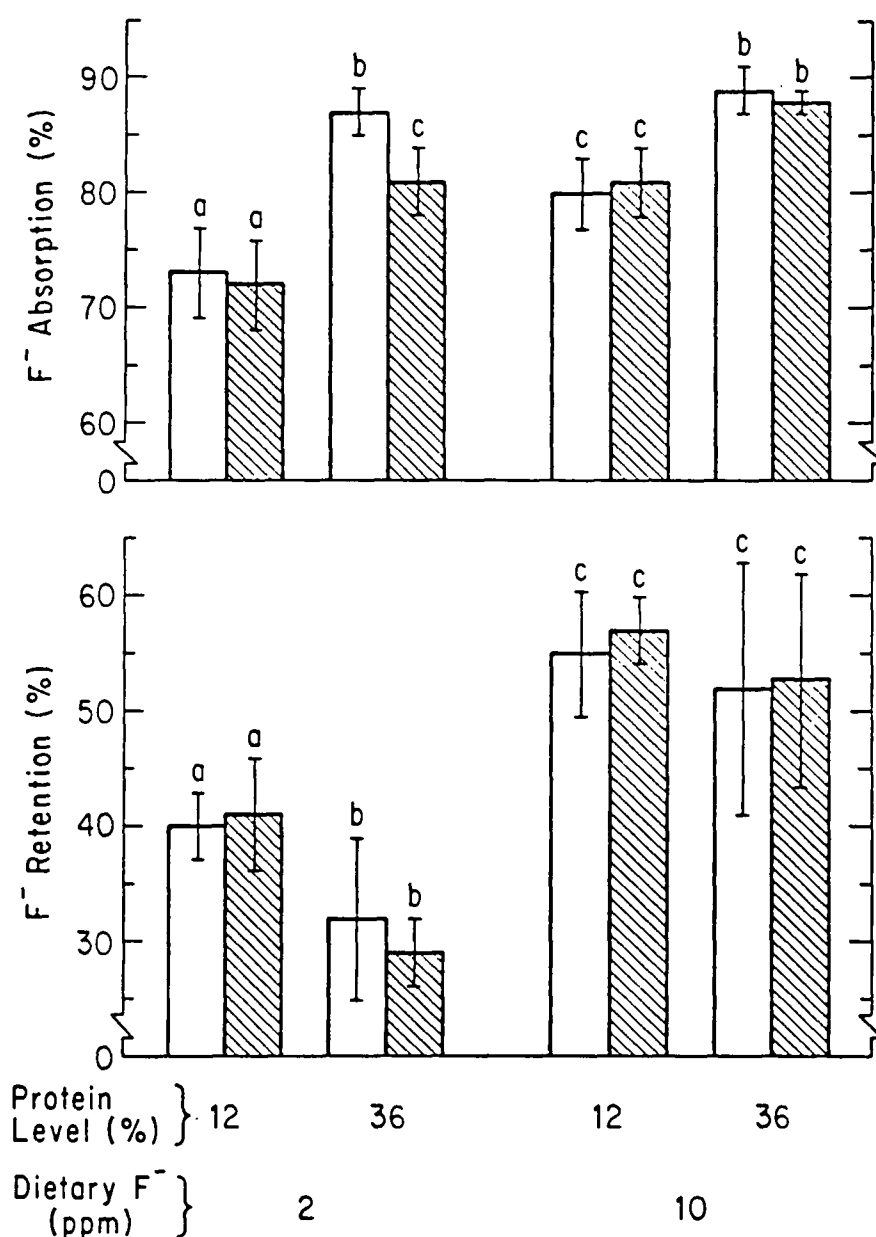
<sup>1</sup>Mean ± SD, n = 6.

<sup>2</sup>Cas = casein; lac = lactalbumin; F = fluoride.

lactalbumin - 2 ppm fluoride, 12% lactalbumin - 10 ppm fluoride and 36% lactalbumin - 10 ppm fluoride) reduced their food intake somewhat during the final week of the study, resulting in slightly lower body weights in these groups. Rats fed 12% lactalbumin had significantly lower final body weight ( $P < 0.05$ ) at either level of fluoride. In addition, variability in food intake was generally greater among rats fed diets containing lactalbumin than in the rats fed diets containing casein. There was no effect of fluoride level on food intake or growth.

#### Fluoride Absorption and Retention

Results for apparent absorption and retention of dietary fluoride are summarized in figure 1. Apparent absorption of fluoride increased significantly ( $P < 0.001$ ) with a fivefold increase in dietary fluoride at 12% protein, regardless of protein type. Although apparent fluoride absorption was also increased at 36% protein at the higher level of dietary fluoride, rats fed diets containing 10 ppm fluoride and 36% protein appeared to absorb similar amounts of fluoride as rats fed diets containing 2 ppm fluoride and 36% protein. Fluoride retention increased significantly ( $P < 0.001$ ) with a fivefold increase in dietary fluoride at the 2 ppm level of fluoride, but not at the 10 ppm level. The enhancing effect of increased dietary



**Figure 1.** Effect of protein level, protein type and dietary fluoride on fluoride absorption and retention. Clear bars = casein, hatched bars = lactalbumin. Values are mean  $\pm$  SD (n=6). Values not sharing a common superscript are significantly different ( $P < 0.05$ ) from each other.



fluoride on the apparent absorption and retention of fluoride is consistent with similar results in both animals (71, 112) and humans (63). The observed absorption and retention values in this study are similar to those reported by others (112, 113).

Feeding high protein diets significantly increased apparent absorption of fluoride ( $P < 0.001$ ) compared to normal protein groups. This protein effect was greatest in rats fed the diet providing 36% casein and 2 ppm fluoride (figure 1). In contrast, fluoride retention was significantly reduced ( $P < 0.001$ ) in high protein groups compared to normal protein groups at the 2 ppm fluoride level. A consistent trend in reduction of fluoride retention among rats fed high protein diets was also evident at the 10 ppm level of fluoride, compared to normal protein groups. When the extent of utilization of absorbed fluoride was expressed proportionally as micrograms of fluoride retained over micrograms of fluoride absorbed (figure 2), a 30-40% lower ratio was observed in high protein groups compared to normal protein groups at 2 ppm fluoride. Although this ratio was reduced by approximately 20% in high protein groups at 10 ppm fluoride, a similar value was observed for rats fed diets containing 36% protein and 10 ppm fluoride as rats fed diets containing 12% protein and 2 ppm fluoride. At both fluoride levels,

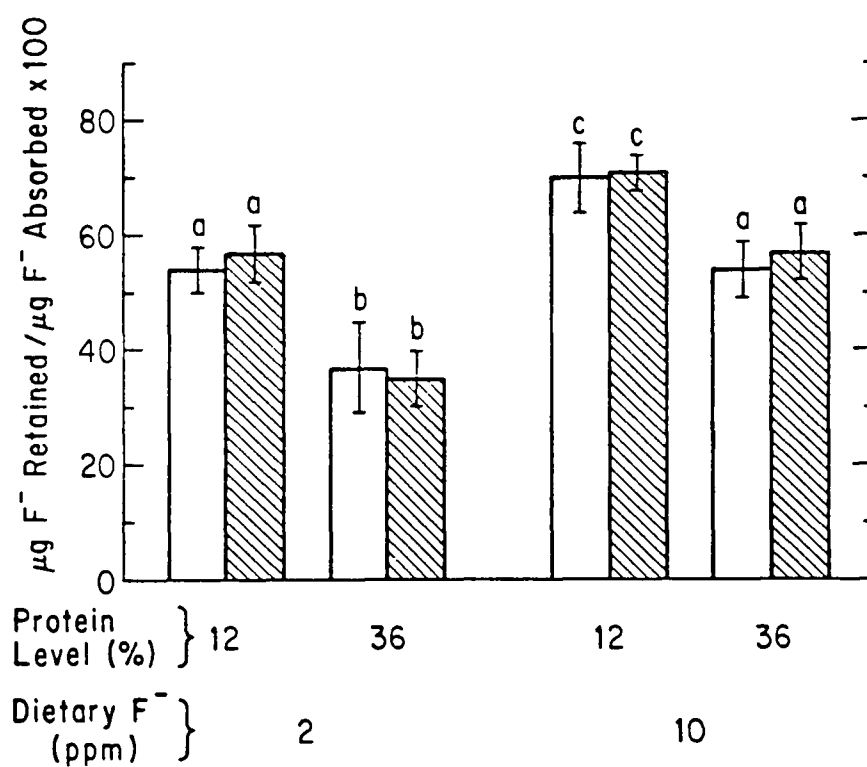


Figure 2. Effect of protein level, protein type and dietary fluoride on the proportion of absorbed fluoride retained by rats. Clear bars = casein, hatched bars = lactalbumin. Values are mean  $\pm$  SD ( $n = 6$ ). Values not sharing a common superscript are significantly different ( $P < 0.05$ ) from each other.

the reduction in fluoride utilization occurred regardless of protein type. The recovery of fluoride standards added as NaF during fecal analysis was 94-100% complete, with a mean of 95%. Good agreement ( $\pm$  5% of the mean) between replicates was observed.

### Bone Fluoride

There were no significant differences between the right and left femurs for any of the indices evaluated as determined by paired t-tests (table 7). Values for left femurs are presented as an indicator of fluoride bioavailability in this study. Although there were no significant differences between right and left tibia ash weights, significant differences ( $P < 0.001$ ) were found between right and left tibias for both fluoride concentration ( $\mu\text{g F/g ash}$ ) and total fluoride content ( $\mu\text{g F/tibia}$ ) (table 7). In every case, values for both of these measures of fluoride content for the right tibia exceeded those of the left tibias. Although both measures of femur fluoride content were similar to tibia fluoride content, variability was generally greater among tibias than femurs.

Femur fluoride increased significantly ( $P < 0.001$ ) with a fivefold increase in dietary fluoride (figure 3, table 7), when expressed as either concentration ( $\mu\text{g/g ash}$ ) or total fluoride

Table 7. Effect of protein level, protein type and dietary fluoride on rat femur and tibia fluoride content.<sup>1-4</sup>

Measures	2 ppm F				10 ppm F			
	12% protein		36% protein		12% protein		36% protein	
	cas	lac	cas	lac	cas	lac	cas	lac
<u>µg/g ash</u>								
L femur	264 ± 25 <sup>a,1</sup>	253 ± 24 <sup>a,1</sup>	206 ± 14 <sup>b,1</sup>	211 ± 7 <sup>b,1</sup>	597 ± 24 <sup>c,1</sup>	596 ± 30 <sup>c,1</sup>	508 ± 36 <sup>d,1</sup>	508 ± 30 <sup>d,1</sup>
R femur	261 ± 27 <sup>a,1</sup>	254 ± 24 <sup>a,1</sup>	205 ± 14 <sup>b,1</sup>	217 ± 11 <sup>b,1</sup>	591 ± 28 <sup>c,1</sup>	598 ± 34 <sup>c,1</sup>	504 ± 32 <sup>d,1</sup>	517 ± 28 <sup>d,1</sup>
L tibia	240 ± 21 <sup>a,2</sup>	236 ± 16 <sup>a,2</sup>	186 ± 17 <sup>b,2</sup>	211 ± 16 <sup>a,b,2</sup>	567 ± 23 <sup>c,2</sup>	569 ± 40 <sup>c,2</sup>	486 ± 32 <sup>d,2</sup>	490 ± 29 <sup>d,2</sup>
R tibia	277 ± 22 <sup>a,3</sup>	269 ± 24 <sup>a,3</sup>	221 ± 18 <sup>b,3</sup>	230 ± 10 <sup>b,3</sup>	597 ± 24 <sup>c,3</sup>	589 ± 41 <sup>c,3</sup>	510 ± 35 <sup>d,3</sup>	510 ± 29 <sup>d,3</sup>
<u>µg/bone</u>								
L femur	40 ± 3 <sup>a,1</sup>	39 ± 5 <sup>a,1</sup>	34 ± 4 <sup>a,1</sup>	38 ± 2 <sup>a,1</sup>	97 ± 5 <sup>b,1</sup>	85 ± 8 <sup>c,1</sup>	86 ± 6 <sup>c,1</sup>	84 ± 6 <sup>c,1</sup>
R femur	41 ± 4 <sup>a,1</sup>	39 ± 5 <sup>a,1</sup>	36 ± 4 <sup>a,1</sup>	39 ± 2 <sup>a,1</sup>	97 ± 5 <sup>b,1</sup>	85 ± 9 <sup>c,1</sup>	86 ± 6 <sup>c,1</sup>	88 ± 4 <sup>c,1</sup>
L tibia	32 ± 1 <sup>a,2</sup>	30 ± 4 <sup>a,2</sup>	27 ± 3 <sup>a,2</sup>	31 ± 2 <sup>a,2</sup>	78 ± 7 <sup>b,2</sup>	68 ± 8 <sup>c,2</sup>	70 ± 5 <sup>c,2</sup>	71 ± 2 <sup>c,2</sup>
R tibia	36 ± 2 <sup>a,3</sup>	34 ± 6 <sup>a,3</sup>	32 ± 4 <sup>a,3</sup>	34 ± 2 <sup>a,3</sup>	82 ± 5 <sup>b,3</sup>	70 ± 8 <sup>c,3</sup>	73 ± 5 <sup>c,3</sup>	74 ± 3 <sup>c,3</sup>

<sup>1</sup>Mean ± SD, n = 6.

<sup>2</sup>Cas = casein; lac = lactalbumin; F = fluoride; L = left; R = right.

<sup>3</sup>For each type of calculation, values in the same row with different superscript letters are significantly different (P < 0.05) from each other.

<sup>4</sup>For each type of calculation, values in the same column with different superscript numbers are significantly different (P < 0.05) from each other.

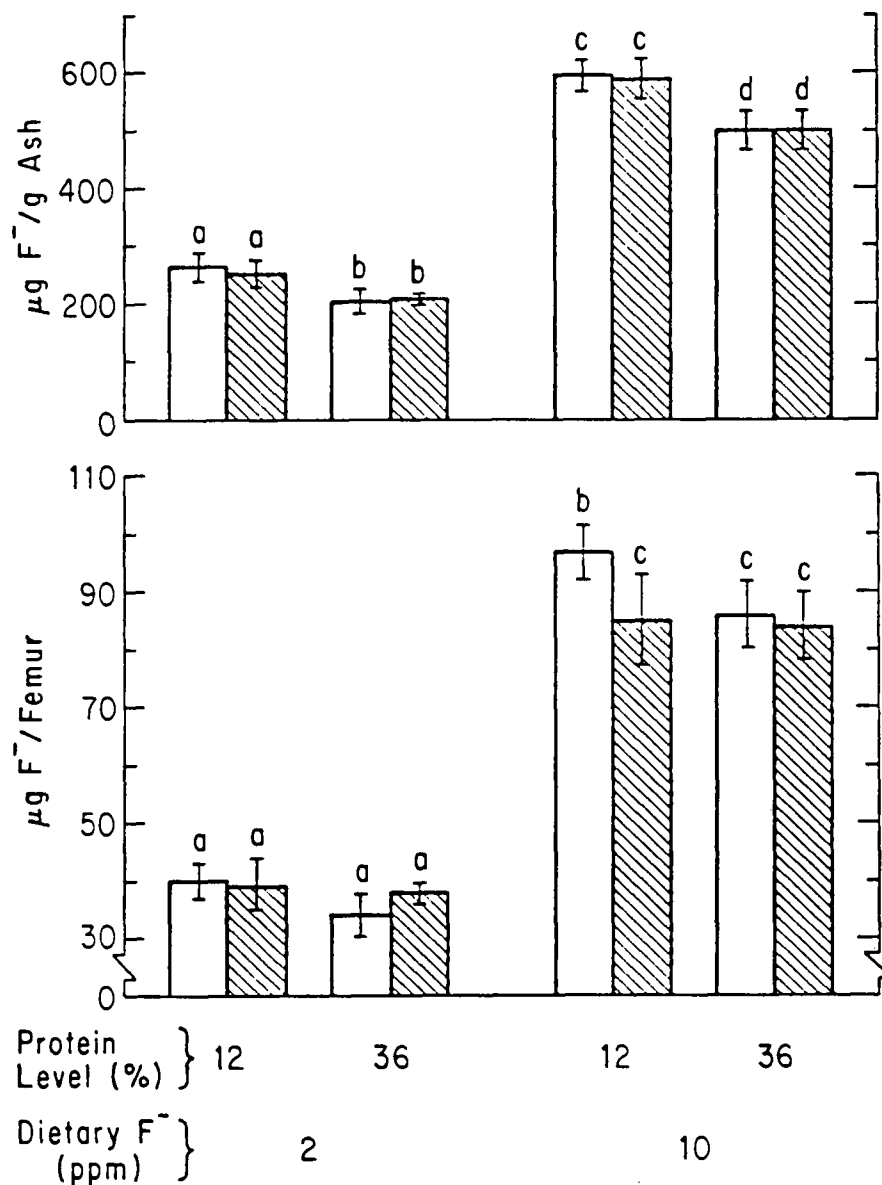


Figure 3. Effect of protein level, protein type and dietary fluoride on femur fluoride concentration and total fluoride content of rat femur. Clear bars = casein, hatched bars = lactalbumin. Values are mean  $\pm$  SD ( $n = 6$ ). Values not sharing a common superscript are significantly different ( $P < 0.05$ ) from each other.

( $\mu\text{g F/femur}$ ). These results agree with the findings of other investigators in demonstrating an increase in skeletal uptake of fluoride with increased concentration of dietary fluoride in similar studies (62, 71, 112, 113).

Femur fluoride concentration decreased significantly ( $P < 0.001$ ) as a result of a threefold increase in dietary protein (figure 3, table 7). However, when expressed on a per femur basis, a significant reduction in femur fluoride was observed only in the high casein group at 10 ppm fluoride ( $P < 0.001$ ). A trend for total femur fluoride to be reduced among rats fed high casein diets and 2 ppm fluoride was evident. In contrast, no significant differences in total femur fluoride were observed between rats fed diets containing either 12% or 36% lactalbumin at either level of fluoride. A significant interaction between protein level and type ( $P < 0.05$ ) was observed when femur fluoride content was expressed on a per femur basis. Although femur fluoride concentration generally agrees with total femur fluoride in casein groups, this was not the case with the lactalbumin groups. Significant interactive effects of fluoride and protein level were observed when fluoride content was expressed on a concentration basis ( $P < 0.05$ ). This occurred consistently with both left and right femurs

and tibias. The reduction of femur fluoride content, expressed as either concentration or on a per femur basis was consistent with the observed reduction in fluoride retention ( $r = .85$ ;  $P < 0.001$ ).

#### Molar Fluoride

Both second and third molar fluoride concentration increased significantly ( $P < 0.001$ ) with a fivefold increase in dietary fluoride (figure 4). Because attempts to recover all molars were not successful in every case, total molar fluoride is not shown. In general, third molars had slightly higher fluoride concentration than second molars, especially at the 10 ppm fluoride level. The fluoride concentration of second molars was significantly reduced in rats fed high lactalbumin diets ( $P < 0.01$ ), as compared to rats fed normal lactalbumin diets at 10 ppm fluoride. In contrast, third molar fluoride concentration was significantly reduced in rats fed high casein diets compared to normal casein diets at 10 ppm fluoride ( $P < 0.01$ ). Although significant differences could not be demonstrated between all low and high protein groups, a trend in reduction of molar fluoride uptake among high protein groups was evident.

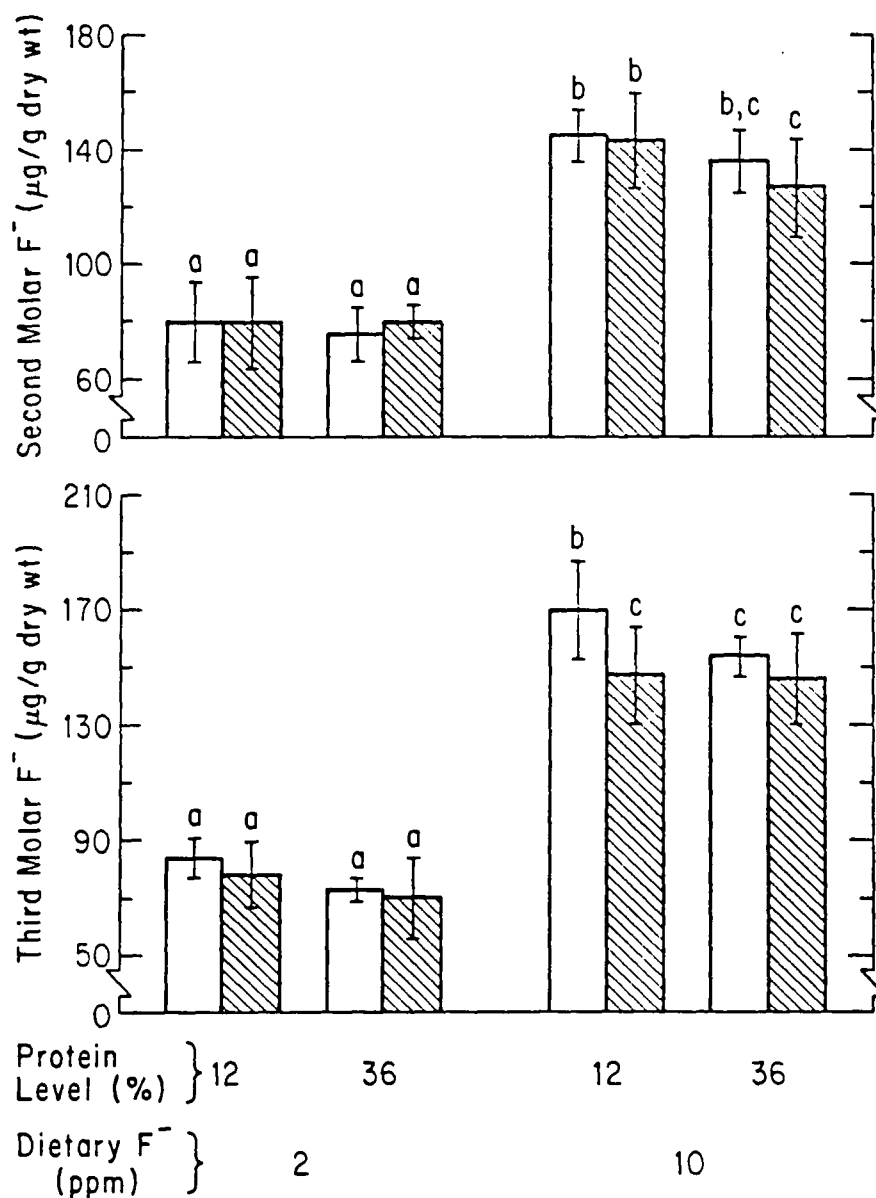


Figure 4. Effect of protein level, protein type and dietary fluoride on rat molar fluoride concentration. Clear bars = casein, hatched bars = lactalbumin. Values are mean  $\pm$  SD ( $n = 6$ ). Values not sharing a common superscript are significantly different ( $P < 0.05$ ) from each other.



## DISCUSSION

In a recent review, Rao (8) states that protein affects fluoride bioavailability. However, no direct evidence for this statement is available, indicating that the influence of protein on fluoride bioavailability has not been specifically investigated. In the present study, a threefold increase in dietary protein level significantly reduced overall fluoride bioavailability in the rat. High protein diets caused increased urinary excretion of fluoride which was reflected in a reduction in femur and tibia fluoride concentration. This reduction in fluoride uptake occurred despite the fact that high dietary protein enhanced apparent fluoride absorption. Increased urinary fluoride excretion in rats fed high protein diets was apparently of sufficient magnitude to overcome the enhancing effect of high protein diets on fluoride absorption. Thus, three main effects observed in the present study were increased apparent fluoride absorption, decreased fluoride retention and decreased skeletal fluoride uptake when high protein diets were fed to rats. These results cannot be attributed to unequal food intake because there were no significant differences in total food intake over the four weeks of the study.

The results of this study provide the first evidence for increased fluoride absorption due to high protein diets. This effect is consistent with previous reports in which excess dietary protein enhanced the absorption of selenium, zinc, and copper (23, 24, 25, 26). However, there are two important differences between these other trace elements and fluoride. Specifically, fluoride is an anion, and fluoride absorption begins in the stomach rather than the small intestine (14, 80, 81, 82). Because of uncertainty regarding the mechanism of fluoride absorption, it is difficult to propose a mechanism by which fluoride absorption is increased in rats fed high protein diets. It is generally considered that dietary fluoride is absorbed in ionic form (14). Recent evidence (67) however suggests that fluoride may also be absorbed as non-ionic hydrogen fluoride (HF,  $pK_a$  3.45). Whitford and Pashley (67) suggest that an inverse relationship may exist between fluoride absorption and gastric pH, where fluoride absorption is enhanced when pH is reduced. Because gastric acid secretion is promoted by dietary protein (97, 114), it is possible that the high protein diets fed to rats in this study resulted in a more acidic environment in the stomach, thus enhancing fluoride absorption. In addition, proteins leave the stomach more slowly than carbohydrates (97). As a

result, the high protein diets used in this study may have allowed a prolonged exposure of fluoride to its absorptive site in the stomach compared to the low protein diets. Thus high protein diets may not only reduce gastric pH, but may also promote a prolonged period of gastric acidity, allowing more opportunity for the passive diffusion of either HF or ionic fluoride across the gastric mucosa. This would not necessarily be true in the intestine where the pH is much higher. In addition, since the time food remains in the stomach is short compared to intestinal transit, further studies are needed to determine the importance of gastric fluoride absorption relative to that in the intestine. Although the hypothesis that pH plays a role in gastric fluoride absorption may help explain enhanced apparent fluoride absorption observed with high protein diets in this study, it is uncertain how much fluoride is absorbed as HF and what factors contribute to its formation. For example, the permeability coefficient of HF relative to ionic fluoride is not known (85). More information is required in order to define what conditions and fluoride levels are important in the absorption of either form of fluoride.

The observation that an increase in dietary protein level increases urinary fluoride excretion has

not been previously reported. The high degree of correlation ( $r = 0.85$ ;  $p < 0.001$ ) between per cent fluoride retention and femur fluoride content indicates that renal fluoride excretion was great enough to overcome the effect of enhanced absorption, causing significant reductions in skeletal uptake in all rats fed high protein diets. An important factor regulating urinary fluoride excretion is urine flow rate (76, 80, 82). High levels of dietary protein are known to alter certain aspects of kidney function such as increasing glomerular filtration rate (GFR) and thus urine flow rate (96, 97). The greater GFR and enhanced urine flow rate associated with high protein diets has been attributed to enhanced synthesis and excretion of urea which is accompanied by increased water consumption and increased urine volume (96, 97). Although neither water intake nor urine volume was measured in this study, it appeared by observation of urine collection bottles that the rats fed 36% protein diets excreted at least two to three times as much urine as rats fed 12% protein diets. This is consistent with reports of increased GFR and urine volume when high protein diets were fed to both rats and humans (115, 116, 117, 118, 119). Urine flow rate may therefore be an important

factor in the increased renal fluoride excretion observed in rats fed high protein diets in the present study.

Another well-known effect of high protein diets is increased net renal acid excretion (120), which results from increased catabolism of sulfur-containing amino acids and conversion of neutral food substances to organic acids. In addition, oxidative deamination of amino acids produces a greater quantity of ammonium ions which must be excreted. Ammonium ion production has been reported to double or triple with high levels of dietary protein in human subjects (117, 118). It is possible that excess ammonium ions may interfere with fluoride reabsorption through the formation of a complex between ammonium ions and ionic fluoride present in kidney tubules causing this fraction to be less well reabsorbed by the tubular cells. However, the mechanism of fluoride reabsorption in the kidney tubules is incompletely understood. Although ionic fluoride has generally been considered the major form in which fluoride is reabsorbed (15, 37), recent evidence suggests that non-ionic HF may be involved (84). The hypothesis that fluoride reabsorption as HF increases as urine pH decreases (84) would predict that urinary fluoride excretion would decrease with the feeding of high protein diets. The results of the

present study, however, contradict this hypothesis by demonstrating increased urinary excretion of fluoride with the feeding of high protein diets.

The combination of increased urine flow rate coupled with decreased reabsorption may account for the observed increase in urinary fluoride excretion of rats fed high protein diets in this study. More specifically, fluoride reabsorption in kidney tubules may not keep pace with the increased filtration rate induced by high levels of dietary protein. This effect would be consistent with the well-documented effect of increased urinary calcium excretion when high protein diets are fed to rats or humans (115, 116, 117, 118, 119, 121). The increase in urinary calcium seen in adults fed high protein diets can be accounted for by an increase in GFR as well as a decrease in fractional renal tubular reabsorption of calcium (117, 118, 121). Thus, the results of the present study suggest that the renal handling of fluoride may be similar to that of calcium with regard to overall effect when high protein diets are consumed.

The enhancement of weight gain by high levels of dietary protein despite similar food intake found in this study has been observed in some studies (24, 111), but not in others (115). The significant increase in

weight gain for rats fed 36% protein diets as compared to those fed 12% protein in this study may raise the question of whether the 12% protein diets were marginally deficient in one or more amino acids. The present study, however, confirmed that 12% protein is adequate for the weanling rat as indicated by the fact that rats fed diets containing either 12% casein or lactalbumin had normal weight gain and food intake for rats of this age (100). Furthermore, Bunce and King (122) reported that maximal growth rates were achieved with a dietary protein level of 12% lactalbumin in male weanling Sprague-Dawley albino rats, as compared to protein levels as high as 25% lactalbumin. Similar results were reported for casein (123).

Increased growth rate in rats fed high protein diets was reflected in greater ash weights for all bone samples of rats fed high protein diets. Because greater skeletal accretion with the same fluoride intake would have the effect of diluting the fluoride present in a particular bone sample (78, 124, 125), the present study has reported bone fluoride in terms of both concentration ( $\mu\text{g}/\text{g}$  ash) and total fluoride content of bones ( $\mu\text{g}/\text{bone}$ ). Due to these differences in ash weight, total bone fluoride may be a better indicator of fluoride bioavailability than bone

fluoride concentration in evaluating the effect of high protein diets on skeletal uptake of fluoride in this study. When bone fluoride content is expressed on a concentration basis, rats with larger bones appear to have less fluoride in their femurs and tibias due to the greater bone size attributable to high protein diets. When expressed on a per femur basis, the effect of bone size differences is removed. The observation that bone fluoride appears to be reduced in the high lactalbumin groups when expressed on a concentration basis, where no effect is evident when expressed as total fluoride, is further indication that fluoride concentration may be an inappropriate index for bone fluoride content in this study.

Skeletal uptake of fluoride is considered to be an excellent indicator of fluoride bioavailability (8, 33). In the present study, reduction in fluoride retention in rats fed high protein diets was reflected in a reduction of femur fluoride uptake when expressed on a concentration basis. Tibia fluoride values generally agreed with femur fluoride values. However, unexplained differences between right and left tibias were observed, suggesting that the femur may be a better criterion of skeletal fluoride uptake in the rat than tibias, as suggested by others (8, 21, 60, 62). Molars have also been shown to be a reliable indicator



of fluoride bioavailability (75). Although the molar data generally agreed with femur data in reflecting a reduction in fluoride retention, variability was greater than in either femurs or tibias.

Increasing the level of dietary fluoride significantly increased skeletal and molar uptake of fluoride, as expected, for both low and high protein groups. These results are consistent with the findings of others (62, 71, 112). Cerklewski et al. (71) recently found a threefold increase in femur fluoride when dietary fluoride as NaF was increased fivefold, compared to the twofold increase in femur fluoride observed in this study. However, these authors fed rats for six weeks, whereas rats were fed for a four week period in the present study. This two week difference in feeding period probably accounts for the higher femur values they observed since fluoride continues to be incorporated into the skeleton with age, especially while rats are still in the rapid growth phase (33). In addition, the fact that rats fed high protein diets providing 10 ppm fluoride in this study appeared to utilize only as much fluoride as rats fed 12% protein and 2 ppm fluoride (figures 1 and 2)

suggests that high protein diets may somehow prevent an increase in apparent fluoride absorption at higher levels of dietary intake.

High protein diets tended to produce a greater depression of fluoride retention in rats fed diets containing 2 ppm fluoride than in rats fed diets containing 10 ppm fluoride. This suggests that a protein-fluoride interaction is dependent on both the level of protein and the level of fluoride in the diet. It is possible that the larger amount of fluoride provided by the diets containing 10 ppm fluoride was partially able to overcome the negative effect of the high protein diets, resulting in smaller increases in urinary fluoride excretion. This observation may also reflect the fact that a higher positive fluoride balance results when dietary fluoride intake increases (18, 63, 64, 71, 112).

Close agreement between bone fluoride and determinations of apparent fluoride retention was observed among all treatment groups fed casein diets. The reduction in fluoride retention due to high protein diets paralleled decreases in bone and molar fluoride content among casein groups at either level of fluoride. However, there is incomplete agreement between retention data and bone data among lactalbumin

groups. Although lactalbumin appeared to influence apparent absorption and retention in the same manner as casein, this was not always reflected in the bone fluoride content. For example, when the proportion of absorbed fluoride which was retained was considered (figure 2), a consistent decrease among high protein treatment groups was seen, regardless of protein type. This suggests that the type of protein is not a factor influencing fluoride bioavailability. The reason for this lack of agreement is not clear. One possible explanation may be that although total food intake was similar between all treatment groups, three of the four lactalbumin groups did lag behind slightly toward the end of the experimental period. Consequently, small differences in fluoride intake as well as differences in growth rate may have affected fluoride uptake in rats fed lactalbumin-containing diets compared to casein-based diets. This could help explain the lower bone fluoride for rats fed diets containing 12% lactalbumin and 10 ppm fluoride compared to rats fed diets containing 12% casein and 10 ppm fluoride, despite similar absorption and retention values. Another possible explanation may involve fecal analysis. For unknown reasons, the feces of rats fed lactalbumin diets appeared to have a higher moisture content than the feces of rats fed casein diets. There

may thus be a matrix effect unique to the feces of rats fed lactalbumin diets which may have introduced either positive or negative interference during fecal analysis. It may be advisable to dry fecal samples to a constant weight prior to diffusion procedures and subsequent fluoride analysis with the electrode to avoid any possible influence of moisture on fluoride determination.

From the data presented in this study, it can be concluded that increased levels of dietary protein have a net negative effect on fluoride bioavailability in the rat. A threefold increase in dietary protein promoted greater losses of fluoride in the urine, which resulted in less fluoride available for uptake by the calcified tissues. Although the intent of this study was to compare the effect of two protein types on fluoride bioavailability, inconsistencies with the lactalbumin groups did not allow for this comparison to be made. If further investigations of this problem were to be conducted, efforts should be directed at identifying mechanisms involved in both increased fluoride absorption and increased renal excretion caused by the consumption of high protein diets. Recommendations which may facilitate these efforts include measuring water intake, urine volume and urine pH. To determine whether protein type is a factor

influencing fluoride absorption or retention, other protein sources should be examined, including other sources of lactalbumin. In addition, testing other levels of both protein and fluoride may help define the level at which a protein-fluoride interaction becomes important.

Although the effect of excess dietary protein on fluoride bioavailability in humans as a consequence of high protein diets cannot be defined by the present study, sufficient evidence has been provided to warrant studying the relationship between protein and fluoride intake in more detail. Considering the prevalence of high protein diets in the United States (27, 28, 29), as well as variable fluoride intake, which is often below the optimum recommended level (1), these results strongly suggest that an interaction between dietary protein and dietary fluoride may be of practical importance in human nutrition. The implications of an adverse effect of dietary protein on fluoride bioavailability would be greatest among small children in low fluoride regions, since it may reduce the benefits afforded by optimal daily fluoride intake with regard to resistance to dental caries.

## SUMMARY AND CONCLUSIONS

The essentiality of the trace element fluoride has recently been reaffirmed (1), based on its proven role in reducing the incidence of dental caries. According to recent estimates, foods can contribute significantly to total daily fluoride intake when processed or prepared in the home with fluoridated water (8, 9, 43). In non-fluoridated areas, foods may be the only source of fluoride intake. However, only 50-80% of the fluoride present in foods is available for absorption and utilization (8, 12). The term bioavailability is used to describe these properties (13).

The bioavailability of dietary fluoride is influenced by a variety of physiological and dietary factors. Although it has been suggested that protein affects fluoride bioavailability, a review of the literature indicates that the relationship between protein and fluoride has not previously been investigated. The present study was designed to determine the effect of dietary protein type and level on the bioavailability of dietary fluoride. In order to accomplish this, a factorial experiment was conducted with male weanling albino rats fed a purified diet. Diets providing 12% or 36% protein, as either casein or lactalbumin combined with 2 or 10 ppm

fluoride as NaF were fed for a four week study period. Criteria for fluoride bioavailability were skeletal and molar fluoride uptake, apparent fluoride absorption and fluoride retention calculated from fecal and urinary fluoride content. The high protein diets caused a significant reduction in fluoride retention at the lower level of dietary fluoride which was reflected by decreased femur and tibia fluoride concentration. A trend for total femur fluoride to be reduced among rats fed high casein diets was also observed. Although there was a tendency for molar fluoride content to be reduced among rats fed high protein diets, results for molar fluoride content were less reliable than either femur or tibia in this regard. The high protein diets providing 2 ppm fluoride resulted in the greatest depression of fluoride retention. This effect occurred despite the fact that high protein diets enhanced the apparent absorption of fluoride.

All rats fed high protein diets excreted significantly more fluoride in their urine which was great enough to overcome the enhancing effect of high protein diets on fluoride absorption. These results suggest that the reduction in skeletal and molar fluoride uptake due to increased urinary fluoride excretion is the result of altered kidney function attributed to factors associated with the consumption

of high protein diets (96, 97). Increased fluoride excretion due to high levels of dietary protein is therefore best explained by increased glomerular filtration rate coupled with decreased renal tubular fluoride reabsorption. This interpretation would be consistent with the previously documented effect of increased urinary calcium excretion with the consumption of high protein diets (117). The fact that the greatest treatment effects were observed at the low fluoride level combined with the high protein diets suggests that an interaction between protein and dietary fluoride may be dependent upon the levels of both protein and fluoride. However, inconsistencies associated with lactalbumin-fed groups do not permit definitive evaluation of the effect of protein type on fluoride bioavailability under the conditions of this study.

The results of the present study provide the first direct evidence that dietary protein is a nutritional factor which can negatively influence the utilization of dietary fluoride. Although the effect of excess dietary protein on fluoride bioavailability in humans as a result of high protein diets cannot be defined by the present study, these results strongly suggest that the potential for an adverse effect exists. An



interaction between dietary protein and dietary fluoride may be of practical importance in human nutrition, considering the prevalence of high protein diets in the United States (27, 28, 29), as well as variable fluoride intake, which is often below the optimum recommended level (1). The results of this study suggest that less efficient utilization of dietary fluoride in conjunction with high protein diets may be particularly important in marginal fluoride intake regions, where most fluoride is provided by foods alone. The combination of excess dietary protein and marginal fluoride intake may reduce the amount of fluoride available for incorporation into developing bones and teeth. This may have important implications for small children where optimal daily fluoride intake is critical for the prevention of dental caries.

In conclusion, the present results support the hypothesis that dietary protein can reduce net fluoride bioavailability. These results contribute to our understanding of fluoride metabolism in general, and provide information regarding factors affecting the renal excretion of this important trace element. This is particularly important since renal excretion is a major factor regulating fluoride homeostasis. Further research is indicated in order to not only identify the mechanism for the protein-fluoride relationship more

clearly, but also to determine the impact of excess dietary protein on fluoride absorption and retention in human subjects under normal dietary conditions. This could be accomplished by conducting metabolic balance studies where various levels and types of protein and fluoride are provided in the diet, and fluoride absorption and retention is calculated.

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