SOME EFFECTS OF CHRONIC AND ACUTE FLUORIDE LEVELS ON METABOLISM AND DISTRIBUTION OF F^{18} IN SELECTED TISSUES OF CATTLE

by

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INTRODUCTION

Fluorides are usually considered as toxic agents in livestock rations. The normal source of fluorides are: (1) from raw rock phosphate used as a source of phosphorous, (2) from grazing areas adjacent to certain industrial plants, and (3) from water containing large quantities of fluoride.

Search for a cheap source of phosphorous to be used as a mineral supplement led to the use of raw rock phosphate. This material may contain as much as four per cent fluorides. When added to the concentrate ration even at low levels it eventually builds up a level within the animal body that results in toxicity. This may take several years. During this time the animal appears to be normal, and then rather abruptly there is a loss of milk production, loss of appetite, loss of body weight and the other symptoms of fluoride toxicity.

Many industrial plants in their processing of raw materials release fluoride. Such processes include aluminum ore reduction; processing raw rock phosphate for the manufacture of phosphoric acid, feed supplements, and fertilizers; manufacture of bricks from fluoride containing clays; calcining limestone; and from certain enameling processes. The area of damage is usually confined to a rather small region adjacent to the plant emitting the fluoride. The

extent of the area of damage would depend upon a number of variables such as the quantity of fluoride emitted, prevailing wind direction, topography, the type and density of vegetation, and weather conditions.

When water passes through rock containing large quantities of fluoride some of it is dissolved. This water containing fluoride then seeps to lower levels. It may either surface in the form of seepage water or streams, or be brought to the surface in the form of well water. Animals drinking such waters can receive sufficient quantities of fluoride to result in toxicity symptoms.

Fluorides have been used in studying the use of carbohydrate in living tissue. This work has been largely with microorganisms and laboratory animals. In the studies with microorganisms it has been demonstrated that fluorides interfere with the use of carbohydrate by forming insoluble complex compounds that precipitate some of the necessary minerals and/or enzymes and effectively remove them from the biological system. These were usually magnesium-fluoro-phosphor-protein complexes. As a result one or more of the intermediate metabolites accumulated in the system and either slowed the whole system or in some cases stopped further metabolism.

Most of the work with laboratory animals has been with acute doses of fluorides that resulted in death within a few hours. It was shown to be involved in a complex system

involving insulin, the anterior pituitary, the enzyme hexokinase and the metabolism of carbohydrate. There was a decided lack of information, however, as to whether the effects in animals on chronic intake were the same as with those given acute dosages.

There is also a lack of information on the blood constituents of laboratory and farm animals, when fed chronic intakes, as to the progressive changes, if any, that occur. It has been demonstrated that in neither laboratory nor farm animals is there any appreciable change in the calcium, phosphorous, or hemoglobin content of the blood. This is in spite of the fact that there is a marked increase in the fluoride content of the bone. As the level of fluoride in the ration increases, the length of time necessary to demonstrate reduction in feed intake and loss of body weight decreases. There was little indication of any alteration in the blood carbohydrate picture to coincide with this reduction in appetite or body weight.

It has been demonstrated that a number of minerals have a marked effect on the ability of the rumen microorganisms to digest cellulose. Fluorides have had little effect on the apparent digestibilities of feed nutrients when either intact cattle or sheep have been studied. This would seem to indicate that the rumen microflora are only slightly disturbed by the presence of chronic fluoride intakes. Information is lacking as to whether there is any shift in the types of

microorganisms or in their functional ability in either the intact animal or in *in vitro* studies. However, the very marked refusal of feed within a matter of two to four days on high fluoride intakes raises the possibility that there is an effect on the rumen microflora that normal digestion techniques will not demonstrate.

This study was undertaken in an attempt to obtain information that is lacking concerning the effects of fluoride in animals. While the digestibility of the ration has not been lowered in the intact animal by relatively low levels of fluoride intake it was not known what effect higher levels of fluoride would have on either the numbers or types of microorganisms. Very high levels of fluoride ingested by the intact animal caused a marked reduction in appetite with a resultant loss in weight. Whether this was due to the accumulation of undigested material or the loss of certain types of bacteria was not known. It was felt that the use of *in vitro* techniques would give some indication as to what level of fluoride was necessary to materially effect cellulose digestion and the types of rumen organisms present.

Changes in the levels of blood glucose and lactate, and liver glycogen have been demonstrated using acute, lethal dosages of fluoride in laboratory animals. On the other hand the ingestion of relatively low levels of fluoride even for extended periods of time had not resulted in the blood

glucose level being changed in cattle or sheep. Information was needed to determine whether there were progressive changes in the blood glucose and lactate and liver glycogen of animals fed chronic fluoride intake after first ingesting fluoride. These determinations were needed in order to determine whether there is a similarity in the alteration of the carbohydrate metabolism between chronic and acute doses of fluoride.

Numerous tissue studies using chemical methods of fluoride analysis have been made. However, there was a decided lack of information as to the distribution in various tissues in relationship to both length of time the fluoride was ingested and/or the level of fluoride. The use of radio-isotope techniques to clarify mineral metabolism has been well established. The use of the fluoride isotope should help to clarify many of the obscurities or unknowns of fluoride distribution and metabolism.

REVIEW OF LITERATURE

There have been a number of reviews of the literature concerning fluorine. These vary in emphasis depending upon the objective of the authors. Roholm (46) has a complete review of the basic literature concerning fluorine intoxication. In it he describes a condition present in sheep grazing in Iceland as early as 1100 A.D. following volcanic eruptions. The symptoms described are similar to those which have been later described as fluorosis. It is pointed out that as early as 1670 fluorspar, treated with sulfuric acid, produced fumes that attacked glass, and that in 1802 this glass test was used to demonstrate the presence of fluorine in animal tissues. McClure (30) summarizes the literature concerning fluoride content of foods and water. Mitchell and Edman (36) emphasized the relative toxicities of various fluoride compounds in reviewing the literature concerning the danger in fluoride feeding to livestock.

Chronic fluorosis produced experimentally in cattle has been reported in a number of papers: Reed and Huffman (45), Phillips et al. (40), Elmslie (12), Majumdar et al. (31, 32, 33), Hobbs et al. (25). These reports concern a variety of fluoride containing compounds, some were natural feeding compounds and others were compounds used specifically to produce chronic fluorosis under carefully controlled

conditions. Relative toxicities of the different fluoride compounds, symptoms and the quantities of fluoride required to induce them in various species have been reported by Dean (8), McClure (29), Pierce (42), De Eads and Thomas (7), Gettler and Ellerbrook (14), Greenwood (17), Phillips et al. (40), who, in most cases, describe effects on various organs and tissues.

One of the difficulties in evaluating the fluoride literature is to distinguish between acute and chronic fluorosis, and between fluorosis as such, and fluorosis complicated by other factors. This has led to considerable variation in the reports as to the actual symptoms that are due to fluorides themselves.

Hobbs et al. (25) reported that cattle fed 600 to 1200 p.p.m. of fluoride refused feed within forty-eight to ninety-six hours. When this level of fluoride intake was maintained by giving the fluoride in a gelatin capsule daily on the basis of the feed consumed the animal showed a reduction of feed intake to less than forty per cent of normal. This was accompanied by a very rapid weight loss so that within sixty to one hundred twenty days the animals were unable to rise and had to be sacrificed. Handler (18, 19) reported marked elevation and depression in blood glucose and lactate and liver glycogen when sufficient sodium fluoride was injected into rats and rabbits to cause death within two to six hours. The direction of the alteration,

however, was dependent upon whether the animal had been fasted or not prior to the injection of sodium fluoride.

Schmidt and Rand (47) stated that:

> Many of the findings reported in the literature are symptomatic of other maladies as well as fluorosis. A number of symptoms reported in the literature are secondary in nature, dependent on primary effects that must also be present for the secondary symptoms to have any validity, as far as fluorosis is concerned.

They reported that mottling, staining, and excessive wearing of permanent teeth forming at the time of fluoride ingestion occurred both under field conditions and in controlled experiments. A bilateral lameness accompanied by exostosis was often the first noticed symptom reported, yet they point out that other dietary deficiencies such as calcium, phosphorous or vitamin D as well as mechanical injury could result in either lameness or exostosis. Hobbs et al. (25) reported very little exostosis on the rib, or bilaterial lameness in the cattle fed chronic levels of fluoride up to seven years when there was adequate calcium and phosphorous in the ration.

Most workers report one or more of the following four symptoms as appearing in animals receiving chronic fluoride intake: (1) tooth effects which include mottling, chalkiness, and abnormal wear; (2) skeletal abnormalities, particularly exostosis; (3) reduction of feed intake with a resultant loss of weight; and (4) increased concentrations of fluorine in the urine, bones and teeth. In addition,

other abnormalities have been reported such as diarrhea, elongated hoofs, brittle bones, dry and stiffened hide, decreased weight gain and milk yield, emaciation and anemia. These latter abnormalities have not been observed in carefully controlled experiments, and are believed by most workers to be secondary conditions brought about by other nutritional inadequacies. An excellent study of the tooth effects accompanied by color plates showing progressive changes is that of Hobbs et al. (25).

Much of this confusion has arisen from reports of the findings obtained under field conditions rather than under carefully controlled feeding experiments. The study by Schmidt and Rand (47) has done much to point out these differences and to distinguish between the differences due to fluorosis itself and those that could possibly be due to complicating factors. Critical study of the review by Roholm (46), McClure (29), Pierce (42), Greenwood (17), Mitchell and Edman (36), in addition to the one mentioned above, will do much to clear up the discrepancies that seem to appear in a casual review of the literature.

The trouble that may arise by only casually checking symptoms is clearly pointed out by Phillips et al. (39) in the National Research Council report on fluorosis.

> No single sign or symptom of fluorosis is definitely diagnostic. Hence it is necessary to observe two or more of the physiologic effects of fluorine for accurate diagnosis of the disease.

> For example, mottling of teeth provides evidence only that excessive fluorine intake occurred during the developmental period of the particular teeth affected. The likelihood of fluorosis in an adult animal suspect would be nil even though it had mottled and worn teeth if it was otherwise in good physical condition. On the other hand, if the animal in question was in a semi-starved condition fluorosis would be indicated but not assured, since starvation may be caused by other metabolic disturbances. In the latter case, a clear-cut diagnosis of fluorosis would be indicated if, in addition to the physiologic symptoms, analyses of the bones, the urine, the feed and water, or all of these, showed abnormally high concentrations of fluorine. The converse is also true: an adult with normal teeth may have fluorosis.

There are many factors that influence the degree to which a particular animal will exhibit fluorosis: (1) the type of fluoride compound fed which in turn effects the availability of the compound, (2) the level of the ingested fluoride, (3) the length of time that the animal was exposed, (4) the age of the animal at initial exposure, (5) the age of the animal when observed, (6) physiological stress factors, and (7) the effectiveness of the defense symptoms. These defense mechanisms are: (1) the urinary and fecal excretion, (2) withdrawal and deposition in the bones and teeth, and (3) the loss of appetite or reduction of food intake when the elimination ceilings are reached.

For the safe levels of fluorides that may occur in feeds without resulting in fluorosis one should refer to the suggested levels given in the National Research Council publication on fluorosis (39).

In studying abnormalities of carbohydrate metabolism fluorides have often been used at acute dosage levels. Most of this work has been done with microorganisms and laboratory animals. Stone and Werkman (48) surveyed a large group of bacteria to determine if they all produced the phosphoglyceric acid postulated in the Embden-Myerhoff scheme. By using fluoride as the inhibitor, they were able to demonstrate that all bacteria, with the exception of the clostridium cultures, did produce phosphoglyceric acid. Differences in sensitivity to fluorides when cultured in the presence of sodium fluoride was demonstrated by Wiggert and Werkman (51) using propionibacterium pentosaceum. This demonstrated the possibility of an organism developing more than one pathway in the dissimulation of glucose depending, of course, upon environmental conditions. The work by Utter and Werkman (49) showed one effect of the fluorides in interfering with this metabolic pattern. Using E. coli they found that the magnesium ion necessary to activate the enzyme, enolase, was precipitated as an insoluble magnesium-fluoro-phosphor-protein complex. This prevented the normal breakdown of 2-phosphoglyceric acid into phosphopyruvic acid. Knowing that manganese in some cases could replace magnesium, they made the substitution and found that inhibition did not occur since the manganese did not form a sufficiently insoluble residual compound to inactivate the

enolase. It was further found that in the absence of phosphorous, fluoride did not block the action of enolase since the insoluble complex was not formed. Gourley (16) demonstrated that the presence of fluorides reduced the ability of human erythrocytes to take up phosphate to about one-fifth of the normal rate. It also decreased the concentration of ATP to about 10 per cent of the level in normal blood within three hours. There was significant increase in the concentration of adenylic acid, suggesting that ATP was decomposed to this point but no further, and the turnover rate of this compound was actually decreased. Glucose-1-phosphate was found to remain in about the same concentration during the duration of the experiment, but the turnover rate was depressed. In the degradation of starch to glucose-1-phosphate the enzymes, phosphorylase and phosphatase, are both involved. Rapp and Sliwinski (44) demonstrated that the presence of monofluorophosphate brought about a 50 per cent reduction in the reaction compared to a 6.4 per cent reduction when the fluoride ion was used.

The magnesium ion is also involved in the formation of adenosine triphosphate. Kaplan and Greenberg (27) reported that fluoride reduced or stopped the formation of this important enzyme. Using rats they reported that sub-lethal doses produced a hyperglycemia, and that lethal doses produced a hypoglycemia when the rats were fasted 72 hours

prior to dosage. Handler et al. (18, 19) reported similar results using fasted rabbits, but animals not fasted did not behave the same as those fasted. The blood sugar in animals allowed to eat up to the time of dosage continued to rise until about 90 minutes before death. From this point until death there was a gradual decline. At death, however, the blood sugar levels were still about 200 mg. per cent compared to normal levels of about 120 mg. per cent. It was noted that there was also a marked rise in the level of blood lactic acid. This rise, which was about fifteen times normal, was postulated to be due to a greater fluoride inhibition of liver glycogenesis from blood lactic acid rather than the glycolytic breakdown of glycogen. The differences observed between fasted and non-fasted animals was reconciled when it was demonstrated that there was a severe depletion of the liver glycogen in the fasted animals. Actually, the primary interest of these workers was to study the mechanism whereby insulin prevented the rise in blood sugar that normally accompanied fluoride toxicity. During the time that this work was in progress Price et al. (43) reported that the effect of insulin was to overcome the inhibitory effect of the anterior pituitary extract on the action of hexokinase. This enzyme catalyzes the formation of glucose-6-phosphate along with phosphoglucomutase in the presence of magnesium ions.

Najjar (37) reported that phosphoglucomutase, an enzyme necessary to convert glucose-1-phosphate to glucose-6-phosphate, was inhibited by fluorides in the presence of either organic or inorganic phosphate. As with the enolase reaction reported earlier it was found that the magnesium ion was necessary as a catalytic agent, and that there was formed an insoluble magnesium-fluro-phosphor-protein complex that effectively removed the magnesium ion necessary for the catalytic formation of glucose-6-phosphate.

Thus it can be seen that there are several places in the Embden-Myerhoff scheme where fluorides will inhibit or block the use of carbohydrate. Some of these points of fluoride interferences are schematically diagrammed in in Figure 1. While it is now recognized that there are numerous other metabolic pathways, which have been well reviewed by Wood (53), the clear demonstration of this inhibition offers one possible explanation to the loss of appetite and reduction in weight observed in fluorosis.

Figure 1. Some points of fluoride inhibition in the use of carbohydrate

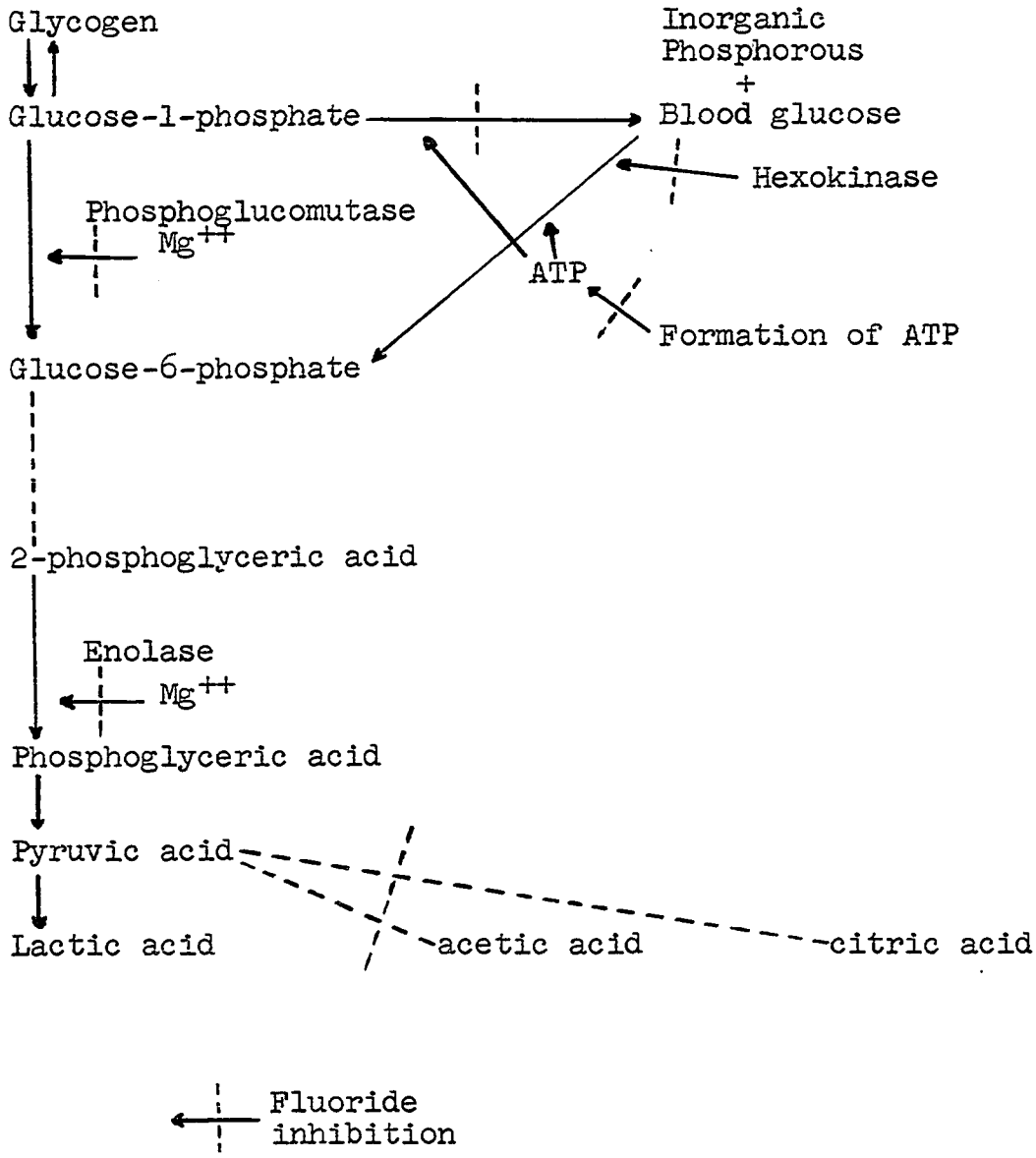


Figure 1. Some points of fluoride inhibition in the use of carbohydrate

EXPERIMENTAL

Artificial Rumen Techniques Used to Study the Digestion of Cellulose by Rumen Microorganisms

The large population of microorganisms, both as to number and the varied types normally found present in the healthy ruminal animal, present numerous problems in studying the effect of a single nutrient on cellulose digestion. They will synthesize nutrients missing from the ration of the animal and seem to work in cooperation with each other as well as their host. In an attempt to study the effect of various nutrients under more carefully controlled conditions than exist in the intact animal by using procedures that would be more definitive than digestion trials, several methods have been developed for studying *in vitro* digestion of cellulose by Marston (34), Louw *et al.* (28), and Huhtanen *et al.* (26) among others.

The method developed by Burroughs *et al.* (3) has the advantage of being rather simple and economical. Whole rumen fluid, collected by straining it through cheesecloth, was diluted each day so that the nutrients originally present were gradually removed. In this way the effect of the nutrient being studied usually became more pronounced with the passage of time. It has the disadvantage of requiring five to ten days to run, during which time the population

of microorganisms may change considerably, and the effect of the nutrient being studied may be different *in vivo* than in the *in vitro* studies.

The purpose for this phase of the study was to determine the level at which fluoride would inhibit the digestion of cellulose using the above method.

Materials and methods - 10 day digestions

Rumen contents were collected from an 800 pound steer fed roughage *ad libitum* and about six pounds of grain concentrate per day. The fluid was expressed through four layers of number 50 grade cheesecloth and placed in thermal-neutral containers to take back to the laboratory. The digestion flasks used were 500 cc. Erlenmeyers maintained in a water bath and kept at 37° C. These flasks were charged as follows:

Cellulose (Sulka-floc)	4.5 gms.
Dehydrated alfalfa	1.5 gms.
Urea (84 gms./liter)	5.0 ml.
Mineral mixture (see below)	30.0 ml.
10 per cent glucose solution	5.0 ml.
Rumen inoculum	225.0 ml.
Fluoride as indicated below	
Distilled water to make total	450.0 ml.

The mineral mixture was made as follows:

$NaHCO_3$	52.500 gms.
$(NH_4)_2SO_4$	37.500 gms.
NaH_2PO_4	52.500 gms.
$ZnSO_4$	.080 gms.
$MnSO_4$	.080 gms.
$MgSO_4$	2.250 gms.
$FeSO_4 \cdot 7H_2O$	.125 gms.

NaCl	7.500 gms.
$CaCl_2$	.020 gms.
$CuSO_4 \cdot 5H_2O$	.040 gms.
KCl	7.500 gms.
Distilled water to make total	2000.000 ml.

The fluoride solution was made by dissolving 11.0125 gms. of NaF in distilled water and making a total volume of 1,000 ml. This was then added to the digestion flask as follows for the initial charge:

10 p.p.m.	.90 ml.
25 p.p.m.	2.25 ml.
50 p.p.m.	4.50 ml.
100 p.p.m.	9.00 ml.
200 p.p.m.	18.00 ml.
400 p.p.m.	36.00 ml.

When the flasks were recharged each day one-half of the above amounts of fluoride were used. At the end of each 24 hours one-half of the remaining material was removed, and the flask was recharged. The removed material was sampled and duplicates were analyzed for cellulose by the method of Crampton (6).

Results and discussion

Two 10 day runs were made. However, for the statistical analysis, only the last four days of each run are included in the data. At the start of the seventh day only 1.56 per cent of the original inocculum remained, and by the tenth day only 0.195 per cent of the original inocculum was present. This procedure should reduce the effect from factors other than fluoride.

The data was analyzed statistically using the multiple range rest as described by Duncan (11). The fluoride levels were considered as treatments and the individual days digestion was considered as a duplicate. The per cent of actual cellulose digestion is given in Table 1. At the bottom of the table the mean digestibilities are arranged with the highest mean being on the left. Any two means not underlined by the same line are considered to be significantly different (p-0.05).

The addition of fluoride, up to levels of 100 p.p.m., did not show any differences in the digestibility of cellulose in this experiment. At 200 p.p.m. the cellulose digestion was reduced by about 50 per cent. This reduction was highly significant (p-0.01). Raising the fluoride level to 400 p.p.m. resulted in another highly significant reduction of the digestion of cellulose. The low rate of cellulose digestion with the addition of 400 p.p.m. of fluoride resulted in the buildup of the cellulose so that no additional cellulose was added on two of the four days reported.

For ease in calculating the quantity of fluoride to add it was assumed that each milliliter of solution in the digestion flask weighed one gram and the fluoride was added on the basis of 450 grams. The solution in the digestion flasks was more fluid than normal rumen contents. In an

Table 1. Per cent cellulose digestion during the last four days of a 10 day run using varying levels of fluoride

Day	Parts per million of fluoride added							
	0	0	10	25	50	100	200	400
7	61.3	61.5	62.0	65.7	62.5	56.3	21.5	18.0
8	68.0	70.5	71.9	70.9	75.3	53.2	21.8	4.4
9	52.5	51.4	44.8	43.6	67.6	71.8	25.6	3.3
10	65.1	68.3	62.1	51.2	55.1	73.6	21.7	6.1
7	64.5	65.0	56.2	53.3	39.2	55.6	28.7	3.2
8	41.1	47.9	45.4	43.6	51.1	43.9	32.7	3.6
9	47.1	55.4	48.8	59.6	62.1	75.3	32.2	3.8
10	60.5	60.9	48.5	52.3	58.2	56.5	40.6	14.1
Treatment mean	57.5	60.1	55.0	55.0	58.9	60.8	28.1	7.1
p.p.m. F added	100	0	50	0	10	25	200	400
Treatment mean[a]	60.8	60.1	58.9	57.5	55.0	55.0	28.1	7.1

[a]Difference of 8.46 needed between any two means to be significant.

attempt to convert the levels used in this experiment to feed intake levels reported in animal feeding experiments, dry matter was determined on the material in the flasks, and on the material taken from the fistulated steer that provided the original innoculum. While it was difficult to obtain a uniformly homogenous sample from the steer due to the layers of material in the rumen it was found that the digestion flasks contained approximately 20 per cent as much dry matter as the rumen contents. Thus to convert the levels

reported for the digestion flasks to approximate equivalent feed intake levels it is necessary to multiply the flask levels by five. Thus the level necessary to obtain a significant reduction in the cellulose *in vitro* was approximately equivalent to 1,000 p.p.m. in a ration. This level has been shown by Hobbs *et al.* (25) to be toxic to the animal resulting in death within 60 to 120 days.

To determine whether this reduction in cellulose digestion at the 200 p.p.m. fluoride level was due to the loss of the ability of the microorganisms to digest cellulose, the contents of the flask on the tenth day were divided after taking samples for cellulose and the digestion continued. In one flask no further fluoride was added so that the fluoride level was halved each day of additional digestion. In the other flask the equivalent of 122 p.p.m. of magnesium was added to determine if it would have any effect. The results are given below.

	Per cent cellulose digestion	
Days digestion	No F added	Mg added, no additional F
11	35.9	32.5
12	52.7	53.2
13	59.6	57.6
14	57.1	58.8

By the end of 48 hours the digestion of cellulose had returned to nearly the level of the control observed prior to stopping the regular run, and the 72 and 96 hour determinations were between the means given for the zero levels

in Table 1. The addition of magnesium at the level of 122 p.p.m. did not result in speeding the rate of recovery. The addition of fluoride at the 200 p.p.m. level for 10 days apparently did not damage the organisms digesting cellulose, as the level of cellulose digestion returned to nearly normal by the time the fluoride content had been reduced to 50 p.p.m.

Materials and methods - washed cell technique

In making further determinations of the effect of fluoride on cellulose digestion the washed cell technique described by Cheng et al. (5) was used in place of the 10 day runs described above. It had the advantage of taking much less time, and of removing the unknown material present in rumen fluid so that the conditions may be more clearly defined.

Utter and Werkman (49) had shown that one of the effects of fluoride on bacterial metabolism was to form an insoluble magnesium complex which removed this ion from solution. This was one of the ways fluoride interfered with the normal metabolic pathway (see Figure 1). They also showed that when manganese was used to replace the magnesium the complex formed was not as insoluble and so the manganese ions remained in solution. The manganese ions then replaced, partially at least, the magnesium ions and the normal

metabolic pathway was continued. To determine if this same effect could be observed using mixed cultures of rumen microorganisms, rather than a purified single strain, a series of tests was made using complete medium, magnesium deficient medium, manganese deficient medium, and magnesium-manganese deficient medium. Also a medium with double and ten times the amount of magnesium was used to determine if an excess of magnesium ions would precipitate the fluoride and permit normal cellulose digestion.

Rumen contents were obtained from a 1,000 pound steer fed approximately six pounds of grain a day and hay ad libitum. Twelve hundred ml. of rumen liquid was obtained by straining rumen contents through four layers of number 50 grade cheesecloth. This fluid was taken to the laboratory in thermal-neutral containers. It was then centrifuged for one and one-half minutes at 1,000 r.p.m. in a Servall angle centrifuge. This caused the feed particles and protozoa to be accumulated on the bottom of the tube. The supernatant was carefully poured off and the accumulation at the bottom of the tube discarded. The supernate was then centrifuged at 5,000 r.p.m. for 20 minutes. The supernatant in this case was discarded and the bacteria which were on the bottom were placed in 150 ml. of distilled water saturated with carbon dioxide gas and placed in a Waring blender for one minute to disperse any clumps of bacteria. The blender was then

washed with 210 ml. of distilled water saturated with carbon dioxide gas to make a volume of 360 ml. The solution was again centrifuged at 5,000 r.p.m. for another 20 minutes and the washing and dispersing process repeated, except that the final volume was made to 400 ml. To 200 ml. of this innoculum was added 180 ml. of the stock mineral solution given below, plus 1.9 grams of cellulose (Solka-floc) to give a level of 0.5 per cent cellulose in the mixture. Carbon dioxide was then bubbled through this innoculum for five minutes and the pH then adjusted to 7.1 using Na_2CO_3. One ml. of either distilled water or fluoride solution was placed in the digestion tubes and 19 ml. of the complete innoculum added. The various levels of fluoride were then run in triplicate and the results of the individual tubes averaged to give the per cent cellulose digestion reported in the following tables. To determine the exact quantity of cellulose in the tubes at the start of the trial three tubes of the complete innoculum were made and cellulose determined without any further bacterial action.

The basal medium used in this experiment was made as follows:

$Na_2HPO_4 \cdot 7H_2O$	12.0000 gms.
KH_2PO_4	6.0000 gms.
$NaHCO_3$	35.0000 gms.
KCl	7.5000 gms.
NaCl	7.5000 gms.
$FeSO_4 \cdot 7H_2O$	0.7500 gms.
Urea	20.0000 gms.
Distilled water to	8,000.0000 mls.

Because of the small quantities of zinc, copper and cobalt required these were made up as separate solutions as follows:

$ZnSO_4 \cdot 7H_2O$	0.080 gms./100 ml.
$CuSO_4 \cdot 5H_2O$	0.200 gms./100 ml.
$CoCl_2 \cdot 6H_2O$	0.200 gms./100 ml.

The magnesium and manganese were also mixed separately as part of the experiment was the study of deficiencies of these ions, and also the excess of magnesium ions.

$MgSO_4$	1.500 gms./100 ml.
$MnSO_4$	0.040 gms./100 ml.

The basal media was divided into 2,000 ml. volumes and the various treatment solutions were made as follows:

Complete medium	2,000.00 ml. basal media
	25.00 ml. $MgSO_4$ media
	2.50 ml. $MnSO_4$ media
	0.25 ml. $ZnSO_4$ media
	2.50 ml. $CuSO_4$ media
	2.50 ml. $CoSO_4$ media
distilled water to	2,250.00 ml. total volume
Magnesium deficient medium	2,000.00 ml. basal media
	2.50 ml. $MnSO_4$ media
	0.25 ml. $ZnSO_4$ media
	2.50 ml. $CuSO_4$ media
	2.50 ml. $CoSO_4$ media
Distilled water to	2,250.00 ml. total volume
Manganese deficient medium	2,000.00 ml. basal media
	25.00 ml. $MgSO_4$ media
	0.25 ml. $ZnSO_4$ media
	2.50 ml. $CuSO_4$ media
	2.50 ml. $CoSO_4$ media
Distilled water to	2,250.00 ml. total volume
Manganese-magnesium deficient medium	2,000.00 ml. basal media
	0.25 ml. $ZnSO_4$ media
	2.50 ml. $CuSO_4$ media
	2.50 ml. $CoSO_4$ media
Distilled water to	2,250.00 ml. total volume

Double magnesium medium	Same as the complete media except that 50.00 ml. of the $MgSO_4$ media was used in place of 25.00 ml.
Ten times the magnesium medium	Same as the complete media except that 250.00 ml. of the $MgSO_4$ media was used in place of 25.00 ml.

This resulted in a total volume of 2,257.75 ml. in this solution rather than the 2,250.00 in the others, but this difference was disregarded in calculating cellulose digestion as the error induced was only 0.0034 per cent.

Because no differences were found in the previous experiment in the cellulose digestion with fluoride levels up to 100 p.p.m., the digestion at 200 p.p.m. was significantly less than the lower levels, and the digestibility at 400 p.p.m. was only 7.1 per cent, the four fluoride levels used in this experiment were 50, 100, 150, and 200 p.p.m. These fluoride concentrations were obtained by using one milliliter of the following solutions added to 19 ml. of the complete innoculum:

50 p.p.m. of fluoride	NaF	0.5531 gms.
	Distilled water to	250.0000 ml.
100 p.p.m. of fluoride	NaF	1.1062 gms.
	Distilled water to	250.0000 ml.
150 p.p.m. of fluoride	NaF	1.6594 gms.
	Distilled water to	250.0000 ml.
200 p.p.m. of fluoride	NaF	2.2125 gms.
	Distilled water to	250.0000 ml.

Results and discussion

During this experiment the level of fluoride was the

only variable five different times. These five replications were grouped together and the results are given in Table 2. Cellulose was determined on each of the triplicate tubes in each trial and the digestion figures given in the table represent an average of these individual analyses. As can be seen in the table there was variation in the digestibility between the various trials. This can be partially accounted for by variation in the concentration of organisms in the innoculum. The multiple range test (11) was used in analyzing the data, and as in the ten day trials the fluoride levels were considered as treatments and the various trials as replications. The mean digestibilities of all trials are then arranged at the bottom of the table with the highest mean on the left. Any two means underlined by the same line are considered the same, while those not underlined by the same line are considered to be significantly different (p-0.05). This same procedure will also be used in presentation of the other data in this experiment.

When the combined values of all five trials were analyzed together no significant difference in cellulose digestion was found between the control and the 100 p.p.m. of fluoride added. Likewise no differences were found between the 50 and 150 p.p.m. levels of fluoride, but the cellulose digestion at these levels was significantly lower than the digestibility of the control and the 100 p.p.m. level, and significantly higher than the 200 p.p.m. level.

Table 2. Per cent cellulose digestion with washed cell technique using varying fluoride levels

Trial	Parts per million of fluoride added				
	0	50	100	150	200
1	61.2	37.4	41.5	35.2	36.8
	56.1	37.1	54.6	37.9	31.4
	51.0	46.1	46.8	36.6	37.0
2	50.4	35.0	50.7	34.5	39.6
	48.0	47.3	49.1	38.9	34.9
	46.2	54.0	53.3	34.3	32.6
3	62.3	68.8	65.7	57.6	42.0
	67.9	63.6	55.8	64.1	51.5
	73.3	58.9	69.0	55.6	46.8
4	54.2	38.6	55.5	52.2	47.3
	49.4	44.0	61.2	45.3	45.7
	57.0	41.2	58.1	55.8	41.1
5	50.3	43.1	44.9	38.6	11.3
	49.1	36.6	53.2	32.5	16.5
	50.7	36.6	51.0	30.1	7.8
Treatment mean	55.14	45.89	54.03	43.28	34.82
p.p.m. F added	0	100	50	150	200
Treatment mean[a]	55.14	54.03	45.89	43.28	34.82

[a]Difference of 4.96 needed between any two means to be significant.

Each individual trial was analyzed with the same technique using the triplicate tubes as replication. There was considerable variation in the pattern obtained between trials as well as variation from the combined results.

However, in four of the five trials the level of digestibility of the 100 p.p.m. level was higher than the 50 p.p.m. level, and in two of the trials this difference was significantly different. When the entire results were then grouped this difference was significant as mentioned above. No attempt was made to determine why the 100 p.p.m. level of fluoride resulted in higher cellulose digestion than did the 50 p.p.m. level. This same pattern had been noted in the 10 day trials but was not significantly different there. The addition of 200 p.p.m. of fluoride resulted in a highly significant reduction in cellulose digestion compared to the control and 100 p.p.m. levels. This difference was similar to the difference observed with the ten day run.

The addition of 50 and 150 p.p.m. of fluoride apparently results in some alteration of the metabolic pattern of the cellulose digesting microorganisms, and the addition of 200 p.p.m. in still further alteration. At least part of this difference may be explained in the following section concerning the effect of magnesium, manganese, or a combination of the two.

In studying the effect of the magnesium, manganese, and magnesium-manganese deficient media, and the media containing two times and ten times the normal level of magnesium two separate trials were used in each case. As in the trials discussed above cellulose analysis was made on each of the

triplicate tubes and these treated as separate replications. The data on these different trials are given in Tables 3 to 7. The method of presentation is the same as that previously described.

Magnesium deficient medium

The cellulose digestion in the control, magnesium deficient, and magnesium deficient plus 100 p.p.m. of fluoride tubes was similar. The difference between the control and the 100 p.p.m. of fluoride added was approaching significance. There was an actual difference of 8.7 per cent between the means compared to a difference of 9.28 per cent needed for significance. The mean digestibilities of the 50 and 100 p.p.m. fluoride added were shown to be the same although the 50 p.p.m. fluoride level was different from the control and the magnesium deficient treatments. The addition of 150 and 200 p.p.m. of fluoride resulted in mean digestibilities that were similar but significantly different from the other treatments.

The discussion of the relationship between the minerals will be made at the end of this section rather than with each treatment individually as there are some relationships that can best be brought in this manner.

Manganese deficient medium

The pattern obtained here was different from that with

Table 3. Per cent cellulose digestion with washed cell technique using varying fluoride levels in a magnesium deficient medium

	Parts per million of fluoride added					
Trial	0	0	50	100	150	200
	Normal mg.	No magnesium				
1	61.2	47.0	37.3	45.9	19.2	16.2
	56.1	45.5	38.9	40.5	24.7	18.8
	51.0	49.6	38.1	43.6	10.5	15.9
2	50.4	50.4	27.6	40.2	39.5	30.0
	48.0	47.0	44.3	44.4	33.7	32.5
	46.2	48.0	34.6	46.4	38.1	31.2
Treatment mean	52.2	47.9	36.8	43.5	27.6	24.1
	Normal mg.	No magnesium				
p.p.m. F added	0	0	100	50	150	200
Treatment mean[a]	52.2	47.9	43.5	36.8	27.6	24.1

[a]Difference of 9.28 needed between any two means to be significant.

the magnesium deficient medium. There was a significant reduction in the digestion of cellulose in all the treatments in which manganese was omitted. The addition of fluoride caused a further reduction in the amount of cellulose digested. The 100 and 200 p.p.m. levels were significantly difference from the control and manganese deficient rations,

Table 4. Per cent cellulose digestion with washed cell technique using varying fluoride levels in a manganese deficient medium

	Parts per million of fluoride added					
Trial	0	0	50	100	150	200
	Normal Mn.	No manganese				
1	61.2	19.3	25.5	30.1	27.5	17.4
	56.1	15.2	23.6	20.3	28.2	20.7
	51.0	23.5	21.4	24.2	22.5	20.5
2	50.4	60.6	46.5	30.1	35.7	22.6
	48.0	58.7	39.6	28.5	40.9	28.4
	46.2	60.1	45.9	32.3	37.9	31.4
Treatment mean	52.2	39.6	33.7	27.6	32.1	23.5
	Normal Mn.	No manganese				
p.p.m. F added	0	0	50	150	100	200
Treatment mean[a]	52.2	39.6	33.7	32.1	27.6	23.5

[a]Difference of 11.23 needed between any two means to be significant.

but not from the 50 and 150 p.p.m. of fluoride levels.

Magnesium-manganese deficient medium

When both magnesium and manganese were omitted from the medium there was a highly significant reduction in the digestion of cellulose. The addition of fluoride at any of the

Table 5. Per cent cellulose digestion with washed cell technique using varying fluoride levels in a magnesium-manganese deficient medium

	Parts per million of fluoride added					
Trial	0	0	50	100	150	200
	Normal Mg.-Mn.	No magnesium-manganese				
1	61.2	27.8	29.7	34.4	26.0	24.2
	56.1	23.4	25.5	26.4	22.9	26.8
	51.0	27.2	32.8	26.0	28.6	18.6
2	50.4	29.0	29.0	30.9	35.9	31.6
	48.0	29.6	31.9	32.2	31.8	30.4
	46.2	29.4	29.6	27.7	31.8	29.6
Treatment mean	52.2	27.8	29.7	29.8	25.2	26.9
	Normal Mg.-Mn.	No magnesium-manganese				
p.p.m. F added	0	100	50	0	200	150
Treatment mean[a]	52.2	29.8	29.7	27.8	26.9	25.2

[a]Difference of 4.74 needed between any two means to be significant.

levels used did not further reduce cellulose digestibility. Removing both ions lowered the cellulose digested by nearly half, but it appears that the metabolic pathway used in digesting this quantity of cellulose is not influenced by fluoride. Thus it appears that there may be two metabolic systems used in the digestion of cellulose. One makes use

Table 6. Per cent cellulose digestion with washed cell technique using varying fluoride levels with two times the magnesium level

	Parts per million of fluoride added					
Trial	0	0	50	100	150	200
	Normal mg.		Two times magnesium			
1	54.2	60.2	52.4	55.4	60.0	42.8
	49.4	64.3	53.7	53.0	53.7	38.6
	57.0	62.2	54.3	54.2	60.4	38.0
2	50.3	51.2	54.0	53.4	49.1	34.2
	49.1	53.4	49.2	59.7	40.3	34.0
	50.7	59.5	58.4	57.0	48.4	31.7
Treatment mean	51.8	58.4	53.7	55.4	52.0	36.6
			Two times magnesium			
p.p.m. F added	0	100	50	150	Normal mg.	200
Treatment mean[a]	58.4	55.4	53.7	52.0	51.8	36.6

[a]Difference of 4.77 needed between any two means to be significant.

of magnesium and/or manganese ions and is influenced by the presence of fluoride ions. The other apparently does not make use of magnesium or manganese ions and is not influenced by the presence of fluoride.

Medium containing two and ten times as much magnesium

The marked reduction in cellulose digestion in the

Table 7. Per cent cellulose digestion with washed cell technique using varying fluoride levels with ten times the magnesium level

	Parts per million of fluoride added					
Trial	0	0	50	100	150	200
	Normal mg.		Ten times magnesium			
1	54.2	59.3	58.4	62.9	55.3	36.1
	49.4	58.9	58.9	63.1	55.1	29.2
	57.0	57.5	63.8	64.4	48.6	27.0
2	50.3	58.3	59.9	54.2	59.4	11.3
	49.1	53.9	63.1	52.4	56.2	8.8
	50.7	47.5	61.6	48.7	55.2	5.6
Treatment mean	51.8	55.9	60.9	57.6	55.0	19.7
p.p.m. F added	50	100	Ten times mg.	150	Normal mg.	200
Treatment mean[a]	60.9	57.6	55.9	55.0	51.8	19.7

[a]Difference of 6.80 needed between any two means to be significant.

presence of fluoride, and the report of Utter and Werkman (49) that magnesium and fluoride form an insoluble complex raised the question whether the inhibitory effect of fluoride could be overcome with the addition of additional quantities of magnesium. Media were used containing two and ten times the normally used quantities of this ion. When two times as much magnesium was added the amount of

cellulose digested was significantly higher than the control with the normal levels. This could be interpreted as indicating that the level of the ion in the normal medium was not high enough to obtain maximum cellulose digestion. The addition of 50, 100, and 150 p.p.m. of fluoride resulted in a slight reduction in the cellulose digestion compared to the two times level but still slightly higher than the control. The addition of 200 p.p.m. of fluoride did result in highly significantly reduced cellulose digestion. The extra magnesium apparently exerted a sparing effect in regards to the fluoride ion.

When the level was raised to ten times normal the cellulose digestion was slightly higher than the control but not significantly so. When 50 p.p.m. of fluoride was added there was a significant increase in the cellulose digestion compared to the normal level. The addition of both 50 and 100 p.p.m. of fluoride to ten times normal magnesium level resulted in a higher percentage digestion of cellulose than did the ten times level. If one assumes that an insoluble fluro-magnesium complex is formed in this medium similar to that reported by Utter and Werkman (49) it would indicate that the level of magnesium desired to obtain maximum cellulose digestion is somewhere between two and ten times the level used in this experiment.

The exceptionally low digestibilities obtained in the

second experiment when 200 p.p.m. of fluoride was added would raise some question about whether these were really true values. If one only takes the values from the first trial the mean digestibility is 30.8 per cent rather than the 19.7 per cent figure given as the average for the two trials. This would be more in line with what one might expect looking at the figures from the two times magnesium level.

General discussion of interaction of fluoride, magnesium and manganese

The mean digestibilities of all the trials are given in Table 8. There appears to be some difference in the effect of fluoride between the ten day and twenty-four hour digestion trials. However, in general the trends are somewhat similar. Using both procedures the addition of 100 p.p.m. of fluoride resulted in slightly more cellulose digestion than did the addition of 50 p.p.m. When fluoride was added at levels of 200 p.p.m. or better there was a significant reduction in cellulose digestion. In terms of the intake of fluoride on a dry matter intake basis by the animal this would be equivalent to approximately 1,000 p.p.m. A level of 600 p.p.m. has been shown by Hobbs et al. (25) to be toxic in the animal within 60 to 120 days.

The cellulose digestion in the magnesium deficient medium with 150 and 200 p.p.m. of fluoride added, the

Table 8. Per cent cellulose digested - summary of Tables 1 through 7

10 DAY RUN								
p.p.m. F	100	0	50	0	10	25	200	400
Treatment mean	60.8	60.1	58.9	57.5	55.0	55.0	28.1	7.1
WASHED CELL TECHNIQUE								
p.p.m. F	0	100	50	150	200			
Treatment mean	55.14	54.03	45.89	43.28	34.82			
Magnesium deficient medium								
p.p.m. F	0	Mg.def.	100	50	150	200		
Treatment mean	52.2	47.9	43.5	36.8	27.6	24.1		
Manganese deficient medium								
p.p.m. F	0	Mn.def.	50	150	100	200		
Treatment mean	52.2	39.6	33.7	32.1	27.6	23.5		
Magnesium-manganese deficient medium								
p.p.m. F	0	100	50	Mg.-Mn. def.	200	150		
Treatment mean	52.2	29.8	29.7	27.8	26.9	25.2		
Two times magnesium medium								
p.p.m. F	Two times	100	50	150	0	200		
Treatment mean	58.4	55.4	53.7	52.0	51.8	36.6		
Ten times magnesium medium								
p.p.m. F	50	100	Ten times	150	0	200		
Treatment mean	60.9	57.6	55.9	55.0	51.8	19.7		

manganese deficient medium with 100 and 200 p.p.m. added and the magnesium-manganese deficient medium with all levels of fluoride added are approximately similar. The consistent level of cellulose digestibility in the absence of magnesium and manganese ions, and the reduction to these same levels with the addition of the larger quantities of fluoride strongly suggests that there are two pathways used by the mixed cultures of rumen microorganisms in breaking down the cellulose. One makes use of the magnesium and manganese ions. The presence of fluoride in sufficient quantity will reduce the quantity of cellulose digested. The other apparently does not need the presence of these two ions and remains rather constant in spite of fluoride levels up to the 200 p.p.m. added. When the fluoride level was increased to 400 p.p.m. in the ten day run there was a further highly significant reduction in the quantity of cellulose digested. This quantity of fluoride was not used in the twenty-four hour trials, but it suggests that this second system may also be effected when the quantity of fluoride is high enough.

Although no attempt was made to determine if there was the formation of an insoluble complex of magnesium and fluoride as reported by Utter and Werkman (49), the data presented when the magnesium levels were raised suggests such a possibility. In these cases the addition of fluoride

at the 50, 100, and 150 p.p.m. levels did not reduce the cellulose digestion. Thus even though such a complex may have been formed there was a sufficient quantity of the magnesium ion present to obtain cellulose digestion about equal to the controls. It may be that the level of magnesium in the control was not quite high enough to obtain maximum digestion. Doubling the quantity resulted in a significant increase over the control. When ten times the normal level was added there was still some increase in digestion over the control, but it was not significant. However, when 50 p.p.m. of fluoride was added the difference was significant. If the possibility of the formation of the insoluble magnesium-fluoro complex is accepted, this quantity of fluoride precipated sufficient magnesium to reduce the ion concentration to a point where larger quantities of cellulose were digested. It would seem that the level of magnesium ion needed to obtain maximum cellulose digestion is higher than that used in the control digestion, and is probably between two and ten times this amount.

This work shows that fluoride, added in sufficient quantity, will reduce the quantity of cellulose digested by mixed cultures of rumen microorganisms. It also demonstrates that an interrelationship exists between magnesium, manganese, and fluoride. The removal of the manganese resulted in less cellulose being digested than did the

removal of magnesium. This was expected due to the fact that the magnesium-fluoro complex had been demonstrated to be less soluble than the manganese-fluoro complex. When both ions were omitted, however, the microorganisms still digested about half as much cellulose as in the control ration, indicating that there was another pathway in degrading cellulose other than the one using magnesium and/or manganese ions. The addition of added quantities of magnesium maintained the level of cellulose digestion similar to the control, and in the absence of fluoride significantly increased the amount of cellulose digested. The level of magnesium used in the control is probably below the quantity needed for optimum cellulose digestion.

Effect of fluoride, magnesium and manganese on the relative numbers of microorganisms at the completion of a twenty-four hour digestion

After observing the pronounced effect of the addition of fluoride at high levels, bacteriological studies were undertaken to determine the effect of this ion on the total number and the various groups of microorganisms present in the mixed culture of rumen innoculum both with and without the presence of magnesium and manganese. One ml. of the contents at the end of the twenty-fourth hour digestion was removed and serially diluted. The experimental procedure

used will be discussed separately by the various media used.

Methods and procedures

Chapman Stone - urease test medium. The Chapman Stone medium was made according to the directions in the Difco Manual (9) and then poured. When it had hardened sufficiently the surface was inoculated with 0.10 ml. of the diluted inoculum and spread with a surface spreader. The urease test medium was then poured over the inoculum in a thin layer. The urease test medium used was that described in the B. B. L. Manuel (2) with the following modifications. The urease medium was made to double strength as was the agar medium. These were mixed, cooled to 50 to 55° C. and then poured over the inoculated plate. The plates were then incubated for 24 hours, observed for a change in color, flooded with a saturated solution of ammonium sulfate, and the colonies of proteolytic bacteria in the Chapman-Stone medium counted.

Results and discussion

The urease test medium was used in an attempt to find the bacteria that were using urea nitrogen. This would have been demonstrated by a change of color in the medium. No such reaction was observed. There are several possible

explanations for this: (1) the organisms utilizing the urea nitrogen did not convert it to ammonia and thus no alkaline reaction occurred; (2) the organisms may have removed the amino group from the urea and utilized it directly; (3) the organisms may have produced ammonia but at a slow enough rate that they could retain it intracellularly until they could convert it into other compounds; (4) the organisms may have preferred to use the nitrogen in the Chapman Stone medium to that in the urease test medium; (5) the urealytic bacteria were suppressed by the high content of NaCl in the medium. These possibilities could be checked by using a urease test solution. This was not done. After observing the urease test medium the plates were flooded with a saturated solution of ammonium sulfate to demonstrate the presence of staphylocci. None of the colonies observed contained the yellow or orange color considered indicative of pathogenicity.

The data on the number of colonies observed, and the serial dilutions used appears in Table 9. The number of colonies obtained with the dilutions of 10^5 and 10^4 is smaller than needed to obtain good results or to demonstrate differences. The addition of fluoride to the complete media did not appear to cause any change in the number of colonies. The omission of magnesium may have reduced the number of colonies some. The omission of manganese and the omission of both magnesium and manganese from the medium did result

Table 9a. Chapman Stone - protolytic bacteria treatment

Fluorine level	Complete media		Mg. deficient		Mn. deficient		Mn., Mg. deficient	
	No. of colonies	Dilution factor	No. of colonies	Dilution factor	No. of colonies	Dilution factor	No. of colonies	Dilution factor
0	7	10^4	3	10^4	3	10^3	2	10^3
50	5	10^4	4	10^4	4	10^3	1	10^3
100	2	10^4	3	10^4	0	10^3	0	10^3
150	4	10^4	4	10^4	1	10^3	1	10^3
200	2	10^4	3	10^4	0	10^3	0	10^3
			Violet red bile - synthesizing group					
0	498	10^2	332	10^2	390	10^2	226	10^2
50	270	10^2	592	10^2	288	10^2	232	10^2
100	240	10^2	326	10^2	224	10^2	72	10^2
150	24	10^2	328	10^2	280	10^2	48	10^2
200	2	10^2	134	10^2	134	10^2	52	10^2

Table 9b. Littman's Oxgall - fungi treatment

Fluorine level	Complete media		Mg. deficient		Mn. deficient		Mn., Mg. deficient	
	No. of colonies	Dilution factor	No. of colonies	Dilution factor	No. of colonies	Dilution factor	No. of colonies	Dilution factor
0	24	10^2	19	10^2	5	10^3	17	10^3
50	24	10^2	28	10^2	9	10^3	22	10^3
100	21	10^2	19	10^2	6	10^3	9	10^3
150	9	10^2	9	10^2	9	10^3	18	10^3
200	10	10^2	9	10^2	10	10^3	18	10^3
	Thioglycollate - total anaerobic count							
0	160	10^5	148	10^5	340	10^5	216	10^5
50	148	10^5	34	10^5	38	10^5	32	10^5
100	186	10^5	31	10^5	120	10^5	53	10^5
150	120	10^5	85	10^5	82	10^5	88	10^5
200	85	10^5	120	10^5	70	10^5	55	10^5

in the reduction of the number of colonies observed. This was particularly true when fluoride was added to the medium. Inasmuch as the numbers observed were small, additional work is indicated before any definite conclusions may be made. It does suggest the possibility though that the proteolytic bacteria need magnesium and manganese in order to develop properly, and that they are somewhat more sensitive to fluoride in the absence of these ions than when they are present.

Methods and procedures

Violet red bile medium. The violet red bile medium was made according to the directions given in the Difco Manual (9). Two methods of inoculation were tried. The medium was cooled to about 45° C. and poured into the petri dish containing 0.1 ml. of inoculum. The two were then mixed by gentle rotation and the surface then covered with another thin layer of the violet red bile medium. The second method consisted of pouring a thin layer of the medium, inoculating by use of a surface spreader, and then pouring a second thin layer over the inoculum. In the first case many of the colonies had the tendency to grow vertically giving the appearance of saucer viewed edgeways. Using the second method the colonies grew horizontally and were much easier to count.

Results and discussion

In the first trial a serial dilution of 10^5 was used. Growth was obtained on only one plate and this only had two colonies. In the second trial a serial dilution of 10^2 was used. This gave some rather large numbers and made counting somewhat difficult. The results indicate that the presence of fluoride in the complete medium resulted in a reduction in the number of aerobic, gram negative colonies. The presence of fluoride also appeared to alter the metabolic pattern of this group of organisms. In the complete medium, the magnesium, manganese and the magnesium-manganese deficient media the colonies all had a characteristic dark red color. At the 50 and 100 p.p.m. levels of fluoride the color changed to an orange, and at the 150 and 200 p.p.m. levels the color was an orange-yellow. The pH range of neutral red is 7 to 8.2 with a color change from red to yellow. The orange to orange-yellow color observed was indicative of the inability of the colony to use lactose. It was further noted that as the fluoride level was increased the colony size appeared to decrease. At the 50 and 200 p.p.m. level of fluoride added many of the colonies appeared to be little more than pinpoint in size and were difficult to count.

The absence of magnesium in the medium caused a reduction in the number of colonies by about 35 per cent. The

addition of fluoride did not cause much change in this number until the 200 p.p.m. level was reached when there was a marked reduction in the number of colonies. A similar but slightly larger reduction in numbers was observed when manganese was omitted. This is similar to the pattern observed in the twenty-four hour cellulose digestion. However, when both magnesium and manganese were omitted there was a very marked reduction in the number of colonies when 100 or more p.p.m. of fluoride were added.

Methods and procedures

Littmen's Oxgall medium. The medium was made according to the directions in the Difco Manual (9). When the level of 30 micrograms of streptomycin per ml. of medium was used there was so much bacterial overgrowth that counting the colonies of fungi was nearly impossible. On the second trial with 60 micrograms of streptomycin bacterial overgrowth was still observed so the level of streptomycin was finally raised to 90 micrograms per ml. At this level there were still a few bacterial colonies, but they did not interfere with the fungal growth. These colonies were considerably smaller than the colonies observed in the medium with either the 30 or 60 microgram level. Incubation at room temperature for three to five days was found to be the best method of developing fungal colonies.

Results and discussion

The fungal growths were of varied types and no attempt was made to differentiate between them. The addition of 150 and 200 p.p.m. of fluoride in the complete medium appeared to cause some reduction in the number of colonies. This was also true in the magnesium deficient medium. The number of colonies obtained with the use of the manganese deficient media appears to be somewhat smaller, and the addition of fluoride seems to have no influence. However, when both magnesium and manganese were omitted the total number of mold colonies observed was about the same as in the complete medium, and the addition of fluoride, even at the 150 and 200 p.p.m. level, did not cause any change in the number of colonies.

Methods and procedures

Thioglycollate medium without dextrose. The thioglycollate medium was made according to the directions in the B. B. L. Manual (2) with the following modification. The medium was made double strength. An equal quantity of double strength agar was made that contained 0.2 per cent of cellulose. The two solutions were then cooled to about 50° C., thoroughly mixed and then poured into the top of a petri dish. When the plates were poured and allowed to harden it was difficult to get all the air out and large

air bubbles would become entrapped. To overcome this difficulty the medium was poured just prior to inoculation and as soon as it was sufficiently firm the surface was inoculated with 0.1 ml. of inoculum and spread with a surface spreader. The bottom of the petri dish was inverted, one side placed on the medium and then slowly worked down onto the full surface. With a little care and practice nearly all air bubbles could be eliminated. The space between the two parts of the petri dish was then sealed with a mixture of equal parts of paraffin and vasoline to prevent air from entering. When air was entrapped there was a tendency toward syneresis that may have spread some colonies.

It was hoped that by using the cellulose in this medium that, in addition to obtaining the total anaerobic count, a count of the cellulose digesting organisms could also be obtained. This medium did give good results in obtaining total anaerobic count but no cellulotic digestion was observed. Other methods tried to obtain this count will be discussed later.

Results and discussion

The results are shown in Table 10. The addition of fluoride did not have much effect on the total count until the 200 p.p.m. level was reached. The omission of magnesium and manganese did result in some lowering of the total

count. The omission of both magnesium and manganese in the presence of fluoride did result in appreciable lowering of the anaerobic count. The results were rather erratic in some of the counts and considerable work needs to be done in this area before any definite conclusions can be made.

Methods and procedures

Medium for cellulose digesting organisms. A medium that would show the presence of the cellulolytic organisms was desired. The basic premise was to develop a medium without readily available carbohydrate and force the organisms to attack the cellulose at the 0.2 per cent level. This would leave a clear area in the plate which could then be counted.

The first medium used was the thioglycollate mentioned above and no cellulolytic organisms could be demonstrated.

The second medium used was one made of 1-1/2 per cent agar, 1 per cent proteose-peptone, and 0.2 per cent cellulose. The medium was incubated for 24, 48 and 72 hours at both 37° and 55° C. without any demonstration of cellulose digestion. The addition of 1 per cent, 5 per cent or 10 per cent autoclaved rumen liquor, or 5 per cent or 10 per cent sterile filtered rumen liquor to the above medium did not result in cellulose digestion either.

After the two media above failed to demonstrate the

desired cellulose digestion a third medium was tried. A basic solution of agar with 0.2 per cent cellulose was made and to each 100 ml. the following was added:

A. 25 micrograms of B_{12} and 5 micrograms of Biotin.

B. 0.3 per cent yeast extract.

C. Combination of A and B.

D. Combination of A and B plus 5 per cent autoclaved rumen liquor.

E. Replace half the distilled water with the mineral solution used in the digestion tubes.

Dilutions of 10^4 and 10^5 were then incubated for 72 hours and checked each 24 hours. The incubation was conducted at both 37° and 55° C. None of the treatments used in this phase of the experiment permitted cellulose digestion. It would appear that the cellulolytic organisms are very fastidious in their requirements, or that the Eh may have risen too rapidly. Also a small quantity of oxygen may have been left between the medium and the petri dish cover and this was sufficient to suppress the growth of the cellulolytic organisms.

The Progressive Changes in Blood Glucose and Lactate of Weanling and Adult Albino Rats Fed 300 p.p.m. of Fluoride

Hansard (21) demonstrated differences between young and old animals in their use of calcium and phosphorous using

the isotope tracer technique. The similarity between calcium and fluoride in metabolic usage has been previously discussed. Handler (18) had shown that acute dosages of fluoride would result in marked alterations of the metabolic pattern. Hobbs et al. (25) had shown marked elevations in the amount of fluoride in the bone tissue when fed at levels up to 300 p.p.m. but this level did not result in a marked reduction in growth rate of the rat. The same workers found no difference in the blood glucose levels of cattle fed fluoride for five to seven years up to 100 p.p.m.

It was felt that, due to the similarity of fluoride and calcium, there might be differences in the way young and old animals adjusted their metabolic pattern to a chronic intake of fluoride. This phase of the work was designed to see if there were differences between weanling and adult albino rats and if these differences could be measured in terms of differences in blood glucose, lactate and liver and muscle glycogen.

Methods and procedures

Weanling rats, 21 to 28 days old, and adult male rats, 15 to 19 months old were used. The animals were randomly lotted to the control or fluoride rations. The control ration used was the standard Ca-3 used in the laboratory as given below.

Ca-3 rat ration

Corn starch	643.0 gms.
Casein (V.F.)	180.0 gms.
Irr. yeast (360 I.U./gms.)	10.0 gms.
CLO (90 I.U.D. + 900 I.U. A/gms.)	2.0 gms.
Wesson oil	40.0 gms.
Cellulose	40.0 gms.
Vitamin B supp.	40.0 gms.
KH_2PO_4	13.2 gms.
$CaCO_3$	12.2 gms.
Min. mix (see below)	20.0 gms.

Mineral mix

NaCl	39.84 gms.
KCl	18.20 gms.
$KHCO_3$	26.20 gms.
$MgSO_4$ (anhydrous)	12.00 gms.
$CuSO_4 \cdot 5H_2O$	0.40 gms.
$MnSO_4 \cdot 4H_2O$	0.20 gms.
$ZnSO_4 \cdot 7H_2O$	0.60 gms.
$FeCl_3 \cdot 6H_2O$	2.50 gms.
$K_2SO_4 \cdot 2H_2O$	0.02 gms.
KI	0.03 gms.
NaF	0.01 gms.

The fluoride ration was the same plus the addition of sufficient sodium fluoride to bring the fluoride level to 300 p.p.m. Three animals were used in each lot. The lots were sacrificed at periodic intervals as indicated in Tables 10 to 12. The animals were anesthetized by placing them in a half gallon jar which contained cotton saturated with ether. As soon as they were unconscious blood samples were taken for blood glucose and lactate analysis, and liver and g. muscle removed for analysis of liver and muscle glycogen. Blood glucose and lactate, and liver and muscle glycogen were determined on each individual animal and the figures reported in the tables in this section are averages of the

Table 10. Blood glucose levels of control and fluoride fed weanling albino rats

Days after start of fluoride feeding	Trial I		Trial II		Trial I Glucose level in fluoride fed rats as a per cent of the controls	Trial II
	Control	Fluoride	Control	Fluoride		
	(Mg. of glucose per 100 ml. blood)					
2	102	106			104	
3	114	86**	137	137	75	100
4		103	138	116*		84
7	106	92*	116	110	87	95
10		84	131	122*		93
14	113	85**	109	115	75	106
18		136	102	109		107
21	148	130*	120	119	88	99
28			133	121		91
35	119	110*			93	
49	127	113*			89	
63	121	110*			92	
84	101	93*			92	

* Significant at 0.05 level.

** Significant at 0.01 level.

Table 11. Blood lactate levels of control and fluoride fed weanling albino rats

Days after start of fluoride feeding	Trial I		Trial II		Trial I Lactate level in fluoride fed rats as a per cent of the controls	Trial II Lactate level in fluoride fed rats as a per cent of the controls
	Control	Fluoride	Control	Fluoride		
	(Mg. of lactate per 100 ml. blood)					
2	18	41**			228	
3	27	53**	35	34	196	97
4		34	50	50		100
7	58	34**	22	20	59	91
10		52	22	38**		173
14	40	41	19	30*	102	158
18		41	26	29		111
21	31	29	26	20	94	77
28			24	26		108
35	65	41**			63	
49	58	46*			79	
63	46	57			124	
84	65	34**			52	

* Significant at 0.05 level.

** Significant at 0.01 level.

Table 12. Liver and muscle glycogen level of control and fluoride fed weanling albino rats

Days after start of fluoride feeding	Trial I		Trial II		Trial I		Trial II	
	Control	Fluoride	Control	Fluoride	Control	Fluoride	Control	Fluoride
	(Mg. per gram of fresh liver tissue)				(Mg. per gram of fresh muscle tissue)			
2	20	25			1.7	1.5		
3	48	91**	11	5*	4.9	8.9**	2.7	2.5
4		38	11	7		4.1	3.2	4.4
7	47	42	4	5	3.2	6.8**	2.7	2.8
10		47	25	39*		4.0	2.8	3.4*
14	14	32**	34	32	3.8	3.2	3.0	4.1
18		23	12	13		2.6	1.3	1.4
21	18	25	2	7*	0.6	1.2*	2.5	2.8
28			3	4			2.0	2.1
35	13	17			4.3	2.8**		
49	17	16			2.3	3.3*		
63	13	12			2.3	2.2		
84	12	8			1.1	1.8		

* Significant at 0.05 level.

** Significant at 0.01 level.

three animals. Due to the fact that there might be changes in the level of metabolites with changes in age from 3 to 15 weeks, control weanlings were sacrificed periodically with the fluoride fed weanlings. It was assumed that the adult male had stabilized its metabolic pattern and animals were sacrificed only at the beginning, middle and end of the experiment. Blood glucose was determined by the method of Folin and Wu (13), blood lactate according to the method of Barker and Summerson (1); and glycogen by the method of Good, Kramer and Somogyi (15).

The adult males had been used in the breeding colony, weighed approximately 500 grams, and were rather obese. Growth curves were similar between the controls and fluoride fed rats in both adults and weanlings.

Results and discussion

The data on the blood glucose, blood lactate, and liver and muscle glycogen for the weanling rats are presented in Tables 10, 11 and 12. The feed was not removed from the rats prior to sacrifice. This may account for some of the variation in the blood glucose and lactate, and the liver and muscle glycogen noted in the various control lots. However, these are all within the normal range.

In the first trial there was a marked drop in the blood glucose level of the weanlings during the first fourteen

days of the experiment, followed by a gradual recovery during the next seven days to approximately 90 per cent of the control animals. The last two columns in Table 10 show the milligrams of glucose in the fluoride fed rats as a per cent of the glucose in the control animals sacrificed at the same time. By using this technique variations due to feed ingestion or slight differences in time of sacrifice could be minimized. Trial II, in general, showed a somewhat similar pattern, although the reduction in the blood glucose was not as marked, and the recovery was more rapid and more complete. The blood glucose level during the second trial on the fourteen the and eighteenth days was higher in the fluoride fed lots than in the controls. This difference, however, was not significant.

This reduction in blood glucose following the ingestion of fluoride could be due to the interference of the fluoride in converting muscle and liver glycogen into glucose. This interference has been pointed out previously in Figure 1. The subsequent recovery to nearly normal levels could be due to either (1) the reduction of fluoride in the animal tissue to a lower level by more adequate elimination of the material or (2) the development of an alternate metabolic pathway in which fluoride did not exert as great a degree of interference.

The decline in blood glucose level in the adult rats

was much faster and to a lower level than in the weanling rats. The recovery period was shorter and the level finally attained was approximately 105 per cent to 110 per cent of the pre-treatment level. This slight elevation above the pre-treatment level persisted from the third week until the conclusion of the experiment at twelve weeks. The pattern in Trial II was similar to Trial I although the decline was not as great and the recovery somewhat more rapid. This is similar to the pattern in the second trial with the weanling rats.

These differences in time response to fluoride ingestion, the degree of reduction in blood glucose, the length of the recovery period and the difference in degrees of recovery to pre-ingestion levels of glucose indicate differences in the effect of fluoride on the metabolic utilization of glucose between weanling and mature albino rats.

During Trial I the blood lactate level of the weanling rats showed a very marked elevation within forty-eight hours after the start of fluoride ingestion. It then declined within one to two weeks to the control level and continued to decline below the control level where it remained for the duration of the trial, with the exception of the ninth week. This latter difference, however, was not significant. During Trial II the blood lactate levels of the weanling

rats were near the controls for the first week, showed a marked rise during the second week followed by a return to normal levels during the third and fourth weeks.

The adult male rats did not show the extreme alteration in blood lactate shown by the weanling rats. In Trial I there was some elevation of blood lactate levels during the latter part of the first week followed by a reduction in blood lactate to below control levels during the second week. After the seventh week the lactate level was considerably above the control level. In Trial II the elevation to above control level occurred during the second week followed by a return to normal for the duration of this trial.

The level of liver glycogen in the controls during the first week of the experiment was higher than from the second week to the end of the experiment. This marked elevation might have been due to the sudden change in ration from the stock adult pelleted ration used in the breeding colony to the high energy Ca-3 ration used in this experiment. The liver glycogen of the weanling fluoride fed rats showed a marked increase above the control during the first two weeks after which it returned to about the level of the control. A similar elevation in Trial II was not noted in the control ration, however, until the tenth to fourteenth days. In Trial II there was a depression in liver glycogen during the first week compared to the elevation in Trial I.

With the adult male rats there was a marked elevation in the liver glycogen following the ingestion of fluoride which continued throughout the entire experiment. In Trial II there was a reduction in liver glycogen during the early part of the experiment followed by recovery to normal and above normal level.

The variation of muscle glycogen in both the weanling and adult was somewhat varied and did not show any consistent pattern in either trial.

The differences between Trial I and Trial II are difficult to explain. These trials were conducted approximately six months apart. However, the animals used, the rations, temperature and humidity within the laboratory were all reasonably similar. The data would indicate that there are differences between weanling and adult albino rats in the effect that 300 p.p.m. of fluoride in the ration has in the alteration of the levels of blood glucose and lactate, and liver glycogen. These data would also indicate that there is a relationship between blood glucose and lactate levels and liver glycogen. In the first trial there was a somewhat higher level of liver glycogen and a lower level of blood glucose and lactate in the weanling rats. In the second trial there was a lower initial liver glycogen but a higher level of blood glucose and lactate. The pattern between the liver glycogen level and blood glucose and lactate was

Table 13. Liver and muscle glycogen level of control and fluoride fed adult male albino rats

Days after start of fluoride feeding	Trial I	Trial II	Trial I	Trial II
	(Mg. glycogen per gm. of liver)		(Mg. glycogen per gm. of muscle)	
0	12[a]	16[b]	2.9[a]	2.5[b]
1	14		1.5	
3	22	6	2.4	1.5
4	27	10	5.6	2.5
7	17	4	1.6	1.9
10	21	14	2.0	2.6
14	20	4	3.0	2.1
18		18		3.1
21		12		3.4
28		30		3.2
35	12		3.0	
49	21		3.7	
63	20		3.3	
84	19		3.5	

[a]Average of 11 rats sacrificed at 0, 28 and 84 days.

[b]Average of 12 rats sacrificed at 0, 7, 14 and 28 days.

Table 14. Blood glucose and lactate levels of control and fluoride fed adult male albino rats

Days after start of fluoride feeding	Trial I	Trial II	Trial I	Trial II
	(Mg. glucose per 100 ml. blood)		(Mg. lactate per 100 ml. blood)	
0	108[a]	125[b]	28[a]	24[b]
1	129		26	
3	77	96	23	24
4	71	99	36	26
7	112	111	40	45
10	60	125	20	42
14	106	115	22	35
18		117		23
21		130		21
28		122		29
35	112		42	
49	97		83	
63	122		43	
84	120		37	

[a]Average of 11 rats sacrificed at 0, 28 and 84 days.

[b]Average of 12 rats sacrificed at 0, 7, 14 and 28 days.

not as pronounced in the adults as in the weanlings. This is further evidence of the difference in metabolic patterns of weanlings and adults. It would also suggest that some of the variations normally observed in blood glucose and lactate levels might be due to a corresponding variation in the liver glycogen level. The conversion of excess blood glucose to liver glycogen and the release of liver glycogen as glucose to elevate low blood glucose levels is a continuous body process. The production of lactate during the breakdown of glucose is also a continuous process. The length of time from the last ingestion of food and/or the amount of physical activity prior to sacrifice could materially effect the level of all three of these compounds. Handler et al. (19) previously demonstrated that the reaction of the fasted rat and the non-fasted rat was quite different when fluoride was administered at the acute toxic level due to variation in the liver glycogen level.

The effect of adding 300 p.p.m. of fluoride to the ration of weanling and adult albino rats demonstrated marked alterations in the blood glucose and lactate and liver glycogen levels. It also demonstrated that there were differences in response between weanling and adult albino rats. These differences within age groups and/or between age groups were not always consistent in the two trials. No explanation is offered for the variation between the two

trials inasmuch as conditions were as similar as could be obtained. It would probably be nessary to determine blood, soft tissue and bone tissue level of fluoride, the total excretion rate of fluoride and some of the other intermediate products of metabolism before a satisfactory explanation could be found.

Effect of Acute Toxic Levels of Fluoride on Weanling Pigs and Lambs

Handler (18) with rats and Utter and Werkman (49) with bacteria have demonstrated among others that fluorides are toxic to biological systems when administered in sufficient quantity. In both of these cases there was interference with the metabolic use of carbohydrate and elevated levels of one or more of the intermediate metabolic products.

Hobbs et al. (25) were not able to demonstrate alteration in the blood glucose of cattle on long time chronic intakes of fluorides. They had, however, shown that when animals were fed sufficiently high quantities of fluoride death resulted in 60 to 120 days. There was no information in the literature on the effect that acute toxic doses would have on the blood constituents of farm animals.

This part of the study was undertaken to determine if acute massive doses of fluoride would have the same effect

on farm animals that had been reported by Handler et al. (19) and others on laboratory animals.

Materials and methods

Eight pigs and two lambs, all of which were still on their dams, were used in this phase of the experiment. With the first several pigs it was noted that there was considerable variation in the initial blood sugar and lactate. Handler et al. (19) had reported stabilizing the blood sugar and lactate of rats with the use of nembutal. He had also reported that there was an immediate rise in both the glucose and lactate levels. In the lambs and pigs taken directly from their dams there was a depression in some of the animals and a rise in others. Nembutal helped to correct these variations but did not completely eliminate them. It was felt that this variation might be due to differences in the length of time between the last nursing of the animal and the time of fluoride injection. Subsequently, animals were taken from their dams and placed on cows milk for 48 hours. They were then given their last nursing four hours before injection of the fluoride. When this technique was followed fairly uniform blood glucose and lactate levels were obtained and the immediate rise reported by Handler et al. (19) with rats was obtained in the lambs and pigs.

A predosage blood sample was taken before the animals

were dosed and they were then bled periodically. When it was evident that the animal would not survive to the next bleeding it was sacrificed and samples of the liver and muscle tissue were taken for glycogen analysis.

The pigs used in this phase weighed from 10 to 15 pounds and were from three to five weeks of age. The lambs weighed 18 and 21 pounds and were approximately six weeks old.

The fluoride was administered as a solution of sodium fluoride injected at the rate of 250 milligrams per kilogram of body weight in the first two pigs and the first lamb. It was then reduced to 200 milligrams per kilogram, and was administered both intraparateonitally and subcutaneously into the pigs.

Results and discussion

When the fluoride was administered to the pigs intraparateonitally they very obviously showed a great deal of discomfort within a period of just a few minutes. Three of the pigs died within 35 minutes and the fourth in 60 minutes. The data presented in Table 15 shows that one of these pigs had an increase of approximately 50 per cent in blood glucose, one about doubled the pre-injection level and the third had about a threefold increase. The blood lactate in all three cases increased about two and a quarter to

Table 15. Effect of NaF on the blood glucose level of weanling pigs

Animal	1[a]	2[a]	961	963	964	962	951	952
Injection site[b]	S.Q.	S.Q.	I.P.	I.P.	I.P.	S.Q.	S.Q.	S.Q.
Mg. of NaF per kilo of body wt.	200	200	250	250	200	200	200	200
Minutes after injection	Milligrams of glucose per 100 ml. of blood							
0	88	85	130	121	121	108	130	142
30	76[c]	66	258	358	197	132	153	142
60		64			187	133	165	141
90		71				135	165	144
120		75				148	158	157
150		93				153	170	169
180		101				159	380	218
210		125				170	396	257
240		150				192	197	345
270		197				182	195	350
300							241	354
330							258	

[a]Taken directly from dam.

[b]I.P. = Interparateonitally; S.Q. = Subcutaneously.

[c]35 minutes at death.

two and a half times as shown in Table 16. There seemed to be no correlation between the glucose and lactate levels in the pigs given sodium fluoride interparateonitally.

The pigs injected interparateonitally not only had a short survival time but when posted showed massive pin point hemorrhages all along the gastro-intestinal tract.

Table 16. Effect of NaF on the blood lactate level of weanling pigs

Animal	1[a]	2	961	963	964	962	951	952
Injection site[b]	S.Q.	S.Q.	I.P.	I.P.	I.P.	S.Q.	S.Q.	S.Q.
Mg. of NaF per kilo of body wt.	200	200	250	250	200	200	200	200
Minutes after injection	Milligrams of glucose per 100 ml. of blood							
0	58	92	69	80	61	30	50	48
30	151	28	160	174	146	40	39	52
60		25			189	31	33	38
90		22				18	27	35
120		12				43	19	34
150		52				54	43	6
180		77				73	61	36
210		124				92	72	65
240		132				88	89	82
270		252				90	77	98
300							52	233
330							48	

[a]Taken directly from dam.

[b]I.P. = Interparateonitally; S.Q. = Subcutaneously.

[c]35 minutes at death.

Both lambs were injected interparateonitally and had a considerably longer survival time and did not show the extensive pin point hemorrhages along the gastro intestinal tract shown by the pigs. Both species showed considerable salivation shortly after injection. This is characteristics of many toxic materials.

When the method of administration was changed to subcutaneous injection, the pigs did not show the obvious discomfort nor the excessive salivation noted with the interparatenional injection. When the animals were posted following death there was only slight evidence of hemorrhaging along the gastro-intestinal tract. There was an area of considerable dark purplish-red discoloration in the subcutaneous fat layer surrounding the injection site that indicated massive pin point hemorrhage in this tissue. This latter method of injection would release the fluoride into the bloodstream of the animal at a slower rate accounting for the increased survival time. The difference in the tolerance of the lambs and the pigs to the method of injection of the fluoride should be noted. While the numbers involved are small it would seem to give some indication of a species difference in the tolerance of fluoride.

The level of glycogen in the liver and muscle is presented in Table 18. There is one point of interest here that should be noted. Pig 1 taken directly from his dam and injected with fluoride had about four and a half times the amount of liver glycogen and nearly twice the amount of muscle glycogen as the next highest levels in the pigs taken from their dams 48 hours before sacrifice and without any feeding for four hours before injection. This variation in the glycogen levels between fasted and non-fasted animals

Table 17. Effect of NaF on the blood glucose and lactate levels of weanling lambs

Animal	705		697	
Mg. of NaF per kilo of body wt.	250		200	
Minutes after injection	Milligrams per 100 ml. of blood			
	Glucose	Lactate	Glucose	Lactate
0	91	25	106	35
30	119	56	97	41
60	216	79	141	56
90	224	137	151	76
120	234	154	294	89
150			311	108
180			284	119
210			242	169
220			234	131

is similar to that reported by Handler (19) with rats and indicates further the difficulty of standardizing metabolic conditions in the body. It could also indicate that the young frequently nursing animal maintains fairly high levels of glycogen and that even such a short fasting time as four hours depletes this level materially. A much larger number of animals than used in this experiment would have to be studied before any definite conclusions could be made, however.

When the level of fluoride administered was reduced to 200 milligrams per kilogram of body weight and injected

Table 18. Effect of NaF on the liver and muscle glycogen of weanling pigs and lambs

	Pigs							Lambs	
Animal	1[a]	961	963	964	962	951	952	705	697
Injection site[b]	S.Q.	I.P.	I.P.	I.P.	S.Q.	S.Q.	S.Q.	I.P.	I.P.
Mg. of NaF per kilo of body wt.	200	250	250	200	200	200	200	250	200
Minutes from injection to sacrifice	35	30	30	60	270	330	300	120	220
Glycogen in mg./gm. of wet tissue									
Liver	44	9	4	3	9	6	1	3	1
Muscle	21	12	10	12	12	5	7	12	13

[a]Directly from dam.

[b]I.P. = Interparateonially; S.Q. = Subcutaneously.

subcutaneously in the abdominal fat layer of the pig survival time increased to 270 to 330 minutes. There was considerable variation in the quantity of blood glucose in the four pigs at any given time and also in the pattern of increase. In general there was a considerable rise in the level of glucose from administration until sacrifice. Pig 2, taken directly from his dam, had a decrease in blood sugar to about 75 per cent of the predosage level, recovered to predosage levels after about two and one-half hours, and then increased to about 230 per cent of the predosage level at death at four and a half hours. The blood lactate on this pig followed the same general pattern. However, the decrease was much greater and the recovery took about an hour longer.

The three pigs taken from their dams for 48 hours and fasted for four hours prior to injection did not show this decline in blood sugar. One of them, 952, did not show any increase for two hours while the other two, 962 and 951, showed an immediate rise. The rate of increase and the level of glucose at sacrifice varied markedly and neither of these factors in themselves seemed to account for the animals' death. However, it did show that the pig has an elevation in blood sugar when treated with a massive dose of fluoride. It also demonstrates the difficulty in standardizing the metabolic status of the pig of this age. A much larger number of animals would have to be used

before drawing definite conclusions.

The blood lactate level in the pigs injected subcutaneously showed a decline from the pre-injection levels and then a recovery and rapid rise to high blood lactate levels. The level at death was variable and seemed to be independent of the blood glucose level. As with the glucose, larger numbers would have to be used before definite conclusions could be drawn. However, the general trend is similar to that reported by Handler (18).

Standardization of the metabolic pattern of weanling lambs and pigs is difficult. Taken directly from their dams there was a wide variation in the blood glucose and lactate levels. Removal of the pigs from their dams and feeding with cow's milk for 48 hours and then removal of the cow's milk four hours prior to injection of the fluoride stabilized the blood levels somewhat. There may be more variability in the metabolic pattern of weanling animals than in the adult animals that were used by Handler (18). In general, the lambs and the pigs showed a response similar to the rats used by Handler (18), when injected with massive doses of fluoride. It should be noted that there is an apparent difference in the tolerance of fluoride between lambs and pigs. The lambs tolerated doses of 250 milligrams per kilogram of body weight injected intraparateonitally. This level and dose killed the pigs within 30 to 60 minutes.

When the level of fluoride was reduced to 200 milligrams per kilogram of body weight and injected subcutaneously the pigs had a longer survival time. This caused some alteration in the curve obtained compared to the rats used by Handler (18). However, this may be explained on the rate of absorption from the subcutaneous fat layer compared to absorption from the intraparateonial cavity.

Fluorides injected in farm animals in massive doses will cause an alteration in the metabolic pattern as shown by changes in the blood glucose and lactate levels and in muscle and liver glycogen.

Distribution of Radioactive Fluoride in Cattle Fed a Sub-lethal Dose of Fluoride

Tracer quantities of radioactive materials have been used rather widely in recent years to study problems in animal metabolism. They have proven successful with minerals that are required only in trace quantities and where the resulting chemical studies are limited by validity of the method for the low concentration present. There are problems encountered in the use of radioactive fluorine, designated hereafter as F^{18}. This isotope only has a half life of 112 minutes (50) and is a positron emitter (0.64 mev.) which results in two high speed gamma rays which places severe limitations on its usage as a biological

tool when employed with farm animals. It necessitates use of multimillicurie doses and then only allows a usable time of 10 to 16 hours from time of preparation in the cyclotron to the final measurement of biological samples. Further limitations involve the required high specific activity to furnish concentrations which can be used and still stay within the safety requirements as established by the AEC.

Chemical analysis of fluorides of tissues of farm animals (4, 25, 33, 40) has been done largely with animals that have been on fluoride intake for a considerable period of time or with animals given a sufficiently high dose to be toxic within a short time. This work indicated that there was a large concentration in the skeleton and a relatively low concentration in the soft tissues. There was little information concerning the difference in distribution that might occur due to the ingestion of varying levels of fluorides.

It was known that animals of different physiological ages have different rates of metabolizing mineral matter (21). It has also been shown that there is some similarity in the metabolic pattern of both calcium and fluoride (25). Whether this similarity between calcium and fluoride was the same in both young and older animals was not known, nor was there any information that the metabolism of fluorine was the same in animals of differing ages. This phase of

the work was designed to study these age differences in the metabolism of fluoride, for a clearer picture of the tissue distribution of fluoride, and to determine the progressive blood changes with respect to glucose and lactate with animals that were fed varying levels of fluoride.

Materials and methods

Animals, rations and records. Two groups of nine grade Hereford heifers were used in this phase of the study. The range in age of the calf group was seven to eleven months, and the yearling group seventeen to twenty-two months. Each group was then subdivided into three lots. The weight ranges in these groups were from 280 to 346 pounds for the calves, and 543 to 710 pounds for the yearlings. These animals were brought in from pasture and given two weeks to adjust to a ration of ad libitum hay feeding plus four pounds of grain mixture per day. The hay used was orchard grass of fair to good quality. The composition of the grain mixture is given below.

Ground yellow corn	340 lbs.
Crushed oats	110 lbs.
Cottonseed meal	50 lbs.
Calcium carbonate	5 lbs.
Vit. A-D supplement	50 grams.

Feed records were maintained during the adjustment period so that at the end of the time the rations could be standardized. The hay offered for each of three lots within each

group was the quantity consumed by the lot consuming the least amount during the pre-experimental period. During the experiment, feed records of the offering and weigh-back of both the hay and the grain were carefully maintained and checked daily. When one of the lots within an age group reduced its feed intake of either hay or grain for three consecutive days the feed was reduced by a like amount in the other two lots. This occurred only once during the experiment. In this way it was possible to keep all three lots within each group on approximately the same feed intake so that differences due to feed consumption were kept to a minimum. The calf group had both the grain and the hay reduced from four to three pounds per head per day on the twelfth day due to the reduced intake of the lots receiving 300 p.p.m. of fluoride. The yearlings had the grain reduced from four to three and a third pounds and the hay from eight to six and two-thirds pounds per animal per day also on the twelfth day. This quantity of feed resulted in the slight loss of weight for the cattle as shown in Table 19 indicating that the ration was slightly below maintenance requirements.

At the start of the experiment the animals were lotted three to a group so that the lot starting weights were as nearly alike as possible. The only difference among the lots was in the amount of fluoride fed. Sufficient sodium

Table 19. Weight changes of cattle fed fluoride

Lot	Animal	F level	12/14/56	12/28/56	1/11/57	2/1/57[a]	2/8/57[a]
			Calves				
1	1718	control	325	335	332		
	1721	control	346	351	351	366	
	1688	control	280	290	297	298	296
		Lot avr.	317	325	327	331	
2	1731	100 p.p.m.	325	327	324		
	1698	100 p.p.m.	326	345	349	362	344
	1669	100 p.p.m.	302	313	316	310	312
		Lot avr.	318	328	330	336	319
3	1695	300 p.p.m.	324	309	315		
	1686	300 p.p.m.	315	294	317	324	
	1676	300 p.p.m.	308	286	295	310	304
		Lot avr.	316	296	309	311	
			Yearlings				
4	1214	control	710	680	669		
	1295	control	561	550	523	520	520
	1298	control	560	538	533	530	526
		Lot avr.	610	589	575	525	523
5	1240	100 p.p.m.	692	700	665		
	1311	100 p.p.m.	543	548	537	532	530
	1171	100 p.p.m.	656	641	630	628	622
		Lot avr.	630	630	611	580	576
6	1219	300 p.p.m.	592	575	578	562	
	1330	300 p.p.m.	704	680	674	650	
	1267	300 p.p.m.	560	555	540	522	504
		Lot avr.	619	603	597	578	

[a]Blanks in the weights on 2/1/57 and 2/8/57 indicate the cattle had been removed, dosed with F^{18} and sacrificed.

fluoride was added to the grain to bring the levels of fluoride up to the quantity given below. When feed intake was changed within a group on the twelfth day the amount of fluoride added to the grain was changed to keep the intake of fluoride at the same level in terms of the total ration.

	Lot number	
Fluoride intake	Calves	Yearlings
control	1	4
100 p.p.m. added	2	5
300 p.p.m. added	3	6

Radioactive procedures and measurements

The F^{18} used in this experiment was produced in the cyclotron at Oak Ridge, Tennessee according to the method described by Martin and Green (35). Four-tenths of a gram of sodium fluoride was placed in the above described capsule for bombardment to produce F^{18}. The total quantity of F^{18} radioactivity obtained varied with the various shipments. Standards were made of each shipment and measured routinely during the entire time that counting was conducted. The decay curve in every case coincided closely with the expected 112 minute half life, indicating no significant amounts of extraneous radioactivities. The day following dosing both the standards and some of the samples where sodium would tend to be concentrated were checked for Na^{22}. In no case was there any indication of Na^{22}. The latter has a half life of 2.6 years (50) and so would be emitting

radioactivity long after the decay of the F^{18}. Due to the variation of activity of the F^{18} between shipments all the results of each study are reported as a per cent of the total radioactive dose administered.

The F^{18} was taken from the cyclotron to the U.T.-A.E.C. facilities in heavy lead shielding where it was prepared for administration. The sample was dissolved in water by radio chemical procedures and made up to a volume of 25 ml. Dose aliquots of 10 to 175 millicuries were then placed in either a gelatin capsule for oral administration or drawn into a shielded syringe for intravenous injection into the jugular vein. These procedures have been described in detail by Hansard et al. (22). Extreme care was necessary in handling the F^{18} due to the heavy gamma irridiation in order not to exceed established safety levels. The four-tenths of a gram of sodium fluoride was usually divided into four doses. This resulted in either oral dosage or intravenous injections that did not exceed the equivalent of one-tenth of a gram of sodium fluoride or forty-four thousandths of a gram of fluoride. For the animal given an oral dose this was equivalent to less than one-tenth of a part per million based on eleven pounds of total daily feed intake. To compare this to the lethal dose of 250 milligrams of sodium fluoride per kilogram of body weight reported earlier in young swine and lambs, 2.7 to 7.9

milligrams of fluoride per kilogram of body weight were injected into the calves and yearlings. This was calculated on the basis of injecting 1000 milligrams of sodium fluoride into the calves and yearlings ranging in weight from 127 to 364 kilograms.

Blood samples from the jugular vein were withdrawn periodically during the day. Urine was collected by means of a retaining catheter and continuous samples for counting were taken. Saliva was collected shortly after each bleeding by placing a 1 inch x 1 inch x 4 inch piece of cellulose sponge, tied to a string, in the mouth of the animal and allowing her to chew it until at least 4 milliliters of saliva were collected. Feces was collected as excreted by conventional procedures (22) in pans placed behind the digestion stalls. Animals dosed orally in most cases were not sacrificed but were returned to their lots after 36 hours in the metabolism units. This allowed sufficient time for the decay of the F^{18} administered by oral dosage and permitted reuse in subsequent studies. These animals were redosed intravenously seven to ten days later and sacrificed for tissue distribution study. By following this procedure each animal dosed orally and then intravenously served as its own control. All animals dosed intravenously were sacrificed, and a few animals dosed orally were also sacrificed in order to check the absorption

values and to compare the differences in distribution of F^{18} between the two methods of administration. All tissue samples obtained after sacrifice were weighed and counted in a well-type scintillation counter. Blood and urine radioactivity was measured by directly pipetting 4 milliliters of the material into a counting tube. All radioactive values are reported as per cent of total dose per gram or milliliter of sample.

The method of Hansard (20) was used to determine the blood volume of each animal. The total quantity of radioactive material present in the blood at any given time was the product of the radioactivity per ml. times the ml. of blood calculated to be present in the animal. Urine volumes were measured and the activity per ml. multiplied by the volume to obtain the total quantity of F^{18} excreted. Fecal weights were obtained and multiplied by the activity per gram to get the total quantity of F^{18} excreted.

Autoradiograms of F^{18} were unsuccessfully attempted according to the method of Perkinson et al. (38). It was determined that the gamma rays produced by the decay of F^{18} were not sufficiently ionizing to react appreciably with the emulsion of the x-ray film. However, by placing three layers of lead foil between the bone slice and the x-ray film, the rays were slowed sufficiently to give adequate ionization when in contact with the film as shown in Plate 1.

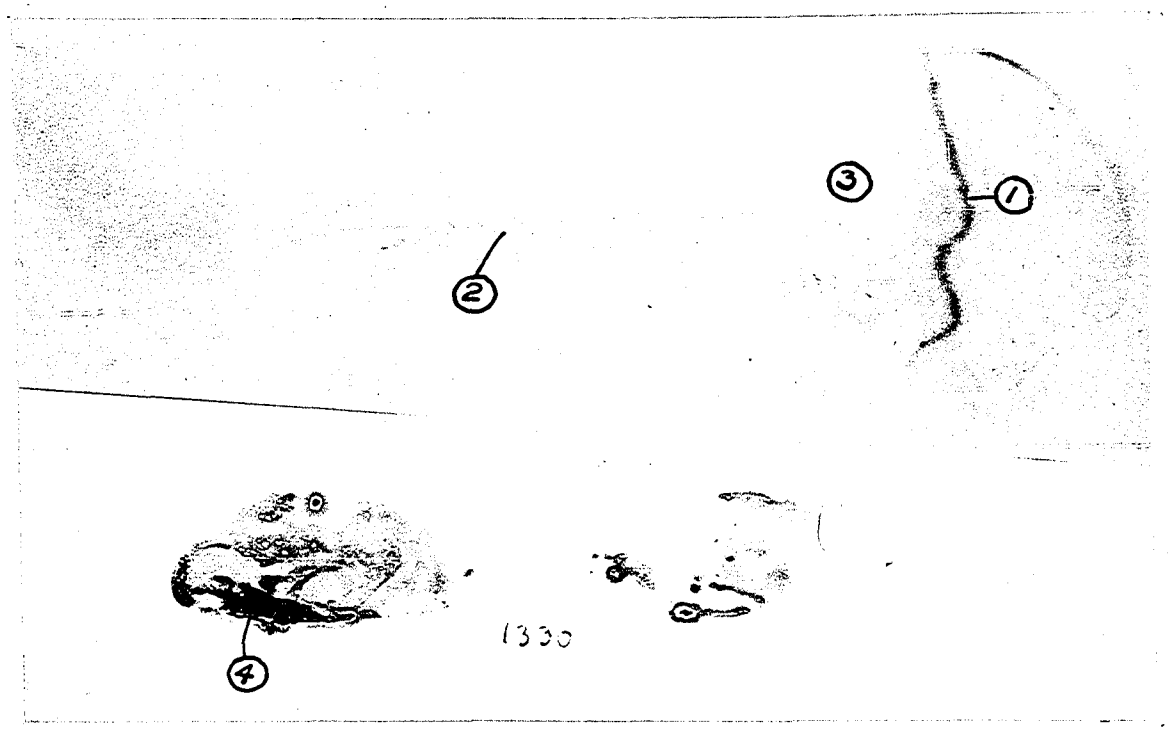


Plate 1. Distribution of F^{18} in bone tissue

From yearling 1211:
1. Epiphysis
2. Periosteum
3. Trabecular bone

From yearling 1330:
4. Unerrupted incisor

All calves and yearlings were bled periodically from the time the experiment was initiated for both blood glucose and lactate determinations. The method of determination used for the blood glucose was that of Folin and Wu (13), and lactate was measured by the method of Barker and Summerson (1).

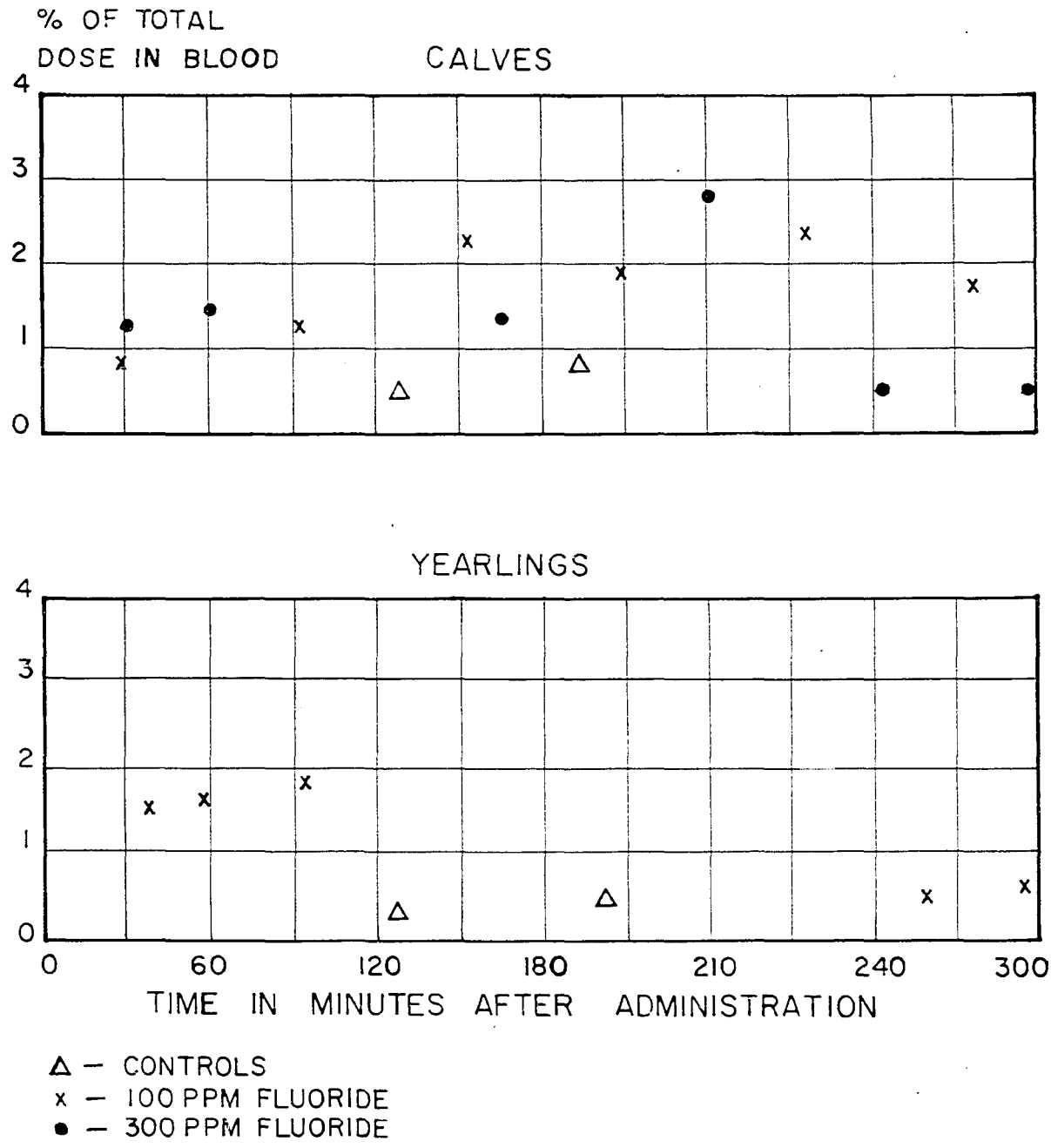
Results and discussion

Animals' weights as shown in Table 19 indicate that these animals were consuming amounts of feed to approximately maintain weight equilibrium. The calves in all three lots remained at essentially the same weight during the experiment. The yearlings in all three lots lost some weight. The intake of 300 p.p.m. of fluoride from sodium fluoride effected the appetite so that only sufficient nutrients were ingested to maintain weight or result in some weight loss. However, there were no significant differences in the body weights on the different levels of fluoride intake. Thus the maintenance or slight loss in weight encountered in the fifty-five days of this experiment was probably due to the reduction of feed and not due to the fluoride *per se*.

Oral dosage with F^{18}

The data presented in Graph 1 shows the appearance of F^{18} in the bloodstream of calves and yearlings as a function

Graph 1. Concentration of F^{18} in the bloodstream of cattle after oral dosage


% OF TOTAL
DOSE IN BLOOD
CALVES
4
3
2
1
0
YEARLINGS
4
3
2
1
0
0
60
120
180
210
240
300
TIME IN MINUTES AFTER ADMINISTRATION
Δ — CONTROLS
x — 100 PPM FLUORIDE
● — 300 PPM FLUORIDE

of time after oral administration of the isotope. While the data is rather limited it does graphically illustrate the absorption of F^{18} and show that the level of the bloodstream was never high in either age group. This no doubt was due to several factors. In the first place dilution with the rumen contents would keep absorption at any given time at a very low level. Again the fluoride may have combined with some of the material in the rumen so that it was not readily available for absorption, and then it must be assumed that the absorbed fluoride was removed from the bloodstream by the bone, kidney or other organs at the same rapid rate indicated by the intravenous curve (Graph 4, Tables 21 and 23). This data would indicate that, when given orally, fluorides do not appear in any great concentration in the bloodstream at any given time up to 380 minutes and were independent of the dietary fluoride level.

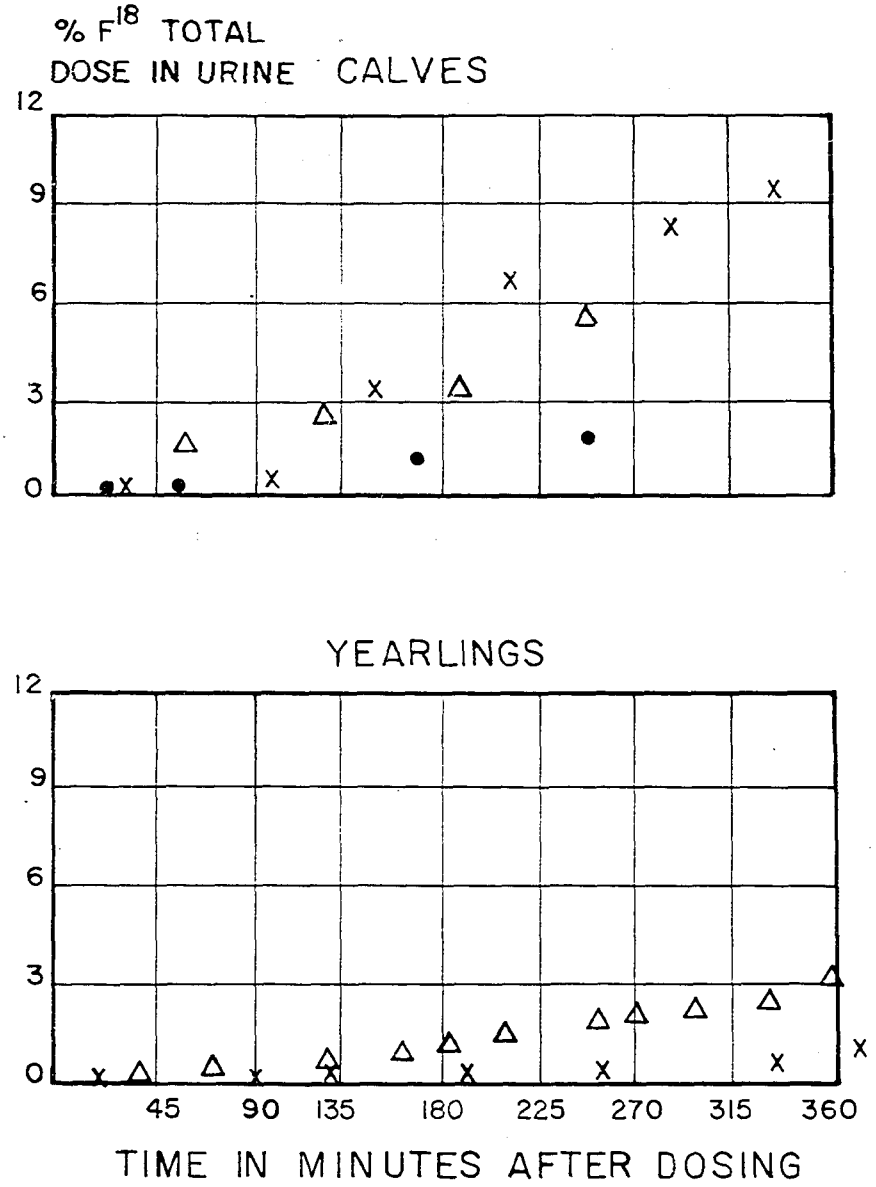
Data secured from the animals receiving the oral doses of radioactivity indicated absorbed fluoride was in an active state of metabolism. Measurable quantities of F^{18} appeared in the urine eleven minutes after dose administration. There was considerable variation in the total quantity excreted by the various animals, however, ranging from a low of 1.11 per cent of the total dose administered in a period of 377 minutes for a yearling receiving 100 p.p.m. of fluoride to a high of 9.53 per cent of the total

dose within 290 minutes by a calf receiving 100 p.p.m. This data is presented in Graph 2. The points on the curves are cumulative totals for the individual animals rather than the level at any given time interval.

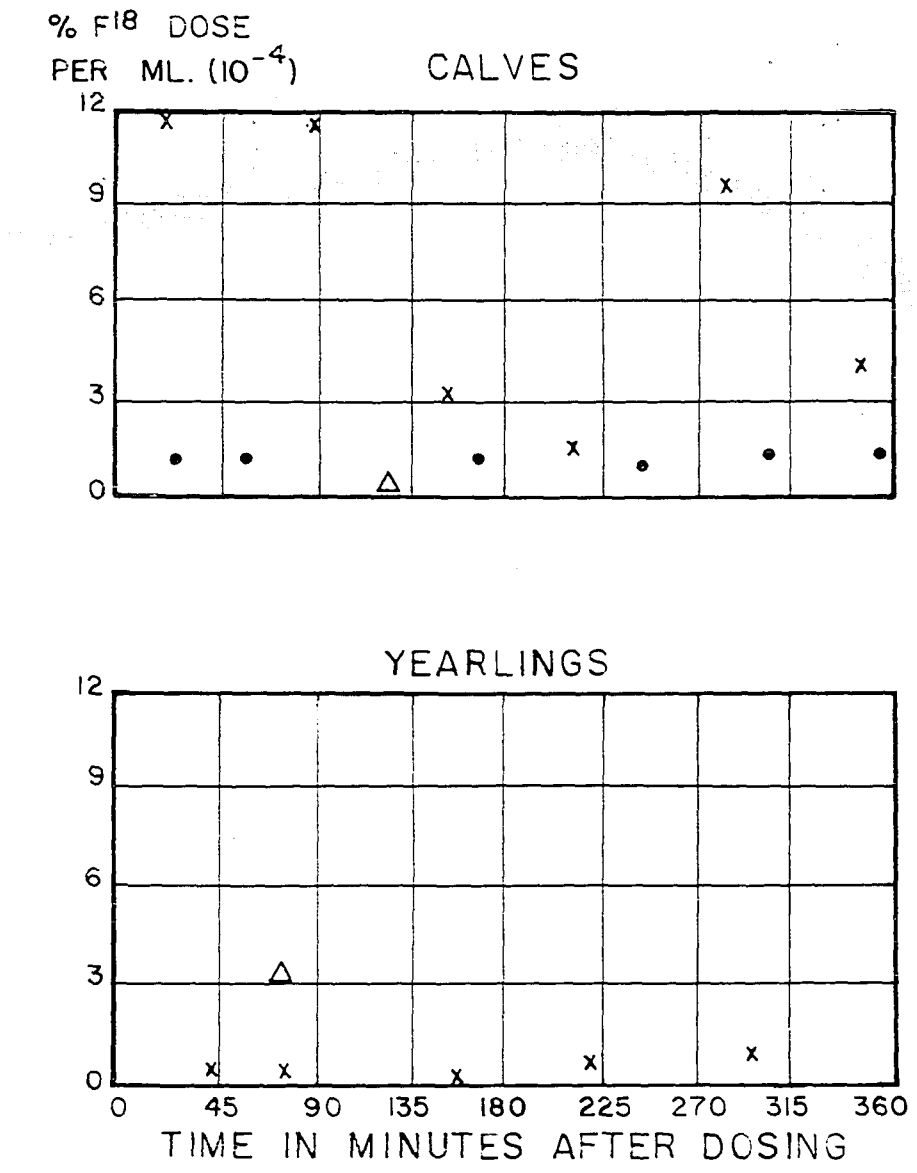
There was a great deal of variation in the concentration of radioactive fluoride whether measured as the concentration per ml. or as the total amount collected between any given time interval. This was due to the fact that both the concentration per ml. and the volume of urine excreted varied widely between animals and with time. These data do show the urine to be an important excretion pathway for absorbed fluoride.

The data presented in Graph 3 indicates that in addition to the elimination of fluoride through the urine there is some recycling of the material back into the gastro-intestinal tract by way of saliva. The levels given in this graph are in concentration per ml. of saliva, inasmuch as no attempt was made to determine the rate of saliva secretion. They do show considerable variation in concentration, but they also show that fluoride can and does re-enter the digestive tract after it is absorbed. Due to the short half life of the isotope it was impossible to study in detail the kinetics of this action. However, the blood curves demonstrate similarities with that of the physiological behavior of calcium as shown by Hansard et al. (21).

Graph 2. Elimination of F^{18} in urine of calves and yearlings dosed orally


% F18 TOTAL
DOSE IN URINE CALVES
12
9
6
3
0
YEARLINGS
12
9
6
3
0
45
90
135
180
225
270
315
360
TIME IN MINUTES AFTER DOSING
• 300 PPM FLUORIDE
X 100 PPM FLUORIDE
Δ CONTROL

Graph 3. Elimination of F^{18} in saliva of calves and yearlings dosed orally



• 300 PPM FLUORIDE
x 100 PPM FLUORIDE
△ CONTROL

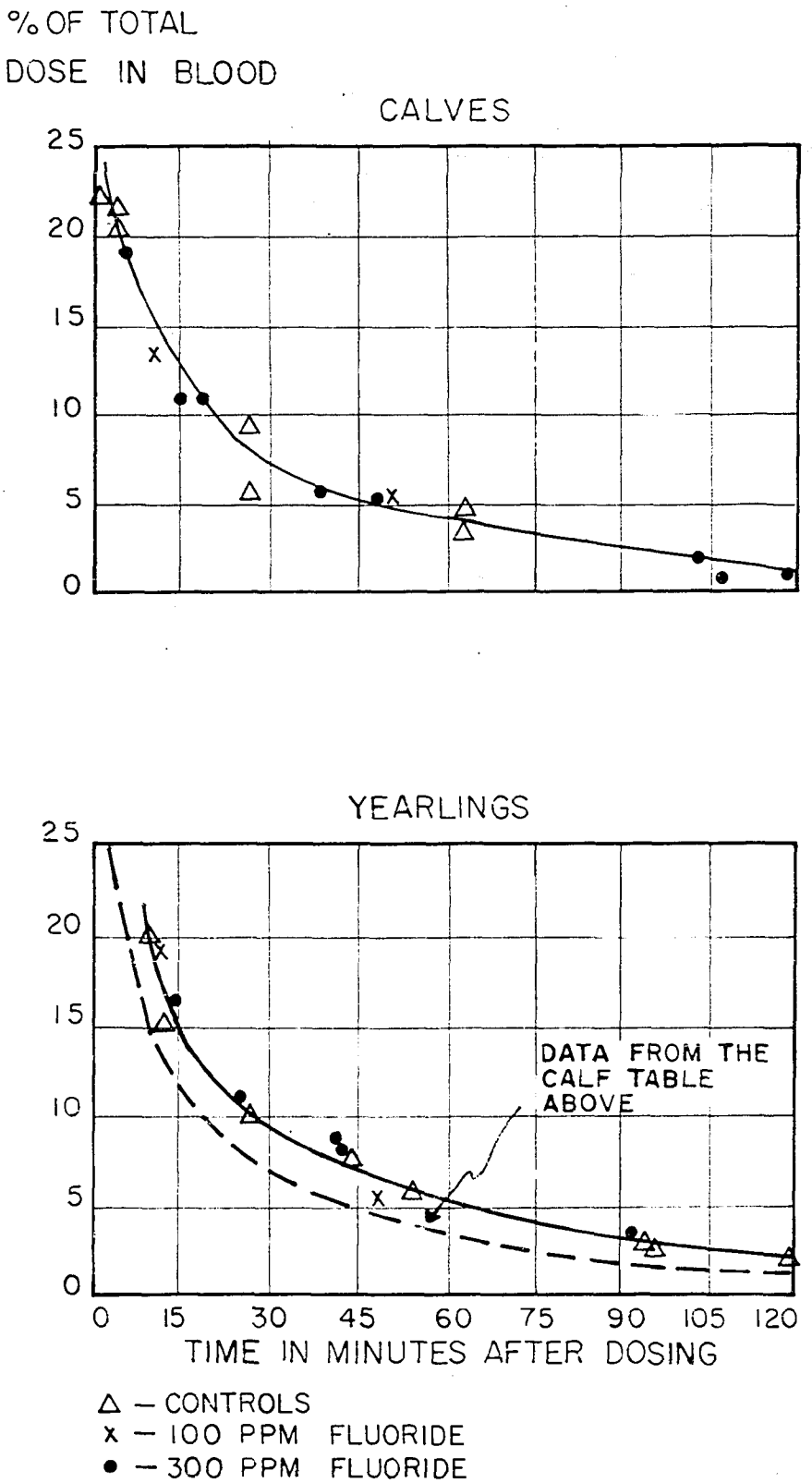
In several of the animals measurable quantities of the isotope were detected in the feces. The maximum time interval was 281 minutes from dosage until collection. It was improbable, however, that the fluoride would have had time to traverse the digestive tract in this limited time. It was more probable that the isotope appearing in the feces entered the large intestine from the bloodstream. Following re-excretion from the intestinal lining into the lumen the F^{18} was subsequently eliminated with the fecal material.

Radioactive fluoride is not a good biological tool when administered orally. The short half life requiring multimillicurie dosage, slow movement and the mass dilution with digestive contents present problems difficult to overcome. The data does show, however, that there was a rapid absorption from the gastro-intestinal tract and subsequent excretion via both urine and feces indicating that both of these pathways are used in removing the element from the body. The concentration in either of these pathways cannot be used, however, as an indication of the intake level.

Intravenous dosage with F^{18}

The disappearance of F^{18} from the blood after intravenous administration is presented in Graph 4. One of the primary objectives of this experiment was to determine the similarities and/or differences in fluoride behavior between

Graph 4. Disappearance of intravenously administered F^{18} from the bloodstream of cattle


% OF TOTAL
DOSE IN BLOOD
CALVES
25
20
15
10
5
0
YEARLINGS
DATA FROM THE CALF TABLE ABOVE
0
15
30
45
60
75
90
105
120
TIME IN MINUTES AFTER DOSING
△ — CONTROLS
x — 100 PPM FLUORIDE
● — 300 PPM FLUORIDE

animals of different physiological ages. Therefore, for ease of comparison, the data from both the calves and yearlings were presented in the same graph. Although data were secured over a period of 345 minutes, results for the first 135 minutes only are presented since the curve beyond this point is very nearly a straight line. The points on these curves represent data from all the animals on all fluoride intake levels.

The curves on both the calves and the yearlings approach closely those for radiocalcium and radiophosphorous obtained by Hansard et al. (23). It is further evidence that the metabolism of fluoride was, in many ways, similar to that of calcium and phosphorous. This similarity has been pointed out by Hobbs et al. (25) when they observed that the disappearance rate of fluoride from the bone of rats after the cessation of fluoride feeding was similar to that of radioactive calcium as reported by Hansard (21).

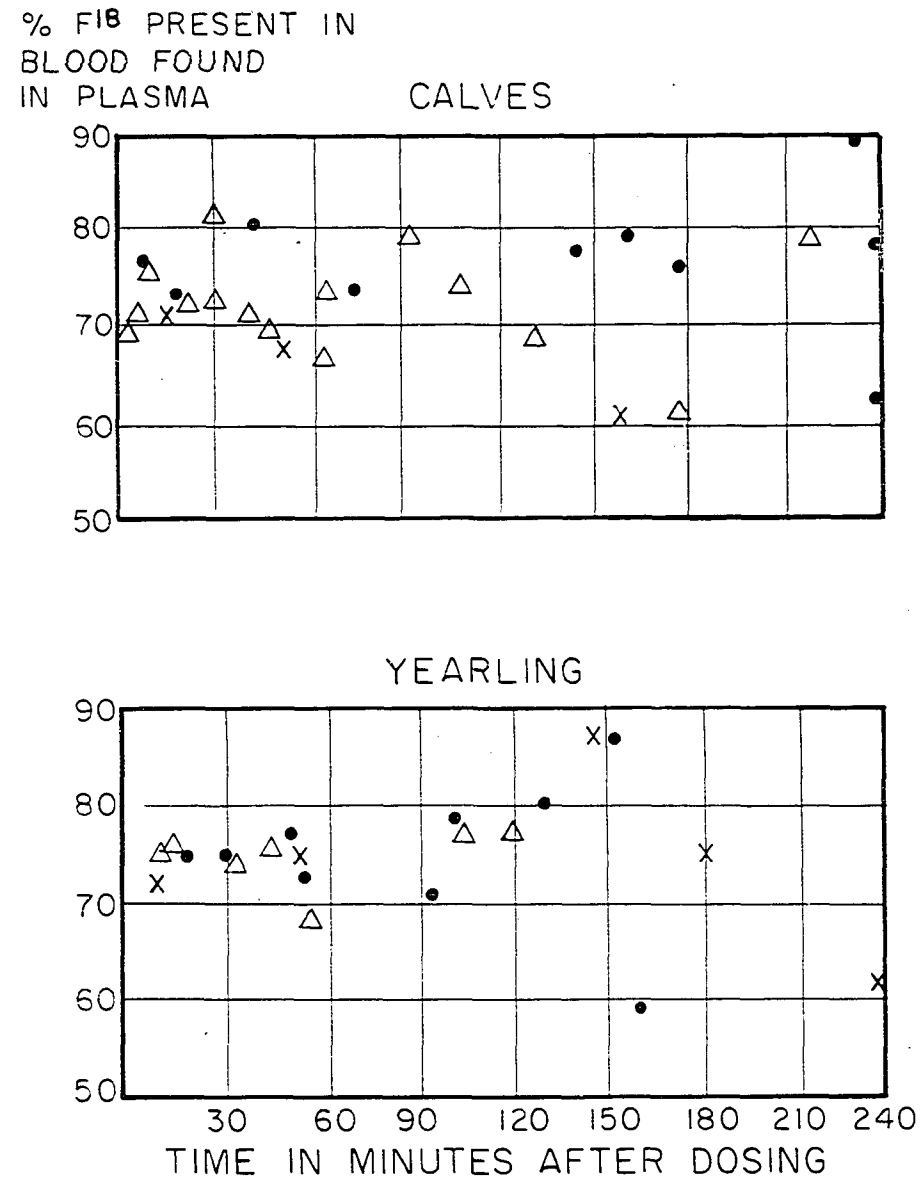
Hansard et al. (21) have pointed out a definite difference in the disappearance rate of Ca^{45} due to age itself. The older the animal becomes, the slower the exchange rate of calcium ions between blood and other body components, especially bone. This difference is evidenced in the blood disappearance rate of F^{18} from calves and the yearlings. The F^{18} was removed at a slightly faster rate by the calves than by the yearlings. This difference was significant

(p-0.05) and is shown on Graph 4 for yearlings as the distance between the solid line and the broken line. This can be accounted for, at least in part, by the increased F^{18} secretion in the saliva and urine, and by a faster removal by the bone tissue of calves, and will be discussed later. In addition to the rate differences between that of calves and yearlings it should be noted that the individually plotted points on the yearling blood curve, representing several animals on three different levels of fluoride intake, cluster very closely about the mean curve, indicating little individual variation. The calves, however, showed more variation in F^{18} disappearance and demonstrated a greater range in the metabolic pattern of the calves with F^{18} in the age range of seven to eleven months than shown in yearlings, ranging from seventeen to twenty-two months of age.

Blood distribution of F^{18}

When the animals were dosed intravenously an attempt was made to determine in which fraction of the blood the fluoride was concentrated. After the sample of the whole blood was taken for radioactivity measurement the remainder was centrifuged and a sample of the plasma taken for counting. The data are presented in Graph 5. This shows that in both the calves and the yearlings about 75 per cent of the

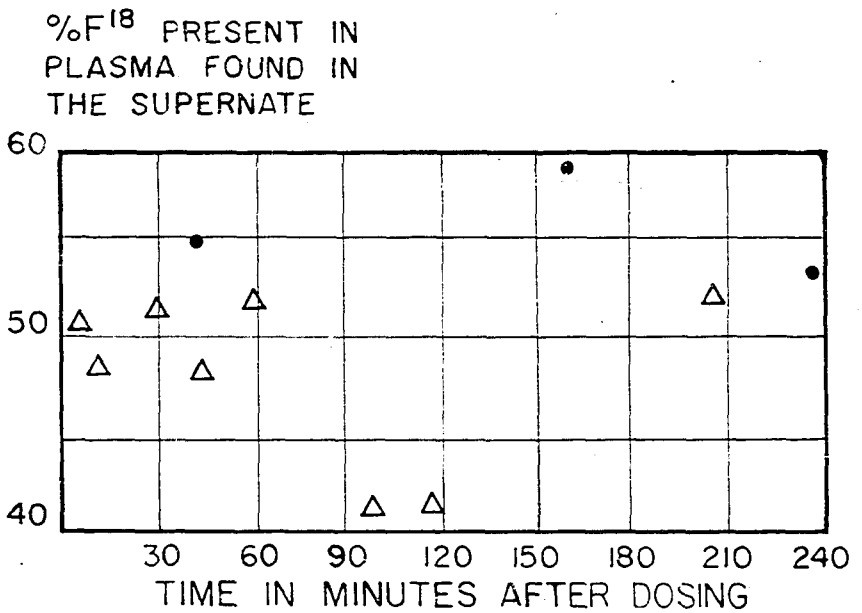
Graph 5. Plasma percentage of the total F^{18} in the blood from intravenous administration


% F18 PRESENT IN
BLOOD FOUND
IN PLASMA
CALVES
90
80
70
60
50
YEARLING
90
80
70
60
50
30
60
90
120
150
180
210
240
TIME IN MINUTES AFTER DOSING
• 300 PPM FLUORIDE
X 100 PPM FLUORIDE
△ CONTROL

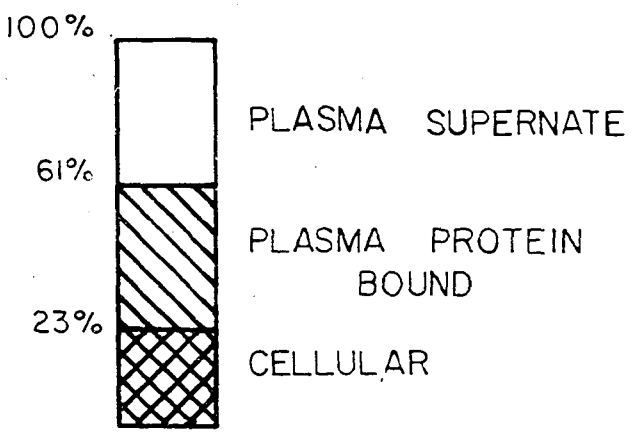
total blood fluoride appears in the plasma. There was little variation in this average level between the calves and the yearlings. However, as has been discussed previously the calves show more variation than do the yearlings. The longer after dosing that samples were taken the greater the variability in the quantity of radioisotope in the various fractions. This would seem to indicate that fluoride was in a highly dynamic state resulting in a good deal of individual variation in the way that different animals handle fluorides. This variability in handling toxic materials below the lethal level is not uncommon.

After noting that the level of fluoride in the plasma was rather constant at about 75 per cent a further attempt was made with the last two groups dosed to determine the quantity of F^{18} in the bound or unbound fraction of the plasma. Following the measurement of activity in whole blood it was centrifuged and samples of the plasma were precipitated with trichloracetic acid, centrifuged and radioactivity measured in the supernatant fraction. As in all the previous work there was no difference between blood samples from animals on the various dietary levels of fluoride. There was very nearly an even distribution between the two fractions indicating that fluoride like calcium was only 50 per cent bound to protein. This data was presented in Graph 6. The bar graph in this figure

Graph 6. Per cent of plasma F^{18} in the plasma supernate from intravenous administration



DISTRIBUTION OF TOTAL BLOOD F^{18} AMONG THE BLOOD COMPONENTS FROM INTRAVENOUS ADMINISTRATION



presents the distribution of the isotope in the total blood 30 to 60 minutes after dose administration.

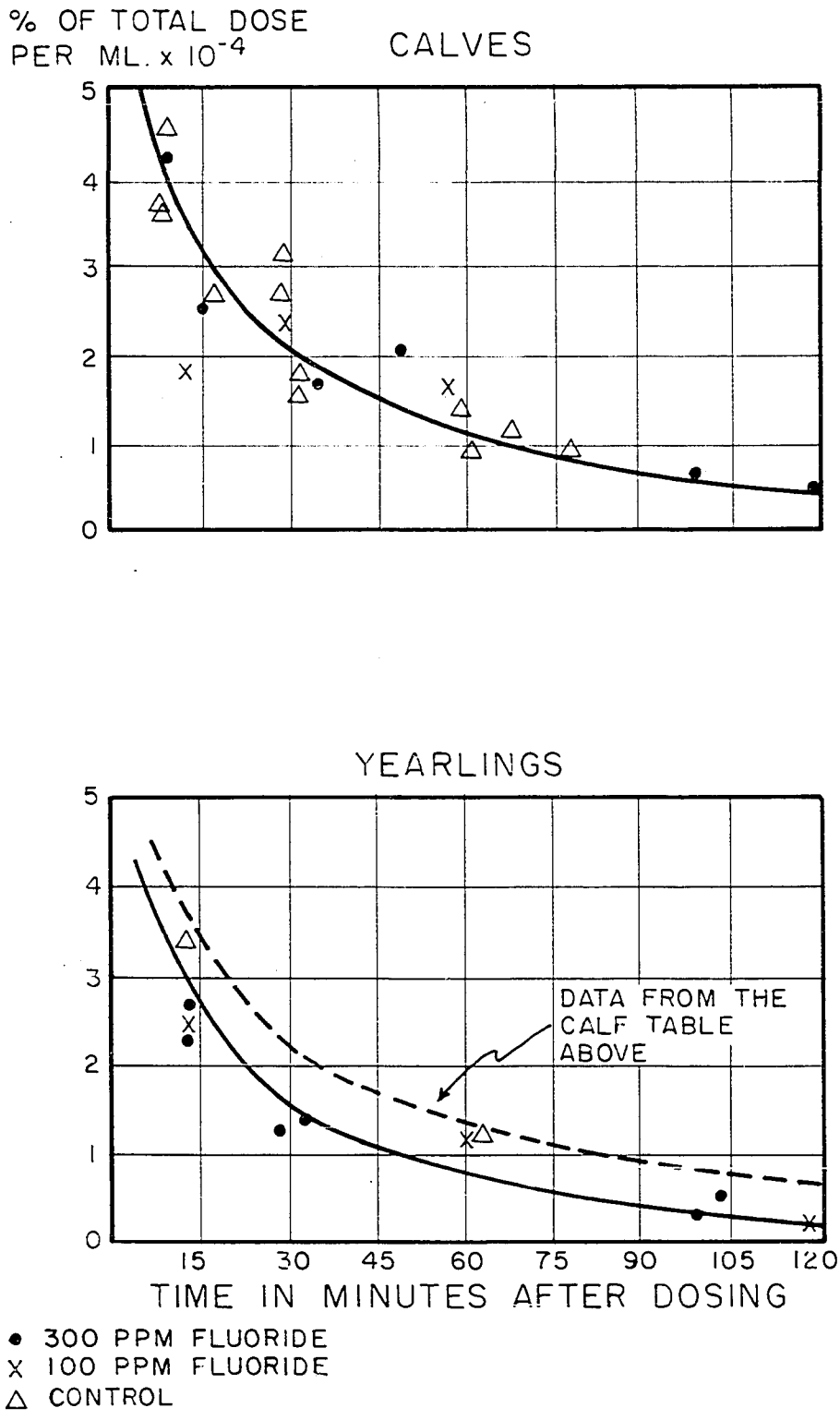
Concentration of F^{18} in saliva

Although there is considerable literature on the behavior of various radioactive compounds following intravenous administration, there is very little data on the amount that appears in the saliva. An attempt was made, therefore, to determine the amount of radioactive fluoride that appeared in the saliva because of the well established effect on the teeth. These effects and the explanations for them have already been discussed in detail by Hobbs et al. (25). In addition there has been a great deal of interest in topical application of fluoride to children's teeth in recent years as a method of preventing tooth decay.

The procedure used in this experiment had the advantages of being simple, requiring little extra in the way of equipment or materials, and did not require surgery nor did it upset the animal. The saliva obtained was usually a mixture of both the heavy mucoid and the thin viscous type. Only, occasionally was it contaminated with rumen contents that were regurgitated previous to or during the collection. When this occurred the sample was discarded and a new sample obtained. No attempt was made to determine the total quantity of saliva excreted. Thus all the

data given in Graph 7 was in terms of the per cent of total dose administered per ml. of saliva (10^{-4}). This data not only illustrates one of the metabolic pathways of the fluoride leaving the bloodstream, but also indicates that there was a very rapid appearance of the isotope in saliva after absorption occurred. There was a high concentration within eleven minutes after dosage. These data help to explain some of the differences noted between the calves and yearlings in the disappearance of fluoride from the bloodstream. The calves had a higher concentration of F^{18} in the saliva than did the yearlings, but more variability. This variability could well be an age difference, but is more probably due to the variation in saliva composition. There was considerable variation in the viscosity of the saliva from sample to sample. There was also considerable variation in the length of time that it took to collect any given sample. Because of this variability, the probability that the difference between the calves and the yearlings being a real difference was only 0.80. It is a further demonstration of the differences in metabolism within the seven to eleven month old age group. The fluoride in the saliva would normally bathe the teeth and then go into the rumen. From this point it could be reabsorbed and again re-enter the metabolic pool. The short half life of the fluoride isotope does not permit the type of study undertaken

Graph 7. Concentration of IV administration F^{18} in the saliva of cattle


% OF TOTAL DOSE
PER ML. x 10^{-4}
CALVES
5
4
3
2
1
0
YEARLINGS
DATA FROM THE
CALF TABLE
ABOVE
15
30
45
60
75
90
105
120
TIME IN MINUTES AFTER DOSING
• 300 PPM FLUORIDE
X 100 PPM FLUORIDE
△ CONTROL

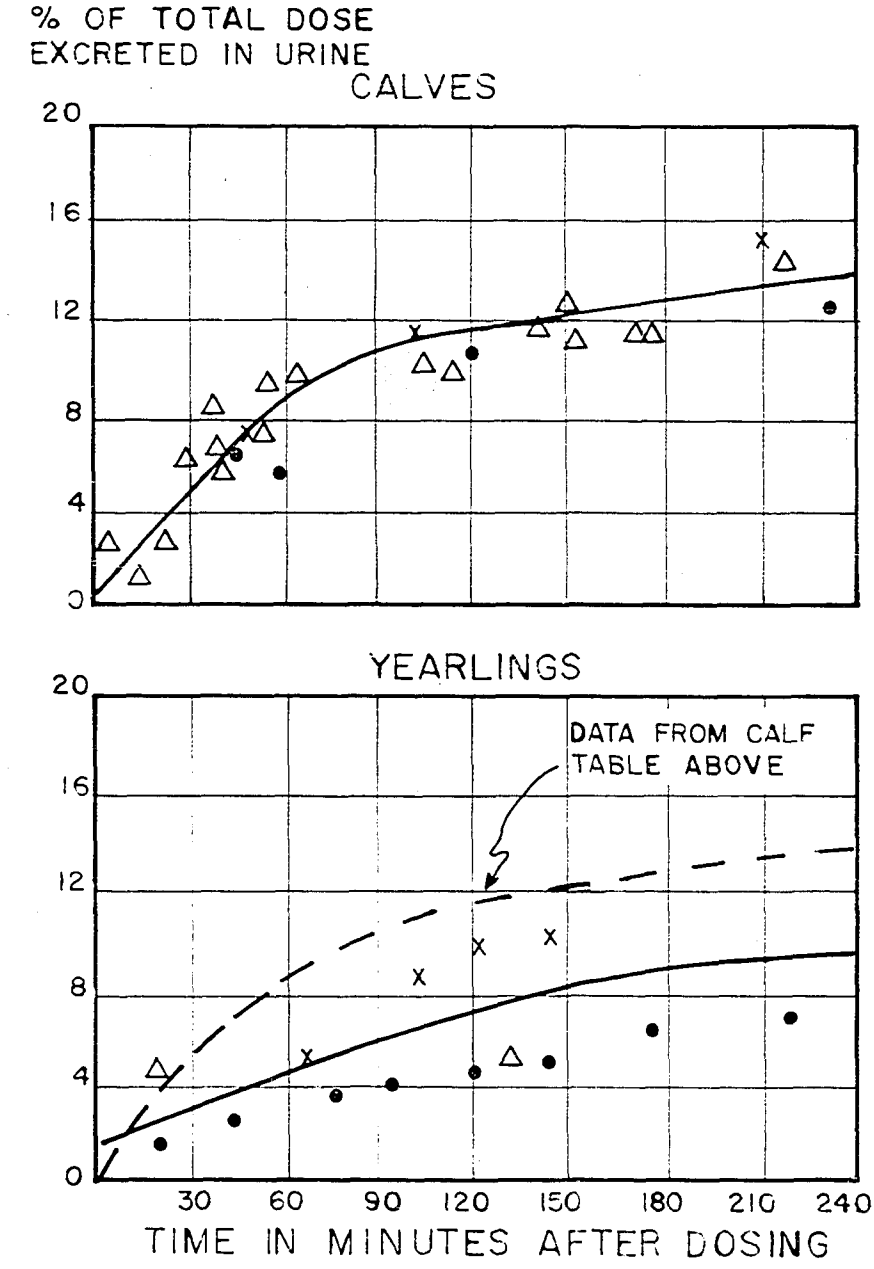
by Hansard et al. (23) in determining metabolic turnover of calcium and phosphorous.

Renal clearance of F^{18}

The renal clearance of F^{18} in the urine is graphically illustrated in Graph 8. The data represents the accumulative total excretion and not the amount in the individual collections. The activity per ml. was multiplied by the quantity of urine excreted to obtain the per cent of the total administered dose in a given collection. This amount was then added to the previous total quantity excreted to obtain the total excretion at any given time. By this method a fairly uniform curve was obtained for all animals. The level of activity in any given collection, however, varied widely, as did the quantity of urine over various periods of time. When an attempt was made to plot the total quantity of radioactivity excreted in any given collection the resulting curve was so erratic as to have little meaning. It was further evidence that the fluoride level of an individual urinary voidation measured either as concentration or total excretion was not an adequate criteria for the level of fluoride ingested at any given time. This substantiates the findings of Hobbs et al. (24, 25).

The calves demonstrated a more uniform total excretion rate than did the yearlings, and as has been shown in the

Graph 8. Renal clearance of F^{18} intravenously administered


% OF TOTAL DOSE
EXCRETED IN URINE
CALVES
20
16
12
8
4
0
YEARLINGS
DATA FROM CALF
TABLE ABOVE
30
60
90
120
150
180
210
240
TIME IN MINUTES AFTER DOSING
● 300 PPM FLUORIDE
x 100 PPM FLUORIDE
△ CONTROL

blood and saliva curves there was no difference due to the level of fluoride ingested. The yearlings presented a somewhat different picture. Although the curve in this case was drawn from the average of all yearling excretions, there appeared to be some difference due to dietary level of fluoride. These differences did not appear to be directly related to the fluoride intake, however. Individual animal variation could have contributed since more difficulty was encountered in properly positioning and adjusting the retention catheters in the yearlings than encountered with the calves. The calves excreted twelve to sixteen per cent of the total injected dose in the urine within the first four hours compared to eight to twelve per cent in the yearlings. This was an additional indication of a somewhat faster rate of metabolism in the calves than in the yearlings. The urine appeared to account for an appreciable quantity of the fluoride excreted by the animal, and may reflect the rapid drop observed in the blood level of F^{18}.

The renal clearance test used on both the oral and intravenously dosed animals showed a similar pattern of urinary excretion. There was an appreciable elimination of fluoride by the urinary pathway, and in both cases it reflected the blood level. The quantity at any given time was variable both as to concentration per milliliter and/or as to the total quantity excreted, so that the urinary level

in a single voidation was not a reliable indicator of the fluoride intake of the animal.

Concentration of F^{18} in selected bone tissue

Selected bones were taken from the animals after slaughter. In addition to the routine counting procedures for the radioactivity some of these tissues were analyzed for total fluoride by chemical analysis (52). This permitted calculation of specific activity and a comparison between the behavior of the radioactive fluoride and the fluoride ingested as sodium fluoride. Because of the variation in total activity between fluoride shipments and the variation in weights of the animals employed no attempt was made to dose the animals according to body weight. The actual concentrations of F^{18} in the tissues, expressed as per cent of the total dose per gram of tissue, and the amount by chemical analysis are presented in Table 20. However, to standardize the concentrations of radioactivity in the bone and remove variation due to dilution of the isotope because of variability in the size of the tissues involved, these values were computed to a 500 pound animal according to the following formula:

$$\text{Quantity in a 500 pound animal} = \text{quantity obtained} \times \frac{\text{actual weight}}{500} .$$

These data and the specific activity values are presented in

Table 20. Concentration of F^{18} in selected bone tissue of calves and yearlings compared to concentration of F as determined by chemical analysis

	Calves					Yearlings			
	Controls		100 p.p.m.	300 p.p.m.		Controls	100 p.p.m.	300 p.p.m.	
Minutes after dosing	394	131	420	360	240	295	403	347	120
Days on fluoride	39	55	39	46	55	39	46	55	55
F^{18} concentration[a]									
Rib shaft	32.1	39.2	41.8	37.1	45.8	15.9	16.4	21.4	19.5
Rib epiphysis	316.0	370.0	225.0	323.0	346.0	64.8	85.9	109.4	120.0
Femur shaft	24.8	19.5	18.0	52.4	6.3	18.6	14.5	6.4	5.0
Femur epiphysis	231.0	65.6	35.9	54.6	233.0	21.1	16.4	37.1	38.1
Mandible	66.6	46.4	29.6	75.2	38.5	41.1	36.8	30.8	50.3
Fluoride by chemical analysis[b]									
Rib shaft	.03		.15	.35		.03	.04	.11	
Rib epiphysis	.07		.49	1.10		.08	.56	1.20	
Femur shaft	.02		.05	.19		.02	.05	.09	
Femur epiphysis	.04		.21	.43		.04	.11	.19	
Mandible	.04		.15	.39		.07	.18	.24	

[a]Given as per cent of the total dose per gram of fresh tissue x 10^{-4}.

[b]Given as per cent.

Table 21. Concentration of F^{18} in selected bone tissue of calves and yearlings corrected to 500 pound animal

	Calves			Yearlings		
	Controls	100 p.p.m.	300 p.p.m.	Controls	100 p.p.m.	300 p.p.m.
Minutes after dosing	394	420	360	295	403	347
Days on fluoride	39	39	46	31	46	55
F^{18} concentration[a]						
Rib shaft	22.5	27.6	23.7	21.9	22.1	27.8
Rib epiphysis	221.2	148.5	206.7	94.4	115.1	142.2
Femur shaft	17.4	11.9	33.5	25.7	19.4	8.3
Femur epiphysis	161.7	23.6	34.9	29.1	22.0	48.2
Mandible	46.6	19.6	48.1	56.7	49.3	40.0
Specific activity[b]						
Rib shaft	.075	.018	.007	.073	.055	.025
Rib epiphysis	.316	.030	.019	.118	.020	.012
Femur shaft	.087	.024	.018	.128	.039	.009
Femur epiphysis	.404	.011	.008	.073	.020	.025
Mandible	.116	.013	.012	.081	.027	.017

[a]Given as per cent of the total dose per gram of fresh tissue x 10^{-4} corrected to 500 pound animal.

[b]Calculated as $\frac{\text{corrected total dose per gram of fresh tissue x } 10^{-4}}{\text{per cent F by chemical analysis}}$.

Table 21. Concentration and relative concentration of fluoride in the bone, as determined by chemical analysis, compared favorably with those reported by Hobbs et al. (25). There was little difference in the fluoride level of the selected bone tissues between the control calves and yearlings. Generally, however, there was a greater concentration of fluoride in selected bones of calves receiving 100 and 300 p.p.m. of dietary fluoride than in the yearlings receiving a similar amount. This was further evidence that the calves in this experiment had a somewhat faster metabolic rate than did the yearlings, and was similar to the pattern for Ca^{45} shown by Hansard et al. (21).

The concentration of fluoride in bone, as measured chemically, did not effect the rate of F^{18} uptake. There were no consistent differences between the animals on the three levels of fluoride in the ration. For example, at the 300 p.p.m. intake level, the rib epiphysis of the calf contained 11,000 p.p.m. of fluoride by chemical analysis and the yearling contained 12,000 p.p.m. of fluoride, and yet in both these animals the concentration of the isotope was about the same as that of the control animals having only 700 and 800 p.p.m. of fluoride in the bone. It is possible that part of this may have been due to the lack of time for equilibrium to be established. The rib epiphysis was the most actively metabolizing bone tissue studied,

having a concentration of from two to ten times that of the shaft or any other bone tissue studied. This is again similar to the pattern for calcium reported by Hansard et al. (21) and is further evidence of the similarity of metabolic behavior of calcium and fluoride.

Generally there was a slightly higher concentration of the isotope in the bone tissue of the calves on the weight corrected basis. The difference in rib epiphysis concentration was significant ($p=0.05$). However, there were no other significant differences between bone tissues of calves and that of yearlings.

The interval of time between dosing and slaughter did not seem to have much effect on the concentration of the isotope in the bone tissue. Bone appeared to remove the fluoride very rapidly from the bloodstream, and once the blood level was reduced little further concentration occurred in the bone. This reflects the disappearance rate of the isotope from the bloodstream mentioned earlier, and shown in Graph 4. This would indicate also the rapid rate that bone removes excess fluoride from the bloodstream and the speed at which equilibrium was established in the animal body. Due to the short half life of F^{18} it was not possible to determine the exchange rate between the bone and blood as it would have been with an isotope of longer half life.

The ratio of radioactive fluoride to total fluoride in

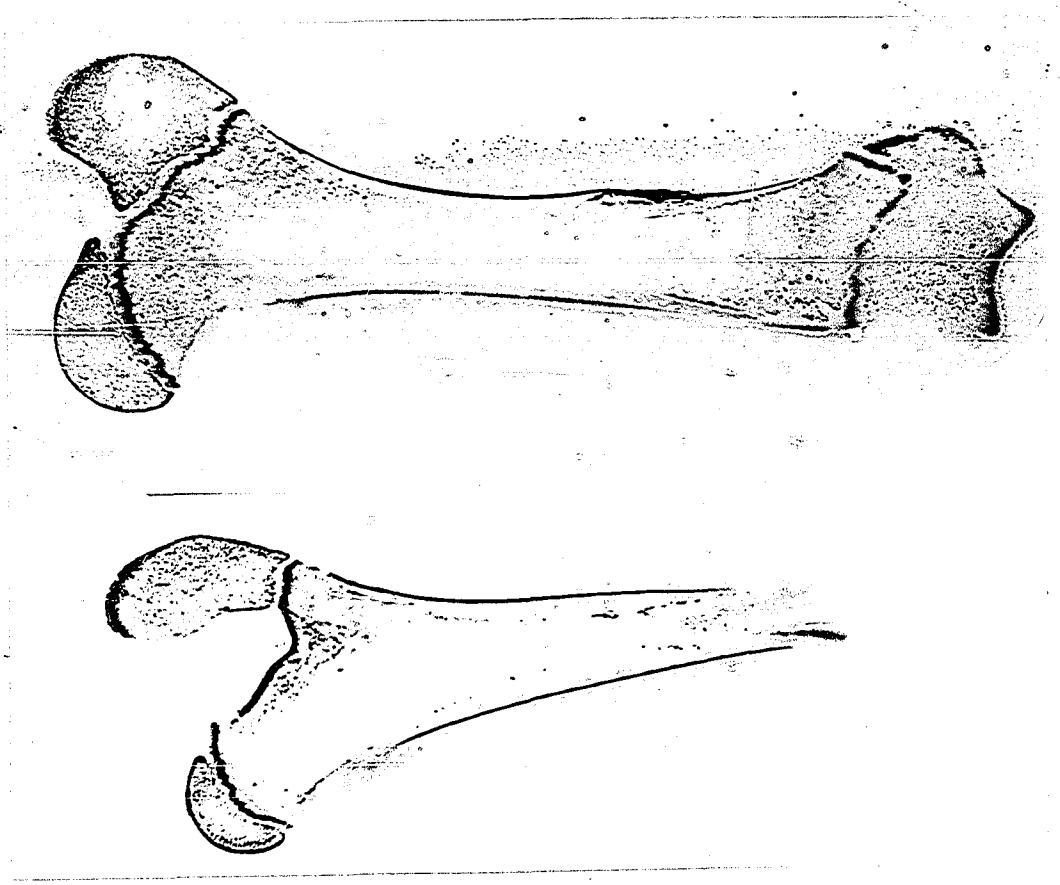
the bone is given in Table 21 expressed as specific activity. It is noted that specific activity decreased with increased fluoride intake in both the calves and the yearlings in every instance except the femur epiphysis of the yearling on the 300 p.p.m. intake level. This was a reflection of total fluoride present since F^{18} values were apparently not affected and would indicate that the bone was not saturated with fluoride at the time of slaughter. Thus the role often assigned to bone tissue (36), that of being a safety mechanism for removal of fluorides from the bloodstream to reduce its harmful effects, still would seem to be in operation with the bone tissue studied in this experiment.

Autoradiograms in Plate 1 produced by the F^{18} in the bone further exemplify these values and illustrate the pattern of laydown in bone. These show the laydown of fluoride to be greatest in the more active portion of the bone and thereby was similar to the laydown of calcium. The epiphysis showed the greatest concentration followed by the periostium and then the trabecular bone area. In one yearling receiving 300 p.p.m. of fluoride, it was possible to obtain a picture from a molar that had not yet erupted. This tooth showed a very heavy concentration of the isotope indicating that this unerupted tooth was in a very active state of metabolism, and that fluoride tends to be concentrated in this tissue. Hobbs et al. (25) have

shown that when cattle ingest fluorides before the eruption of their permanent molars, these teeth tend to be softer and wear much faster than those teeth that have already erupted when fluoride ingestion begins. If, as it has been suggested, this wear is due to a softening of the enamel by the interference of fluorides with regular calcium and/or enamel laydown, this autoradiogram would lend support to the theory by showing a very heavy concentration of fluoride in this tissue.

One animal in each lot was initially given a tracer dose of Ca^{45} in an attempt to determine if there was any difference in the bone growth rate of the animals receiving fluoride, and to see if there were any differences in the calcium laydown pattern. At sacrifice the bones from these animals were set aside for forty-eight hours to permit the decay of all radioactive fluoride and then exposed to x-ray film for the autoradiograms of Ca^{45}. These are presented in Plates 2 and 3 which are of the calves and yearlings respectively. In neither the calves nor the yearlings was there any measurable difference in the growth rate of the bone for the duration of this experiment. The laydown pattern of the calcium isotope was very similar to those presented by Hansard (21) indicating that there is little if any difference in the calcium laydown pattern. When the pattern of calcium laydown in Plate 2 is compared to the

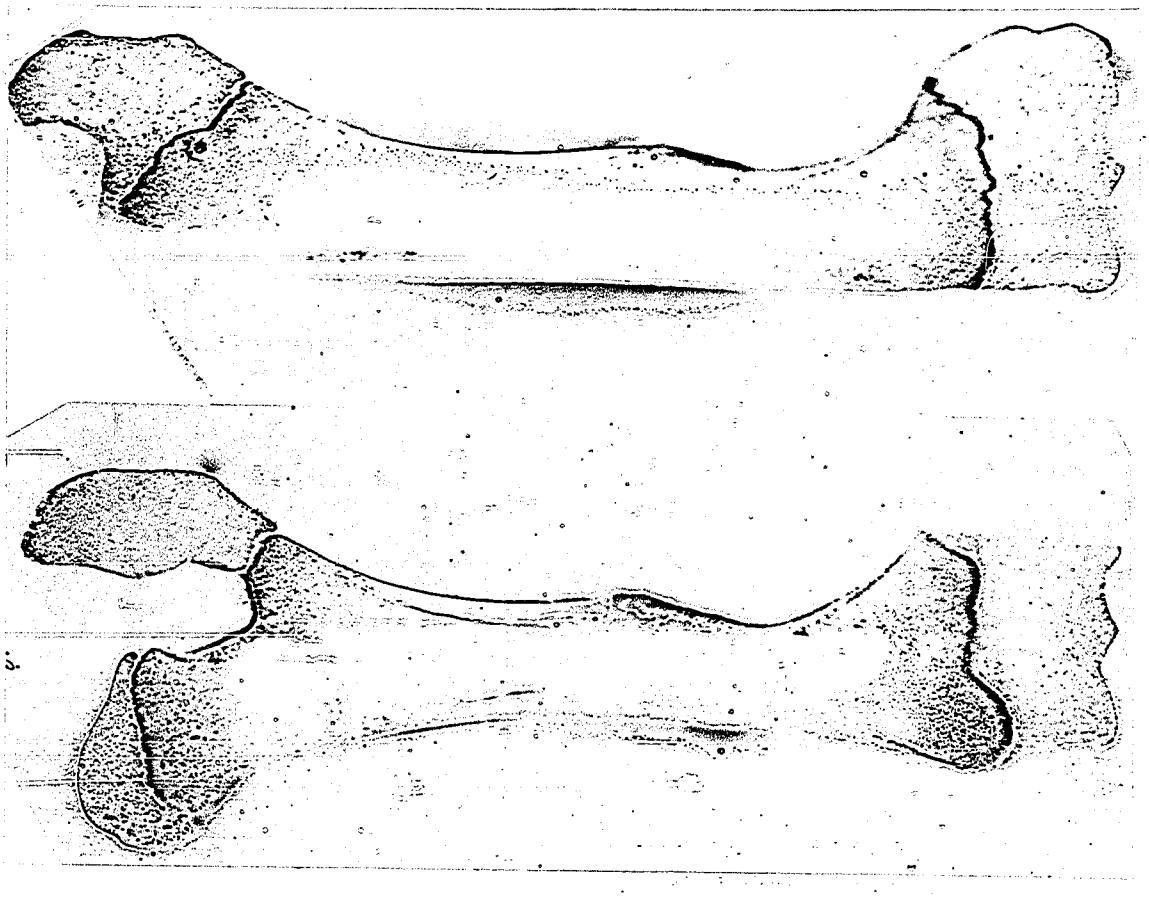
Calf 1718 Control



Calf 1731 receiving 100 p.p.m. fluoride

Plate 2. Distribution of Ca^{45} in a calf bone

Yearling 1214 Control



Yearling 1330 receiving 300 p.p.m. fluoride

Plate 3. Distribution of Ca^{45} in a yearling bone

pattern of fluoride laydown in Plate 1, it is noted that it is hard to tell any difference between the two. This is still further support to the similar behavior of fluoride and calcium.

Concentration of F^{18} in selected soft tissues

The level of the isotope in selected soft tissues obtained is presented in Table 22 and the concentration corrected to a 500 pound animal in Table 23. This correction was made in the same manner as reported earlier in the section on bone tissue. The data in these tables were arranged as a function of time after sacrifice. It is noted, generally, that the concentration in a tissue goes down with time, regardless of the dietary level of intake. This reflects the blood disappearance curve data reported earlier.

The difference in level of the isotope in the gastroneimus muscle between calves and yearlings is pronounced. This lower tissue level in the calves reflects the higher metabolic rate, the lower level in the blood observed earlier, the faster elimination through the urine, and the slightly higher level in the bone at sacrifice. If there was to be interference of fluoride with the carbohydrate metabolism and/or an alteration in the level of the metabolites by the fluoride, it appears feasible that the

Table 22. Concentration[a] of F^{18} in selected soft tissues of calves and yearlings

Treatment	Minutes from dosing to sacrifice	G. muscle	Thyroid	Adrenal	Liver	Bile	Spleen	Kidney
			Calves					
Control	131	2.02	1.87	.92	1.75	2.65	1.02	5.32
300 p.p.m.	240	.53	1.39	.43	[b]	.59	.55	2.82
300 p.p.m.	360	.00	1.60	.00	1.05	.85	1.15	2.07
Control	394	.23	.88	.19	.66	.42	1.01	.98
100 p.p.m.	420	.43	1.34	.00	.95	.89	.97	1.67
			Yearlings					
300 p.p.m.	120	1.84	1.07	.72	.97	2.36	.76	4.69
Direct from pasture	120	1.64	1.15	.99	1.99	.99	1.29	3.64
300 p.p.m.	347	.53	1.08	.29	.51	1.72	.37	1.46
100 p.p.m.	403	.51	.90	.00	.45	.88	.34	2.16

[a]Per cent of total F^{18} dose per gram of tissue x 10^{-4}.

[b]Because of mechanical difficulties the sample could not be counted.

Table 23. Concentration[a] of F^{18} in selected soft tissues of calves and yearlings corrected to 500 pound animal

Treatment	Minutes from dosing to sacrifice	G. muscle	Thyroid	Adrenal	Liver	Bile	Spleen	Kidney
			Calves					
Control	131	1.51	1.40	.69	1.31	1.99	.76	3.99
300 p.p.m.	240	.34	.90	.28	[b]	.38	.36	1.83
300 p.p.m.	360	.00	1.02	.00	.67	.54	.74	1.32
Control	394	.16	.62	.13	.46	.29	.71	.69
100 p.p.m.	420	.28	.88	.00	.63	.59	.64	1.10
			Yearlings					
300 p.p.m.	120	2.72	1.58	1.06	1.43	3.49	1.12	6.94
Direct from pasture	120	1.89	1.32	1.14	2.29	1.13	1.48	4.19
300 p.p.m.	347	.69	1.40	.38	.66	2.24	.48	1.90
100 p.p.m.	403	.79	1.39	.00	.69	1.35	.52	3.33

[a]Per cent of total F^{18} dose per gram of tissue x 10^{-4}.

[b]Because of mechanical difficulties these samples could not be counted.

higher concentration present in the muscle of the yearlings would be expected to give more pronounced differences in the older group. The blood glucose levels, to be discussed later, did not show these expected differences. Thus the level of fluoride in the muscle, as indicated by the isotope, did not seem to interfere with the blood glucose level.

Three of the tissues studied, thyroid, liver, and spleen, seemed to have some retention of the isotope independent of the blood level. The rather consistent residual level in the thyroid is interesting. Fluoride and iodide are both members of the halogen family, and the latter is part of the thyroxine which aids in regulating body activity. Whether some of the fluoride may have replaced some of the iodide with a resulting effect on body activity was not studied. However, if this happened it might help to explain the reduced feed intake and lower body weight of mature animals reported by Hobbs et al. (25). This thyroid fluoride level was particularly higher in the yearlings on a weight corrected basis, and was significant ($p<0.05$).

The liver appeared to aid in removal of the fluoride from the bloodstream and concentrate it in the bile. The yearlings had a higher level of the isotope in the liver shortly after administration, but after approximately six hours there was little difference. This difference in the concentration in bile on a weight corrected basis was highly

significant (p-0.01) between calves and yearlings. The bile enters the small intestine and this would help account for the rapid appearance of the isotope in the intestinal tract.

There seemed to be a difference in the behavior of the spleen in concentrating the isotope between calves and yearlings. The calves had a rather consistent level of the isotope in the spleen over the two to seven hour period. The yearlings on the other hand had about two and a half times as much at two hours as at approximately six hours. This is another example of the difference in the metabolic patterns between calves and yearlings.

The lower concentration of F^{18} in the kidney of the calves compared to the yearlings was significant (p-0.05) on the weight corrected basis. The lower level in the kidney of the calves at sacrifice would be expected inasmuch as there was a higher level in the urine and more total F^{18} excreted by the calves. The calves appeared to remove the isotope at a faster rate than did the yearlings. However, the difficulty encountered with the retention catheters in the yearlings could be partially responsible for the higher residual levels in the kidneys of the yearlings.

The actual level of the isotope in the walls of the gastro-intestinal tract is presented in Table 24 and the level in the contents is presented in Table 26. Tables 25 and 27 show the data corrected to a standard 500 pound

Table 24. Concentration[a] of F^{18} in walls of the gastro-intestinal tract of calves and yearlings

Treatment	Minutes from dosing to sacrifice	Rumen	Omasum	Abomasum	First 1/2 S. inst.	Last 1/3 S. inst.	Large inst.
			Calves				
Control	131	1.72	2.81	1.34	1.26	1.47	1.43
300 p.p.m.	240	1.50	1.64	.63	.58	.60	1.18
300 p.p.m.	360	1.76	5.70	.45	.48	.32	.60
Control	394	1.22	.27	2.64	.21	.25	.35
100 p.p.m.	420	.27	1.46	.60	.28	.33	.59
			Yearlings				
300 p.p.m.	120	.83	.97	.00	.00	.00	.43
Direct from pasture	120	1.99	2.54	1.02	1.00	2.01	1.10
300 p.p.m.	347	1.15	1.12	.44	.35	.32	.57
100 p.p.m.	403	2.64	1.68	1.01	1.32	1.09	.92

[a]Per cent of total F^{18} dose per gram of tissue x 10^{-4}.

Table 25. Concentration[a] of F^{18} in walls of the gastro-intestinal tract of calves and yearlings corrected to 500 pound animal

Treatment	Minutes from dosing to sacrifice	Rumen	Omasum	Abomasum	First 1/2 S. inst.	Last 1/3 S. inst.	Large inst.
			Calves				
Control	131	1.28	2.09	1.00	.94	1.10	1.07
300 p.p.m.	240	.97	1.06	.41	.38	.39	.76
300 p.p.m.	360	1.13	3.65	.29	.31	.20	.38
Control	394	.85	.19	1.83	.15	.17	.24
100 p.p.m.	420	.18	.96	.40	.18	.22	.39
			Yearlings				
300 p.p.m.	120	.93	1.08	.00	.00	.00	.48
Direct from pasture	120	2.29	2.93	1.17	1.15	2.32	1.27
300 p.p.m.	347	1.50	1.47	.57	.46	.42	.74
100 p.p.m.	403	3.02	1.92	1.16	1.15	1.24	1.05

[a]Per cent of total F^{18} dose per gram of tissue x 10^{-4}.

Table 26. Concentration[a] of F^{18} in contents of the gastro-intestinal tract of calves and yearlings

Treatment	Minutes from dosing to sacrifice	Rumen	Omasum	Abomasum	First 1/2 S. inst.	Last 1/3 S. inst.	Large inst.
			Calves				
Control	131	1.30	1.88	1.99	1.38	3.34	2.80
300 p.p.m.	240	1.06	1.45	.46	.56	.71	5.03
300 p.p.m.	360	.93	.00	.00	.50	1.08	2.86
Control	394	.57	1.04	.34	.30	.33	1.60
100 p.p.m.	420	1.31	1.49	.57	.42	.56	1.43
			Yearlings				
300 p.p.m.	120	.94	1.15	1.20	1.01	4.90	1.98
Direct from pasture	120	.60	.26	.42	5.68	1.62	5.05
300 p.p.m.	347	.72	1.18	.46	.44	.79	2.28
100 p.p.m.	403	.39	.00	.00	.33	.75	1.51

[a]Per cent of total F^{18} dose per gram of contents x 10^{-4}.

Table 27. Concentration[a] of F^{18} in contents of the gastro-intestinal tract of calves and yearlings corrected to 500 pound animal

Treatment	Minutes from dosing to sacrifice	Rumen	Omasum	Abomasum	First 1/2 S. inst.	Last 1/3 S. inst.	Large inst.
			Calves				
Control	131	.97	1.40	1.48	1.03	2.48	2.08
300 p.p.m.	240	.68	.94	.30	.36	.46	3.26
300 p.p.m.	360	.60	.00	.00	.34	.69	1.83
Control	394	.40	.72	.24	.21	.23	1.11
100 p.p.m.	420	.86	.98	.38	.28	.37	.94
			Yearlings				
300 p.p.m.	120	1.04	1.28	1.34	1.13	5.47	2.21
Direct from pasture	120	.69	.30	.48	6.54	6.54	5.82
300 p.p.m.	347	.94	1.53	.60	.57	1.03	2.96
100 p.p.m.	403	.45	.00	.00	.38	.86	1.73

[a]Per cent of total F^{18} dose per gram of contents x 10^{-4}.

animal. The isotope appeared in appreciable quantities in both the wall of the intestinal tract and the contents. The quantity in the walls, particularly in the forepart of the tract, was probably due to the blood supply to these organs. The levels in the gastro-intestinal walls were erratic either when arranged according to time or by the concentration of fluoride in the ration. This no doubt was partially due to individual animal variation. Generally the levels in the intestinal wall seemed to be a little higher in the yearlings than in the calves. The contents of the tract showed measurable levels within two hours following intravenous administration, the shortest time interval between dosing and slaughter, and for the duration of the experiment to seven hours. This would indicate the isotope enters the gastro-intestinal tract directly from the bloodstream. This behavior is similar to that of Ca^{45} previously reported by Hansard (21). The difference in the concentration in the last third of the small intestine was significant (p-0.05) while the probability of the difference in the large intestine being a real difference was p-0.80. This indicated the elimination of fluoride by way of the fecal material was one of the pathways for excretion of absorbed material. Yearlings seem to make more use of this pathway than do the calves.

These differences in the behavior of the isotope between calves and yearlings indicate a real age difference.

The calves apparently are metabolizing at a somewhat faster rate than the yearlings. The higher level of the isotope in the rib epiphysis, the more rapid removal from the blood-stream, the greater total quantity eliminated in the urine are all evidences pointing in this direction. The yearlings showed a somewhat higher level of the isotope in the blood-stream, at least for the time intervals studied in this experiment reflecting the slower methods for removal of fluoride. This would indicate that the tissue level should remain somewhat higher for the yearlings. This trend was observed in the muscle tissue studied. In addition to the cumulative effect of fluoride in the older animals, this reduction in removal could be a contributing factor to the appetite depression and reduction in weight reported by Hobbs (25). This increase in level of isotope in the muscle tissue studied did not, however, interfere with the level of blood glucose which will be reported in the next section.

The use of the isotope has demonstrated the rapid removal of fluoride from the body tissues either through the elimination pathways of the urine, or into the storage areas of the bone. The animal appears to try to remove this from the body proper at a rapid rate. It confirmed some of the ideas previously held concerning fluoride; that the fluoride behaves in many ways similar to calcium,

that single voidations of urine are not adequate criteria of the fluoride intake of the animal, and that the fluorides tend to be concentrated in the bone. It also demonstrated some new ideas concerning the behavior of fluoride; that there are differences in the behavior of the material depending upon the physiological age of the animal, that the absorption from the gastro-intestinal tract is rather slow to aid in keeping the blood level low, that removal of fluoride from the bloodstream is fairly rapid, that fluorides appear in the saliva in appreciable quantities within a very short space of time after entering the bloodstream, that fluorides reenter the gastro-intestinal tract in rather appreciable quantities after getting into the bloodstream, and that fluorides appear to be concentrated in the thyroid, liver and spleen regardless of the blood level. It has also demonstrated the F^{18} has serious limitations as a biological tool due to its short half life and high levels of radiation. This makes it impossible to study many of the equilibrium reactions that would give a great deal more information concerning the behavior of fluoride.

Glucose level of calves and yearlings fed fluoride

Average blood glucose levels for calves used in this study are presented in Table 28. The animals were bled approximately four to five hours after the morning feeding.

Table 28. Blood glucose levels of calves and yearlings

Treatment	Pre F feeding	1 day	2 day	3 day	4 day	5 day	8 day	11[a] day	15 day	18 day	22 day	25 day
						Calves[b]						
Control	69.5	75.7	75.3	64.0	64.3	64.6	50.9	51.2	47.7	49.2	46.7	48.5
100 p.p.m.	68.7	85.3	71.0	72.2	63.5	62.4	48.7	52.3	50.7	48.5	50.2	47.8
300 p.p.m.	67.8	76.1	80.2	68.1	67.6	37.8**	61.1	54.5	50.2	48.6	43.2	48.2
						Yearlings[c]						
Control	83.2	73.5	74.7	62.2	63.4	71.5	52.5	58.6	51.1	47.9	51.8	47.9
100 p.p.m.	78.3	74.2	73.8	71.9	60.6	50.3**	46.0	44.6	49.6	48.3	47.3	49.2
300 p.p.m.	76.3	78.5	74.1	67.5	56.1	52.9*	48.1	51.4	49.2	50.7	50.5	46.4

[a]Grain reduced from 4 pounds to 3 pounds per head daily due to failure of 300 p.p.m. lot to consume it all.

[b]Average intake per calf was 4 pounds grain, 4 pounds hay per day.

[c]Average intake per yearling was 4 pounds grain, 8 pounds hay per day.

* Significant at the 0.05 level.

** Significant at the 0.01 level.

It was assumed that the two weeks adjustment period from pasture to the hay and grain feeding would also stabilize the blood glucose levels. These data, however, indicate a steady decline in the glucose blood level until the end of the first week of study. At this time the levels seemed to stabilize at about 45 to 50 milligrams per 100 cc. of blood which is within the normal limits indicated by Dukes (10), but toward the lower limits of the normal range. This decline in blood glucose level in all lots might be due to the adjustment involved in going from a gaining condition to a maintenance condition. This decline was about the same in all lots until about the fifth day. At this time the three lots showing significant differences (p-0.05) consumed practically no grain during the morning. They then consumed the grain for several days before again starting to leave some of the grain, necessitating adjustment of the grain intake of the twelfth day. Inasmuch as these lots showed no further significant differences, it is believed that the differences in glucose level shown on the fifth day are due to the reduction in grain intake and not to the added dietary fluoride. It was noted that the data presented showed no differences in the blood glucose levels between the lots on the varying fluoride levels during the first 25 days of fluoride feeding. This substantiated the work reported by Hobbs (25) in which no blood glucose

differences could be demonstrated on cattle that had been on 100 p.p.m. of fluoride added for several years. While there were differences in the behavior of the radioactive fluoride between calves and yearlings and differences in the level of the isotope in the blood and the muscle tissue, these differences were not reflected in blood glucose level.

The high level of fluoride intake, 300 p.p.m. in the ration, reduced the feed intake to the maintenance level or slightly below. This adjustment was accomplished by a consistent drop in the blood glucose level by all lots and this decline appeared to be due to the reduction of feed intake rather than the dietary intake of fluoride.

Blood lactate was determined on individual animals during the first five days. The level variation for the nine calves on the day before starting the fluoride feeding was from 13.1 to 55.1 milligrams per cent, and from 16.5 to 84.2 milligrams per cent for the yearlings. This individual variation, although not usually as pronounced, continued during the five days blood lactate was determined. In comparing the values obtained with the notes made during the bleeding, it was found that the more excited the animal became during the bleeding time, the higher was the lactate level. The determinations were, therefore, discontinued and the values obtained are not included in this report.

GENERAL DISCUSSION AND SUMMARY

The results of the experiments reported in this thesis show that (1) the effect of fluoride concentration on cellulose digestion by rumen microorganisms *in vitro* was not a straight line relationship, (2) that there was an interrelationship between fluoride, magnesium and manganese on the digestion of cellulose by rumen microorganisms *in vitro*, (3) that the ingestion of 300 p.p.m. of fluoride by weanling and adult albino rats resulted in alterations in the level of blood glucose and lactate, and liver glycogen in both age groups but that there were differences in these alterations dependent upon age, (4) that the administration of acute massive doses of fluoride in nursing lambs and pigs resulted in marked alterations in the level of blood glucose and lactate and liver glycogen, (5) that there was a species difference between the lambs and the pigs in the tolerance of acute massive doses of fluoride both as to level and site of injection, (6) that there were differences between calves and yearling cattle in the metabolism of radioactive fluoride, and (7) that the use of radioactive fluoride confirmed previous reports of similarities between fluoride and calcium.

It is difficult to explain why the addition of 100 p.p.m. of fluoride added to *in vitro* suspensions of rumen

microorganisms resulted in cellulose digestion similar to the control and significantly higher than the 50 and 150 p.p.m. additions which in turn were essentially the same. Fluoride has been shown to interfere with carbohydrate metabolism at a number of points. However, it has usually been used at high levels in order to demonstrate accumulation of intermediate metabolites. It might be that the concentration of fluoride needed to accomplish this blocking effect varies at the different possible blockage points. It has also been shown that there are several alternate metabolic pathways. The concentration of fluoride present may influence the degree to which these alternatives are used. An extensive study, using many levels between 0 and 150 p.p.m. of fluoride, would be necessary to clarify this pattern. It would also require the study of intermediate metabolites and end products of the digestion present at different times to clarify the problem. This failure of the microorganisms to react in a direct straightline relationship to fluoride concentration does help, however, to explain some of the differences noted between various workers as to the response of animals ingesting similar levels of fluoride.

The interrelationship between magnesium, manganese and fluoride shown by workers using single strains of microorganisms was also demonstrated using mixed suspensions of

rumen microorganisms. Manganese was used by these mixed cultures to replace magnesium to a large degree indicating that the manganese ion can be used to replace the magnesium ion partially in the metabolic cycle. When both manganese and magnesium were omitted from the inoculum there was a highly significant reduction in cellulose digestion. The addition of fluoride up to 200 p.p.m., did not then show any additional changes in cellulose digestion. When the level of magnesium was doubled there was a significant increase in cellulose digestion compared to the control. With this increased concentration of magnesium the digestion of cellulose obtained with the addition of 50, 100, and 150 p.p.m. of fluoride was similar to the control with normal magnesium. However, when the magnesium content was increased to ten times that of the control the highest digestibility obtained was with the addition of 50 p.p.m. of fluoride. This interaction between fluoride, magnesium and manganese can be explained, in part at least, by the degree of insolubility of the magnesium-fluro and manganese-fluro complexes formed.

Other workers had demonstrated that the albino rat could consume 300 p.p.m. of fluoride in the ration without seriously effecting the growth rate and that this level would cause large accumulations of fluoride in the bone tissue. This work demonstrated there was an alteration in the blood glucose and lactate and liver glycogen of rats

receiving this level of fluoride. These changes normally occurred within the first three to four weeks and then returned to nearly normal levels. This would indicate that when fluoride is first ingested either the elimination mechanism does not function immediately and consequently the body concentration of fluoride is high enough to interfere with carbohydrate metabolism, or else there is a time lag in the animal in shifting to an alternate metabolic pathway. It also demonstrated that there was a difference between the weanling and adult albino rat in adjusting to chronic fluoride intake. This could be due to the difference in metabolic rate between the two age groups or it might be explained by the fact that the adult rats were obese and had well developed metabolic pathways for the use of body fat as an alternative to the utilization of ingested carbohydrate.

The failure to obtain nearly identical results between the two trials and the differences noted between the blood glucose and lactate levels and liver glycogen levels between the trials would indicate that the fluoride effect in carbohydrate metabolism varies somewhat with the metabolic condition of the animal. Previous work showing large differences between fasted and non-fasted animals would lend support to this hypothesis. Thus when comparisons are being made between various experiments concerning the toxic effect of materials it is necessary to know, insofar as possible, the

metabolic condition of the animal before drawing comparisons.

The use of acute toxic doses of fluoride 200 to 250 mgs. per kilogram of body weight with lambs and piglets resulted in marked changes in blood glucose and lactate and smaller changes in liver glycogen. Differences in the tolerance to administration site of the fluoride were demonstrated between suckling lambs and suckling pigs. The lambs used lived from two to four hours after the injection of fluoride into the intraparateneal cavity. Pigs injected with this same dosage level, 250 mg. per kilogram, lived for only thirty to sixty minutes. It was necessary to reduce the dosage to 200 mg. per kilogram and inject it subcutaneously in the abdominal fat layer in order that the pigs might survive for four hours. A further difference noted was that there was only light pinpoint hemorrhage along the gastro-intestinal tract of the lambs while there was evidence of massive pinpoint hemorrhage along the gastro-intestinal tract or in the fat layer immediately surrounding the injection site in the pigs. The reasons for this difference in reaction between lambs and pigs is not known. The marked elevation of blood glucose and lactate and the reduction of liver glycogen indicates a somewhat similar behavior pattern between these farm species and the adult albino rat that was used by Handler. It further served to illustrate, however, as did the work mentioned above that it is necessary to

establish a similar metabolic base before making comparisons.

Hereford heifer calves, seven to eleven months of age, and Hereford yearling heifers, seventeen to twenty-two months of age, were used to determine whether there was an alteration in the blood glucose and lactate as there had been in the rats when placed on a ration containing 300 p.p.m. of fluoride. No differences were noted between the controls and the animals receiving 300 p.p.m. of fluoride in either group. There was a decline in the blood glucose levels but this decline was uniform in both the controls and fluoride fed animals and seemed to be correlated to the level of feed intake. The fluoride did reduce the feed intake but the control animal being pair-lot fed had a corresponding caloric reduction.

Cattle from both the controls and fluoride fed lots were then dosed with F^{18} and studies of the distribution pattern of the isotope were made. Oral dosage of the isotope resulted in very low blood levels. However, measurable quantities of the dose appeared in the urine within eleven minutes after administration. The isotope, because of its short half life and high radiation rate, is not a good biological tool for oral administration and therefore was used, in the majority of this work, by means of intravenous injection.

The age differences noted between the weanling and adult rats were also noted between the calves and the yearlings. This was evidenced by a significant difference between the age groups in the removal of the isotope from the bloodstream. This disappearance curve in both age groups was similar to the disappearance curve obtained by Hansard with Ca^{45}.

The technique used to determine the amount of isotope that would appear in the saliva did not subject the animal to surgery. It demonstrated that fluoride, once absorbed into the bloodstream, returns to the gastro-intestinal tract by way of the saliva in appreciable quantities within eleven minutes, and can again become a part of the metabolic pool. Due to the very short half life of the isotope it was not possible to determine metabolic turnover as has been done with isotopes of longer half life.

Renal clearance studies were made to determine both the rate and extent of elimination of fluoride in the urine. Individual accumulations in which the isotope concentration was multiplied by the volume of urine eliminated resulted in very erratic readings. This substantiated other work that reported single voidations were no criteria of current fluoride intake. However, when the voidations were cumulatively totaled a rather smooth, uniform curve was obtained. Twelve to sixteen per cent of the fluoride injected into the

calves appeared in the urine within the first four hours compared to eight to twelve per cent in the yearlings.

Separation of the blood showed that about seventy-five per cent of the total blood fluoride appeared in the plasma and that about half of the isotope in the plasma was present in the protein-free fraction.

Due to differences in body weight the tissue concentration was corrected to the equivalent of a five hundred pound animal. The length of time that these animals had been on intakes of 100 and 300 p.p.m. of fluoride did not interfere with the rate of uptake of the isotope by bone tissue even though the animals receiving 300 p.p.m. of fluoride at the time of injection of the isotope had approximately fifteen times as much fluoride in their bones when measured by chemical analysis. The length of time between administration of the isotope and sacrifice of the animal, two to six hours, did not appear to affect the fluoride level of the bone indicating that the bone removes fluoride from the bloodstream very rapidly and that once blood levels are reduced there is little further concentration of the isotope in the bone.

New techniques to develop autoradiograms of the distribution of the isotope in bone tissue were developed. This was necessitated by the production of high speed gamma rays by the isotope and its short half life. It again

demonstrated the similarity of metabolism of both calcium and fluoride.

Three of the soft tissue studies, thyroid, liver and spleen, appear to have some retention of the isotope independent of the blood level. The yearlings had a significantly higher retention of the isotope in the thyroid than did the calves and a highly significant difference in the concentration in the bile. The yearlings also had a significantly higher concentration of the isotope in the kidneys. This may have been accounted for in part by the difficulties encountered with the catheters, however.

Both the lining of the gastro-intestinal tract and the contents of the tract showed appreciable levels of the isotope within the shortest time intervals studies between administration and sacrifice, two hours. This would indicate that the isotope reentered the gastro-intestinal tract from the bloodstream.

The use of F^{18} confirmed previous studies that had demonstrated that fluoride and calcium behaved similarly in many respects, that single urinary voidations are not adequate criteria of current fluoride intake and that fluoride tends to be concentrated in bone tissue. In addition it was demonstrated that there are differences in the metabolic pattern of fluoride depending upon physiological age, absorption from the gastro-intestinal tract is slow, that

fluoride is rapidly removed from the bloodstream, that fluoride rapidly appears in the saliva once it gets into the bloodstream and that fluoride appears to be concentrated in the thyroid, liver and spleen.

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