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# FLUORIDE IN THE NUTRITION AND METABOLISM OF EXPERIMENTAL ANIMALS

by

Charles W. Weber

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THE UNIVERSITY OF ARIZONA

GRADUATE COLLEGE

I hereby recommend that this dissertation prepared under my direction by Charles W. Weber

entitled FLUORIDE IN THE NUTRITION AND METABOLISM OF EXPERIMENTAL ANIMALS

be accepted as fulfilling the dissertation requirement of the degree of Doctor of Philosophy

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After inspection of the dissertation, the following members of the Final Examination Committee concur in its approval and recommend its acceptance:*

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|---|---|
| A. R. Kemmerer | 27 April 1966 |
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| W. F. McCaughey | 4-28-66 |
| James W. Berry | 28 April 1966 |

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ABSTRACT

Chicks, of a New Hampshire x Delaware cross, fed toxic levels of NaF showed reduced growth rates at dietary levels of 500 ppm fluoride or above. No significant differences were found in feed efficiency, total plasma protein levels, total plasma lipoproteins, dietary energy or percent fat utilization. The fatty acid concentrations of several body organs were investigated, and no significant changes were found to occur as a result of the dietary fluoride at levels up to 1000 ppm. Enzyme activities measured in liver and kidney homogenates were not altered by dietary fluoride. Heart cytochrome oxidase levels were significantly increased with 500 ppm dietary fluoride when compared with the unsupplemented birds. Plasma alkaline phosphatase levels were increased with 1000 ppm fluoride, but not at the 500 ppm level.

White mice fed dietary fluoride levels up to 900 ppm failed to show a significant depression in growth rate. No marked changes in digestible energy or digestible fat were observed at the highest level of dietary fluoride. A significant increase in succinic dehydrogenase activity in liver and heart homogenates was obtained in mice fed 225 ppm fluoride. Kidney levels of this enzyme were not significantly affected by dietary fluoride. Cytochrome oxidase levels were not significantly altered in heart and liver tissue, but were increased in the kidney. Isocitric dehydrogenase activities of heart, kidney and liver did not exhibit a significant change with fluoride treatment.

White mice were fed three dietary treatments of 1) low-fluoride; 2) low-fluoride plus 6 ppm F added and 3) a control diet. The results show that an absence of dietary fluoride through two generations of mice did not significantly alter growth rate, reproduction, or protein and fat digestion. Bone fluoride levels were decreased from 36 ppm in parental mice to a low of 9 ppm in the second generation. Bone citric acid levels remained unaltered. The majority of enzyme systems evaluated were unaffected by the feeding of the low-fluoride diet. Significant increases in activity were found in liver cytochrome oxidase levels while malic dehydrogenase activity was significantly depressed in mice of the second generation as a result of feeding the low-fluoride diet. These results suggest that dietary fluoride is not essential for normal growth and reproduction, but may be required for maintenance of enzyme activity at optimum rates.

## CHAPTER I

### INTRODUCTION and REVIEW OF LITERATURE

Fluoride is found in great abundance throughout the world in soil, plants, animals and in water. It occurs in the soil chiefly as fluorspar ($CaF_2$) and as cryolite ($Na_3Al\ F_6$), and is found in various forms in practically all foods and mineral waters (Gautier and Clausman, 1916). The skeletal structures of animals, especially teeth, contain the greatest amounts of fluorine present in physiological material. Although fluorine was discovered by Scheele in 1771, it was not until 1886 that it was isolated by a chemist named Moissan. About 1883, Morichini found fluoride in the bones of a fossil elephant. The findings of other early workers such as Gay-Lussac; Blaizot,(1893); Berzelius, (1806); Carnot, (1892); Brandl and Tappeiner, (1891); confirmed the presence of fluorine in animal skeletons.

In the early twentieth century the problem of fluorine toxicity became prominent. It was discovered that the usage of feed additives, such as rock phosphates, and airborne pollution, from manufacturing plants, could produce both acute or chronic poisoning, which could lead to stunting or death of animals.

Increased interest in the physiological significance of fluoride occurred in the 1930's as a result of its reported beneficial effects in the prevention of dental caries. McCollum et. al. (1925) had previously demonstrated an association between fluoride and dental caries in rats.

Other early studies indicated that the margin between beneficial and detrimental effects of fluoride was extremely small in comparison with other halides and minerals. As a result of such studies, an attempt was made to prevent dental caries through the artificial fluoridation of community water supplies and the addition of fluoride compounds to toothpaste. Fluoride additions to public water supplies has become controversial throughout the nation and the possibility of error in artificial fluoridation of public water supplies has caused concern about fluoride toxicity in humans.

Toxicity

Fluorosis in animals has been thoroughly reviewed by several authors: Cass, (1961); Hodge (1961), Phillips and Suttie, (1960), and the induced pathological changes have been reported for the kidney, thyroid and other soft tissues in mammals by Phillips and Lamb (1934). Gardiner and associates (1959), using chicks, have found hypertrophy and hyperplasia of the columnar epithelium of the proventriculus with high levels of fluoride.

The different chemical forms of fluoride exert different relative toxicities. The toxicity also is influenced by the level of fluoride intake and the physical form in which the compound is administered. The more soluble forms of fluoride are more readily absorbed and therefore, are more toxic per unit of fluoride ingested. The manner of administration in relation to the chemical form, whether via the drinking water or in the feed, also contributes to the relative toxicity. Mitchell and Edman (1945) using balance studies found the percent fluoride absorption was as follows: NaF in solution 97, $CaF_2$

in solid form 62, and bone meal 37 percent. They also found that 30 to 60 percent of ingested fluoride was retained in the body, and of this amount, 95 percent was retained in the skeleton, while the remainder was divided approximately equally between teeth and soft tissues.

In rats it was found that 44 percent of ingested fluoride crossed the membrane of excised gut sections into the surrounding medium in the first hour while an additional 14 percent was found in the medium after a total of two hours (Wagner, 1962; Foster and Rush, 1961). Wallace, (1954) in a previous experiment had found that in rats the cell membranes were freely permeable to sodium fluoride.

The toxicity of fluoride was greatly increased by increased fat level in the diet (Phillips and Hart, 1935; and Phillips, 1955).

Transport of the fluoride ion after absorption has been studied in the horse by Seppilli et al. (1957). Their experiments showed that a relatively large fraction of the fluoride which reached the blood was found in serum albumins. Using high levels of NaF (0.01 molar), it was found that 44 percent of the circulating ion occurred in the free ionic state while 56 percent of it was found to be protein bound. At a lower level of NaF, (0.003 molar), only 37 percent was protein bound, while 63 percent was in the free ionic form. These workers postulated that the mechanism of blood fluoride circulation was such that the ionic fluoride dialyzed across the capillary walls and was taken up by the bone with a resultant dissociation of the albumin-fluoride complex to free more fluoride ions.

The use of radioactive halides has demonstrated that $F^{18}$ disappeared very rapidly from the blood (Ullberg et al., 1965). Bone and

cartilage were found to have the highest concentrations of $F^{18}$ in the mineralized portions, while in unmineralized cartilage, no $F^{18}$ was detected. In soft tissues of blood vessels, central nervous system, eye, pituitary gland, muscle, thyroid, gastric mucosa, intestinal mucosa and liver there was little or no accumulation of the $F^{18}$ ions. The soft tissues of the heart and kidneys were affected by high levels of fluoride. Lindemann et al., (1950) demonstrated that in rat kidneys, the loops of Henle and convoluted tubules became dilated and inflamed when the animals were fed a 0.05 percent NaF diet but, showed a slow and partial recovery upon withdrawal of fluoride. Rats used in another study showed that no histologic changes occurred until after 28 days on a diet level of 0.05 percent NaF (Pendborg, 1957). Rats fed 50 ppm fluoride showed severe myocardial changes after one month, which included regressive degeneration of myocardial fibers with cloudy swelling and vacuolar degeneration, infiltration of round cells into the interstitial tissue, and small hemorrhages.

The uptake of blood fluoride is apparently different for each body organ. Buttner and Muhler observed an increased retention of fluoride in the soft tissues of chickens fed fat supplemented diets. Additional values for fluoride concentration in soft tissues and bone are listed by several authors (Gautier, 1917; Suttie et al., 1958; Ullberg, 1964; Delga and Fournier, 1950).

Fluoride accumulation has been shown to have unfavorable effects on the reproduction of albino rats when fed a level of 0.025 percent fluoride in the diet (Schulz and Lamb, 1925). Maze (1925) using rats, found that 0.01 percent NaF and KI (0.005 percent) were required to

insure normal reproduction. Reproductive performance was remarkably resistant to toxic levels of fluoride as long as appetite (feed consumption) was not severely depressed (Peirce, 1959). Signs of fluoride toxicity in newborn animals rarely occur because both placental and mammary transfer of fluoride ion is limited.

The process by which fluoride is incorporated into bones and teeth has been investigated by Smith and Lantz, (1933); Neuman et al., (1950); Miller and Phillips (1956). There is a reciprocal effect of fluoride on bone citrate such that as the citrate concentration decreases bone fluoride concentration increases. (Zipkin and Gold, 1963; Zipkin et al., 1965). An apparent explanation for this relationship is the influence of the fluorine ions on the crystallinity of bone apatite.

Enzyme Inhibition with Fluoride

The effects of fluoride on enzyme systems are many and varied. For example, recent in vitro studies on the enzymes of the digestive tract have indicated that the esterases and lipases of the pancreas and intestinal musoca are inhibited by fluorine (Amberg and Loevenhart, 1908; Cedrangolo, 1938). The inhibition of the lipases is greatest for the shortest chain fatty acid substrates and decreases as chain length is increased. The intestinal phosphatases in mice are apparently resistant to fluoride inhibition in comparison with the esterases and lipases in the rat (Cloetens, 1939; Burt et al., 1957).

The fluoride ion has been shown to affect some of the enzymes of the blood, causing inhibition of serum esterases, and the probable blockage of pyruvic dehydrogenase, acetic thiokinase, and alpha-ketoglutaric dehydrogenase in vitro. This was also indicated by increased levels of

pyruvate, acetoacetic and alpha-ketoglutaric acid found in the blood of rats fed fluoride (El Hawary, 1955; Skorepa and Todorovicova, 1957). An opposite affect was found in rabbit blood; cholinesterase activity was increased with higher intakes of NaF (Kutateladze and Antelava, 1960). Enolase and glyceraldehyde-3-phosphate dehydrogenase activities were inhibited in rat erythrocytes in the presence of fluoride (Mills and Jones, 1961).

The liver is probably the organ used most in enzyme studies on the in vitro effects of fluoride on enzyme systems. Liver lipase and esterase in several species are inhibited by concentrations of fluoride ions. Liver succinic dehydrogenase and succinic oxidase activities were decreased by 0.02 M NaF, while catalase activity was enhanced at this level (Sullivan and Knobelsdroff, 1962; Engelbrecht, 1962). The substrates of pyruvate and acetate were inhibited from reacting in rats by a 0.0001 molar level of fluoride (Aisenberg and Potter, 1955). Other liver enzymes inhibited by fluoride were inosinediphosphatase (Plant, 1955; Cloetens, 1939) acid and alkaline phosphatases (Tanaka, 1958); ornithine transcarbamylase which was stimulated by low levels of NaF and inhibited at higher levels (Cohen and Hayano, 1947) and takasulfatase which was entirely inhibited by fluoride (Tanaka, 1938). The conversion of kynurenine into 3-hydroxykynurenine (Ito et al., 1956) and the reaction of cysteine via cysteine sulfinic acid to sulfate was blocked by NaF in liver homogenates. Elliott (1955) using liver microsomes of guinea pigs, found with the addition of fluorine, that the reaction of cholic acid with co-enzyme A to yield hydroxamic acid was blocked. Flavin and Ochoa (1957) have reported an ATP-dependent enzyme

which catalyzed the $CO_2$-dependent phosphorylation of fluoride to yield monofluorophosphate and referred to it as "fluorokinase." In kidney homogenates fluoride inhibited the phosphatases and succinic dehydrogenases and also caused blockage of acetate metabolism. Very little research has been conducted on the effects of fluoride on enzymes of the heart. In a study in 1949, Hegglin et al., found that heart adenosine triphosphatase was inhibited by NaF.

A number of studies has indicated that the following muscle enzymes were inhibited by fluoride: creatine phosphokinase (Ennor and Rosenberg, 1954); adenosine triphosphatase (Hegglin et al., 1949); and 5-adenylic acid deaminase (Nikiforuk and Colowick, 1956). Lipman (1928) found that the hydrolysis of glycerophosphoric acid, hexosediphosphoric acid, and hexosemonophosphoric acid in muscle pulp was inhibited by the fluoride ion. The skeletal muscle conversion of glucose-1-phosphate to glycogen was activated by fluoride ions in the rabbit.

Enzymes of the bone have been found to be affected in different ways according to the enzyme involved. In rabbits, alkaline phosphatase increased and acid phosphatase decreased in the presence of fluorine (Tanaka, 1958). Abe (1959) found low levels of fluoride accelerated alkaline phosphatase activity and high levels decreased the activity of rat epiphyseal cartilage. Brustone (1957) observed partial to complete inhibition of esterase activity in bones and teeth by fluoride.

Additional enzyme systems influenced by fluoride ions are as follows: enolase, phosphoglucomutase, phosphorylase (Boser, 1957); glycerophosphatase (Imouye, 1927); urease (Jacoby, 1928); tyrosinase

(Lerner, 1952); glutamine synthetase (Denes, 1954); glycerate dehydrogenase (Willis and Sallach, 1962); Peroxidase of the thyroid gland (Hosoya, 1963).

In vivo fatty acid metabolism was decreased by fluoride ion inhibition of beta-hydroxybutyric dehydrogenase (Cheldelin and Beinert, 1952) and of the esterases of fatty tissues (Cedrangolo, 1938), and by blockage of fatty acid oxidation (Johnson and Lardy, 1950).

The mammary gland has also been shown to be sensitive to NaF through blockage of citric acid formation from substrates of fumarate and pyruvate or fumarate and acetate (Terner, 1955).

The above studies represent a partial list of enzymes which are inhibited in vitro and indicate that the effects of fluoride differ with organ and species. A careful analysis of the enzymes inhibited by fluoride, as found in the literature, demonstrates one clear point; each animal, each tissue and preparation procedure influence the results obtained.

## Dietary Essentiality of Fluoride

A very limited amount of work has been done with low fluoride diets. These studies, dating from the early thirties, were stimulated by the question of whether fluoride is an essential element in animal nutrition.

Sharpless and McCollum (1933) fed rats a semipurified diet with and without 10 ppm added fluorine. After a period of 120 days on the diet, no differences were noted in growth, reproduction and bone or tooth structure. The amount of fluoride in the diet is not known, but the occurrence of 150 ppm fluoride in femurs of rats fed the

unsupplemented diet indicated that it must have contained a sizable amount. Phillips, Hart and Bohstedt (1934) used a mineralized milk diet containing 0.2 ppm of fluoride; this diet was fed to rats for a period of 140 days. At the end of the feeding period, no significant differences were found between control rats and those fed low fluoride diet, but the control rats also had relatively higher bone fluoride levels. In 1937, Marcovith, Shuey and Stanley fed rats a mixture of rice and milk which contained 0.6 ppm fluoride for a period of 35 days. The results of bone fluoride analysis were as follows: low fluoride diet of rice and milk, 50 ppm; control diet of cryolite plus 4 ppm F added, 117 ppm; while the control diet of cryolite plus 7 ppm added had a value of 239 ppm fluoride. In a study by Evans and Phillips (1939), a diet containing 1.6 ppm F was fed to rats for a period of five generations, and at the end of this time, no differences in growth, reproduction or tooth structure were evident. Lawrenz, in 1945, formulated a purified diet which contained 0.47 ppm of fluoride and fed this to the offspring of female rats which had been maintained on the same diet. The low-fluoride diet was fed to young rats from weaning for a period of 207 days. At the end of the experiment, it was found that no significant differences in growth or tooth structure existed. Muhler (1954) fed a purified diet containing 0.1 ppm F to rats and found that it interrupted the reproductive cycle. However, it could not be determined whether this reproductive failure was due to the lack of fluoride in the diet or to the absence of some other dietary factor. Maurer and Day (1957) fed rats a highly purified corn starch-casien diet which contained no detectable amounts of fluoride. The

carcass analyses extrapulated to diet intake indicated a digestable fluoride content of about 0.007 ppm. The low-fluoride and supplemented animals showed no significant differences in liver, kidney and bone alkaline and acid phosphatase. The teeth of the low fluoride group showed no evidence of caries, and appeared no different from those of the supplemented rats. The fact that they were able to wean only half of the pups born could have been due not only to the low fluoride level but also to other dietary imbalances. A purified diet of sucrose, crisco, lactalbumin, liver extract, vitamins and salts, which was calculated to contain not more than 0.03 ppm F, was fed to rats by Pothapragada, 1962. The second generation rats had levels of 43 ppm F in the humerus bones. The authors found that a reproductive failure occurred in the third generation. Doberenz (1963) fed a minimal fluoride diet containing less than 0.005 ppm F to rats for 10 weeks and found a significant depletion in bone fluoride concentration. Three diets were used as follows: low-fluoride, low-fluoride plus 2.0 ppm F added and a control diet. Bone fluoride values were 2.90 ppm for low-fluoride, 34.63 ppm for low-fluoride plus 2 ppm F added and 12.54 ppm F for the control diet. The body weights at the end of 10 weeks were not significantly different. Enzyme studies showed a significant increase in serum isocitric dehydrogenase activity and a decrease in the liver activity of this enzyme for the low-fluoride fed rats. Other enzyme systems studied failed to show any significant differences due to fluoride.

The above papers failed to demonstrate an essential role for fluroide in the metabolism of rats. The effects of fluoride on reproduction is questionable and there is a good chance that dietary factors

other than fluoride have been involved in the results. The purification of diets to remove the fluoride raises the question of what else may have been removed in the process.

The evidence in support of fluoride as an essential dietary element comes from three different authors. In 1925, Maze used a whole-milk powder ration to maintain rats. When the animals were fed this diet, reproductive ability was lost and it was found necessary to add NaF (0.01 percent) and KI (0.005 percent) to obtain normal reproduction. Even then skimmed-milk diets failed to produce normal lactation or normal growth in the young. Information concerning the protein content and other essential dietary ingredients is unavailable. The authors stated that since the NaF and KI were added together, the beneficial effects could not be attributed to one or the other. Chaneles (1929) found that young rats which received 50 mg. of fluoride per kg. of body weight daily, did grow more rapidly than controls during a three to four month period. After this growth was depressed and at six months of age their body weight was 20 percent less than that of the controls. In addition, the reproductive cycle of the female rats was impaired in this study. In a 1945 study, McClendon and Foster used corn, sunflower seed, soybeans and yeast as dietary ingredients. The nutrient salts were purified by recrystallization. Two experimental rats were used, and after 48 days on the purified diet the animals showed signs of dying. One was treated with milk containing fluoride and lived an additional 70 days. The authors stated that the low fluoride in the mother's diet caused the birth of young and a limited amount of milk secretion. The rats had extensive decay of the molars and in some cases all the crowns

were lost. McClendon and Gershon-Cohen (1953) later fed a diet of hydroponically cultured corn, sunflower seeds and yeast and obtained a marked growth reduction which was attributed to a dietary deficiency of fluoride. The weights of the rats at 88 days of age were 51 grams for the low-fluoride diet and 129 grams for the control animals. A still later study demonstrated that the fluoride deficient diets failed to maintain normal growth rates as compared with the control rats (McClendon and Gershon-Cohen, 1954). Roentgenograms of the lower jaw of rats receiving the fluoride-deficient diet showed extensive caries of the molars. The authors concluded that the caries could be prevented by the presence of fluoride. No data for dietary or carcass fluoride levels were presented in any of the three papers by McClendon and associates.

CHAPTER II

# FLUORIDE TOXICITY IN THE CHICK

## INTRODUCTION

Young chicks and adult poultry have exhibited higher tolerance levels for fluoride than most mammalian species. The chick's ability to tolerate larger quantities of fluoride is credited to a lower intestinal absorption, and perhaps a more effective elimination of fluoride (Haman, et al., 1936; Phillips, et al., 1935). Kick et al., (1933), found that chicks could not tolerate fluoride levels higher than 3600 ppm in the diet. Gerry et al., (1947), found that in the growing chick the maximum safe dietary level of fluoride was 300 to 400 ppm of fluoride when fed as rock phosphate.

Phillips et al., (1935), demonstrated that the growth of chicks was inhibited by feeding 70 mg of fluoride per kg of body weight per day. The authors postulated that this level of fluoride caused growth inhibition by restriction of feed consumption. In addition, intraperitoneal injections of fluoride also restricted feed consumption. The restriction of feed intake by both methods indicated that the action of fluoride was systemic in nature and independent of any action in the digestive tract. Bixler and Muhler (1960) found that an increase in dietary fat enhanced body fluoride retention in the chick. Suttie and Phillips (1960), using rats fed a fat-free diet, found that ingested fluoride had no effect on the level of fecal fat

excretion. This would appear to eliminate the possibility that an increase in metabolic fat was responsible for the poor utilization of dietary fat by the fluorotic rat. Furthermore, dietary-free-fatty acids were efficiently utilized by fluoride-fed rats. At equal fluoride intakes in rats fed diets of normal fat content, more fat was observed in the feces of animals receiving fluoride by stomach intubation than by intraperitoneal injection. The authors concluded that the high levels of fecal lipid in fluorotic rats could be explained in part by inhibition of intestinal lipase activity.

These studies were undertaken to evaluate the effects of dietary fluoride on fat utilization and various enzyme systems in the chicks.

## EXPERIMENTAL PROCEDURES

Day-old chicks (New Hampshire x Delaware cross) were used in a series of dietary fluoride toxicity experiments and were fed for periods of four weeks in each experiment.. The chicks were selected at random and housed in electrically-heated batteries with raised wire floors at one day of age. Feed and water were supplied *ad libitum*. Table I lists the composition of the experimental diets. The vitamin mixture supplied essential vitamins at levels recommended by the National Research Council (1960). Fluoride was added to the diet in the form of the sodium salt. Records of mortality, feed consumption and weight gain were maintained for the duration of the four-week experimental period.

The methods for analysis of enzyme activities were as follows: isocitric dehydrogenase (Sigma, 1961); alkaline phosphatase (Sigma,

1961); succinic dehydrogenase (Cooperstein et al., 1950); cytochrome oxidase (Cooperstein and Lazarow, 1951); and lactic dehydrogenase (Sigma, 1957). Total plasma proteins were determined by TS Meter (American Optical Company). Lipoproteins were determined by the method of Straus and Wurm, 1958. Fatty acids were analyzed statistically by the Duncan multiple range test (Duncan, 1955).

RESULTS and CONCLUSIONS

The feeding of fluoride produced significant depressions in growth rate at levels of 500 ppm F or above. Results of four experiments showed essentially the same degree of weight depression with fluoride (Table 2). The relationship between growth depression and fluoride concentration was statistically significant for both male and female chicks and amounted to 8 percent at the 500 ppm F level; while a growth depression of 21 percent was obtained at 1000 ppm F. No significant differences were obtained in feed conversion, total plasma protein, total lipoprotein, dietary metabolizable energy or percent fat retentions (Tables 2 and 3). Suttie and Phillips (1960) demonstrated that high levels of NaF caused an alteration in fat absorption in rats; this finding could not be confirmed with the chick in the present experiments. A small decrease was noted in percent protein retention at the 1000 ppm F level (Table 3).

The effect of dietary fluoride on several enzyme systems was studied in connection with these experiments but little or no effect was obtained. Liver and kidney homogenates showed no significant differences in activities of lactic dehydrogenase, cytochrome oxidase and succinic dehydrogenase as a result of feeding 0 ppm, 500 ppm or

1000 ppm added fluoride (Table 4). Liver isocitric dehydrogenase activity was also not significantly affected by the three dietary levels of fluoride. The heart homogenates examined for succinic dehydrogenase activity failed to show a significant change attributable to dietary treatment (Table 4). Cytochrome oxidase activities, measured on heart muscle, were significantly elevated at the 500 ppm dietary F level but not at the 1000 ppm F level. This may be explained on the basis of a lower level of fluoride being stimulatory while a still higher level is not, as suggested by Cohen and Hayano (1947). It was found that 0.007 M NaF stimulated rat liver ornithine transcarboxylase activity, while 0.05 M NaF caused complete inhibition of the enzyme. Further, Abe (1959) found 5 ppm fluoride accelerated bone alkaline phosphatase, while 100 ppm F and above was inhibiting.

Plasma alkaline phosphatase levels rose as the dietary fluoride level was increased. Significantly higher levels of plasma alkaline phosphatase were obtained with 1000 ppm added fluoride in the diet compared with the unsupplemented control group (Table 4).

The fatty acid compositions of the different body organs were apparently not altered by the feeding of 500 or 1000 ppm F levels in comparison with the 0 ppm added F level of the basal diet (Tables 5 and 6). The fecal fat samples from the fluoride-treated chicks did not show a significant change in the individual fatty acid ratio or the ratio between the saturated and unsaturated fatty acids when compared to the control samples. The liver, kidney and heart fatty acids showed no significant alterations in the levels of fatty acids or the ratio of saturated to unsaturated fatty acids at either the 500 or 1000 ppm F

levels. A small difference in the liver fatty acids was noted. Liver linoleic acid decreased slightly while arachidonic acid was increased at the 1000 ppm F level; but, this change was not proven to be statistically significant.

The data on tissue fatty acid levels and percent fat retentions would indicate that fluoride had little or no effect on either fat digestion or cellular metabolism of fatty acids in the chick in contrast to the report of Suttie and Phillips (1960) with the rat.

The results obtained suggest that other than the reduction in growth rate there was no impairment in dietary fat or protein utilization; since fat and protein retentions were not significantly altered by dietary fluoride level. Total dietary metabolizable energy was essentially the same for all treatment groups, also suggesting no impairment in energy metabolism.

The data do show that feed intake was reduced either as a result of lowered energy needs resulting from fluoride feeding or an impairment in the appetite centers of the brain. The usage of consumed nutrients was equally efficient for all fluoride-treated chicks as evidenced by the feed conversion data.

## SUMMARY

Chicks fed toxic levels of NaF showed reduced growth rates at levels of 500 ppm added F or above. A 21 percent depression in growth rate was demonstrated for both males and females fed 1000 ppm added fluoride; however, no significant differences were found in feed conversion, total plasma protein, total plasma lipoproteins, dietary metabolizable energy or percent fat retentions.

The fatty acid concentrations of several body organs were investigated, and no significant changes were found to be caused by either 500 or 1000 ppm of dietary fluoride. Liver samples from chicks fed 1000 ppm fluoride did show a decrease in linoleic acid and an increase in arachidonic acid but this was not apparent at the 500 ppm F level. Since the liver samples were pooled for analysis, no statistical measurements were possible. It can be postulated that dietary fluoride levels up to and including 1000 ppm had no detectable effect on fat digestion or cellular fatty acid metabolism.

Dietary fluoride did not alter the enzyme activities measured in liver and kidney samples. No significant differences were found in succinic dehydrogenase and cytochrome oxidase activities of the liver and kidney tissues. Heart cytochrome oxidase levels were significantly increased with 500 ppm dietary fluoride. Plasma alkaline phosphatase showed increased activity at 1000 ppm fluoride, but not at the 500 ppm F level. Isocitric dehydrogenase showed no changes which could be associated with the dietary fluoride treatments.

Table 1. Basal diets used for all chick experiments.

| Ingredients | Dietary Levels of NaF | | |
|---|---|---|---|
| | 0 ppm F | 500 ppm F | 1000 ppm F |
| Fish Meal | 5.00 | 5.00 | 5.00 |
| Alfalfa Meal (dehy.) | 2.00 | 2.00 | 2.00 |
| Dried Whey | 1.00 | 1.00 | 1.00 |
| Distillers Dried Solubles | 1.00 | 1.00 | 1.00 |
| Animal Fat | 5.00 | 5.00 | 5.00 |
| Ground Yellow Corn | 49.78 | 49.67 | 49.56 |
| Soybean Meal | 31.15 | 31.15 | 31.15 |
| Dicalcium Phosphate | 1.00 | 1.00 | 1.00 |
| Calcium Carbonate | 0.75 | 0.75 | 0.75 |
| Salt (trace) | 0.20 | 0.20 | 0.20 |
| Techmangan | 0.02 | 0.02 | 0.02 |
| Methionine Hydr. Analogue | 0.10 | 0.10 | 0.10 |
| Vitamin Premix (PR-9)[1] | 2.50 | 2.50 | 2.50 |
| $Cr_2O_3$ | 0.30 | 0.30 | 0.30 |
| NaF | - | 0.11 | 0.22 |

[1]Pr-9 vitamin premix supplied the following per pound of diet: 4,5000 I.U. vitamin A, 700 I.C.U. vitamin $D_3$, 2 mg. riboflavin, 12.5 mg. niacin, 5 mg. d-calcium pantothenate, 400 mg. choline chloride, 6 mcg. vitamin $B_{12}$, 2.5 I.U. d-alpha-tocopheryl acetate, 1 mg. menadione sodium bisulfite and 56.75 mg. ethoxyquin in a soybean meal carrier.

Table 2--Effect of dietary fluoride on growth and feed conversion.[1]

| Dietary Treatments | Initial Weight | Average body weight at 4 wks., gms. | Percent Reduction in body weight | Feed Consumption/ bird, gm. | Feed Conversion |
|---|---|---|---|---|---|
| 0 ppm | 38.0 | 413.8[a2] | -- | 708 | 1.706[a2] |
| 500 ppm | 37.9 | 385.4[b] | 8.73 | 680 | 1.768[a] |
| 1000 ppm | 38.2 | 313.3[c] | 20.71 | 574 | 1.826[a] |

[1]Average values for the combined 4 experiments.

[2]Means having different superscripts are statistically different at the 0.05 level of probability.

Table 3--Effect of dietary fluoride on plasma protein, lipoprotein and dietary nutrient utilization.[1]

| Dietary Treatments | Total Plasma Protein | Total Lipo-Protein | Dietary Metabol-izable Energy | Percent Fat Reten-tion | Percent Protein Retained |
|---|---|---|---|---|---|
| 0 ppm | 3.09[a2] | 134 | 3.0230 | 93.56 | 79.30 |
| 500 ppm | 3.08[a] | 126 | 3.0975 | 93.79 | 79.86 |
| 1000 ppm | 3.04[a] | 133 | 3.0153 | 92.34 | 75.31 |

[1]Average values for the combined 4 experiments.

[2]Means having different superscripts are statistically different at the 0.05 level of probability.

Table 4--The effect of dietary fluoride on enzymes systems of chicks.

| Enzyme systems | Tissue | Enzyme Activity 0 ppm F | 500 ppm F | 1000 ppm F |
|---|---|---|---|---|
| Lactic dehydrogenase[2] | Liver | 668.5[a1] | - | 734.4[a] |
| | Kidney | 270.7[a] | - | 271.8[a] |
| Cytochrome oxidase[3] | Liver | 4.272[a] | 4.002[a] | 4.794[a] |
| | Kidney | 3.150[a] | 3.258[a] | 2.958[a] |
| | Heart | 3.618[a] | 4.878[b] | 4.290[ab] |
| Succinic dehydrogenase[3] | Liver | 1.656[a] | 1.740[a] | 1.842[a] |
| | Kidney | 1.902[a] | 1.902[a] | 1.878[a] |
| Isocitric dehydrogenase[3] | Liver | 20.04[a] | 15.60[a] | 15.78[a] |
| Alkaline phosphatase[4] | Plasma | 27.78[a] | 34.14[ab] | 40.07[b] |

[1]Means having different superscripts are statistically different at the 0.05 level of probability.

[2]Enzyme activity is given as delta O.D./mg/min.

[3]Enzymes activity is given as delta O.D./mg./hour.

[4]Alkaline phosphatase activity is given Sigma Units/ml.

Table 5--The effect of dietary fluoride on fatty acid utilization in chicks.[1]

| Dietary Treatment | Samples Analyzed | Fatty Acids, as percent Total Fat | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | C[2] 10:0 | C 12:0 | C 14:0 | C 16:0 | C 16:1 | C 16:2 | C 18:0 | C 18:1 | C 18:2 | C 18:3 | C 20:0 | C 20:4 | Ratio Sat. / unsat. |
| | Feed | T | 0.5 | 1.5 | 19.4 | 1.4 | T | 10.9 | 43.2. | 23.2 | 0.2 | - | - | 0.47:1 |
| 0 ppm F | Feces | T | T | 1.2 | 28.3 | 1.0 | 0.5 | 30.9 | 24.3 | 12.2 | T | 1.4 | - | 1.6:1 |
| 500 ppm F | " | T | T | 1.6 | 29.1 | 1.3 | 0.7 | 28.9 | 22.7 | 13.1 | - | 1.4 | - | 1.6:1 |
| 1000 ppm F | " | T | T | 1.5 | 27.9 | 1.1 | 0.6 | 26.7 | 24.1 | 14.3 | T | 1.8 | - | 1.4:1 |
| 0 ppm F | Liver | - | - | 0.7 | 22.4 | 2.1 | T | 22.5 | 25.9 | 17.9 | - | - | 8.6 | 0.84:1 |
| 500 ppm F | " | - | - | 0.6 | 20.0 | 1.7 | T | 23.6 | 22.9 | 21.0 | - | - | 10.3 | 0.79:1 |
| 1000 ppm F | " | - | - | 0.5 | 20.0 | 0.6 | T | 26.1 | 18.9 | 18.1 | - | - | 13.3 | 0.92:1 |

[1]Average of experiments 2, 3, 4 is given on fatty acid values.

[2]Length of the carbon chain and number of double bonds per chain.

[3]Trace.

[4]Ratio of total saturated and unsaturated fatty acids.

Table 6--The effect of dietary fluoride on the fatty acid levels in the heart of the chick.[1]

| Dietary Treatment | Samples Analyzed | Fatty Acids, as Percent Total Fat | | | | | | | | | | | | Ratio Sat. unsat. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 10:0 | 12:0 | 14:0 | 16:0 | 16:1 | 16:2 | 18:0 | 18:1 | 18:2 | 18:3 | 20:0 | 20:4 | |
| 0 ppm F | Kidney | - | T | 1.4 | 17.3 | 3.5 | 1.5 | 13.3 | 27.2 | 25.9 | T | - | 9.1 | 0:48:1 |
| 500 ppm F | " | - | T | 1.6 | 16.5 | 3.0 | 1.7 | 13.7 | 25.7 | 22.6 | - | - | 11.4 | 0.49:1 |
| 1000 ppm F | " | - | T | 1.3 | 16.6 | 3.5 | 0.6 | 12.6 | 28.7 | 22.5 | T | - | 11.2 | 0.46:1 |
| 0 ppm F | Heart | T | T | 1.4 | 19.2 | 6.1 | 1.0 | 7.3 | 39.2 | 19.5 | 1.8 | - | 2.7 | 0.40:1 |
| 500 ppm F | " | T | T | 1.6 | 16.0 | 5.6 | 1.2 | 8.2 | 36.6 | 22.7 | 1.6 | - | 4.7 | 0.36:1 |
| 1000 ppm F | " | T | T | 1.4 | 19.3 | 5.2 | 0.7 | 8.5 | 38.1 | 20.8 | 1.6 | - | 4.6 | 0.41:1 |

[1]Average of experiments 2, 3, 4 is given on fatty acid values.

[2]Length of the carbon chain and number of double bonds per chain.

[3]Trace.

[4]Ratio of total saturated and unsaturated fatty acids.

CHAPTER III

# FLUORIDE TOXICITY IN THE MOUSE

## INTRODUCTION

Mice have shown a surprising tolerance to high levels of fluoride in comparison with other mammalian species. Leone et al., (1956) found that 46 mg fluoride (as NaF) per kg of body weight orally or 23 mg per kg intravenously, produced acute toxicity in mice. Segreto et al., (1961), using $SnF_2$ administered as a 10 percent solution via stomach tube, reported an Ld-50/24 hours of 3.54 mg. in mice. These workers observed no changes at autopsy and histologic examination which could be considered pathognomonic. The kidneys showed marked hyperemia but no tubular changes which could be associated with chronic sodium fluoride poisoning. Segawa (1955) reported that the toxic symptoms of NaF in mice were excitation and convulsions. The use of larger doses caused severe convulsions followed by tonic paralysis. It was assumed that these effects of NaF were due to activation of cell membranes by NaF itself or through an anticholinesterase action. Fleming and Greenfield (1954) working with pregnant mice found that the oral or parenteral administration of NaF or $CaF_2$ during gestation caused changes in the structure of the jaws and teeth of the neonatal mice. The changes which occurred were retardation of calcification in the jaw bones and enamel matrix plus alteration in the cell structure of the ameloblasts. $CaF_2$ seemed to be more toxic to the fetus than NaF.

The fluoride was given in the drinking water at ingestion rates of 60 to 80 mcg. per day, and injections were at a level of 100 mcg. per day. Levels which could be tolerated without causing resorption of fetuses or stillbirths were approximately 600 to 700 mcg. of $CaF_2$ and 1000 to 1200 mcg. of NaF.

In view of the lack of information concerning the toxic effects of fluoride in mice, the present studies were undertaken to evaluate the effects of dietary fluoride on growth, digestion and selected enzyme activities.

## EXPERIMENTAL PROCEDURES

Weanling mice from the Charles River Farms were selected randomly and housed in raised wire screen cages at a room temperature of 26 + 2°C. Feed and water were supplied ad libitum. The dietary fluoride was administered as the sodium salt.

In the first experiment eleven mice were used per dietary treatment, and the duration of the study was three weeks. The dietary treatments employed included a basal diet (Table 7) and two levels of added NaF to supply 225 and 450 ppm F. The mice were weighed and sacrificed at the end of the third experimental week at which time samples of blood, liver, kidney, and heart were obtained and frozen immediately in a dry ice-acetone solution and stored at -10°C until analyzed for fatty acids and enzyme activities.

The fatty acids were determined by the method of Metcalfe and Schmitz (1959). Isocitric dehydrogenase activity was determined as outlined by Sigma (1961), while succinic dehydrogenase was estimated by the method of Cooperstein et al., (1950) and cytochrome

dehydrogenase by the Cooperstein and Lazarow (1951) method. Bone citric acid levels were determined by the method of Hess and White (1955).

In the second experiment 450 and 900 ppm added dietary fluoride were fed for a period of four weeks. The mice were selected at random and alloted by sex to the dietary treatments. The 12 animals of each treatment group were divided into either two males or two females per cage and were weighed weekly. Feed and fecal samples were collected during the last two weeks of the experiment for $Cr_2O_3$, fat and bomb calorimeter analyses. These values were used to calculate the percent fat digestion and digestible energy values. The data were analyzed statistically by the multiple range test (Duncan, 1955).

## RESULTS AND DISCUSSION

Two toxicity studies were carried out with mice to determine the effects of fluoride on growth, enzyme systems, bone citric acid levels, fatty acid metabolism, digestible energy and total dietary fat utilization. No significant differences in body weight gain for growing mice were obtained using levels of fluroide up to 900 ppm (Tables 8 and 9). No marked changes in digestible energy or digestible fat were noted with the feeding of sodium fluoride and there were no gross symptoms of fluoride toxicity noted. A significant increase was obtained in succinic dehydrogenase activity in liver and heart homogenates from mice fed the 225 ppm fluoride level (Table 10). Kidney levels of this enzyme were not significantly affected by the fluoride treatments. Cytochrome oxidase levels were not significantly altered in heart and liver tissue, but were increased by dietary fluoride in

the kidney (Table 10). Isocitric dehydrogenase activities of heart, kidney and liver did not exhibit a significant change with the feeding of fluoride.

It has been reported by Suttie and Phillips (1960) that the feeding of high levels of fluoride to rats resulted in an impairment of fat digestion and an increase in fecal fat. The results obtained with mice in our studies showed fat digestions ranging from 90.0 to 92.8% (Table 9). No significant differences could be attributed to dietary fluoride. Digestible energy measurements also failed to indicate a significant alteration of energy digestibility which could be attributed to the fluoride treatments.

Further examination of fat metabolism was undertaken through analysis of liver fatty acid compositions (Table 11). The results obtained suggest the possibility of an alteration in fat metabolism; however, the number of samples involved were not sufficient to allow a statistical evaluation of the differences. Liver oleic acid values were decreased from 44% of total liver lipids to a level of 35.2% with the feeding of 450 ppm fluoride. A linear increase in arachidonic acid (20:4) was obtained with the respective fluoride treatments (Table 11). The work of Zipkin et al., (1963) indicated a decrease in bone citric acid levels in rats with the feeding of fluoride. The results obtained in the first experiment (Table 2) failed to substantiate the above findings. There were no significant differences in bone citrate which could be attributed to the dietary fluoride treatments. In view of the marked differences in fluoride tolerance between rats and mice, the possibility exists that the

treatment levels employed (up to 450 ppm fluoride) were not high enough to elicit the reported response in bone citrate in the mice.

SUMMARY

Two experiments were conducted with weanling mice in order to evaluate the effects of dietary fluoride on growth rates, energy and fat digestion, bone citric acid levels and tissue enzyme activities. The results of these studies indicate that mice are relatively tolerant to the ingestion of fluoride. Levels of up to 900 ppm fluoride failed to produce a significant depression in growth rate. A number of workers have reported that as little as 500 ppm fluoride in the diet of rats produces a marked depression in growth.

Significant alterations in tissue enzyme activities were obtained with as little as 225 ppm fluoride. Succinic dehydrogenase activities were increased in both liver and heart tissue with the feeding of added fluoride but no significant change in the activity of this enzyme was noted in kidney homogenates. Kidney cytochrome oxidase levels were increased with the feeding of fluoride; while isocitric dehydrogenase activities in these three tissues were not altered.

The present studies failed to detect a significant change in the levels of bone citric acid in mice. A suggested *in vivo* effect on fat metabolism was indicated from measurements of liver fatty acid compositions. There was a linear increase in arachidonic acid with the feeding of 250 and 450 ppm fluoride as compared with mice fed the unsupplemented control diet.

Table 7--Composition of mouse diets.

| Ingredients | 0 ppm F % | 225 ppm F % | 450 ppm F % | 900 ppm F % |
|---|---|---|---|---|
| Soybean protein | 28.00 | 28.00 | 28.00 | 28.00 |
| Corn oil | 6.00 | 6.00 | 6.00 | 6.00 |
| Sucrose | 56.96 | 56.91 | 56.86 | 56.76 |
| Vitamin mixture[1] | 0.14 | 0.14 | 0.14 | 0.14 |
| Salt mixture[2] | 8.00 | 8.00 | 8.00 | 8.00 |
| Choline Chloride | 0.20 | 0.20 | 0.20 | 0.20 |
| DL-Methionine | 0.40 | 0.40 | 0.40 | 0.40 |
| $Cr_2O_3$ | 0.30 | 0.30 | 0.30 | 0.30 |
| NaF | 0 | 0.05 | 0.10 | 0.20 |
| TOTAL | 100.00 | 100.00 | 100.00 | 100.00 |

[1]Supplied the following as mg. per kgm. of diet: 12.5 ascorbic acid, 12.5 thiamine • HCl, 100.0 niacin, 20.0 riboflavin, 12.5 pyridoxine • HCl, 1.25 d-biotin, 75.0 d-calcium pantothenate, 10.0 vitamin $B_{12}$ (0.1%), 14,000 I.U. vitamin A, 4.00 folic acid, 1,500 I.U. vitamin $D_2$, 200.0 d-alpha-tochopheryl-acetate, 1.25 menadione (2-methyl-naptho-quinone), 500.0 ethoxyquin, 500.0 i-inositol, 25.0 para-aminobenzoic acid, 25.0 oxytetracycline.

[2]Supplied the following as percent of diet: 0.8200 $Ca(OH)_2$, 1.9760 $KH_2PO_4$, 0.5000 NaCl, 0.0336 $MnSO_4$. $H_2O$, 0.1320 $FeSO_4 \cdot 7H_2O$, 0.0240 $ZnSO_7$, 0.0020 $CuSO_4 \cdot 8H_2O$, 0.0052 KI, 0.0100 $CoCl_2 \cdot 6H_2O$, 0.5000 KCl, 0.5800 $MgSO_4 \cdot 7H_2O$, 0.0003 $H_2MoO_4 \cdot H_2OMoO_4 \cdot H_2O$, 0.0007 KBr.

Table 8--Effect of dietary fluoride on bone citric acid levels and body and liver weights in mice.

| Dietary Treatments | Average initial body weights, gms. | Average 3rd week body weights gms.[1] | Average 3rd week liver weights, gms. | Liver weights as % of body weight | Bone citric acid, mcg/gm. |
|---|---|---|---|---|---|
| 0 ppm F | 9.18 | 22.09[a2] | 1.4786 | 6.69 | 14.05[a] |
| 225 ppm F | 9.73 | 23.64[a] | 1.6290 | 6.89 | 8.84[a] |
| 450 ppm F | 9.91 | 22.73[a] | 1.5443 | 6.79 | 14.74[a] |

[1] Average weights are for both males and female mice.

[2] Means having different superscripts are statistically different at the 0.05 level of probability.

Table 9--Effect of dietary fluoride on body weights, digestible energy and fat digestion in mice.

| Dietary Treatments | Sex of Mice | Average 4th week weights, gms. | Percent Digestible energy | Percent Fat Digestion |
|---|---|---|---|---|
| 0 ppm F | Male | $26.5^{a1}$ | 3.8409 | 90.04 |
| 450 ppm F | Male | $26.5^{a}$ | 3.7756 | 90.94 |
| 900 ppm F | Male | $22.9^{a}$ | 3.8457 | 92.24 |
| 0 ppm F | Female | $20.7^{a}$ | 3.8577 | 92.06 |
| 450 ppm F | Female | $18.4^{a}$ | 3.7725 | 92.51 |
| 900 ppm F | Female | $19.7^{a}$ | 3.8284 | 92.84 |
| 0 ppm F | Male and Female | $23.6^{a}$ | 3.8493 | 91.05 |
| 450 ppm F | Male and Female | $22.5^{a}$ | 3.7741 | 91.73 |
| 900 ppm F | Male and Female | $21.3^{a}$ | 3.8371 | 92.54 |

[1]Means having different superscripts are statistically different at the 0.05 level of probability.

Table 10--Effect of feeding NaF on the enzyme activity of body organs of mice.

| Enzyme system | Tissue | Dietary levels of added fluoride | | |
|---|---|---|---|---|
| | | 0 ppm F[1] | 225 ppm F | 450 ppm F |
| Succinic dehydrogenase | Liver | 024.64[b]2 | 039.73[a] | 034.36[ab] |
| | Kidney | 090.64[a] | 102.64[a] | 104.8[a] |
| | Heart | 168.86[c] | 756.18[a] | 463.30[b] |
| Cytochrome oxidase | Liver | 810.42[a] | 900.09[a] | 904.64[a] |
| | Kidney | 123.00[b] | 476.73[a] | 477.55[a] |
| | Heart | 875.5[a] | 865.8[a] | 679.8[a] |
| Isocitric dehydrogenase | Liver | 90.44[a] | 79.87[a] | 97.21[a] |
| | Kidney | 34.84[a] | 43.92[a] | 39.65[a] |
| | Heart | 38.88[a] | 46.03[a] | 47.11[a] |

[1]Enzyme activities are given in units of change in O.D/gm tissues/ min. for succinic dehydrogenase and cytochrome oxidase and as change in O.D/mg. tissue/minute for isocitric dehydrogenase.

[2]Means having different superscripts are statistically different at the 0.05 level of probability.

Table 11--Effect of dietary fluoride on the fatty acid composition of liver and feces in mice.

| Dietary treatments ppm F | Samples Analyzed | Fatty acids percent of total fat | | | | | | | | | Sat.[3] Unsat. Ratio |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | C[1] 14 | C 16 | C 16:1 | C 18 | C 18:1 | C 18:2 | C 18:3 | C 20 | C 20:4 | |
| 0 | Feed | | 11.0 | T[2] | 1.9 | 28.2 | 55.6 | 2.3 | 1.0 | | 0.16:1 |
| 0 | Feces | | 20.5 | 5.1 | 12.3 | 30.0 | 25.3 | - | 6.8 | - | 0.66:1 |
| 250 | Feces | | 24.4 | 7.7 | 9.9 | 29.5 | 28.5 | - | - | - | 0.52:1 |
| 450 | Feces | | 19.1 | 3.8 | 11.4 | 26.4 | 22.3 | - | 9.2 | 7.8 | 0.66:1 |
| 0 | Liver | 0.4 | 22.6 | 4.9 | 7.5 | 44.1 | 13.2 | - | - | 7.3 | 0.44:1 |
| 250 | Liver | 0.3 | 21.8 | 4.2 | 6.5 | 44.7 | 12.3 | - | 1.0 | 9.2 | 0.42:1 |
| 450 | Liver | 0.3 | 21.2 | 3.4 | 10.4 | 35.2 | 14.0 | - | T | 15.5 | 0.47:1 |

[1]The length of the carbon chain and the number of double bonds found per chain length.

[2]Trace amounts of fatty acids which are found.

[3]Ratio of saturated to unsaturated fatty acid.

## CHAPTER IV

## EFFECT OF LOW-FLUORIDE DIETS ON SUCCEEDING GENERATIONS OF MICE

### INTRODUCTION

The early work of Sharpless and McCollum (1933) stimulated the question of whether fluoride was an essential element in animal nutrition. Later experiments have further investigated the possibility of fluoride being a dietary essential (Phillips, et al., 1934; Marcovith, et al., 1937; Evans and Phillips, 1939; Lawrenz, 1945; Muhler, 1945; Maurer and Day, 1957; Pothapragada, 1962; and Doberenz, 1963). None of these investigators conclusively demonstrated that fluoride had an essential physiological function in the body. In most of these experiments either the dietary levels of fluoride in supposedly low-fluoride diets were fairly high or the experimental animals exhibited impaired reproduction which indicated a dietary imbalance other than fluoride.

Three laboratories have published experimental results which support the theory that fluoride is an essential dietary element. Maze (1925) and Chaneles (1929) demonstrated in short term experiments that fluoride was necessary for normal reproduction and growth. A series of articles by McClendon et al., (1945, 1953, 1954) suggested that fluoride was essential to sustain life and to maintain normal growth, reproduction and lactation in rats. The lack of fluoride in the diet also led to increased decay of the molars. These experiments

led to the following study which was an attempt to determine the effects of feeding minimal fluoride diets to mice for an extended period of time.

EXPERIMENTAL PROCEDURES

The hydroponic techniques which were used for the production of sorghum (Sorghum Vulgare, DD38) and soybeans (Glycine Max, Lee) of low-fluoride content have been previously described (Doberenz, 1963). The positive control diet was mixed with grain sorghum and soybeans from a field-grown source while greenhouse-grown material was used in the low-fluoride diet. Both samples of soybeans were ground and autoclaved for one hour at 15 pounds pressure in order to destroy the growth inhibitors present in raw soybeans.

Pure crystalline vitamins were used and, in most cases, needed no further purification. The few vitamins which were fed in larger quantities were purified by recrystallization (Doberenz, 1963). Minerals were likewise recrystallized before use in the experimental diets.

Three dietary treatments were used throughout the experiments: 1) low-fluoride diet with hydroponically grown grain sorghum and soybeans; 2) low-fluoride diet plus 6 ppm added fluoride (as NaF); and 3) basal-control diet with field-grown sorghum and soybeans (Table 12).

White mice from the Charles River Farms were housed individual in stainless steel wire screen cages at a room temperature of 26 $\pm$ 2°C. Feed and deionized-distilled water (more than 18 million ohms resistance) were supplied ad libitum. Five weanling female mice (about 9 gm. in

weight) were placed on each dietary treatment. The experimental period for each generation was 12 to 14 weeks and was sufficient to reproduce and wean the next generation of mice.

During the first four weeks of the experiment, the mice were weighed each week and feed consumption recorded. Feed and fecal samples were collected during the four weeks of the experiment and analyzed for $Cr_2O_3$, fat and gross energy by standard laboratory methods. The values $Cr_2O_3$, fat and gross energy were used to calculate dietary percent fat digestions and digestible energy values.

At parturition, the number of live pups produced was recorded. Previous to the date of delivery, a solid stainless steel plate was placed over the wire cage bottom in order to prevent loss of the young pups through the wire mesh screens.

At weaning, five female offspring from each diet were continued on the respective dietary treatments and the adult female mice were weighed and sacrificed for further study. Samples of blood, liver, kidney and heart were frozen immediately by a dry ice-acetone solution and stored at -10°C until analyzed. The carcases were also frozen immediately for analysis of bone fluoride and citric acid.

The bone citric acid levels were determined by the method of Hess and White (1955). Bone and feed fluoride levels were determined by the methods of Singer and Armstrong (1965) and Frere (1962). The color reaction of Megregian (1954) was used.

Samples of liver, heart, kidney and body fat were analyzed for fatty acids (Metcalfe and Schmitz, 1959). Enzyme activities were determined on suitable tissue homogenates. The enzyme methods used

were as follows: succinic dehydrogenase, Cooperstein et al., (1950); cytochrome oxidase, Cooperstein and Lazarow (1951); isocitric dehydrogenase, Sigma (1961); malic dehydrogenase, Sigma (1957); lipase, Sigma (1961). The data were analyzed statistically by the multiple range test (Duncan, 1955).

## RESULTS AND CONCLUSIONS

Mice reared on low-fluoride diets through three generations failed to show a significant influence of the low-fluoride conditions on body weight gain (Table 13). The results on growth rate do not agree with those of McClendon and Gershon-Cohen (1953), who reported a marked growth depression in rats fed a low-fluoride diet. Organ weights, calculated as percent of body weight, are shown in Table 15 and do not indicate a significant change which is attributable to dietary treatment.

No appreciable differences were noted in the digestible energy levels of the diet in relation to the dietary fluoride content in the first generation of mice (Table 14.) Likewise, fat digestion was not affected in the first generation. However, mice in the second generation on the low-fluoride diet exhibited lower levels of energy utilization, and lower protein utilization. No significant change was noted in the utilization of these by second generation mice fed the control diet. (Table 14).

The data suggest an improvement in protein digestion as a result of feeding the low-fluoride diet (Table 14). Mice of the first generation fed the low-fluoride diet exhibited protein digestibilities of 91.5% as compared with 87.5% for mice fed the same diet to which 6 ppm fluoride

had been added. It was also apparent with mice of the second generation that the feeding of the low fluoride resulted in somewhat higher levels of protein digestion. These figures were 61% protein digestibility on the low fluoride diet, as compared with 57.7% when 6 ppm fluoride was added. The protein utilization of the control diet was not appreciably different among the two generations of mice (Table 14).

The grain sorghum and soybeans employed in these studies were the result of at least 9 consecutive generations of seeds grown under hydroponic conditions in the specially constructed greenhouse under low fluoride conditions. The possibility exists that such culture techniques altered the carbohydrate components and possibly the protein or amino acid makeup of these dietary ingredients.

The dietary effect of fluoride levels on liver enzyme activity, (Table 19) was calculated as a percentage of the total activity.

A relationship was found between the in vitro studies on enzyme inhibition (Table 20a) and the measurements of enzyme activity in mice fed the different dietary fluoride levels (Table 19). The in vitro studies showed cytochrome oxidase to be inhibited 50 percent with 0.1 M fluoride; while malic dehydrogenase activity was stimulated 14 percent. Isocitric dehydrogenase was neither stimulated nor inhibited by fluoride in the in vitro studies. The dietary fluoride apparently stimulated malic dehydrogenase in the second generation of mice but not in the first generation (Table 19). The results for the second generation agree with data obtained in the in vitro studies with isocitric dehydrogenase. Liver isocitric dehydrogenase was significantly decreased in mice of the first generation fed the 6 ppm fluoride supplemented diet or the

control diet, but not in the subsequent generation of mice (Tables 18 and 19). Stookey and Muhler (1963) analyzed liver, kidney and heart tissues of fluoride fed rats and found that only the kidney showed a significant increase when fluoride was fed. The kidney fluoride levels increased by 1000 fold under these conditions. Liver and heart fluoride levels were relatively unaffected . The kidney concentrated fluoride but failed to show a significant change in enzyme activity (Table 18). Enzyme systems of heart, kidney and liver tissue were not significantly altered in activity with diet. Those studied were: glucose-t-phosphate dehydrogenase, NADH dehydrogenase, phosphohexose isomerase, and glutamic-oxalacetic and glutamic-pyruvic transaminase (Weber and Reid, 1966). The possibility of alterations occurring in intestinal lipase activity with the low-fluoride diet was evaluated (Table 21). These results agree with the dietary fat retention results (Table 14). Intestinal lipase was not significantly altered by the low-fluoride diet.

The influence of dietary fluoride on cellular fatty acid metabolism was investigated. No significant alteration in fatty acid metabolism in body fat, liver, kidney or heart tissue could be attributed to diet.

Although the bone citrate levels tended to decrease through the first and second generations fed the low fluoride diet these differences were not statistically significant (Table 17). Studies by Zipkin et al., (1963) had suggested that there was an inverse relationship between bone fluoride levels and citrate. The results obtained in the present experiments do not suggest such a relationship.

The bone fluoride levels for mice fed the low-fluoride diet decreased with each generation (Table 16). The parental generation mice fed the low-fluoride diet had 35.9 mcg F/gm of bone; this was decreased to a level of 8.7 mcg F/gm in the second generation. The diet with 6 ppm added fluoride and the control diet produced bone fluoride levels which remained relatively constant at 250 - 300 and 104 - 138, respectively (Table 16). An extrapolation of bone fluoride levels to the dietary levels gives an approximation of 0.2 ppm fluoride in the low-fluoride diet; however, fluoride is carried in the milk during lactation and a large portion of the fluoride found in the bones of mice fed the low-fluoride diet could have been from this source.

Mice fed the low-fluoride and 6 ppm fluoride diets exhibited no differences in reproduction rate which could be attributed to dietary fluoride. This was in contrast to the reproductive difficulties encountered by Muhler (1954), Maurer and Day (1957) and Pothapragoda (1962) with the use of purified diets low in fluoride. No significant differences were demonstrated between low-fluoride and 6 ppm F diets in number of young mice born or weaned. This experiment failed to support the results of McClendon et al., (1945, 1953, 1954) to the effect that fluoride was essential to sustain life and to maintain normal reproduction and lactation in rats.

## SUMMARY

White mice fed a low-fluoride diet for three generations and those fed 6 ppm added fluoride showed no significant differences in growth, and in weights of liver, kidney and heart between mice fed

the low-fluoride diet and those supplemented with 6 ppm fluoride. In the first generation no significant differences in digestible energy or fat utilization were caused by the feeding of the low fluoride diet. Lower percent protein and energy digestion was found in the second generation of mice fed the hydroponically grown feedstuffs. The utilization of the control diet by mice in the two generations was not altered. Gross observations of the mice fed the low-fluoride diet in comparison with those fed 6 ppm fluoride failed to demonstrate any noticeable differences.

Measurements of bone citric acid and bone fluoride concentrations failed to demonstrate a significant relationship between them.

Liver enzyme activities were not significantly altered by the low-fluoride dietary treatment. A decrease of 41 percent in activity of liver isocitric dehydrogenase occurred in the first generation, but not the second. Liver cytochrome oxidase exhibited depressed activity (45 percent) in the second generation, but not the first. Mice fed the 6 ppm fluoride diet showed a 75% increase in activity for liver malic dehydrogenase in the second generation, but not in the first. The kidney and heart homogenates showed no significant alterations in succinic dehydrogenase, cytochrome oxidase and malic dehydrogenase activities. Other enzymes systems which showed no significant changes were NADH dehydrogenase, phosphohexose isomerase, glucose-6-phosphate dehydrogenase, alkaline phosphatase, lactic dehydrogenase, and glutamic-oxalacetic and glutamic-pyruvic transaminase in heart, kidney and liver homogenates. The small intestine lipase showed a significant decrease in activity in mice fed the low-fluoride diet in the second generation.

A dietary effect of fluoride on reproduction could not be demonstrated and no significant differences were found between the low-fluoride and 6 ppm added fluoride diets in the number of young born or weaned through two generations of mice.

In conclusion, the feeding of a low-fluoride diet to mice for two generations failed to show a significant alteration in growth or in the biochemical criteria employed to evaluate the essentiality of dietary fluoride.

Table 12--Composition of mouse diets.

| Ingredients | Low-Fluoride percent | Low-Fluoride plus 6 ppm F added percent | Basal percent |
|---|---|---|---|
| Soybean | 35.00[1] | 35.00[1] | 35.00[2] |
| Milo | 47.50 | 47.50 | 47.50 |
| Sucrose | 6.17 | 6.17 | 6.17 |
| Corn oil | 6.00 | 6.00 | 6.00 |
| Vitamin mixture[3] | 0.14 | 0.14 | 0.14 |
| Mineral mixture[4] | 4.58 | 4.58 | 4.58 |
| DL-Methionine | 0.11 | 0.11 | 0.11 |
| Choline Chloride | 0.20 | 0.20 | 0.20 |
| $Cr_2O_3$ | 0.30 | 0.30 | 0.30 |
| NaF | 0 | 0.0015 | 0 |
| TOTAL | 100.00 | 100.00 | 100.00 |

[1]Soybeans and milo are grown under hydroponic low-fluoride conditions.

[2]Soybeans and milo are supplied from field-grown sources.

[3]Supplied the following as mg. per kgm. of diet: 12.5 ascorbic acid, 12.5 thiamine · HCl, 100.0 niacin, 20.0 riboflavin, 12.5 pyridoxine · HCl, 1.25 d-biotin, 75.0 d-calcium pantothenate, 10.0 vitamin $B_{12}$ (0.1%), 14,000 I.U. vitamin A, 4.00 folic acid, 1,500 I.U. vitamin $D_2$, 200.0 d-alpha-tocopheryl-acetate, 1.25 menadione (2-methyl-naptho-quinone), 500.0 ethoxyquin, 500.0 i-inositol, 25.0 para-aminobenzoic acid, 25.0 oxytetra-cycline.

[4]Supplied the following as percent of diet: 0.8200 $Ca(OH)_2$, 1.9760 $KH_2PO_4$, 0.5000 NaCl, 0.0336 $MnSO_7 \cdot H_2O$, 0.1320 $FeSO_4$ $7H_2O$, 0.0240 $ZnSO_7$, 0.0020 $CuSO_4 \cdot 8H_2O$, 0.0052 KI, 0.01100 $CoCl_2 \cdot 6H_2O$, 0.5000 KCl, 0.5800 $MgSO_4 \cdot 7H_2O$, 0.0003, $H_2MoO_4 \cdot H_2O$, 0.0007 KBr.

Table 13--Effect of dietary fluoride content on growth in mice.

| Dietary Treatments | Weight gain from 18-49 days (gms.) | | | |
|---|---|---|---|---|
| | Parental generation | 1st generation | 2nd generation | 3rd generation |
| Low-fluoride | 18.1[a1] | 19.4[a] | 17.7[a] | 17.6[a] |
| Low-fluoride plus 6 ppm F | 17.2[a] | 19.0[a] | 18.1[a] | 19.6[a] |
| Basal (control) | 20.6[b] | 17.5[a] | 17.5[a] | 17.7[a] |

[1] Means having different superscripts are statistically different at the 0.05 level of probability.

Table 14--Effect of dietary fluoride on metabolism of the diet.

| Dietary Treatments | Generation | Percent Digestible energy | Percent Protein Digested | Percent Fat Digested |
|---|---|---|---|---|
| Low-fluoride | 1st[1] | 94.69 | 91.48 | 97.43 |
| Low-fluoride plus 6 ppm F | " | 92.33 | 87.49 | 96.09 |
| Basal (control) | " | 87.38 | 76.71 | 92.98 |
| Low-fluoride | 2nd[2] | 67.21 | 61.68 | 90.41 |
| Low-fluoride plus 6 ppm F | " | 68.83 | 57.75 | 91.90 |
| Basal (control) | " | 83.99 | 72.70 | 94.28 |

[1]Fecal samples collected from six-week-old mice.

[2]Fecal samples collected from three-week-old mice.

Table 15--Effect of dietary fluoride on the body organs size in mice.

| Dietary Treatments | Body organs, percent of body weight | | | |
|---|---|---|---|---|
| | Generation | Livers | Kidneys | Hearts |
| Low-fluoride | Parental | 4.71 | 1.17 | 0.44 |
| Low-fluoride plus 6 ppm F | " | 5.06 | 1.12 | 0.43 |
| Basal (control) | " | 6.95 | 1.39 | 0.50 |
| Low-fluoride | 1st | 5.15 | 1.88 | 0.53 |
| Low-fluoride plus 6 ppm F | " | 5.26 | 1.54 | 0.56 |
| Basal (control) | " | 6.66 | 1.48 | 0.55 |
| Low-fluoride | 2nd | 4.25 | 1.48 | 0.51 |
| Low-fluoride plus 6 ppm F | " | 4.59 | 1.68 | 0.57 |
| Basal (control) | " | 5.13 | 1.80 | 0.53 |

Table 16--Effect of dietary fluoride on femur flouride levels.

| Dietary Treatments | Bone fluoride levels (mcg./F/gm.) | | |
|---|---|---|---|
| | Parental | First | Second |
| Low-fluoride | 35.9[a1] | 18.7[a] | 8.7[a] |
| Low-fluoride plus 6 ppm F | 299.8[c] | 290.1[c] | 249.1[c] |
| Basal (control) | 138.0[b] | 129.8[b] | 104.4[b] |

Table 17--Effect of dietary fluoride on citric acid levels.

| Dietary Treatments | Bone citric acid levels (mcg./mg.) | | |
|---|---|---|---|
| | Parental | First | Second |
| Low-fluoride | 11.9[abl] | 12.2[ab] | 9.1[a] |
| low-fluoride plus 6 ppm F | 14.2[bc] | 16.4[b] | 11.7[ab] |
| Basal (control) | - | 14.8[bc] | 12.5[bc] |

[1]Means having different superscripts are statistically different at the 0.05 level of probability.

Table 18--Effect of dietary fluoride on enzyme activity of different body organs of mice.

| Dietary Treatments | Tissue | ENZYME ACTIVITY | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Cytochrome Oxidase | | | Isocitric Dehydrogenase | | | Malic Dehydrogenase | |
| | | Parental[1] | First[2] | Second[3] | Parental | First | Second | First | Second |
| Low-fluoride | Liver | 0.101[a4] | 6.045[a] | 4.616[a] | 0.079[a] | 8.305[a] | 5.802[a] | 10.076[a] | 10.214[a] |
| Low-fluoride plus 6 ppm F | " | 0.122[a] | 5.561[a] | 2.999[b] | 0.094[a] | 4.935[b] | 6.022[a] | 10.221[a] | 17.841[b] |
| Basal | " | - | 5.662[a] | 3.478[ab] | - | 4.406[b] | 5.334[a] | 8.810[a] | 19.770[b] |
| Low-fluoride | Heart | 0.112[a] | 8.332[a] | 3.[illegible][a] | 0.079[ab] | 4.469[a] | 15.389[a] | 11.306[a] | 42.599[a] |
| Low-fluoride plus 6 ppm F | " | 0.143[a] | 7.910[a] | 2.966[a] | 0.096[a] | 6.558[a] | 13.781[a] | 12.653[a] | 42.922[a] |
| Basal | " | 0.104[a] | 7.189[a] | 3.150[a] | 0.071[b] | 6.841[a] | 10.838[a] | 11.921[a] | 31.424[a] |
| Low-fluoride | Kidney | 0.102[a] | 7.574[a] | 5.089[a] | 0.076[a] | 10.080[a] | 3.816[a] | 10.076[a] | 41.127[a] |
| Low-fluoride plus 6 ppm F | " | 0.116[a] | 8.533[a] | 5.620[a] | 0.075[a] | 8.277[ab] | 3.804[a] | 10.221[a] | 36.741[a] |
| Basal | " | 0.918[a] | 8.798[a] | 3.908[a] | 0.059[a] | 4.146[b] | 2.459[b] | 8.810[a] | 7.431[b] |

Basal

[1]Enzymes activity calculated as delta O.D.per min. per mg. of tissue (wet basis).

[2]Enzymes activity calculated as delta O.D.per min. per gm. of protein (tissue made up in buffer).

[3]Enzymes activity calculated as delta O.D.per min. per gm. of protein (tissue made up in buffer).

[4]Means having different superscripts are statistically different at the 0.05 level of probability.

Table 19--Effect of dietary fluoride on enzyme systems.

| Dietary Treatments | Liver Enzymes Expressed as percent of Low Fluoride | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Cytochrome Oxidase | | | Isocitric Dehydrogenase | | | Malic Dehydrogenase | | |
| | Parental | First | Second | Parental | First | Second | Parental | First | Second |
| Low-fluoride | 100[a1] | 100[a] | 100[a] | 100[a] | 100[a] | 100[a] | - | 100[a] | 100[a] |
| Low-fluoride plus 6 ppm F | 121[a] | 92[a] | 65[a] | 119[a] | 59[b] | 118[a] | - | 101[a] | 175[b] |
| Basal | - | 94[a] | 75[ab] | - | 53[b] | 92[a] | - | 87[a] | 194[b] |

[1] Means having different superscripts are statistically different at the 0.05 level of probability.

Table 20--*In vitro* inhibition of enzyme system using body organs of mice.

| Enzyme System | Tissue | Percent Activity[1] | |
|---|---|---|---|
| Glutamic-oxalacetic Transaminase | | 0.05 M F[2] | 0.1 M F |
| | Liver | 96.79 | 103.27 |
| | Kidney | 101.10 | 100.19 |
| | Heart | 138.60 | 140.70 |
| Glutamic-pyruvic Transaminase | | 0.05 M F | 0.1 M F |
| | Liver | 100.40 | 101.00 |
| | Kidney | 96.95 | 103.97 |
| | Heart | 100.70 | 100.50 |
| Succinic Dehydrogenase | | 0.03 M F | 0.06 M F |
| | Liver | 86.07 | 43.05 |
| | Kidney | 46.18 | 24.90 |
| | Heart | 60.08 | 18.85 |
| Isocitric Dehydrogenase | | 0.05 M F | 0.1 M F |
| | Liver | 95.40 | 94.60 |
| | Kidney | 90.85 | 83.32 |
| | Heart | 96.18 | 75.18 |
| Malic Dehydrogenase | | 0.03 M F | 0.06 M F |
| | Liver | 108.61 | 112.49 |
| | Kidney | 107.81 | 116.67 |
| | Heart | 101.65 | 97.63 |

Table 20--continued

| Enzyme System | Tissue | Percent Activity[1] | |
|---|---|---|---|
| Lactic Dehydrogenase | | 0.05 M F[2] | 0.1 M F |
| | Liver | 109.78 | 87.72 |
| | Kidney | 96.47 | 91.97 |
| | Heart | 92.86 | 83.54 |
| NADH Dehydrogenase | | 0.025 M F | 0.05 M F |
| | Liver | 80.36 | 82.14 |
| | Kidney | 77.09 | 62.85 |
| | Heart | 79.72 | 70.27 |
| Lipase | | 0.05 M F | 0.1 M F |
| | Liver | - | 113.21 |
| | Small Intestine | 103.01 | 102.11 |

[1]Percent activity was evaluated by dividing the control activity into fluoride treated activity.

[2]Numbers represent the molar concentration of fluoride ion used.

Table 21--Effect of dietary fluoride on small intestine lipase activity.

| Dietary Treatments | Lipase activity | | |
|---|---|---|---|
| | Parental[1] | First[1] | Second[1] |
| Low-fluoride | 8.73[a2] | 8.27[a] | 4.43[a] |
| Low-fluoride plus 6 ppm F | 8.46[a] | 8.34[a] | 8.08[b] |

[1]Activity expressed as change in NaOH titration in six hours per mg. tissue (wet weight).

[2]Means having different superscripts are statistically different at the 0.05 level of probability.

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