SOME BIOCHEMICAL EFFECTS OF FLUORINE AND BROMINE

by

Alexander Rudolf Doberenz

A Dissertation Submitted to the Faculty of the

COMMITTEE OF BIOCHEMISTRY AND NUTRITION

In Partial Fulfillment of the Requirements
For the Degree of

DOCTOR OF PHILOSOPHY

In the Graduate College

THE UNIVERSITY OF ARIZONA

1 9 6 4

THE UNIVERSITY OF ARIZONA

GRADUATE COLLEGE

I hereby recommend that this dissertation prepared under my direction by Alexander R. Doberenz

entitled SOME BIOCHEMICAL EFFECTS OF FLUORINE AND BROMINE

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

B. R. Reid — Dissertation Director — Sept. 23, 1963 — Date

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

B. J. Hallett	Sept. 23, 1963
Mitchell G. Vavich	Sept 23, 1963
Wm. J. McCauley	Sept. 24, 1963
Wayne Ferris	Sept. 24, 63

*This approval and acceptance is contingent on the candidate's adequate performance and defense of this dissertation at the final oral examination. The inclusion of this sheet bound into the library copy of the dissertation is evidence of satisfactory performance at the final examination.

STATEMENT BY AUTHOR

This dissertation has been submitted in partial fulfillment of requirements for an advanced degree at The University of Arizona and is deposited in The University Library to be made available to borrowers under rules of the Library.

Brief quotations from this dissertation are allowable without special permission, provided that accurate acknowledgment of source is made. Requests for permission for extended quotation from or reproduction of this manuscript in whole or in part may be granted by the head of the major department or the Dean of the Graduate College when in their judgment the proposed use of the material is in the interests of scholarship. In all other instances, however, permission must be obtained from the author.

SIGNED: Alexander R. Doberenz

ACKNOWLEDGMENTS

The author expresses sincere gratitude to:

Drs. B. L. Reid, A. A. Kurnick, A. R. Kemmerer and E. B. Kurtz for their counsel and guidance during the course of this study and in the preparation of this dissertation.

Drs. M. G. Vavich and B. J. Hullet for their helpful suggestions in preparation of this dissertation.

Mrs. B. A. Seferlis for her aid in typing the rough draft of this dissertation.

My dear wife, Angela, for her patience and encouragement during my years in graduate school.

Departments of Biochemistry and Nutritiion and Poultry Science for their excellent cooperation in making readily available the facilities required for this research.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF ILLUSTRATIONS

DISSERTATION ABSTRACT

Doberenz, Alexander R. Ph.D., The University of Arizona, 1964.

"Some Biochemical Effects of Fluorine and Bromine"

Dissertation Director: B. L. Reid

New Hampshire x Delaware crossbred chicks were employed to evaluate the effects of toxic amounts of fluoride (as sodium fluoride) in diets containing 0 or 10% added fat, high amounts of sodium bromide and/or higher than normal calcium levels. The feeding of 1000 ppm fluoride in the absence of added dietary fat produced an 18.3% reduction in growth to 4 weeks of age. The presence of 10% added dietary fat did not significantly alter the results obtained with the fluoride feeding. In the presence of 10,000 ppm bromide (as sodium bromide) and added dietary fat, a 14.8% reduction in growth occurred. In another study, in the absence of added dietary fluoride, a level of 5,000 ppm bromide produced a reduction in body weight at 4 weeks of age, while a level of 10,000 ppm bromide was required before significant mortality was observed.

Soybean and grain sorghum hydroponically grown under minimal fluoride conditions, using recrystallized

nutrient salts, were used to prepare a rat diet, containing <0.005 ppm F. Weanling rats were fed: I-minimal fluoride diet, II-minimal fluoride plus 2 ppm F as sodium fluoride and III-field-grown soybean-sorghum diet (2.67 ppm F). Whole carcass fluoride analysis of the pups, at the start of the study, yielded an average value of 0.72 ppm. At the end of the ten-week treatment period, the average body weights were: I - 267.8, II - 251.4 and III - 277.7 gm and the tibia and fibula bones contained an average fluoride level in ppm of: I - 2.92, II - 34.68 and III - 12.54.

Enzyme studies showed a significant increase in serum isocitric dehydrogenase activity and a decrease in liver activity of this enzyme in rats fed diet I. No difference in alkaline or acid phosphatases, lactic dehydrogenase, or glutamic-oxalacetic and glutamic-pyruvic transaminases were observed.

CHAPTER I

REVIEW OF LITERATURE

Introduction and History. Fluorine is one of the most widely distributed elements in nature. The literature on the quantitative and qualitative distribution of fluoride in air, water, soil, plant, feed and animal tissue is exceedingly large (Cholak, 1959; Underwood, 1962; McClure, 1949a).

Biological interest of fluoride in the early research was due to the occurrence of fluorosis in livestock (National Research Council, 1960; Cass, 1961) and the industrial uses of fluorides by man (Princi, 1960). Early in the 19th century, fluoride was determined in body tissue (Berzelius, 1806; Carnot, 1892; Heintz, 1849; Hempel and Sheffler, 1899). Hupka and Luy (1929) reported on the illness (stiffness, mild shifting lameness and anorexia) in cattle grazing near a chemical factory that used fluorides. Hupka and Götze (1931) reported on the same type illness in cattle grazing in the area of a phosphate fertilizer company. The use of fluoride compounds for pest control and fluoride-rich fertilizers are potential sources of fluorides (Romanenko, 1954).

Recently, interest has shifted to the relationship of fluoride to the incidence of dental caries (Muhler and

Hine, 1959; Underwood, 1962). The fluoride in the body of animals is derived mainly from the ingestion of food and water; however, a small quantity may be inhaled since air may contain fluoride in gaseous or particulate form (Collings Jr., et al., 1951; Cass, 1961). The absorption of fluoride through the skin is negligible, except for hydrofluoric acid which on contact with the skin may spread into the cutaneous and subcutaneous tissues.

Fluorosis. Numerous investigations, involving several animal species, have been conducted to study biochemical responses to fluoride in the diet and drinking water (Cass, 1961; Weidmann, et al., 1963; McClure, 1949b; Hodge, 1961). The levels of fluoride studied range from minimal to abnormally high.

The animal has two physiological mechanisms which protect it from chronic fluorosis (Stookey, et al., 1963). The first is the ability to increase urinary excretion of fluorides, and second by the deposition of fluorides in the skeleton (rapid at first, and then more slowly until a saturated state is reached). "Flooding" of the soft tissues apparently occurs when the bones have reached 35-45 times the normal concentration of fluoride. Anorexia occurs at this state of fluoride toxicosis (Phillips and Suttie, 1960). In the latter stages of fluorosis, metabolic breakdown occurs and death soons follows. Prior to the eruption of permanent

teeth, chronic endemic dental fluorosis or mottled enamel may occur at rather low levels of dietary intake (Smith, Lantz and Smith, 1931; Schour and Smith, 1942; Armstrong, 1942). The teeth become modified in shape, size, color, orientation and structure. The incisors may become pitted and the molars abraded under these conditions (Smith and Lantz, 1933). These clinical signs of fluorosis often appear in teeth and bones before "flooding" of the soft tissues occurs. Exostosis of the jaw and long bones develop and joints become thickened and stiff. The animal grows at a reduced rate or may lose weight. Anemia occurs, presumably due to the extension of the bone lesions into the marrow (Pendbort, et al., 1946). Pathological changes have been reported in the kidney, thyroid and other soft tissues (Phillips, Hart and Bohstedt, 1934; Phillips and Lamb, 1934; Taylor, et al., 1961). In fluorotic chicks, hypertrophy and hyperplasia of the columnar epithelium of the proventriculus have been reported (Gardiner, et al., 1959).

Biochemical Changes. A direct correlation between the concentration of fluoride in the urine and drinking water has been demonstrated in rabbits (Gardner, et al., 1957), but skeletal decontamination may maintain high urinary levels of fluoride long after removal of the animal from excess of fluoride ingestion (Underwood, 1962). The manner in which fluoride is incorporated into the structure of bones and teeth has been described (McCann and Bullock, 1957; Miller

and Phillips, 1956; Neuman, et al., 1950; Smith and Lantz, 1933). The fluoride ion apparently forms hydroxy-fluorapatite by replacing hydroxyl and bicarbonate ions in the hydroxyapatite (Beevers and McIntyre, 1946; Neuman and Neumann, 1958). The decomposition of fluoride in bones and teeth occurs at a logarithmic rate. Hodge (1953) postulated a rapid ionic exchange of fluoride ions in the tissue fluids with OH or CO_3 ions on the mineral crystal surface followed by a slow process of bone formation leading to storage of the fluoride in the hydroxyapatite lattice. A decrease in carbonate and an increase in magnesium content of fluorotic bone, suggests a replacement of the carbonate group by fluoride ion and possibly a precipitation of some fluoride as MgF (McCann and Bullock, 1957; Weldmann, et al., 1959). As the fluoride content of bone increases, a decrease in citrate content has been noted (Zipkin and Gold, 1963). X-ray diffraction studies of bone indicate that fluoride increased crystal size and decreased crystal imperfections compared to normal bone and decreased the citrate ions which occupy only crystal surface positions in apatites (Schraer, et al., 1962; Zipkin, et al., 1962).

Alkaline phosphatase is closely associated with bone calcification. The serum level of this enzyme was significantly increased in fluorotic cattle (Phillips, 1932), in rabbits (Weidmann, et al., 1959), and in chicks (Moltzok

and Branion, 1958). In other studies with rats and rabbits (Maplesdon, et al., 1960; Mitchell and Edman, 1952) little or no change in serum phosphatase activity was detected when high fluoride diets were fed. Attention to other enzymatic processes sensitive to fluoride, particularly those requiring metal catalysts, have been studied in an attempt to determine biochemical defects associated with fluorosis.

It has been shown that fluoride ions inhibit lipase and fatty acid oxidase activities (Amberg and Hobenhart, 1908; Johnson and Hardy, 1950) and the closely related acetate-activating system (Aisenberg and Potter, 1955). Fecal lipid levels are drastically increased in the fluorotic rat. Lohmann and Meyerhof (1934) showed that fluoride inhibited the conversion of phosphoglyceric acid to phosphopyruvic acid in vitro. Warburg and Christian (1942) demonstrated the inhibition of enolase by fluoride. Many other enzyme systems have shown to be inhibited by fluoride. A partial list (Frajola, 1959) would include, in addition to the already mentioned, potato phosphorylase, acid phosphatase, prolidase, liver esterase, glucose dehydrogenase, pancreatic lipase, cholinesterase, leucyl aminopeptidase and succinic dehydrogenase.

Tolerance. Many factors determine the tolerance of animals to fluoride. The "maximum safe level" of dietary fluoride is only meaningful when the following are known: the form

in which the fluorine is ingested, duration and continuity of intake, influence of other dietary ingredients, age and species.

The more soluble fluoride compounds are more toxic than the insoluble compounds. Mitchell and Edman (1952) reviewed the literature on fluoride toxicity in rats and rated the toxicity of various substances as follows: sodium fluoride 110-190 ppm, cryolite 150-230 ppm, rock phosphate 120-260 ppm, and calcium fluoride 230-2300 ppm. Intermittent dosage of a given level of fluoride was less toxic than continuous dosage or ingestion (Lawrenz, et al., 1940a, 1940b).

Numerous dietary components have been shown to influence the toxicity of fluoride. The mitigating effects of calcium and aluminum salts have been demonstrated in rats (Lawrenz and Mitchell, 1941a; Weddle and Muhler, 1954; Sharpless, 1936). It is believed that these salts decrease the solubility of the fluoride through the formation of insoluble complexes. Molybdenum has been reported to increase the retention of fluoride and to increase toxicity (Stookey and Muhler, 1962; Stookey, et al., 1962a, 1962b). These authors have shown that molybdenum significantly increased the absorption of fluoride from the first portion of the intestine of older rats and acts inversely in young animals. In the stomach there is a decreased absorption of fluoride in older animals and either an increase or no effect in

younger animals. These data demonstrate that the presence of molybdenum increases absorption of fluoride in older animals.

Phillips and Hart (1935) noted that high fat diet with 0.2% sodium fluoride produced more severe fluoride toxicosis in rats, as evidenced by failure to gain weight over a six-week period, than a low fat diet. Miller and Phillips (1955) confirmed the enhancement of fluoride toxicity in the rat by high dietary fat levels.

Buttner and Muhler (1957) demonstrated an increased retention of fluoride in the heart and kidney with high levels of dietary fat. In 1958, Buttner and Muhler showed that feeding 2 mg daily and increasing dietary fat from 5 to 20% resulted in increased fluoride retention in the whole carcass, femur and soft tissues. The presence of fat and fluoride produced a decrease in body weight gain.

Bixler and Muhler (1960) studied the retention of fluoride in soft tissues of the chicken and observed increased retention of fluoride in fat supplemented animals when the fluoride was administered either by injection or by stomach tube. No differences in serum cholesterol levels were obtained with fluoride supplementation. A marked increase in fecal fat, nitrogen and dry matter, and a voluntary reduction in food consumption was demonstrated by Sievert and Phillips (1959), Suttie and Phillips (1960).

Poultry exhibits a much higher tolerance than mammalian species to fluorine, perhaps because of poorer absorption and more effective elimination (Haman, et al., 1936: Phillips, et al., 1935a, 1935b).

Caries Susceptibility. There is little doubt that dental caries are of bacterial origin. Caries-susceptible rats maintained under germ-free conditions do not develop carious lesions (Orland, et al., 1954; Orland, et al., 1955). Inbred strains of rats have been produced which show a strong genetic influence for caries-resistance (Hunt, et al., 1955). The ingestion of appropriate amounts of fluoride during the calcification of teeth promotes a definite increase in caries-resistance due to the presence of fluoride ion in the crystal lattice of the enamel and dentin (Shaw, 1954). Sodium fluoride and stannous fluoride reduced dental caries when administered posteruptively (Zipkin and McClure, 1951; Muhler, et al., 1953).

Epidemiological studies which started in the early 1900's showed a relationship between high fluoride content of domestic waters and the incidence of mottled enamel (Dean, 1942). Later the development of sensitive analytical method (Cholak, 1959) for fluorine confirmed this conclusion. McClure (1951) summarized the results of a major study in the United States, which showed that dental caries in permanent teeth decreased as the fluorine concentration of the drinking water increased. Hodge (1950) calculated that 1.0 ppm F in the

water is the point of "maximum health with maximum safety." Long-term studies on large populations have demonstrated the value of maintaining 1.0 ppm F in the drinking water of communities for improved dental health. An average reduction of 60% in caries for permanent teeth of children born after the fluoride adjustment of the water supply is apparent in all studies (Underwood, 1962).

The mode of action of fluoride inhibition of dental caries is still unknown. McClure (1948) reported fluoride content data on several hundred sound and carious teeth and found no significant differences in fluorine content of dentine or enamel. The theory that fluoride forms a protective layer of acid-resistant fluorapatite on the surface of the enamel is popular today (Underwood, 1962). A more detailed discussion of the theories on the mode of action of fluoride in the reduction of dental caries is given by Jenkins (1963).

Minimal Feed Trials. Over the past 30 years several papers have appeared which attempted to demonstrate an essential function for fluorine. Sharpless and McCollum (1933) fed a semipurified diet with and without 10 ppm added fluoride. At the end of 120 days no differences were noted in growth, reproduction or bone and tooth structure. Based on the fluoride content of the femurs (150 ppm) at the end of the experiment, the unsupplemented diet apparently contained an

appreciable amount of this element. Phillips, Hart and Bohstedt (1934) fed rats for 140 days on a mineralized milk diet containing 0.2 ppm F and found no differences between this diet and their controls. At the end of the experiment, the fluoride content of the animals fed the unsupplemented diet was relatively higher. Evans and Phillips (1939), also using rats fed a mineralized milk diet containing 1.6 ppm F, found that at the end of 5 generations there were no differences in growth, reproduction, or tooth structure. Lawrenz (1945) fed a purified diet which contained 0.47 ppm F to rats obtained from females which had been previously maintained on this diet. The carcasses of the young contained only 0.8 ppm F at birth. The pups raised for 207 days on the low-fluoride diet showed no significant differences in growth or tooth structure. Muhler (1954) used a purified diet containing 0.1 ppm F which failed to support adequate reproductive performance of rats. However, the author stated that he could not determine whether the cause of the poor reproductive performance was due to the lack of fluoride or to the purified nature of the diet. Maurer and Day (1957) used a corn starch-casein diet prepared by exhaustive purification procedures. They were unable to detect any fluoride by direct analyses of the diet. It was estimated from the carcass fluoride contents that the diet had contained less than 0.007 ppm

of utilizable fluoride. No significant differences were observed between the deficient and supplemented animals. Alkaline and acid phosphatase determinations on liver, kidney and bone showed no differences between the rats fed the low fluoride diet and those fed the fluoride supplemented diet. The teeth of rats in both groups appeared sound and no gross evidence of caries was noted. Due, presumably, to other imbalances, only one-half the young were weaned on these diets.

The only paper which does not support the conclusion that fluorine is a nonessential element is that of McClendon and Gershon-Cohen (1953). Their diet consisted of water-cultured crops of corn, sunflower seed and yeast. The results obtained indicated a very marked reduction in growth rate due, apparently, to a lack of dietary fluoride. Control rats weighed an average of 129 grams at 88 days of age compared to 51 grams for the rats fed the fluoride-free diet. Roentgenograms of the lower jaw of rats receiving the deficient diet showed extensive caries of molars (McClendon and Gershon-Cohen, 1954). However, no data was presented on the fluoride content of the diet or the animals.

Bromine. Little work on the metabolism of bromide has been done in comparison to fluoride. Bromine is found in all plant and animal tissues in trace amounts. Radioactive Br^{82} studies support the earlier work that bromide is evenly distributed

in the tissues of the rat (Cole and Patrick, 1958) and man (Hellerstein, et al., 1960). These studies also indicate that bromide is retained for only short periods and is excreted in the urine. The bromide retentions are influenced by the bromide-chloride ratio (Mason, 1936; Winnek and Smith, 1937b; Hellerstein, et al., 1960).

Winnek and Smith (1937a, 1937b) prepared a purified diet containing less than 0.5 ppm Br. This was fed to rats for 11 weeks and no signs of ill health or reproductive abnormalities were noted. There have been two reports of growth responses with bromide in chicks (Huff, et al., 1956) and mice (Bosshardt, et al., 1956).

Borgatti (1947) reported that 0.14 g Br/kg daily had no effect on the growth of the fowl. Only the gonads, particularly those of the male, were affected. Development of the testes was arrested at an infantile stage, but no modification of the secondary sex characteristics was noted.

Several workers have studied the relationship between bromide and iodide (Baumann, et al., 1953). Thyroid glands rendered hyperplastic by lack of iodide were found to be rich in bromide. When iodide is supplied to such animals, bromide was lost and iodide taken up by the gland. Apparently the thyroid takes up larger than normal amounts of bromide only in the absence of iodide. Bromide cannot be

used to synthesize the thyroid hormone *in vivo* and has been shown to be of no value in preventing goiter in iodide deficient animals. Several bromothyroxines have been prepared in the laboratory and shown to be as effective as thyroxine itself.

CHAPTER II

BROMIDE AND FLUORIDE TOXICITIES IN THE CHICK

Introduction. The relationship of certain minerals to toxic levels of fluoride has been studied largely with the rat. Magnesium, calcium, molybdenum, phosphorus and aluminum have been studied (Underwood, 1962). Lawrenz and Mitchell (1941) and Weddle and Muhler (1954) demonstrated that an increase in calcium content of the diet at a constant phosphorus level produced a depression in total retention of fluoride in growing rats.

With chicks, Bixler and Muhler (1960) found that an increase in dietary fat enhanced fluoride retention. Sievert and Phillips (1959) and Suttie and Phillips (1960) reported a marked increase in fecal fat, nitrogen and dry matter excretion as a result of reduced lipase activity when fluoride was fed.

Very little work has been done on the toxicity of bromides in chicks, and apparently none on the inter-relationship of bromide and fluoride.

The effects of calcium and bromide on fluoride toxicity was determined for chicks fed diets with or without dietary fat.

Experimental. New Hampshire x Delaware chicks were used for an experimental period of four weeks in all experiments. Lots contained 10 birds (5 males and 5 females) which were randomized and housed in electrically heated batteries with raised wire floors and fed ad libitum. Supplements of fluoride and bromide were added as the sodium salts and calcium as the carbonate to the basal diet. The sodium content of all diets was equalized with sodium chloride. Two basal diets (Table 1) with and without 10% added fat were employed. The calorie-protein ratio of the basal diet containing 10% added fat was equalized with the basal without added fat (41.4 - 1) by adjustment of the milo, corn and soybean levels. Vitamins were supplied at the levels recommended by the National Research Council. Also included were two antibiotics and an anti-oxidant. The added dietary fat was supplied as hydrolyzed animal fat and vegetable fat.[1] The calcium-phosphorus ratio was 1.6 to 1.

The chicks were weighed at the end of the four week experimental period, except in the last experiment in which birds were weighed weekly. Feed utilization and mortality were calculated for the four week experimental

---

[1]HEF - Proctor and Gamble Co., Inc., Cincinnati, Ohio.

TABLE 1

BASAL DIETS

Ingredients	Without Added Fat %	With 10% Added Fat %
Ground Milo	37.10	15.1
Ground Yellow Corn	24.00	20.0
Soybean Meal (44%)	22.75	38.75
Dehydrated Alfalfa Meal (17%)	3.00	3.00
Dried Whey	3.00	3.00
Fish Meal (65%)	3.00	3.00
Meat and Bone Scraps (50%)	3.00	3.00
Ground Limestone	1.00	1.00
Dicalcium Phosphate	0.50	0.50
$MnSO_4 \cdot 5H_2O$	0.02	0.02
Methionine Hydr. Analogue	0.125	0.15
Vitamin and Antibiotic Mix[1]	2.50	2.50
Fat	--	10.00
TOTAL	100.00	100.00

[1]Supplied the following per pound of diet: 4,500 I.U. vitamin A, 700 I.C.U. vitamin D_3, 2 mg. riboflavin, 12.5 mg. niacin, 5 mg. d-calcium pantothenate, 200 mg. choline chloride, 6 mcg. vitamin B_{12}, 2.5 I.U. d-alpha-tocopheryl acetate, 1 mg. menadione sodium bisulfite, 2 mg. procaine penicillin, 10 mg. chlorotetracycline and 56.75 mg. ethoxyquin in a soybean meal carrier.

periods. The data were analyzed statistically by the Duncan multiple range test (Duncan, 1955).

Results and Discussion. In a series of experiments run to study the effects of bromine, calcium and fat on fluoride toxicity, a detrimental effect of high levels of sodium bromide on the growth rate of chicks was obtained (Table 2). A level of 10,000 ppm bromide produced a significantly lower body weight at 4 weeks of age. The feeding of 20,000 ppm bromide caused 100% mortality by 2 weeks of age. Bromide levels lower than 10,000 ppm did not significantly affect body weight. No marked changes in efficiency of feed utilization were noted with the feeding of sodium bromide at levels up to 10,000 ppm bromide. Groups 4 and 5 showed definite signs of a sedative effect of bromide.

Toxic amounts of fluoride and bromide in the absence of added fat were studied (Table 3). The birds fed the 10,000 ppm bromide level showed a significantly lower (13%) body weight at 4 weeks of age with a mortality of 22.5%. A striking retardation of growth (70% reduction) occurred in the group receiving 2,000 ppm fluoride and birds fed these amounts of fluoride and bromide simultaneously died by three weeks of age. Neither fluoride nor bromide alone was sufficient to produce 100% mortality by 4 weeks of age. Efficiency of feed utilization was decreased by 45% in the fluoride supplemented group, while no substantial change in efficiency of

TABLE 2

EFFECT OF SODIUM BROMIDE ON THE GROWTH OF CHICKS TO 4 WEEKS OF AGE[1]

Groups	ppm Br Added as NaBr[2]	Average Weights at 4 Weeks (gms)	Feed Utilization	% Mortality
1	0	440[a][3]	1.92	10.0
2	2,500	419[a]	1.87	5.0
3	5,000	410[a]	1.91	10.0
4	10,000	345[b]	1.93	20.0
5	20,000	--	--	100.0

[1]Two lots of 10 (5 males and 5 females) New Hampshire x Delaware chicks were fed on each dietary treatment.

[2]Sodium content of all diets was equalized with sodium chloride.

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

TABLE 3

EFFECT OF TOXIC AMOUNTS OF FLUORIDE AND BROMIDE ON CHICK GROWTH IN THE ABSENCE OF ADDED FAT[1]

Added Fluoride[2] (ppm)	Added Bromide[2] (ppm)					
	0			10,000		
	Average wt. at 4 weeks (gms)	Feed Utilization	% Mortality	Average wt. at 4 weeks (gms)	Feed Utilization	% Mortality
0	411	1.76	2.5	358	1.83	22.5
2000	124	2.55	20.0	-	-	100.0

[1]Four lots of 10 (5 males and 5 females) New Hampshire x Delaware chicks fed on each dietary treatment.

[2]Supplied as sodium fluoride and sodium bromide. Sodium content was equalized in all diets with sodium chloride.

feed utilization was noted when a toxic level of sodium bromide was incorporated in the basal diet.

The results of the interaction of fluoride and bromide at various levels of intake without added dietary fat are shown in the upper portion of Table 4. As the bromide level was increased, the body weight at 4 weeks decreased. In this experiment, the group receiving 5,000 ppm bromide had a significiantly lower body weight at 4 weeks than the controls. This is not in agreement with the results of Table 2 and might possibly be explained on the basis that a larger number of birds were used in this experiment. The groups receiving 10,000 ppm bromide in addition to 1,000 ppm fluoride showed a significant reduction in weight compared with the group receiving fluoride only. The group receiving 10,000 ppm bromide, and 2,000 ppm fluoride simultaneously showed 100% mortality by 3 weeks, as in Table 3. However, the groups receiving 5,000 ppm bromide, in addition to either 1,000 or 2,000 ppm fluoride, did not show a significant reduction in body weight below that of the respective groups receiving fluoride only.

Interaction of fluoride and bromide at various levels with 10% added dietary fat are shown in the lower half of Table 4. The results are essentially the same as those groups receiving no added dietary fat. Again, no additive toxic effect was noted in the groups receiving 5,000 ppm bromide with either fluoride levels compared with that of

TABLE 4

EFFECT OF FLUORIDE AND BROMIDE ON CHICK GROWTH WITH AND WITHOUT FAT[1]

Added Fluoride[2] (ppm)	Added Bromide[2] (ppm)					
	0		5,000		10,000	
	Average wt. at 4 weeks (gms)	Feed Utilization	Average wt. at 4 weeks (gms)	Feed Utilization	Average wt. at 4 weeks (gms)	Feed Utilization
	Without Added Fat					
0	450[a][3]	1.79	417[b]	1.84	332[e]	1.91
1000	352[de]	1.96	336[e]	1.84	286[f]	2.71
2000	127[gh]	2.76	113[h]	3.20	-	-
	With 10% Added Fat					
0	459[a][3]	1.61	408[b]	1.84	382[c]	1.87
1000	376[cd]	1.69	366[cd]	1.74	274[f]	1.99
2000	144[g]	2.38	142[g]	2.34	-	-

[1]Three lots of 10 (5 males and 5 females) New Hampshire x Delaware chicks were fed on each dietary treatment.

[2]Supplied as sodium fluoride and sodium bromide. Sodium content was equalized in all diets with sodium chloride.

[3]Weights having different superscripts are statistically different at the 0.05 level of probability (Duncan, 1955).

the respective groups receiving fluoride only. An additive toxic effect was noted in the group receiving the highest bromide level and 1,000 ppm fluoride while the group receiving the highest levels of both fluoride and bromide reached 100% mortality by 3 weeks of age.

Bixler and Muhler (1960) have indicated that the feeding of 20% dietary fat and the administration of fluoride by injection or by stomach tube resulted in increased skeletal retention of the element. These findings substantiated an earlier report by Buttner and Muhler (1958) with rats. Fat has been reported to increase the toxicity of fluoride in rats fed increased amounts of dietary fluoride. When isocaloric diets of varying fat content were fed in combination with the same fluoride level, growth was the same in all groups.

The results of these studies do not confirm the work with rats to the effect that ad libitum feeding of toxic amounts of fluoride in high fat diets resulted in increased toxicity based on weight gain.

Effect of increased calcium on fluoride toxicity without added dietary fat are shown in the upper half of Table 5. By increasing the calcium level of the diet to 3%, a significant reduction in body weight occurred. However, when a toxic level of fluoride was supplemented at this calcium level, no further decrease in weight was noted. Thus, the

TABLE 5

EFFECT OF FLUORIDE ON CHICK GROWTH IN THE PRESENCE OF INCREASED CALCIUM[1]

Added Fluoride[2] (ppm)	% Ca in Diet					
	1.1			3.0		
	Average wt. at 4 weeks (gms)	Feed Utilization	% Mortality	Average wt. at 4 weeks (gms)	Feed Utilization	% Mortality
	Without Added Fat					
0	430	1.72	0.0	310	2.01	10.0
1000	358	1.82	0.0	307	1.99	10.0
	With 10% Addded Fat					
0	403	1.79	10.0	275	2.07	20.0
1000	350	1.79	5.0	313	1.94	0.0

[1]Two lots of 10 (5 males and 5 females) New Hampshire x Delaware chicks fed on each dietary treatment.

[2]Supplied as sodium fluoride. Sodium content was equalized in all diets with sodium chloride. Each diet was supplemented with 100 ppm zinc.

increase in calcium decreased the toxicity of fluoride, probably by the formation of insoluble calcium fluoride which is poorly absorbed. The decrease in weight on the high calcium diet might be due to the fact that not enough zinc was present in the diets with the large calcium-phosphorus ratio.

Effects of increased dietary calcium on fluoride toxicity with added 10% fat are shown in the lower half of Table 5. The results are similar to those of the groups receiving no added fat, indicating that dietary fat had no effect on fluoride-calcium interaction. Again, the high calcium level decreased the body weight gain at four weeks, but the addition of fluoride to the high calcium diet had no further effect on body weight.

The average body weight of chicks fed 2,000 ppm fluoride with and without 10% added fat for different periods of times are shown in Figures 1 and 2. Body weight gains were recorded weekly for four weeks. When fluoride supplementation was started at the end of the first or second week, the growth curve paralleled that of the negative controls (fluoride supplemented for 4 weeks) and when the control diet was given to chicks starting on the high fluoride diet, the growth curve paralleled that of the positive controls (no fluoride supplementation for 4 week). Examination of Figures 1 and 2 show that fat content of the basal diet had

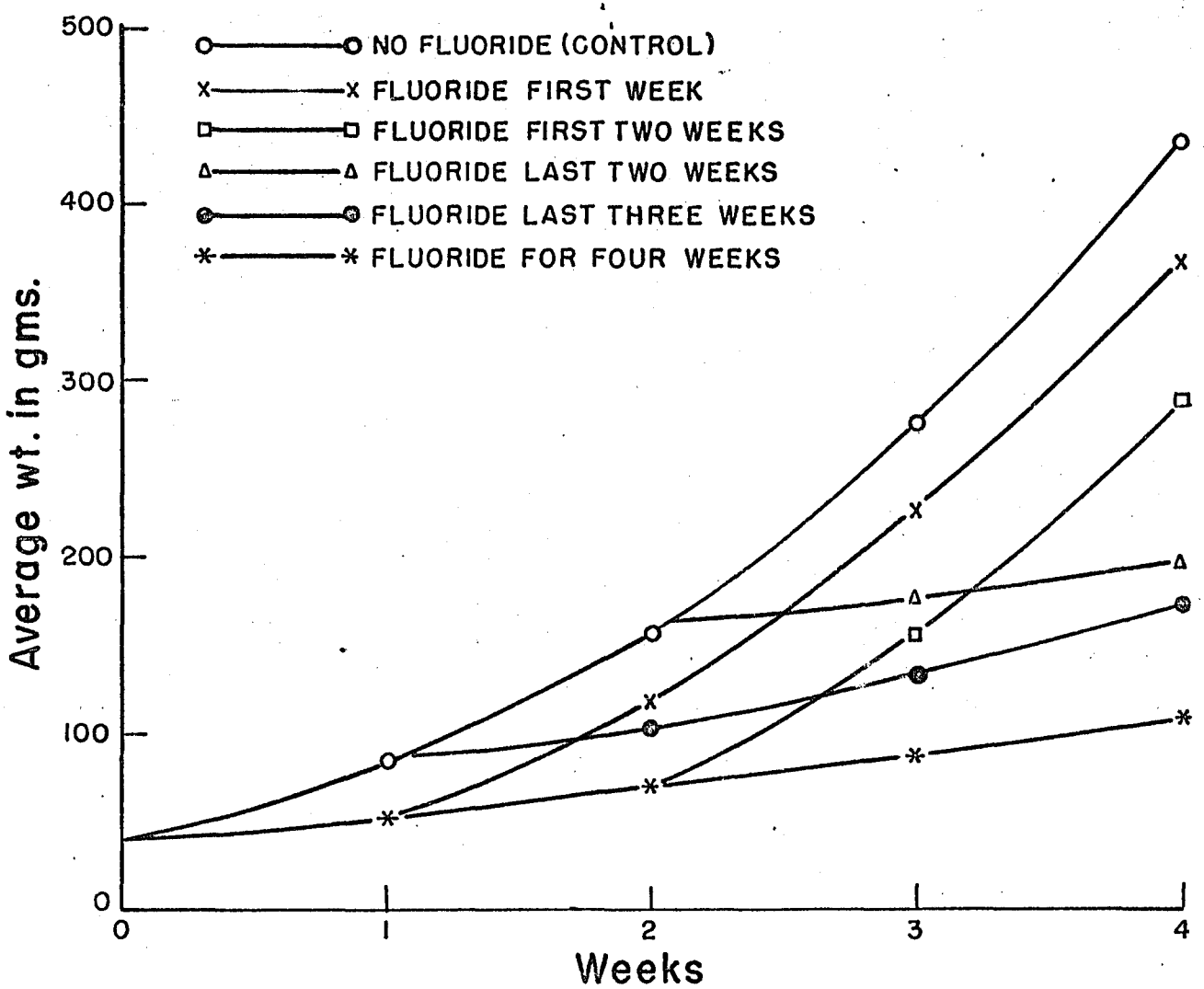

Figure 1.- Average body weight of chicks fed 2000 ppm fluorine for different periods of time without added dietary fat.

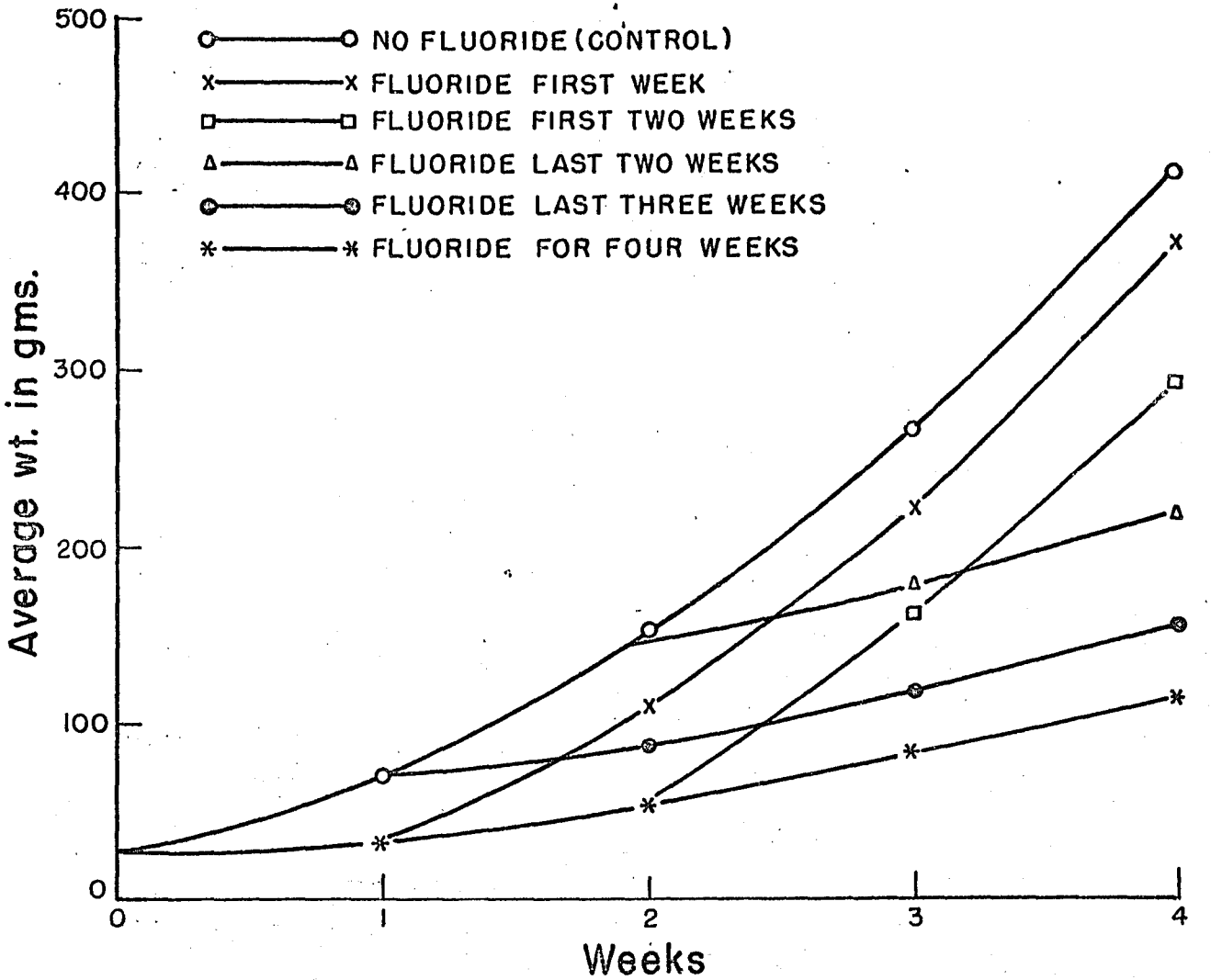

Figure 2.- Average body weight of chicks fed 2000 ppm fluorine for different periods of time with 10% added dietary fat.

no effect on the growth curves. Chick growth, for each week, was representative of the diet fed, regardless of the fluoride content of the diet previously fed. This indicates that no carry-over effect occurred when toxic diets were removed from chicks.

It may be concluded from these results with chicks that no effects were observed by the addition of 10% fat on fluoride or bromide toxicity during a four week period, based on body weight, when the caloric content of the diets are equal.

Summary. The toxic level of bromide, based on body weight gain was found to be between 5,000 and 10,000 ppm. Bromide at a level of 20,000 ppm produced 100% mortality after 2 weeks of treatment.

The inclusion of 1,000 ppm fluoride with 10,000 ppm bromide produced a decrease in body weight greater than that of the fluoride alone. However, 1000 ppm fluoride with 5,000 ppm bromide did not significantly reduce the body weight below that of the fluoride alone. Feeding chicks a diet containing 2,000 ppm fluoride and 10,000 ppm bromide produced 100% mortality within 3 weeks. Neither fluoride nor bromide alone at these levels was sufficient to produce over 25% mortality by 4 weeks of age.

Toxic amounts of fluoride added to a high calcium diet with increased zinc did not produce a significant

reduction in body weight as compared with the high calcium control diet.

No effects were observed by the addition of 10% fat on fluoride or bromide toxicity during a four week period, based on body weight gain.

CHAPTER III

EFFECT OF A MINIMAL FLUORIDE DIET WITH RATS

Introduction. Over the past 30 years several papers have appeared which attempted to demonstrate an essential physiological function for fluorine. Sharpless and McCollum (1933), Phillips, Hart and Bohstedt (1934), Evans and Phillips (1939), Lawrenz (1945) Maurer and Day (1957) and Muhler (1954) were some of the early workers who directly approached this problem. In some cases, the minimal diets must have contained fluorides since the carcass contained fluorides. In other cases, the purified diets employed were inadequate for normal reproductive performance.

The only paper which does not support the conclusion that fluorine is a non-essential element is that of McClendon and Gershon-Cohen (1953). These workers attributed a marked reduction in growth rate and extensive caries of the molars to the lack of dietary fluoride. However, no data were presented on the fluoride content of the tissues of the animals at the beginning or end of the experimental period and the especially prepared diet was not fed in combination with fluoride.

This chapter reports on the effect of feeding natural foodstuffs of minimal fluoride content on growth, feed utilization, apparent nitrogen digestibility and serum, liver and bone enzymes in the rat.

Experimental. A hydroponic technique for the culture and production of sorghum (Sorghum vulgare, DD38) and soybean (Glycine Max, Lee) of minimal fluoride content was employed in a greenhouse constructed for this purpose. The building was equipped with four evaporative coolers and radiant steam heat for temperature control between 65 and 95°F. The coolers were equipped with microfilters to minimize dust.

Silica sand (ASTM C-190) was used as the potting medium after treatment with hydrochloric acid followed by heating at 800°C. for eight hours in 1/2-inch layers to insure removal of fluorides.

All benches were covered with polyethylene sheets and polyethylene was used to cover the ceiling to prevent moisture condensate from dropping on the plants or in the pots. The compositions of the nutrient solutions used are shown in Table 6.

The water purification system consisted of a double distillation followed by passage through an ion exchange deionizer and a millipore filter. The water purity was checked constantly with a resistance meter and averaged

TABLE 6

NUTRIENT SOLUTION COMPOSITION

Ingredient	Gm/Liter of Solution	
	Soybean	Sorghum
$Ca(NO_3)_2 \cdot 4H_2O$	1.17	1.17
KNO_3	0.50	0.50
$MgSO_4 \cdot 7H_2O$	0.50	0.50
KH_2PO_4	0.14	0.14
$MnCl_2 \cdot 4H_2O$	0.00181	0.00181
H_3BO_3	0.00286	0.00286
$ZnSO_4 \cdot 7H_2O$	0.00022	0.00022
$CuSO_4 \cdot 5H_2O$	0.00008	0.00008
$H_3MoO_4 \cdot H_2O$	0.00009	0.00009
$FeSO_4 \cdot 7H_2O$	0.0183	0.0183
pH adjusted with NaOH to	6.0	8.0

15-16 million ohms. According to Bullock, water with a resistance above 2.5 million ohms contains no fluorides.

All chemicals employed were analyzed for fluoride and recrystallized when necessary to remove fluorides. The complete nutrient solution employed contained <0.001 ppm fluorine.

The original seeds of sorghum and soybean contained 3.5 ppm and 3.7 ppm fluoride, respectively (Table 7). The fluoride content of the first crop of seeds produced under the greenhouse conditions contained 0.014 ppm F on a dry weight basis; the second crop contained <0.005 ppm F. The latter crop of seeds, containing less than 0.005 ppm F were used for replanting and for feeding trials.

No differences in germination or growth of the plants were noted when compared with seeds supplied with fluoride in the nutrient solution (Kurtz, 1961). Amino acid analyses of the soybean and sorghum were found to correspond with field-grown seeds.

The soybeans were ground and processed by autoclaving for a period of 40 minutes under 15 pounds pressure. Previous studies had shown such treatment to be adequate for the destruction of inhibitors present in raw soybeans (Reid, et al., 1960).

Fluoride analyses were accomplished by distillation with perchloric acid according to the method of Willard and

TABLE 7

FLUORIDE CONTENT OF SORGHUM AND SOYBEAN SEEDS

Sample	ppm Fluoride		
	Original Seed	Greenhouse-Grown Seed	
		1st Generation	4th Genration
Sorghum Seed	3.540	0.013	<0.005
Soybean Seed	3.720	0.014	<0.005

Winter (1933) using the apparatus described by Hackabay, et al. (1947). The distillates obtained by this procedure were treated by the method of Megregain (1954). Organic samples required ashing at 600°C. with F-"free" calcium oxide (as a fixative) for 4 hours before distillation.

All components of the basal diet were analyzed for fluoride prior to use (Tables 8a, 8b, 8c) and purified when necessary. The vitamins used were in the crystalline form or purest form obtainable and, in most cases, required no further treatment in order to render them free of fluoride.

The calcium source employed required special treatment in order to render it free of fluoride. Calcium carbonate (12.3 ppm F) was mixed with an excess of perchloric acid and the resulting solution heated at 160°C. for 6 hours. Sodium hydroxide was added to the cooled solution and the precipitated calcium hydroxide was filtered and dried.

The three dietary treatments for the rat feeding trials were as follows: (1) Minimal fluoride diet--greenhouse-grown sorghum and soybean meal, with a fluoride content of less than 0.005 ppm; (2) Minimal fluoride diet plus 2.0 ppm F and; (3) Normal control--field-grown sorghum and soybeans, with a fluoride content of 2.67 ppm.

Nine weanling (20 days of age) Sprague-Dawley rats were used per treatment (3 males and 6 females). The experimental period was 10 weeks. The rats were distributed among

TABLE 8a

COMPOSITION OF RAT DIET

Ingredients	%
Soybean (37% protein)	55.00
Sorghum (9% protein)	23.25
Sucrose	9.80
Vitamin Mix F-2	1.50
Salt Mix F-2	8.00
Corn Oil	2.00
Chromic Oxide	0.25
Choline Chloride	0.20
Sodium Bromide Solution[1]	
TOTAL	100.00

[1]To supply 5 ppm bromide.

TABLE 8b

"FLUORIDE-FREE" SALT MIXTURE
F-2

Source	% of Diet
$Ca(OH)_2$	1.6600
KH_2PO_4	3.9500
NaCl	0.5000
$MnSO_4 \cdot H_2O$	0.0336
$FeSO_4 \cdot 7H_2O$	0.1320
$ZnSO_4$	0.0160
$CuSO_4 \cdot 5H_2O$	0.0200
KI	0.0052
$CoCl_2 \cdot 6H_2O$	0.0100
KCl	0.5000
$MgSO_4 \cdot 7H_2O$	0.5800
$H_2MoO_4 \cdot H_2O$	0.0024
Sucrose (carrier)	0.5960
TOTAL	8.0052

TABLE 8c

FLUORINE-FREE VITAMIN MIXTURE
F-2

Ingredient	Mg per Kg of Diet
Ascorbic Acid	12.50
Thiamine HCl	12.50
Niacin	100.00
Riboflavin	20.00
Pyridoxine HCl	12.50
d-Biotin	1.25
Calcium Pantothenate	75.00
Vitamin B_{12} (0.1%)	10.00
Vitamin A Acetate (crystalline)	7000 I.U.
Folic Acid	4.00
Vitamin D_2	1500 I.U.
d-alpha-Tocopheryl Acetate	75.00
Menadione (2-Methyl-Naphthoquinone)	1.25
dl-Methionine	4000.00
i-Inositol	500.00
Para-Aminobenzoic Acid	25.00
Terramycin (895 μ/mg)	25.00
Sucrose (carrier)	9.00 gm

the dietary treatments according to litter, sex and weight. The weanling rats were housed in individual, stainless steel cages in a temperature-controlled environment equipped with microfilters to minimize dust. The average carcass fluoride analysis of the weanling rats was 0.72 ppm F (fresh weight basis).

At the end of the growth period, all rats were anesthetized with ethyl ether and blood samples were collected. Livers were quickly removed, rinsed with saline solution and homogenized in Krebs-Ringer phosphate solution (Umbreit, et al., 1959). The homogenates were quick-frozen with Dry Ice and stored at -10°C until enzyme activities could be measured. The blood was kept in an ice bath for one hour and then centrifuged. The serum enzyme activities were measured the same day. Alkaline and acid phosphatases were measured with p-nitro phenylphosphate as the substrate at pH 9.3 and 4.8, respectively, according to the methods of Bessey, et al. (1946) and Andersch and Szczypinski (1947). The method of Martland and Robinsen (1929) was used to prepare the humerus bone for alkaline phosphatase activities. Glutamic-oxalacetic and glutamic-pyruvic transaminases were determined by the Sigma-Frankel method (1961).

Lactic dehydrogenase was determined by the Berger-Broida method (1960). Isocitric dehydrogenase activities were determined by the Sigma method (1961).

Results and Discussion. The feeding of a low fluoride diet, containing less than 0.005 ppm by analysis, to weanling rats for a period of 10 weeks failed to produce a significant change in weight gains during the experimental period (Table 9). Feed utilization calculated from feed consumption data and weight gains were not significantly different when tested by Duncan's Multiple Range Test (1955). The utilization of dietary nitrogen was in the range of 95.4-96.1% and was not affected by the presence of either 2.0 ppm added fluoride (as NaF) or the 2.67 ppm measurable fluoride present in the field-grown dietary ingredients (Table 9).

Marked differences were noted in the fluoride content of tibiae from the animals fed the respective diets (Table 10). The rats fed the minimal fluoride diet showed an average fluoride content of 2.92 ppm in the tibiae (dry wt.). Muhler (1954) reported that the feeding of a diet which contained not more than 0.1 ppm fluoride resulted in a maximum storage of 23-29 ppm in the femurs of rats at 80 days of age. These data suggest that the availability of the fluoride of the minimal fluoride diet was low. The feeding of 2 ppm fluoride as NaF resulted in a level of 34.6 ppm fluoride in the tibiae at 91 days of age. The natural fluoride which was present in the field-grown soybean and sorghum was less available to the rat than the fluoride supplied as NaF (Table 10).

TABLE 9

EFFECT OF DIETARY FLUORIDE CONTENT ON GROWTH AND FEED UTILIZATION IN THE RAT

Dietary Treatment	Wt. Gain 21-91 Days (gms)	Average Final Wt. (gms)	Feed Utilization (gm feed/ gm gain)	% Apparent Nitrogen Digestability
Minimal Fluoride (<0.005 ppm F)	213.9	267.8	5.27	95.9
Minimal Fluoride plus 2.0 ppm F	202.5	251.4	5.14	96.1
Normal Control (2.67 ppm F)	233.9	277.7	4.87	95.4

TABLE 10

FLUORIDE ACCUMULATION IN TIBIA AND FIBULA OF THE RAT[1] AT 91 DAYS OF AGE

Dietary Treatment	Average ppm Fluoride (fresh weight basis)
Minimal Fluoride (<0.005 ppm F)	2.92 (4)[2]
Minimal Fluoride plus 2.0 ppm F	34.63 (4)
Normal Control (2.65 ppm F)	12.54 (4)

[1]Initial whole carcass fluoride content of weanling rats was 0.72 ppm (fresh weight basis).

[2]Number of determinations.

Measurements of serum alkaline and acid phosphatase levels were made at the end of the ten week feeding period. No significant changes in activity were obtained for either of these enzymes (Table 11). Maplesden, et al., (1960) were unable to demonstrate a change in the serum alkaline phosphatase activity with the feeding of high fluoride diets. Maurer and Day (1957) were also unable to obtain a significant difference in serum alkaline phosphatase activity attributable to the low fluorine diet fed.

Serum and liver levels of the glutamic oxalacetic and glutamic-pyruvic transaminases were estimated at the termination of the study in order to evaluate the effects of the minimal fluoride dietary conditions (Tables 12 and 14). The transaminases have been implicated as indicators of hepatic impairment, heart damage and a number of other conditions associated with tissue damage. As reported by others (Cohen, 1955) the serum activities of the glutamic-oxalacetic system were in the order of four times those obtained for the glutamic-pyruvic system. No statistically significant differences in the activities of these two enzyme systems in either serum or liver could be attributed to the respective dietary treatment employed.

Likewise, lactic dehydrogenase levels of serum or liver were not statistically altered with the feeding of

TABLE 11

EFFECT OF DIETARY FLUORIDE LEVEL
ON SERUM PHOSPHATASES

Dietary Treatment	Alkaline[1]	Acid[1]
Minimal Fluoride (<0.005 ppm F)	7.53	1.54
Minimal Fluoride plus 2.0 ppm F	8.19	1.61
Normal Control (2.67 ppm F)	7.75	1.35

[1]Expressed as micromoles of p-nitrophenol liberated/ hr./ml. of serum.

the minimal fluoride diet. These data might suggest that there was no impairment of heart, liver or kidney due to the low fluoride conditions imposed (Tables 12 and 14).

Significant elevation in isocitric dehydrogenase activities of the serum from animals fed the minimal fluoride diet was observed (Table 13). A concomitant decrease was observed in the isocitric dehydrogenase activity of livers from rats fed the minimal fluoride diet (Table 15). In vitro addition of fluoride (10^{-4} M) to the isocitric dehydrogenase assay system produced a significant inhibition in activities with liver homogenates from each of the dietary treatments.

The only enzymatic changes found to occur were in the isocitric dehydrogenase system. The data on the lowered activity in the liver, along with the elevated serum activity, in the absence of fluoride, may suggest a degree of liver damage associated with the minimal fluoride feeding.

The effect of dietary treatment on alkaline phosphatase activity of the humerus was determined and the addition of fluoride in vitro studied (Table 16). No significant differences were noted. The addition of fluoride in vitro did not significantly change the alkaline phosphatase activity.

Except for the changes noted in isocitric dehydrogenase activity, our results are in agreement with the

TABLE 12

EFFECT OF DIETARY FLUORIDE
ON SERUM TRANSAMINASES

Dietary Treatment	Glutamic-Oxalacetic[1]	Glutamic-Pyruvic[1]
Minimal Fluoride (<0.005 ppm F)	132.2	36.1
Minimal Fluoride plus 2.0 ppm F	152.8	38.0
Normal Control (2.67 ppm F)	126.1	30.9

[1]Sigma-Frankel units/ml. serum/hr.

TABLE 13

EFFECT OF DIETARY FLUORIDE
ON SERUM DEHYDROGENASES

Dietary Treatment	Lactic[1]	Isocitric[2]
Minimal Fluoride (<0.005 ppm F)	819[a]	591[a][3]
Minimal Fluoride plus 2.0 ppm F	793[a]	384[b]
Normal Control (2.67 ppm F)	764[a]	404[b]

[1]Berger-Broida units/ml. of serum.

[2]Millimicromoles of α-ketoglutarate formed/ml. of serum/hr.

[3]Means having different letter superscripts were significantly different at the 0.05 level of probability.

TABLE 14

DIETARY FLUORIDE CONTENT AND LIVER ENZYME ACTIVITY

Dietary Treatment	Lactic Dehydrogenases[1]	Transaminases[2]	
		Glutamic-Oxalacetic	Glutamic-pyruvic
Minimal Fluoride (<0.005 ppm F)	2573	25.3	37.7
Minimal Fluoride plus 2.0 ppm F	2922	33.5	38.7
Normal Control (2.67 ppm F)	2613	40.6	32.9

[1]Berger-Broida units/mg. tissue.

[2]Sigma-Frankel units/mg. tissue/hr.

TABLE 15

EFFECT OF IN VITRO FLUORIDE CONCENTRATION ON ISOCITRIC DEHYDROGENASE ACTIVITY IN LIVER HOMOGENATES[1,2]

Dietary Treatment	Addition of Fluoride in vitro		
	None	10^{-4} M	10^{-2} M
Minimal Fluoride (<0.005 ppm F)	383^{e}	274^{a}	289^{ab}
Minimal Fluoride plus 2.0 ppm F	436^{f}	297^{ab}	313^{abc}
Normal Control (2.67 plus F)	436^{f}	330^{bcd}	359^{cde}

[1]Millimicromoles of α-ketoglutarate formed/mg. tissue/20 min.

[2]Means having different letter superscripts were significantly different at the 0.05 level of probability.

TABLE 16

EFFECT OF DIETARY AND IN VITRO FLUORIDE LEVELS ON BONE ALKALINE PHOSPHATASE ACTIVITIES

Dietary Treatment	Alkaline Phosphatase Activity[1]	
	No. F	4×10^{-4} MF[2]
Minimal Fluoride (<0.005 ppm F)	172	190
Minimal Fluoride plus 2.0 ppm F	183	184
Normal Control (2.67 ppm F)	212	214

[1]Micromoles of p-nitrophenol liberated/gm of bone/hr.

[2]Fluoride added in vitro as sodium fluoride.

reports in the literature favoring a non-essential role for fluoride.

Summary. The feeding of a minimal fluoride (<0.005 ppm F) diet for a ten week period to weanling rats demonstrated a significant depletion in bone fluoride compared to the control group receiving the minimal diet plus 2 ppm fluoride. Both serum and liver enzymes were determined and the only significant differences noted were an increase in serum isocitric dehydrogenase and a decrease in the activity of this enzyme in the liver.

REFERENCES

Aisenberg, A. C. and V. R. Potter, 1955. Effect of fluoride and dinitrophenol on acetate activation in kidney and liver homogenates. J. Biol. Chem. 215:737.

Amberg, S. and A. S. Loevenhart, 1908. Further observations on the inhibiting effect of fluoride on the action of lipase, together with a method for the detection of fluoride in food products. J. Biol. Chem. 4:149.

Andersch, M. A. and A. J. Szczpinski, 1947. Use of p-nitrophenyl phosphate as the substrate in determination of serum acid phosphatase. Am. J. Clin. Path. 17:571.

Armstrong, W. D., 1942. Review of the dental fluorosis studies at the University of Minnesota. In "Fluorine and Dental Health," Ed. F. R. Moulton, American Association for the Advancement of Science, Washington, D. C., p. 54.

Baumann, E. J., D. B. Sprinson and D. Marine, 1941. Bromine and the thryoid. Endocrinology 28:793.

Beevers, C. A. and D. B. McIntyre, 1946. Atomic structure of fluorapatite and its relation to tooth and bone material. Mineral Mag. 27:254.

Berger, L. and R. Broida, 1960. The colorimetric determination of lactic dehydrogenase in serum or other fluids at 400 to 500 mμ. Tech. Bull. No. 500, Sigma Chem. Co., St. Louis, Mo.

Bessey, O. A., O. H. Lowry and M. J. Brock, 1946. A method for the rapid determination of alkaline phosphatase with five cubic millimeters of serum. J. Biol. Chem. 164:321.

Berzelius, J., 1806. Extract d'une letter de M. Brezelius a' M. Berthollet. Ann. Chim. et Phys. 21:246.

Bixler, D. and J. C. Muhler, 1960. Retention of fluoride in soft tissues of chickens receiving different fat diets: J. Nutrition 70:26.

Borgatti, G., 1947. Effect of bromine on the gonads of the fowl. Arch. Sci. Biol. Napoli 32:67.

Bosshardt, D. K., J. W. Hoff, O. P. Miller and R. H. Barnes, 1956. A nutritional requirement for bromine. Proc. Soc. Exptl. Biol. Med. 92:216.

Buttner, W. and J. C. Muhler, 1957. The effect of fluoride administration on fluoride deposition in tissues and on serum cholesterol in the rat. J. Nutrition 63:263.

Buttner, W. and J. C. Muhler, 1958. The retention of fluoride by the skeleton, liver, heart and kidney as a function of dietary fat intake in the rat. J. Nutrition 65:259.

Carnot, A., 1892. Recherche du fluo dans les os modernes et les os fossiles. Compt. rend. 114:1189.

Cass, J. J., 1961, Response of livestock and poultry to absorption of inorganic fluorides. J. Occuptational Med. 3:35.

Cholak, J., 1959. Fluorides: A critical review 1. The occurrence of fluoride in air, food and water. J. Occupational Med. 1:1.

Cohen, P. P., 1959. Estimation of animal transaminases. In "Methods in Enzymology," Eds. S. P. Colowick and N. O. Kaplan, Academic Press, N. Y., p. 178.

Cole, B. T. and H. Patrick, 1958. Tissue uptake and excretion of bromine -82 by rats. Arch. Biochem. Biophys. 74:357.

Collings, G. H., R. B. L. Fleming and R. May, 1951. Absorption and excretion of inhaled fluorides. Arch. Ind. Hyg. 4:585.

Dean, H. T., 1942. The investigation of physiological effects by the epidemiological method. In "Fluorine and Dental Health," Ed. F. R. Moulton, American Association for the Advancement of Science, Washington, D. C., p. 23.

Duncan, D. B., 1955. The new multiple range and F test. Biometrics 11:1.

Evans, R. J. and P. H. Phillips, 1939. A new low fluorine diet and its effect upon the rat. J. Nutrition 18:353.

Frajola, W. J., 1959. Fluoride and enzyme inhibition. In "Fluorine and Dental Health," Eds. J. C. Muhler and M. K. Hine, Indiana University Press, Bloomington, p. 60.

Gardner, D. E., T. G. Scharff, F. A. Smith and H. C. Hodge, 1957. Investigations on the metabolism of fluoride IV. Fluoride balance studies at high levels of intake in rabbits. J. Nutrition 61:207.

Gardner, E. E., F. N. Andrews, R. L. Adams, J. C. Rogler and C. W. Carrick, 1959. The effect of fluorine on the chicken proventriculus. Poultry Sci. 38:1423.

Haman, K., P. H. Phillips and J. G. Halpin, 1936. The distribution and storage of fluorine in the tissue of the laying hen. Poultry Sci. 15:154.

Heintz, W., 1849. Uber die chemische zusamensetzung der knocken. Pogyendorf An. Physik. 77:269.

Hellenstein, S., C. Kaiser, D. DeS. Darrow and D. C. Darrow, 1960. The distribution of bromide and chloride in the body. J. Clin. Invest. 39:282.

Hempel, W. and W. Scheffer, 1899. Uber eine methode zur bestimmung des fluors neben kohlensäure and der fluorgehalt von einigen zähnen. Z. tschr. Anorg. Chem. 20:1.

Hodge, H. C., 1950. The concentration of fluorides in drinking water to give the point of minimum caries with maximum safety. J. Am. Dental Assoc. 40:436.

Hodge, H. C., 1953. Conf. on Metabolic Interrelations Trans. 4th Conf. 1952.

Hodge, H. C., 1961. Metabolism of fluorides. J. Am. Med. Assoc. 177:313.

Huckabay, W. B., E. T. Welch and A. V. Mettler, 1947. Constant-temperature steam-distillation apparatus for isolation of fluorine. Anal. Chem. 19:154.

Huff, J. W., D. K. Bosshardt, O. P. Miller and R. H. Barnes, 1956. A nutrient requirement for bromine. Proc. Soc. Exptl. Biol. Med. 92:216.

Hunt, H. R., C. A. Hoppert and S. Rosen, 1955. Genetic factors in experimental rat caries. In "Advances in Experimental Caries Research," Ed. R. F. Sognnaes, American Association for the Advancement of Science, Washington, D. C., p. 66.

Hupka, E. and P. Luy, 1929. Gehaufters and auftreten von osteomalacie unter weiderindern, vernuscoht durch fluorwasserstoffsäure enthaltenden fabriksauch. Arch. Wiss. Prakt. Tiech. 60:21.

Hupka, E. and E. Götze, 1931. Zur frage der schadlichkeit des fluors beim rinde. Deutsche tierarztl. Wchnschr 39:203.

Jenkins, G. N., 1963. Theories on the mode of action of fluoride in reducing dental decay. Brit. J. Nutrition 22:97.

Johnson, R. J. and H. A. Lardy, 1950. Orthophosphate uptake during the oxidation of fatty acids. J. Biol. Chem. 184:235.

Kurtz, E. B., 1961. Unpublished data. Univ. of Arizona.

Lawrenz, M., 1945. Unpublished data. Quoted by H. H. Mitchell and M. Edman, 1945. Fluorine in soil, plants and animals. Soil Sci. 60:81.

Lawrenz, M. and H. H. Mitchell, 1941. The effect of dietary calcium and phosphorus on the assimilation of dietary fluorine. J. Nutrition 22:91.

Lawrenz, M., H. H. Mitchell and W. A. Ruth, 1940a. Adaptation of the growing rat to the ingestion of a constant concentration of fluorine in the diet. J. Nutrition 19:531.

Lawrenz, M., H. H. Mitchell and W. A. Ruth, 1940b. The comparative assimilation of fluorine by growing rats during continuous and intermittent dosage. J. Nutrition 20:383.

Lohmann, K. and O. Myerhof, 1934. Uber die enzymatische unwandlung von phosphoglycerinsäure in brenztraubensäure und phosphorsäure. Biochem. Z. 273:60.

Maplesden, D. C., I. Motzok, W. T. Oliver and H. D. Branion, 1960. Placental transfer of fluorine to the fetus in rats and rabbits. J. Nutrition 71:70.

Martland, M. and R. Robinson, 1929. The preparation and the use of the bone phosphatase. Biochem. J. 23:237.

Mason, M. F., 1936. Halide distribution in body fluids in chronic bromide intoxication. J. Biol. Chem. 113:61.

Maurer, R. L. and H. G. Day, 1957. The non-essentiality of fluoride in nutrition. J. Nutrition 62:561.

McCann, H. G. and F. A. Bullock, 1957. The effect of fluoride ingestion on the composition and solubility of mineralized tissues of the rat. J. Dent. Res. 36:391.

McClendon, J. F. and J. Gershon-Cohen, 1953. Trace element deficiencies. Water-culture crops designed to study deficiencies in animals. J. Agr. Food Chem. 1:464.

McClendon, J. F. and J. Gershon-Cohen, 1954. The effect of fluorine-free food on dental and preiodental structure as revealed by roentgen studies. Am. J. Roentgenol. Radium Therapy, Nuclear Med. 71:1017.

McClure, F. J., 1948. Observations on induced caries in rats VI Summary results of various modifications of food and drinking water. J. Dental Res. 27:34.

McClure, F. J., 1949a. Fluorine in foods survey of recent data. U. S. Pub. Health Repts. 64:1061.

McClure, F. J., 1949b. Mineral metabolism (fluorine and other trace elements). Ann. Rev. Biochem. 18:335.

McClure, F. J., 1951. AMA Handbook of Nutrition, 2nd ed. Blakiston, N. Y., p. 137.

Megregian, S., 1954. Rapid spectrophotometric determination of fluoride with zirconium-eriochrome cyaine R lake. Anal. Chem. 26:1161.

Miller, R. F. and P. H. Phillips, 1955. The enhancement of the toxicity of sodium fluoride in the rat by high dietary fat, J. Nutrition 56:447.

Miller, R. F. and P. H. Phillips, 1956. The effect of age on the level and metabolism of fluorine in the bones of the fluoridated rat. J. Nutrition 59:425.

Mitchell, H. H. and M. Edman, 1952. The fluorine problem in livestock feeding. Nutrition Abstr. Revs. 21:787.

Motzok, I. and H. D. Branion, 1958. Influence of fluorine on phosphatase activities of plasma and tissues of chicks. Poultry Sci. 37:1469.

Muhler, J. C., 1954. Retention of fluorine in the skeleton of the rat receiving different levels of fluorine in the diet. J. Nutrition 54:481.

Muhler, J. C. and M. K. Hine, 1959. "Fluorine and Dental Health." Indiana University Press, Bloomington.

Muhler, J. C., W. H. Nebergall, and H. G. Day, 1953. Preparations of stannous fluoride compared with sodium fluoride for prevention of dental caries in rats. J. Am. Dental Assoc. 46:290.

National Research Council, 1960. The fluorosis problem in livestock production. Publication 824, Washington, D. C.

Neumann, W. F. and M. W. Neuman, 1958. "The Chemical Dynamics of Bone Mineral." University of Chicago Press, Chicago.

Neumann, W. F., M. W. Neuman, E. R. Main, J. O'Leary and F. A. Smith, 1950. The surface chemistry of bone: II Fluoride deposition. J. Biol. Chem. 187:655.

Orland, F. J., J. R. Blayney and R. W. Harrison, 1954. Use of the germfree animal technique in the study of experimental dental caries. I Basic observations on rats reared free of all microorganisms. J. Dental Res. 33:147.

Orland, F. J., J. R. Blayney, R. W. Harrison, J. A. Reyniers, P. C. Trexler, R. F. Erwin, H. A. Gordon and M. Wagner, 1955. Experimental caries in germfree rats inoculated with interococci. J. Am. Dental Res. 50:259.

Phillips, P. H., 1932. Plasma phosphatase in dairy cows suffering from fluorosis. Sci. 76:239.

Phillips, P. H., H. E. English and E. B. Hart, 1935a. The influence of sodium fluoride upon the basal metabolism of the rat under several experimental conditions. Am. J. Physiol. 113:441.

Phillips, P. H., H. E. English and E. B. Hart, 1935b. The augmentation of the toxicity of fluorosis in the chick by feeding desicated thyroid. J. Nutrition 10:399.

Phillips. P. H. and E. B. Hart. The effect of organic dietary constituents upon chronic fluorine toxicosis in the rat. J. Biol. Chem. 109:657.

Phillips, P. H., E. B. Hart and G. Bohstedt, 1934. Chronic toxicosis in dairy cows due to the ingestion of fluorine. Wisconsin Univer. Agr. Exper. Sta. Res. Bull. No. 123, p. 1.

Phillips, P. H. and A. R. Lamb, 1934. Histology of certain organs and teeth in chronic toxicosis due to fluorine. Arch. Pathol. 17:169.

Phillips, P. H. and J. W. Suttie, 1960. The significance of time in intoxication of domestic animals by fluoride. A. M. A. Arch. Indust. Health 21:343.

Pindborg, E. V., J. J. Pindborg and C. M. Plum, 1946. Studies on incisor pigmentation in relation to liver, iron and blood picture in the white rat. II. The effect of fluorine on the iron metabolism. Acta Pharmacol. Toxiol. 2:294.

Princi, F., 1960. The effects on man of absorption of fluoride. J. Occupational Med. 2:20.

Reid, B. L., A. A. Kurnick and A. R. Doberenz, 1960. Unpublished data, Univ. of Arizona.

Romanenko, K. L., 1954. Poisoning of farm animals with superphosphate. Veterinariya 31:52.

Schour, I. and M. C. Smith, 1942. Experimental dental fluorosis. In "Fluorine and Dental Health," Ed. F. R. Moulton, American Association for the Advancement of Science, Washington, D. C., p. 32.

Schraer, H., A. S. Posner, R. Schraer and I. Zipkin, 1962. Effect of fluorine on bone crystallinity in the growing rat. Biochem. Biophys. Acta 64:565.

Sharpless, G. R., 1936. Limitation of fluorine toxicosis in the rat with aluminum chloride. Proc. Soc. Exptl. Biol. Med. 34:562.

Sharpless, G. R. and E. V. McCollum, 1933. Is fluorine an indispensable element in the diet? J. Nutrition 6:163.

Shaw, J. H. (Ed.), 1954. "Fluoridation as Public Health Measure." American Association for the Advancement of Science, Washington, D. C., p. 232.

Sievert, A. H. and P. H. Phillips, 1959. Metabolic studies on the sodium fluoride-fed rat. J. Nutrition 68:109.

Sigma, 1961. Isocitric dehydrogenase. Tech. Bull. No. 175. Sigma Chem. Co., St. Louis, Mo.

Sigma-Frankel, 1961. A simplified method for the colorimetric determination of glutamic-oxalacetic and glutamic pyruvic transaminases. Tech. Bull. 505, Sigma Chem. Co., St. Louis, Mo.

Smith, M. C. and E. M. Lantz, 1933. Experimental production on mottled enamel. Arizona Sta. Tech. Bull. 45:325.

Smith, M. C., E. M. Lantz and H. V. Smith, 1931. The cause of mottled enamel, a defect of human teeth. Arizona Sta. Tech. Bull. 32:253.

Stookey, G. K., D. B. Crane and J. C. Muhler, 1962a. Effect of molybdenum on fluoride absorption. Proc. Soc. Expt. Biol. Med. 109:580.

Stookey, G. K., R. H. Roberts and J. C. Muhler, 1962b. Synergistic effect of Molybdenum and fluoride on dental caries in rats. Proc. Soc. Exptl. Biol. Med. 109:702.

Stookey, G. K., D. B. Crane and J. C. Muhler, 1963. Role of skeleton and kidney in fluoride absorption in rat. Proc. Soc. Exptl. Biol. Med. 113:366.

Stookey, G. K. and J. C. Muhler, 1962. Effect of molybdenum on fluoride retention in the rat. Proc. Soc. Exptl. Biol. Med. 109:268.

Suttie, J. W. and P. H. Phillips, 1960. Fat utilization in the fluoride-fed rat. J. Nutrition 72:429.

Taylor, J. J., J. K. Scott, E. A. Maynard, F. A. Smith and H. C. Hodge, 1961. Toxic effects of fluoride on the rat kidney. Toxicol. Applied Pharmacol. 3:278.

Umbreit, W. W., R. H. Burris and J. F. Stauffer, 1959. "Manometric Techniques," Burgess Publishing Co., Minn., p. 149.

Underwood, E. J., 1962. "Trace Elements in Human and Animal Nutrition." Academic Press, N. Y., p. 259.

Warburg, O. and W. Christian, 1942. Isollerung and kristallisation des gärungsferments enolase. Biochem. Z. 310:384.

Weddle, D. A. and J. C. Muhler, 1954. The effects of inorganic salts on fluorine storage in the rat. J. Nutrition 54:437.

Weidmann, S. M., J. A. Weatherell and D. Jackson, 1963. The effect of fluoride on bone. Brit. J. Nutrition 22:105.

Weidmann, S. M., J. A. Weatherell and R. G. Whitehead, 1959. The effect of fluorine on the chemical composition and calcification of bone. J. Pathol. Bacteriol. 78:435.

Willard, H. H. and O. B. Winter, 1933. Volumetric method for determination of fluorine. Ind. Eng. Chem., Anal. Ed. 5:7.

Winnek, P. S. and A. H. Smith, 1937a. The determination of bromine in biological substances. J. Biol. Chem. 119:93.

Winnek, P. S. and A. H. Smith, 1937b. Studies on the role of bromine in nutrition. J. Biol. Chem. 121:345.

Zipkin, I. and R. S. Gold, 1963. The citrate content of teeth. Proc. Soc. Exptl. Biol. Med. 113:580.

Zipkin, I. and F. J. McClure, 1951. Complex fluorides: caries reduction and fluorine retention in bones and teeth of white rats. Public Health Rep. 66:1523.

Zipkin, I., A. S. Posner and E. D. Eanes, 1962. The effect of fluoride on the X-ray diffraction pattern of the apatite of human bone. Biochim. Biophys. Acta 59:255.