SOME BIOLOGICAL EFFECTS OF DIETARY FLUORINE IN GUINEA PIGS WITH PARTICULAR REFERENCE TO INCISOR TOOTH AMELOBLAST AND ODONTOBLAST HEIGHTS.

by

DOUGLAS GEORGE DALE

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Department of Nutrition,
Macdonald College of McGill University,
Montreal.

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Douglas George Dale

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ABSTRACT.

Two six-week fluorine feeding trials were conducted with young growing guinea pigs as test animals. Basal diets were prepared that contained 75% hay since the ultimate aim of the experiment was to establish a bioassay for fluorine in forage crops.

Sodium fluoride was added to the basal diets of some groups to achieve levels of fluorine ranging from 19 to 130 p.p.m. To other groups, fluorine-containing hay grown in the vicinity of a large industrial operation was fed, resulting in dietary fluorine levels of the hay-meal ration ranging from 60 to 112 p.p.m.

Fluorine in the diet depressed the feed intake and weight gains of guinea pigs when its level in the complete diet approached 70 p.p.m. The heights of incisor ameloblast and odontoblast cells as measured microscopically from longitudinal sections of the guinea pigs' mandibles were depressed progressively by increasing increments of dietary fluorine, as was also digestion of the crude fibre in the diet. Bone storage of fluorine increased as the level of fluorine in the diet increased. When the p.p.m. of fluorine in the bone ash were plotted against the logarithm of the p.p.m. of fluorine in the diet the relationship was essentially linear. The regression of fluorine in

bone ash on dietary fluorine was of sufficient magnitude to suggest that bone storage could be used as a criterion in a fluorine bioassay. As judged from the various indices examined, fluorine in the form of contaminated hay appeared to be less available to the organism than fluorine in the form of its sodium salt.

INTRODUCTION.

Farm animals are exposed to dietary fluorine from a number of sources. The element is present in small amounts in most natural feedstuffs so that a completely fluorine-free diet probably does not exist outside of the laboratory. Fluorine is also found in higher than average concentrations in certain water supplies, in raw soft phosphates used as mineral supplements and in foliage grown in the vicinity of certain industrial operations.

The various species of domestic animals are thought to exhibit differences in the degree of their fluorine tolerance, and in this regard it is generally accepted that ruminant animals are the most susceptible to chronic fluorine poisoning.

Probably due to economic factors, dairy cattle have received most attention by investigators of chronic fluorosis of domestic animals in North America. In this species structural defects of the permanent teeth that erupt during or immediately following prolonged excessive fluoride intake is the first and most easily recognized symptom. This particular lesion, which is in effect an enamel hypoplasia, frequently is referred to as "dental fluorosis." Dental fluorosis of moderate severity may occur as the only manifestation of elevated fluorine intake and in such cases it is doubtful if the economic value of the animal is seriously affected.

At rather poorly defined higher fluorine intake levels however, skeletal changes, lameness and more generalized symptoms such as decline in appetite, body weight, and milk production occur.

Investigations and literature reviews over a number of years have indicated that under experimental conditions an intake of fluorine, as sodium fluoride, below one milligram per kilogram body weight per day continued over periods of several years has resulted in no measurable economic damage to dairy cows. At intakes above this level, and depending on length of exposure and possibly other factors such as nutritive status, there is the possibility of economically important damage.

It is often necessary for field investigators to decide whether or not animals consuming feed or water with elevated fluorine content are suffering from fluorosis to the extent that economic damage has occurred. An approximation of the daily fluorine intake can be arrived at by calculation based on the chemical analysis of the feed and water for its fluorine content. When this approach is taken it is occasionally observed that animals under field conditions appear to suffer economic damage at levels of fluorine intake that failed to seriously impair the economic value of the animals of the same species under experimental conditions. While it is realized that the uncontrolled factors of heredity, nutrition and management that are difficult to assess in field cases, no doubt play a significant part in this problem, still the conjecture has arisen that perhaps fluorine in the form of contaminated foliage may exert a different biological effect than fluorine in the form of the sodium salt, which is the form of fluorine employed in most feeding experiments.

It is felt that one approach to the question of the relative toxicity of different sources of fluoride would be the development of a suitable bioassay. A bioassay may have advantages over chemical determination of fluorine in feedstuffs, especially in the assessment of the probable toxicity of particular sources of fluoride. The major problem would be to find a numerically expressed criterion of fluorine intake in some readily available laboratory animal. The effect of ingestion of varying levels of different sources of fluoride could then be compared.

There is acceptable evidence that the incisor teeth of the rat and other laboratory and domestic animals are one of the most sensitive biological indicators of elevated fluorine intake. Both the enamel and the dentin of the tooth are affected and since the formation of these structures is a function of the ameloblast and odontoblast cells respectively it would be anticipated that changes in these cells would occur prior to the gross changes in the tooth. Indeed, Schour and Smith (1934) have reported derangements of the ameloblasts and odontoblasts in experimental fluorosis.

A depression in height of the incisor odontoblasts occurs as a result of vitamin C deficiency in guinea pigs and use has been made of this fact in a bioassay technique for vitamin C developed by Crampton et al. (1944) at this laboratory. The guinea pig is able to consume a reasonably large amount of roughage in its diet and since from the practical point of view, one of the most useful aspects of a bioassay for fluorine would be in assessing the toxicity of fluorine-containing forage crops, this species would appear to be ideally suited

as a test animal. It therefore seemed logical to examine histologically the incisor teeth and associated tissues of young growing guinea pigs that had ingested fluorine at several intake levels. It was considered that there was a definite possibility that the heights of the ameloblast and, or, odontoblast cells might be depressed or elevated in linear relationship to the fluorine content of the diet.

In order to test the above hypothesis an experiment was conducted in three phases with the following objects:

I. The development of a satisfactory basal diet that would allow maximum use of forage, since bioassay of fluorine-containing hay was the ultimate aim.

II. The establishment of a satisfactory histological technique that would permit dental cell measurements to be made in well defined areas and to determine the range of dietary fluorine within which guinea pigs might serve as the test animal in a bioassay procedure.

III. The collection of data that would permit the selection of criteria upon which a dosage response curve could be established for fluorine bioassay.

REVIEW OF LITERATURE.

Through the years there has been considerable interest in the biological effects of fluorine with the result that the "fluorine literature" is extensive. A recent compilation by Campbell and Widner (1958) lists over 8500 references in this field. These workers report an interesting trend in the literature on fluorine compounds, in that early reports (up to 1932) deal for the most part with acute toxicity, After this time attention seemed to focus more on the chronic effects of prolonged exposure to low levels of the element. In so far as the dental effects of fluorine are concerned another trend is apparent in that reports up to the early 1940's are concerned with the tooth damaging properties of fluorine. From that time on the emphasis has shifted so that in more recent years the majority of papers have dealt with the beneficial effects on dental health of low level fluorine ingestion.

Although the toxic nature of fluorine compounds for mammals had been reported in the literature from the middle of the nineteenth century one of the first reports of chronic poisoning in farm animals was by Emmerling (1902) who issued a warning against feeding pigs lime phosphate that contained sodium fluoride. Since this time there have been numerous reports of chronic fluorosis in livestock occasioned by the ingestion of fluorides in various forms. The papers by Boddie (1947) from the United Kingdom, Mitchell (1942) from the United States, Walker and Milne (1955) from Africa, Pierce (1939)

from Australia, Dale and Crampton (1955) from Canada to cite just a few, illustrate the world-wide interest in the fluorine problem in livestock health.

The sources of fluorine that might adversely affect livestock have been reported by Phillips et al. (1955) to include the following:

(a) mineral feed supplements.

(b) water contamination both by soluble and particulate fluorides.

(c) forage contamination with soil particle splash of high fluoride content e.g. road dust.

(d) forage containing elevated fluorine content induced by effluent fluorine from industrial processes.

(e) manufacturing by-products containing high fluoride content that may be used as feed concentrates.

There has been considerable interest in the question of the degree of toxicity of various fluorine-containing compounds and also in whether or not a given fluorine compound was more or less toxic when administered in drinking water as compared to its presence in the feed. McClure (1939) indicated that there was no difference from the standpoint of: severity of effects produced, rate of gain, or fluorine storage in the bones and teeth, of rats exposed to sodium fluoride at equivalent levels (22.6, 45.2 and 90.4 p.p.m.) either in drinking water or in feed. Lawrenz and Mitchell (1941) exposed rats to sodium fluoride and calcium fluoride in feed or in water. On the basis of data accumulated on growth and the amount of

fluorine retained in the body, it was concluded that at low levels (8 p.p.m.) the fluorine in sodium fluoride administered in drinking water was 21% more assimilable by the rat than the fluorine of the same compound, consumed in the food, at the same level. The same authors, in a second experiment, fed rats, diets of equivalent calcium content, to which sources of fluorine were added as sodium fluoride, ground tea leaves, or raw rock phosphate. The conclusions derived from both these experiments were that at intakes of 9-12 p.p.m., the fluorine in sodium fluoride was no more assimilable by the animal body and presumably no more toxic, than the fluorine in calcium fluoride. The fluorine in sodium fluoride was, however, 5% more assimilable than the fluorine in green tea, which in turn was somewhat more assimilable than the fluorine in raw rock phosphate.

McClure (1950) reported that there was no difference in amounts of fluorine deposited in the molars, incisors, mandibles or femurs of rats fed sodium fluoride or sodium silica fluoride at daily dietary intake levels of fluorine of 5, 10, 15, 25, and 50 p.p.m. On the other hand, Jackson et al. (1950) fed fluorine to rats as the sodium salt and as fluorine in bone meal and showed that the fluorine retained varied in straight line relationship with the total fluorine consumed in so far as sodium fluoride was concerned. The proportion of fluorine retained from added bone meal decreased as the amount ingested increased. Hobbs et al. (1954) reported that fluorine on a p.p.m. basis in forage from the vicinity of an aluminum plant resulted in a lower bone storage, as well as less severe effects on teeth than was observed

when fluorine was fed as sodium fluoride at an equivalent level. The animals in this experiment were beef cattle continuously exposed to fluorine from one year of age up to three and one-half years of age. The same authors studied the relative toxicity of various fluorine compounds included in the diets of rats at levels of 150, 300, and 600 p.p.m. Comparative storage in bones from the various compounds ranged from high to low in the following order:

(1) K_2SiF_6	(4) NaF	(7) $CaSiF_6$
(2) $NaSiF_6$	(5) rock phosphate	(8) $MgSiF_6$
(3) KF	(6) natural and synthetic cryolite	(9) CaF_2

Storage levels did not always parallel severity of damage in the teeth. When fed at the same level potassium silica fluoride produced slightly earlier and more severe dental damage than did the other compounds. An experiment with rats was also conducted by these workers to compare the toxicity of forage contaminated by industrial effluents with that of sodium fluoride. Groups of rats were fed levels of 15-100 p.p.m. fluorine as sodium fluoride, or as contaminated hay from an "effluent" area that was substituted for the alfalfa meal of the basal ration. Bone storage of fluorine from contaminated hay was comparable to or less than that from sodium fluoride, however the teeth of animals fed the fluorine-contaminated hay showed slightly greater wear.

The results of controlled feeding trials by a number of investigators have suggested that the various species of domestic

animals exhibit different degress of tolerance to the toxic effects of prolonged fluorine ingestion. Phillips et al. (1955) suggested that the following dietary p.p.m. of fluorine as sodium fluoride or other soluble fluorides, and as phosphatic limestone or rock phosphate would not produce evidence of economic damage:

	NaF	Rock Phosphate
(a) Dairy cows	30-50	60-100
(b) Beef cows	40-50	65-100
(c) Sheep	70-100	100-200
(d) Swine	70-100	100-200
(e) Chickens	150-300	300-400
(f) Turkeys	300-400	

Expressed in terms of milligrams of fluorine per kilogram of body weight per day, the work of Rand and Schmidt (1952) and Schmidt et al. (1954), as well as the critical literature review by Schmidt and Rand (1952), suggests that for dairy cattle a maximum innocuous level for the most toxic of the common fluoride salts (sodium fluoride) is in the neighbourhood of one milligram of fluorine per kilogram of body weight per day.

Other workers have attempted to establish the tolerance limits of fluorine for livestock in so far as the occurrence of "economic damage"is concerned. Neeley and Harbaugh (1954) studied a herd of cattle whose source of drinking water had an elevated fluorine content

and agreed with previous workers that one milligram of fluorine per kilogram body weight per day produced only moderate dental fluorosis with no economic damage. Suttie and Phillips (1959) reported on studies with dairy cattle fed graded amounts of sodium fluoride in the diet. Their results suggest that inhibitory effects on milk production and maintenance of body weight might be anticipated in dairy cows receiving fluorine at the rate of 1.7 milligrams per kilogram body weight per day if the animals were exposed from two years of age. When mature animals were exposed to excess dietary fluorine in the same manner they were able to withstand as high as 1.95 milligrams fluorine per kilogram body weight per day for at least two lactations.

In so far as laboratory animals are concerned there has not been the same degree of interest in establishing safe tolerance levels of fluorine. Sollman et al. (1921) added sodium fluoride to the feed of rats in graded amounts so as to provide a fluorine intake of from 0.15 to 151 milligrams fluorine per kilogram body weight per day. He reported that doses of 42 to 151 milligrams per kilogram body weight per day produced immediate and marked retardation of growth. At dosage levels of 0.15 to 8.0 milligrams per kilogram there was no effect on growth or feed consumption during the nine-week feeding period. Cristiani and Chausse (1927) established that the fatal dose of sodium fluoride for the guinea pig was between 240 to 390 milligrams. They reported that five percent of this dose administered daily produced cachexia and death in three to four months. Two percent of the lethal dose per day did not adversely affect the guinea pigs during a ten-month period; in fact their weight gains exceeded that of

the controls.

The small laboratory animals have been utilized to a much greater extent than the domestic farm animals in experiments designed to study the metabolism of fluorine in the animal body and the influence of dietary and other factors on fluorine toxicosis. In this regard Miller and Phillips (1956) reported that the age of rats at the commencement of fluorine feeding had a marked effect on the rate of deposition of fluorine in the femur. Younger animals accumulated fluorine in the skeleton at a faster rate than did older animals. Younger animals also released a greater percentage of their bone storage of fluorine than did older animals when fluorine was removed from their respective diets. Upon reintroducing fluorine into the diets of these animals it was found that the older rats redeposited fluorine in the skeleton to a lesser degree than did the younger animals.

The effect of frequency of fluoride administration in relation to skeletal storage was investigated by Weddle and Muhler (1956). Their data indicated that when young rats ingest fluorine once a day more fluorine is retained in the skeleton than is retained when an equivalent amount is given in divided doses three times a day.

The influence of diet on the toxicity of fluorine has been investigated by several workers. Smith (1935) reported that suboptimal intake of any of the known dietary essentials did not result in increased susceptibility to the destructive action of fluorine on the teeth of rats. He also reported that addition of vitamin C to the diet of guinea pigs exposed to excess dietary fluorine failed to

influence the extent of the damage to the teeth. However, Bodie (1957), reporting on trials with rats, noted that the inclusion of calcium carbonate as 5% of the diet depressed the storage of fluorine in the bones of the experimental animals by as much as 50% when sodium fluoride was included in the diets at levels of 50, 100, and 250 p.p.m.

The sex of the experimental animal has been shown to exert an effect on the susceptibility to fluorine damage in some species. Channels (1930) exposed rats to sodium fluoride and reported that females were more susceptible than males. The effects (weight loss) occurred in females after three months' exposure whereas at similar exposures weight loss did not occur in the males until after four months. Bixler et al. (1954) reported that female rats on a low fluorine diet (0.3 p.p.m.) stored more fluorine than males on the same diet. Shafer and Muhler (1954) reported that female rats, in spite of being smaller in size, stored more fluorine in their femurs than did males on the same diet. These latter workers also administered estradiol and diethylstilbestrol to rats and found that the fluorine concentration in the femurs of males and females receiving the two hormones was appreciably higher than that of corresponding control animals. Anderson et al. (1955), on the other hand, reported that male turkeys had a lower fluorine tolerance than females when exposed to high level fluorine intake in the feed. The criterion in this experiment was growth rate.

Possibly because of the similarity in the dental effects of scorbutus and fluorine intoxication several workers have investigated

the relationship of fluorine poisoning to vitamin C metabolism. Phillips (1933) administered graded levels of orange juice to guinea pigs on a scorbutic diet and noted that several times the anti-scorbutic dose did not prevent the appearance of symptoms similar to, if not identical with those of scurvy when 25-30 milligrams of fluorine per kilogram per day was also included in the diet. He suggested that chronic fluorine poisoning may interfere with the action of vitamin C in the organism. In a later paper Phillips et al. (1934) noted that the decreased ascorbic acid content of the suprarenal gland produced by scurvy was also produced by fluorine toxicosis. On the other hand Hauk (1934) reported that fluorine fed to rats and guinea pigs did not interfere with the storage of vitamin C in their livers or adrenals. Svirbely (1936) administered 15 milligrams of sodium fluoride plus 30 milligrams of thyroid extract per day to male rats for a total of 19 days and reported that there was a markedly decreased ascorbic acid content in their livers, adrenals and small intestines. He further noted that neither substance alone had any appreciable effect. Hobbs (1954) noted a decline in plasma ascorbic acid in rabbits experimentally fed high levels of fluorine. Wadhwani (1952) fed monkeys sodium fluoride at a level of 10 milligrams per kilogram body weight per day for six months. He noted that the inclusion of 20 milligrams of ascorbic acid per day in the diet of the monkeys prevented the occurrence of exostosis. Exostosis that formed due to fluorine in monkeys not receiving the vitamin C supplement did not improve after fluorine withdrawal until ascorbic acid was administered. Although the role of vitamin C in

fluorine toxicosis is not clear, there is evidence to support a conclusion that there is a relationship between the two.

One of the first reports in the literature of an effect of fluorine on the teeth of animals was that of Brandl and Tappeiner (1891). These workers studied the teeth of dogs that had been experimentally fed sodium fluoride. They described porosity of the cement and stated that the roots appeared corroded. McCollum et al. (1925) reported osseus overgrowths (exostosis) and overgrowth and arcing of the maxillary incisors in rats fed a diet containing 226 p.p.m. fluorine. Armstrong (1933) observed broken incisors in young growing albino rats fed a diet containing 0.1 percent sodium fluoride for six weeks. Klein and McCollum (1933) studied decalcified and ground sections of incisors of rats experimentally fed fluorine and described the histological changes as an enamel hypoplasia. They concluded that the changes in the teeth are produced during the developmental stages of tooth formation. Schour and Smith (1934) described in detail the histological changes in the enamel and dentin of the rat's incisor in acute and chronic fluorosis. They concluded that fluorine probably exerts a direct local action on the enamel forming cells (ameloblasts). There are few reports in the literature on the histology of the incisor teeth of guinea pigs following prolonged exposure to elevated dietary fluorine. Fleming (1953) transplanted tooth germs obtained from mice and guinea pigs intraocularly or intracerebrally into guinea pigs and then exposed the recipients to drinking water of elevated fluorine content. He later recovered the transplants at autopsy and described the histological appearance. Calcification of enamel and dentin was retarded

by fluorine and the structure of the ameloblasts was altered. There are no reports in the literature on measurements of the heights of ameloblasts (enamel forming cells) or odontoblasts (dentin forming cells) of animals exposed to elevated levels of fluorine intake.

The possibility of using a biological indicator other than the dental effect as an aid in the diagnosis of chronic fluorine poisoning was suggested by Phillips (1932). He stated that in the absence of other bone diseases the plasma phosphatase in fluorosis formed a sensitive test for the toxic effects of chronic fluorine poisoning. Ellis and Maynard (1936) stated that analysis for fluorine in bone was a more sensitive measure of fluorine damage than the enamel pigment changes that occur in rats exposed to elevated levels of dietary fluorine.

Crampton et al. (1944) reported that the maximum height of the odontoblast cells of guinea pig incisors was related to the daily dose level of vitamin C. When odontoblast heights in microns were plotted against the logarithm of the daily dose of ascorbic acid, the relationship was linear between intakes of 0.5 and 2.0 milligrams of ascorbic acid. A bioassay for ascorbic acid using the guinea pig as the test animal and the height of the incisor odontoblast cells as the criterion was successfully established by these workers (Crampton 1947).

EXPERIMENTAL PROCEDURE.

Phase I. The Determination of a Basal Diet for Fluorine Bioassay.

At the outset of these studies it was necessary to develop a diet that would be nutritionally satisfactory, acceptable to guinea pigs, and at the same time contain a high proportion of roughage in the form of a forage crop. A small scale feeding trial was therefore conducted using three different levels of ground hay incorporated into a complete diet in pellet form. It was proposed that the assay period should be of six weeks duration.

Animals.

Three groups of two female guinea pigs each, about three to four weeks of age, were used for this test. They were housed in individual cages and each group fed one of the three diets to be described, for a period of six weeks. At the end of the feeding period the test animals were killed with chloroform and subjected to a post mortem examination.

Diets.

Three experimental diets were formulated as follows:

Diet	Grain Mix	Hay Mix
A	25%	75%
B	50%	50%
C	75%	25%

The three diets were pelleted for feeding.

Composition of Grain Mix.

The composition of the grain mix was Macdonald College guinea pig diet No.10 with beet pulp omitted. The percentage composition was as follows:

	Percent
Cat groats	25.0
Wheat	23.0
Soybean oilmeal	12.5
Skimmilk powder	15.0
Fishmeal	5.0
Dried Brewers' yeast	10.0
Molasses	5.0
Iodized salt	0.5
20/FOS	1.0
Vitamin Supplement (291-A)*	3.0

Composition of Hay Mix.

The composition of the roughage fraction of the diet consisted of ground Timothy hay with added vitamins and minerals according to the following formula:

	Percent
Ground Timothy hay	95.5
Iodized salt	0.5
20/FOS	1.0
Vitamin Supplement (291-A)*	3.0

In addition to the diet a daily dose of one milligram of ascorbic acid was administered orally to each guinea pig.

The diet and water were supplied ad libitum.

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* The composition of the Vitamin Supplement 291-A was as follows:

Dry Vitamin D (750,000 I.U./lb.)	.097 grams
Dry Vitamin A (5,000 USP units/gm.)	.320 "
Dry Vitamin E (20,000 I.U./lb.)	.227 "
Sucrose to make up	3.000 grams of supplement.

Results.

Post mortem findings.

Necropsy of the guinea pigs at the end of the six-week feeding period did not reveal any gross pathology.

Weight gains and feed consumption.

The average gain in weight in six weeks for each group of guinea pigs, along with their total feed consumption is shown below:

Diet group and hay content (%) of diet	Weight gains in grams after 6 weeks (average of 2 animals)	Feed consumption in grams for 6 weeks (average of 2 animals)
A (75%)	175	1300
B (50%)	230	1375
C (25%)	180	1050

The highest feed consumption and the greatest gain in weight was attained by the guinea pigs on diet B. However the guinea pigs on diet A consumed almost one-third again as much hay as did the animals on diet B. Although the weight gains of animals on diet A were inferior to those achieved by the animals on diet B their rate of gain was comparable with that achieved by a similar group of guinea pigs that had been fed a standard guinea pig colony diet on a previous occasion (see Appendix Fig.I).

Based on the absence of any gross post mortem pathology attributable to the diets fed and the fact that a high consumption of hay was a priority requisite for a fluorine bioassay of forages, diet A, that contained 75% hay, was adopted as a basal diet.

Phase II. Preliminary Fluorine Feeding Trial.

This trial was conducted primarily to gain some insight into the histological problems that might be encountered in a bioassay based on measurement of ameloblast and/or odontoblast cell heights. As a secondary consideration we also wished to learn whether or not fluorine, up to about 75 p.p.m. in the diet, would depress feed consumption to the point where a bioassay would be complicated by "starvation effects" or other complications of suboptimal nutrition.

Animals.

Twenty-eight male and seven female guinea pigs, four to six weeks of age were randomly assigned to seven experimental diet treatments according to the following plan:

Allotment Plan

Lot No.	Diet treatment	No. of guinea pigs
1	Basal diet	5*
2	Basal diet + NaF 7.5 mg/kg air dried feed	5
3	" " + NaF 15.0 " " " "	5
4	" " + NaF 30.0 " " " "	5
5	" " + NaF 60.0 " " " "	5
6	Basal diet made up with Hay A **	5
7	Basal diet made up with Hay B ***	5

* Four males and one female per lot

** F contaminated hay (approx. 50 p.p.m.)

*** F contaminated hay (approx. 100 p.p.m.)

It was realized that this plan confounded the effects of "kind of hay" and "source of fluorine" and that this would render interpretation of weight gain data more difficult. The plan deficiency was accepted however because it did not seem likely that it would interfere with the attainment of the primary objectives of this trial.

The guinea pigs were housed individually in metal cages with perforated floors. Feed and water were provided ad libitum. A weekly record was kept of the animals' body weights. At the start of the trial each guinea pig was assigned a separate feed container which was filled with the allotted diet and then weighed. The cage feeder was filled as often as necessary from this container. At the end of the first week the container and the remaining contained feed was again weighed and the difference in weight entered on the individual record sheet of the animal as feed consumed during the week. This procedure was repeated at each weekly weighing period. Observations on each animal were also recorded at the time of weighing and any abnormalities were noted on the individual record sheets. At the end of the six-week experimental period all animals were killed with chloroform. They were then examined post mortem for any gross pathological changes. At this time the mandibles were removed, dissected free of adhering soft tissue, and transferred to a ten percent formalin solution.

Diets.

The diet previously referred to as diet A and described in Phase I was the basal diet used. In lots six and seven (see allotment plan), hay samples A and B were substituted for the Timothy hay used

to prepare diets 1-5. These two hays were obtained from fluorine hazard areas where previous chemical analysis had revealed a fluorine content of under 50 p.p.m. in the case of hay A and over 100 p.p.m. in the case of hay B.

The sodium fluoride added to the diet of lots 2 to 5 was prepared as a solution containing 2.20 grams of sodium fluoride per litre of tap water. Measured amounts of this solution replaced equivalent portions of 1000 ml. of tap water used to dampen 2660 gram batches of the mixed feed, prior to pelleting, according to the following plan:

Diet Lot No.	NaF solution added to 2660 gm. of feed (ml.)	Estimated p.p.m. F in air dried diet
1	0	X
2	20	X + 7.5
3	40	X + 15.0
4	80	X + 30.0
5	160	X + 60.0

In addition to the assigned diet each animal received an individual oral dose of 0.2 ml. of an ascorbic acid solution that was prepared daily by dissolving 50 mg. of ascorbic acid in 10 ml. of tap water. Thus each animal was assured a daily intake of one mg. of ascorbic acid over and above that which was derived from the diet. Previous work in this laboratory has indicated that a daily supplement of one mg. of ascorbic acid per animal is reasonably reliable insurance against the appearance of scurvy in growing guinea pigs on a non-scorbutogenic diet.

Fluorine levels in the diets.

The chemical analysis of fluorides in the pelleted diets used in this trial were conducted by analysts at the Aluminum Laboratories, Arvida, P.Q. The method used was one proposed by Harvey and Puxley (1955). Values reported for seven test diets and the corresponding calculated values are presented in Table 1.

TABLE 1. Chemically determined and calculated fluorine levels in the diets.

Diet Lot No.	p.p.m. fluorine in air dried diets by chemical analysis	Calculated p.p.m. fluorine in air dried diets
1	24	24.0
2	30	31.5
3	36	39.0
4	66	54.0
5	86	84.0
6	60	-
7	78	-

The calculated amount of fluorine in diets 2 to 5 was estimated by assuming that the chemical analysis of the basal diet (Lot No.1) prepared from Timothy hay was correct and the fluorine content was actually 24 p.p.m.

It will be noted that there is reasonable agreement between the calculated and the chemically determined fluorine levels except in the case of diet No.4 where a discrepancy of 12 p.p.m. exists. For the purpose of plotting fluorine levels against ameloblast and odontoblast heights only, diet No.4 was assigned a fluorine content of 60 p.p.m. since this was the mean of the values obtained by chemical analysis and calculation (see Appendix Figures 5 to 9).

Feed consumption and weight gains.

Table No.2a gives the average feed consumption and weight gains of the guinea pigs in each experimental lot over the six-week feeding period.

At the outset it should be pointed out that the feed consumption and weight gains of the guinea pigs in lots 1-5 as compared to those of the animals in lots 6 and 7 were potentially influenced by two distinct dietary factors as follows:

(1) Lots 1-5 were fed the basal diet to which fluorine was added in the form of sodium fluoride while lots 6 and 7 received diets in which the fluorine content was derived from the presence in the diet of fluorine contaminated hay (nature of fluorine compound unknown).

(2) Lots 1-5 were fed diets that contained "Timothy" hay while lots 6 and 7 received diets that contained a "mixed hay" grown in a fluorine hazard area.

Thus both the "source" of fluorine and the "kind" of hay were different for these two groups and therefore differences in feed consumption and/or weight gains between the groups might have been caused by the operation of either one or both of these factors.

Analysis of variance of the feed consumption data (see Table 2b) indicates that there is no significant differences in feed consumption between any of the seven treatment lots. There was a higher feed consumption by the animals in lots 6 and 7 that received fluorine contaminated "mixed" hay. However when the feed consumption of lots 6 and 7 was compared statistically with the feed consumption of lots 1 to 5 the difference was not significant (see Table 2b).

Reference to Table 2a shows that the weight gains of the guinea pigs in lot 1 (basal diet) were inferior to those observed for any of the other treatment lots except those that received the highest level of added sodium fluoride (lot 5). We are not able to explain the poor weight gain performance of the guinea pigs in this treatment lot. If the poor performance of the guinea pigs that consumed the basal diet is disregarded for the moment there appears to be a progressive decline in weight gains that accompanies increments of sodium fluoride in the diet for lots 2-5. That this effect was not solely due to differences in feed intake was shown by analysis of covariance (see Appendix Table 1) and adjustment of weight gains to average feed intake (see Table 2a).

Analysis of variance of the weight gain data (see Table 2c) showed that there was no significant differences attributable to the

seven fluorine levels in the diet. However when the observed weight gains of lots 6 and 7 were compared statistically with the observed weight gains of lots 1-5 the difference was statistically highly significant (P=.01). However, as previously pointed out, the effects of "kind of hay" and "source of fluorine" were confounded in this trial so that it is not possible to say which factor predominated in producing the observed difference in weight gains.

TABLE 2a. Average feed consumption and weight gains of guinea pigs on various levels and sources of dietary fluorine over a six-week period.

Lot No.	No. animals	p.p.m. F	Observed feed consumption gm/pig (42 days)	Mean of F source groups	Observed weight gain gm/pig (42 days)	Mean of F source groups	Weight gains[1] adjusted to average feed intake gm/pig(42 days)	Adjusted mean of F source group
1	5	24	1900		142		139	
2	5	30	1820		166		171	
3	5	36	1800	1834	161	151[2]	168	155
4	5	66	1860		149		150	
5	5	86	1789		137		145	
6	5	60	1945	1957	185	185[2]	177	175
7	5	78	1970		185		174	

[1] Regression coefficient = 0.1042

[2] Necessary difference between these group means at P=.01 is 13 grams.

TABLE 2b. Analysis of variance of feed consumption data.

Source	D/F	Variance	F Values		
			Obs.	5%	1%
Total	34				
Between 7 F levels	6	24,819	1	2.46	-
Timothy hay + NaF vs. F contaminated mixed hay	1	106,943	2.62	4.21	-
Error	27	40,784			

TABLE 2c. Analysis of variance of weight gain data.

Source	D/F	Variance	F Values		
			Obs.	5%	1%
Total	34				
Between 7 F levels	6	1,844	1.87	2.46	-
Timothy hay + NaF vs. F contaminated mixed hay	1	8,141	8.26	4.21	7.68
Error I	27	985			
Regression	1	2,770			
Error II	26	916			

Ante and Post Mortem Examination.

During the early part of the experimental feeding period the guinea pigs were affected with cutaneous eruptions around the face and legs. There did not appear to be any relationship between the incidence of this dermatitis and the fluorine levels in the diets. The skin condition responded moderately well to local application of a quaternary ammonium solution. The incisor teeth of certain animals presented a chalky white appearance after several weeks on test. Broken incisors were also observed. These teeth abnormalities were considered to be possible fluorine effects but the incidence did not appear to coincide with fluorine levels in the diet. No lesions attributable to fluorine ingestion were found on gross examination of the visceral organs.

Histology and Cell Measurements.

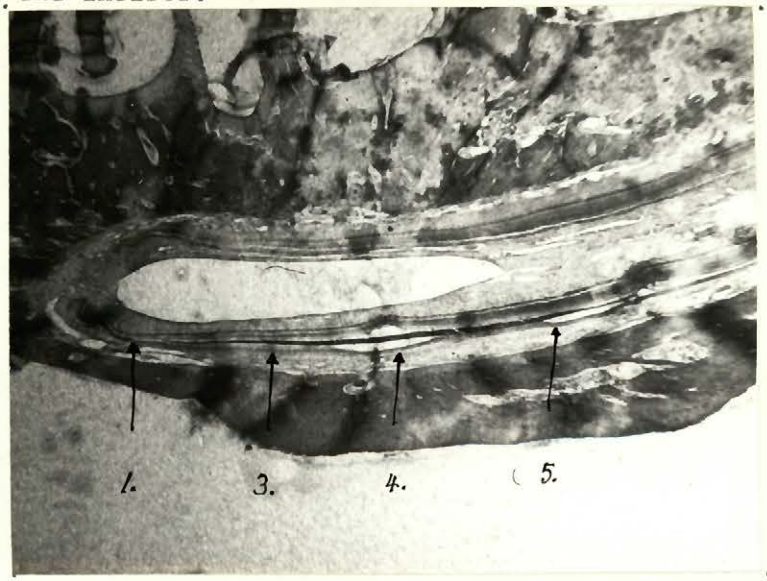
The mandibles were removed from the skull of each animal at the conclusions of the necropsy. After freeing them of adhering soft tissue, the two rami were separated at the symphysis mandibulae and were then transferred to 10% formalin. The general procedure adopted at this laboratory for bringing the jaws through decalcification, dehydration, clearing, infiltration, embedding, sectioning, mounting and staining is described in the Appendix under the heading of Histological Technique.

Two or three slides, each carrying four mounted sections of lower jaw were prepared for each animal. These were then examined under

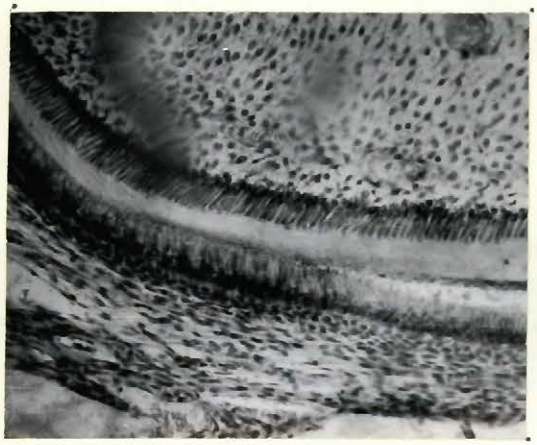
the low (X 100) and high (X 300) power objectives of the microscope and a single jaw section was selected as representative of the particular experimental animal. This selection was made on the basis of correctness of plane of sectioning, presence or absence of ameloblast and odontoblast cells, depth of staining, etc. The thirty-five sections thus selected were then studied more closely using the high power objective of a microscope fitted with a calibrated ocular micrometer. Ameloblast and odontoblast cell heights appeared to vary in any given section depending on their location along the long axis of the incisor tooth. Figure 1 shows micro-photographs of one of the histological sections of a guinea pig mandible and clearly illustrates this difference in ameloblast cell heights. In the case of the ameloblasts it was considered that seven reasonably distinct areas of cell heights were discernible in most sections. A diagrammatic sketch of a typical lower incisor section showing these areas is presented in Appendix Figure 2. Measurements of typical ameloblast and the odontoblasts in direct apposition, were therefore made in each of these seven areas. In many sections it was not possible to obtain measurements at all of the areas. This was due to the loss of certain cells during the preparation of the slides. In the case of the odontoblasts it was usually impossible to find cells in area number 7, because the odontoblast has lost its function and disappeared as a recognizable entity at about the level of the proximal end of the pulp cavity.

The data accumulated from these measurements are summarized in Tables 3 and 4.

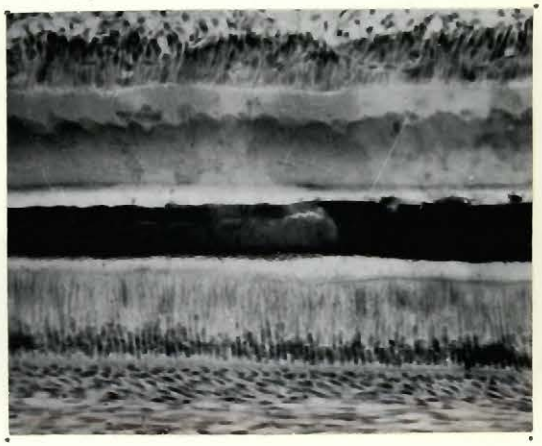
FIG.1.-Microphotographs of a typical mandible section showing differences in ameloblast height along the long axis of the incisor.



Histological section of a guinea pig mandible (X8) showing several measurement areas.

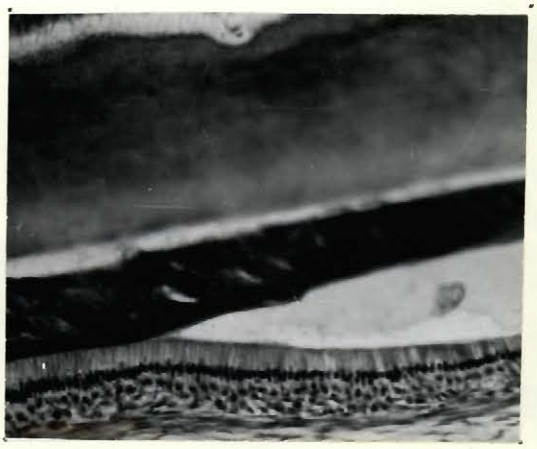


High power (X200) of area No.1.



High power (X200) of area No.3.

High power (X200) of area No.4.



High power (X200) of area No.5.

TABLE 3. Average ameloblast heights in relation to diet and area examined.

Area measured	Treatment lots							Area average
	1	2	3	4	5	6	7	
1	29.6(5)*	28.0(2)	24.0(3)	27.4(5)	18.5(2)	29.3(4)	25.5(4)	26.8(25)
2	32.5(4)	30.8(5)	35.5(4)	35.5(4)	24.0(3)	34.5(4)	25.6(5)	31.2(29)
3	44.7(5)	38.8(5)	39.5(4)	44.8(4)	50.0(2)	49.7(5)	53.0(2)	44.9(28)
4	21.4(5)	17.0(3)	20.0(3)	19.5(4)	16.5(2)	21.3(5)	19.0(1)	19.8(23)
5	31.8(4)	26.0(5)	28.0(4)	25.6(5)	23.0(5)	27.2(5)	23.4(5)	26.2(33)
6	20.3(4)	23.0(5)	24.0(4)	26.7(3)	18.8(4)	23.0(5)	18.2(5)	21.7(30)
7	19.5(4)	23.8(4)	21.3(4)	24.7(3)	17.3(3)	20.2(5)	20.2(5)	20.9(28)
Treatment average	29.4(31)	27.3(29)	27.9(26)	29.2(28)	23.0(21)	29.3(33)	24.3(27)	

*Figures in brackets represent the number of observations contributing to the average. Maximum = 5.

TABLE 4. Average odontoblast height in relation to diet and area examined.

Area measured	Treatment lots							Area average
	1	2	3	4	5	6	7	
1	31.0(5)*	24.8(4)	27.5(4)	26.8(5)	22.5(2)	23.3(4)	26.0(4)	26.4(28)
2	31.8(4)	30.2(5)	27.8(4)	33.7(3)	33.0(3)	30.8(4)	33.2(5)	31.4(28)
3	30.0(5)	30.0(4)	26.8(4)	34.7(3)	44.0(2)	39.4(5)	39.0(2)	33.7(25)
4	32.3(4)	29.0(3)	25.3(3)	32.3(4)	33.0(2)	36.4(5)	59.0(1)	33.1(22)
5	30.0(3)	27.0(4)	23.7(3)	28.0(5)	32.0(4)	34.8(5)	40.0(5)	31.5(29)
6	26.0(2)	24.0(2)	26.0(1)	30.3(3)	28.5(4)	41.0(5)	40.5(5)	33.9(22)
7	36.0(1)	39.0(1)	26.0(1)	38.5(2)	34.0(1)	36.2(5)	47.0(3)	38.1(14)
Treatment average	30.8(24)	28.3(23)	26.4(20)	31.0(25)	32.0(18)	35.0(33)	38.0(25)	

*Figures in brackets represent the number of observations contributing to the average. Maximum = 5.

Analysis of variance for odontoblast heights as shown in Appendix Table 2 indicates that there is a highly significant difference between the fluorine treatment lots, P=.01. As we anticipated, there is also a highly significant difference between areas where measurements were taken. There appears to be no interaction between areas and treatment levels. Thus it would appear that the selection of a definite area for measurement would have an important bearing on the establishment of a possible bioassay technique.

Essentially the same finding applies to the ameloblasts. Analysis of variance for ameloblast height is presented in Appendix Table 3. From the observed F value for locations measured it would appear that the site of measurement is even more critical for this cell type. Since both these analysis reveal significant differences between fluorine levels it was decided to plot ameloblast and odontoblast heights against the fluorine levels in experimental diets 1 to 5. The fluorine in diets 6 and 7 was present as a contaminant of the hay used in the diet preparation. For this reason the cell height values obtained from animals on these diets are indicated separately on the graphs shown in Appendix Figure 3 to 9 inclusive.

Results.

It was considered desirable in the establishment of a bioassay to reduce the histological technique involved to as simple a procedure as possible. Thus the ideal would be to select only one cell type that could be easily measured several times in just one area. The

area should be such that it could be readily found on all sections and preferably one that would allow some latitude in sectioning technique.

With these several considerations in mind it was decided that the ameloblasts in area 5 approached the ideal most closely. Reference to Table 3 shows that 33 out of a possible 35 measurements were obtained in this area. This would indicate that the cells in this location are not so often lost during preparation of the slide. With the exception of the cell height value for the second dietary fluorine level (see Appendix Fig.7) an indication of a straight line relationship between cell height and fluorine level would appear to exist in this case. A somewhat similar relationship is apparent for the ameloblasts and odontoblasts in area one (see Appendix Fig.3); however, the examination of this area demands very careful preparation of the sections especially in proper alignment of the block when cutting ribbon sections.

The odontoblasts in area 3 (Appendix Fig.5) and the ameloblasts in area 4 (Appendix Fig.6) might also have been considered but for the fact that reference to Tables 3 and 4 shows that only about 50% of the possible cell measurements were obtainable in these areas. Thus it would appear that cells in these zones are easily lost during preparation of the slides.

The original premise that fluorine might affect ameloblast height in linear relationship was partially borne out when measurements were made in area 5.

Since levels of fluorine in the diet ranging from 24 to 86 p.p.m. did not significantly affect feed consumption of young growing guinea pigs over a six-week period, it was concluded that this species would be satisfactory as a test animal in which fluorine-containing forages might be assayed.

Statistical analysis of the weight gain data obtained from this trial did not warrant a definite conclusion in regard to the relative effects of fluorides from different sources.

PHASE III. Fluorine Feeding Trial with Two Levels of Ascorbic Acid in the Diets.

Ascorbic acid deficiency in the diet of young growing guinea pigs is known to cause a dimunition in height of their incisor odontoblast cells. The results of Phase II indicated that fluorine excess had a similar effect when cell heights were measured in certain areas. It was therefore considered desirable to examine both of these factors in the one experiment to see if different levels of ascorbic acid might be a complicating factor in a potential fluorine bioassay based on the measurement of ameloblast or odontoblast cells.

While the results of Phase II made it apparent that ameloblast and odonotblast heights were affected in a somewhat linear fashion by dietary fluorine increments it was by no means certain that the range in microns would prove to be of practical use in a bioassay procedure for forages. For this reason several other potentially useful criteria were also examined in Phase III, including the effect of fluorine on feed consumption and body weight gains, digestibility of nutrients and bone storage of fluorine. The fecal excretion of dietary fluoride was also compared for guinea pigs receiving fluorine as NaF and in the form of fluorine-contaminated hay.

Animals.

Forty guinea pigs were weaned at three weeks of age and fed in groups a basal diet high in roughage, as described in Phase I. When each animal had learned to eat the diet and had reached a weight of 250 grams (for females), or 275 grams (for males) it was allotted at

random to one of ten dietary treatments (2 females and 2 males per lot), as shown in the following allotment plan:

Allotment Plan

Type of hay	Diet No.	Level of NaF added to the diet*	Daily dose of ascorbic acid***	
			0.5 mg.	1.0 mg.
Macdonald College Hay	1	nil	lot 1 pens 1-4	lot 6 pens 21-24
	2	X**	lot 2 pens 5-8	lot 7 pens 25-28
	3	2X	lot 3 pens 9-12	lot 8 pens 29-32
	4	4X	lot 4 pens 13-16	lot 9 pens 33-36
Fluorine-contaminated Hay	5	nil	lot 5 pens 17-20	lot 10 pens 37-40

* NaF added in the form of a solution containing 2.2 mg. NaF per ml. tap water.

** X = 80 ml. of this solution per 2660 grams diet (or 30 p.p.m. F).

*** Administered orally as a 0.5% solution of ascorbic acid in tap water in amounts of 0.1 ml. and 0.2 ml. respectively.

Animals were housed individually in cages with perforated floors. Feed and water were provided ad libitum. Animals were weighed weekly and a record kept of live weight changes and feed consumption. At the time of weighing each animal was inspected and any abnormalities noted on individual record sheets. During the second week of the test samples of feces were collected from each lot for determination of digestibility by the Index method, using chromic oxide as the indicator. At the termination of the feeding period the animals were killed with chloroform and examined post mortem. The lower jaws were removed for histological study as described in Phase II. The femurs were also removed, cleaned of soft tissue and stored pending analysis of their fluorine content.

Diets.

Five test diets were fed that had the following general mixing formula.

General mixing formula for test diets.

Ingredient	Amount	
	(grams)	(%)
Hay (ground)	1810	68.0
Vitamin-Mineral Mix No.309	90	3.6
Grain Mix No.2	630	23.6
Molasses	130	4.8
	2660	100.0

Vitamin-Mineral Mix No.309	(%)	Grain Mix No.2	(%)
Dry D (750,000 I.U./lb.)	2	Oat groats	26
Dry A (10,000 USP Units/gm.)	3	Wheat	24
Dry E (20,000 I.U./lb.)	5	Soybean oilmeal	14
CrO_3	30	Skimmilk powder	16
Salt	11	Fishmeal	5
Dynafos	22	Dried Brewers' yeast	10.5
Cornstarch	27	Vit-Min. Mix No.309	4.5

Preparation of Test Diets.

Diet 1. Macdonald College hay was used in this diet after it had been ground to a coarse powder. The dry ingredients were weighed and then mixed in a Hobart mixer. A litre of hot water was then mixed with the 130 grams of molasses and the whole added to the dry ingredients and thoroughly mixed. The mixture was then pelleted and dried.

Diet 2. This diet was prepared exactly as described for diet 1, with the exception that 80 ml. of NaF solution (2.2 grams NaF per litre of tap water) replaced an equal quantity of the hot water.

Diet 3. As for diet 2, but 160 ml. NaF solution was used.

Diet 4. As for diet 2, but 320 ml. NaF solution was used.

Diet 5. As for diet 1, but using hay from a fluorine-hazard area instead of Macdonald College hay.

Ascorbic Acid.

Each guinea pig was dosed daily with an aqueous solution of ascorbic acid. The ascorbic acid solution was prepared daily by dissolving 50 milligrams of ascorbic acid in 10 ml. of tap water. Animals in lots 1-5 were given 0.1 ml. of this solution per day which gave them a dietary supplement of 0.5 mg. of ascorbic acid daily. Lots 6-10 were given 0.2 ml. of the solution daily which gave this group a daily ascorbic acid supplement of 1.0 mg. Both groups were given a double dose on Saturday and none on Sunday.

Fluorine levels in diet and feces.

Chemical determinations of the amount of fluorine present in the diets fed, and in the feces collected during the second week on test, were conducted by analysts at the Aluminum Laboratories Limited, Arvida, P.Q., using a method proposed by Harvey and Puxley (1955).

Unfortunately there was an insufficient amount of feces collected to enable analysis of each experimental lot. It was therefore decided to pool the feces of the two ascorbic acid levels as far as fluorine determination was concerned.

Fluorine levels in the diets and in the feces voided by animals on these diets are shown in Table 5 along with the calculated amount of fluorine in diets 2 to 5.

TABLE 5. Fluorine levels in the air dried diets and feces of guinea pigs.

Diet	Animal Lot No's.	p.p.m. F in diets by chemical analysis	Calculated p.p.m. F in diets	p.p.m. F in feces
1	1 and 6	19	-	10
2	2 and 7	40	49	17
3	3 and 8	70	79	31
4	4 and 9	130	139	35
5	5 and 10	112	-	69

It will be noted that again there is reasonable agreement between the calculated levels of fluorine in the diet and the level determined chemically. The calculated values are all higher by a constant amount, i.e. 9 p.p.m. This might be explained if one makes the assumption that the level in the basal diet was actually lower than that reported. Difficulty in obtaining truly representative samples from a batch of mixed feed may account for the discrepancy.

Ante Mortem and Post Mortem Examinations.

Ante Mortem Inspections.

At weekly inspection of animals the occurrence of chalky white and broken incisors was noted toward the end of the feeding period. This lesion did not appear to be confined to the individuals in any particular group but was observed more frequently in the higher fluorine dietary lots.

Most animals in the highest dietary fluorine lots, i.e. diet lots 4 and 5, had lost weight during the last week on test which was taken to indicate that the cumulative effect of high fluorine intake had begun to exert a deleterious effect on general metabolism.

Post Mortem Observations.

Necropsy did not reveal any specific gross lesions attributable to diet treatments.

Feed Consumption and Weight Gains.

The total feed consumption over the six-week period for each animal in the trial is shown in Table 6a with analysis of variance of this data presented in Table 6b. Fluorine levels in the diet had a highly significant (P=.01) effect on feed consumption as did also the sex of the experimental animal. The analysis also reveals a significant (P=.05) interaction between fluorine ingestion and sex on feed consumption. This interaction is illustrated graphically in Fig.2, and reveals that the males increased their feed intake with increasing fluoride intake up to about 70 p.p.m. in the diet. Females, on the other hand, had diminishing feed intakes with each increment of fluorine in the diet from 20 p.p.m. to 130 p.p.m.

TABLE 6a. Total feed consumption of guinea pigs in grams for a six-week feeding period.

Sex	Ascorbic acid	Diet Lot No.	1	2	3	4	5*
		Animals/lot	8	8	8	8	8
		F p.p.m.	19	40	70	130	112
M	0.5		1386	1408	1615	1185	1537
			1425	1380	1538	1340	1300
	1.0		1393	1625	1503	1292	1320
			1343	1541	1881	1389	1490
Sub-totals			5547	5954	6537	5206	5647
F	0.5		1570	1359	1408	1196	1302
			1420	1196	1296	1123	1399
	1.0		1464	1314	1275	1173	1402
			1369	1575	1381	1171	1429
Sub-totals			5823	5444	5360	4663	5532
Totals			11370	11398	11897	9869	11179

* Not included in analysis of variance since F present in diet as contaminated hay.

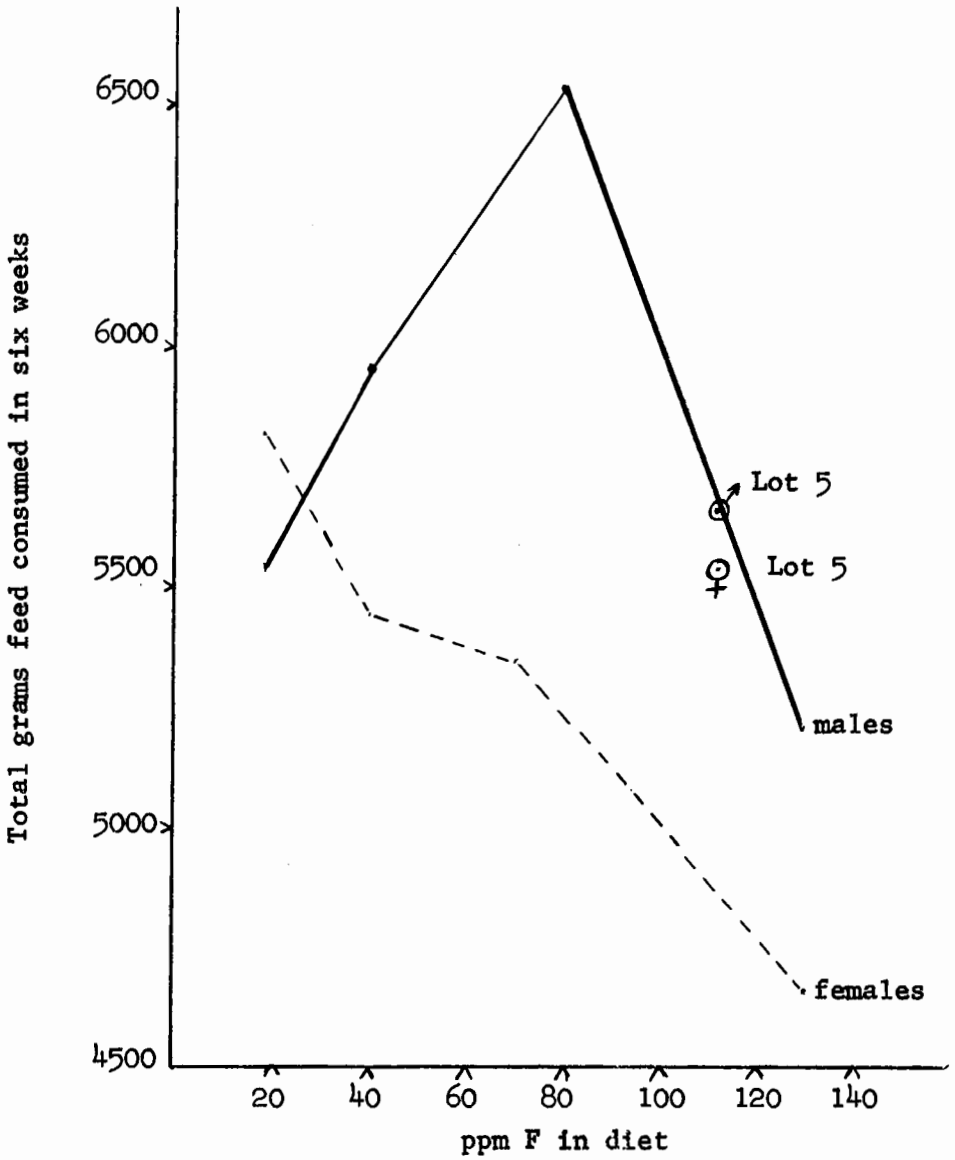
TABLE 6b. Analysis of variance of total feed consumption.

Source	D/F	Mean square variance	F Value		
			Obs.	5%	1%
Total	31				
Fluorine levels**	3	96,157	8.80	3.24	5.29
Ascorbic acid	1	22,261	2.03	4.49	8.53
Sexes**	1	119,316	10.93	4.49	8.53
F × AA	3	16,753	1.53	3.24	5.29
F × Sex*	3	44,247	4.05	3.24	5.29
AA × Sex	1	8,977	< 1		
F × AA × Sex	3	3,279	< 1		
Error	16	10,920			

* Significant (P=.05)

** Highly significant (P=.01)

FIG.2. The interaction of fluorine and sex on feed consumption of Guinea Pigs.



The total weight gain achieved by each animal is shown in Table 7a, with the analysis of variance for this data presented in Table 7b. As would be anticipated from the feed consumption analysis, fluorine levels in the diet had a highly significant effect on the animals' total weight gain (P=.01). Although the effect of sex was not significant according to this analysis, when the weight gains of males and females are plotted separately against fluorine in the diet, there is again a trend toward the males being able to tolerate higher levels of fluorine (see Fig.3).

TABLE 7a. Total weight gains of guinea pigs in grams for six-week feeding period.

Sex	Ascorbic acid	Diet Lot No.	1	2	3	4	5*
		Animals/lot	8	8	8	8	8
		F p.p.m.	19	40	70	130	112
M	0.5		121	36	134	56	137
			122	117	166	52	115
	1.0		122	195	159	-20	95
			123	152	207	92	161
Sub-totals			488	500	666	180	508
F	0.5		127	97	126	71	80
			141	143	182	91	136
	1.0		147	160	106	66	95
			179	176	150	24	145
Sub-totals			594	576	564	252	456
Totals			1082	1076	1230	432	964

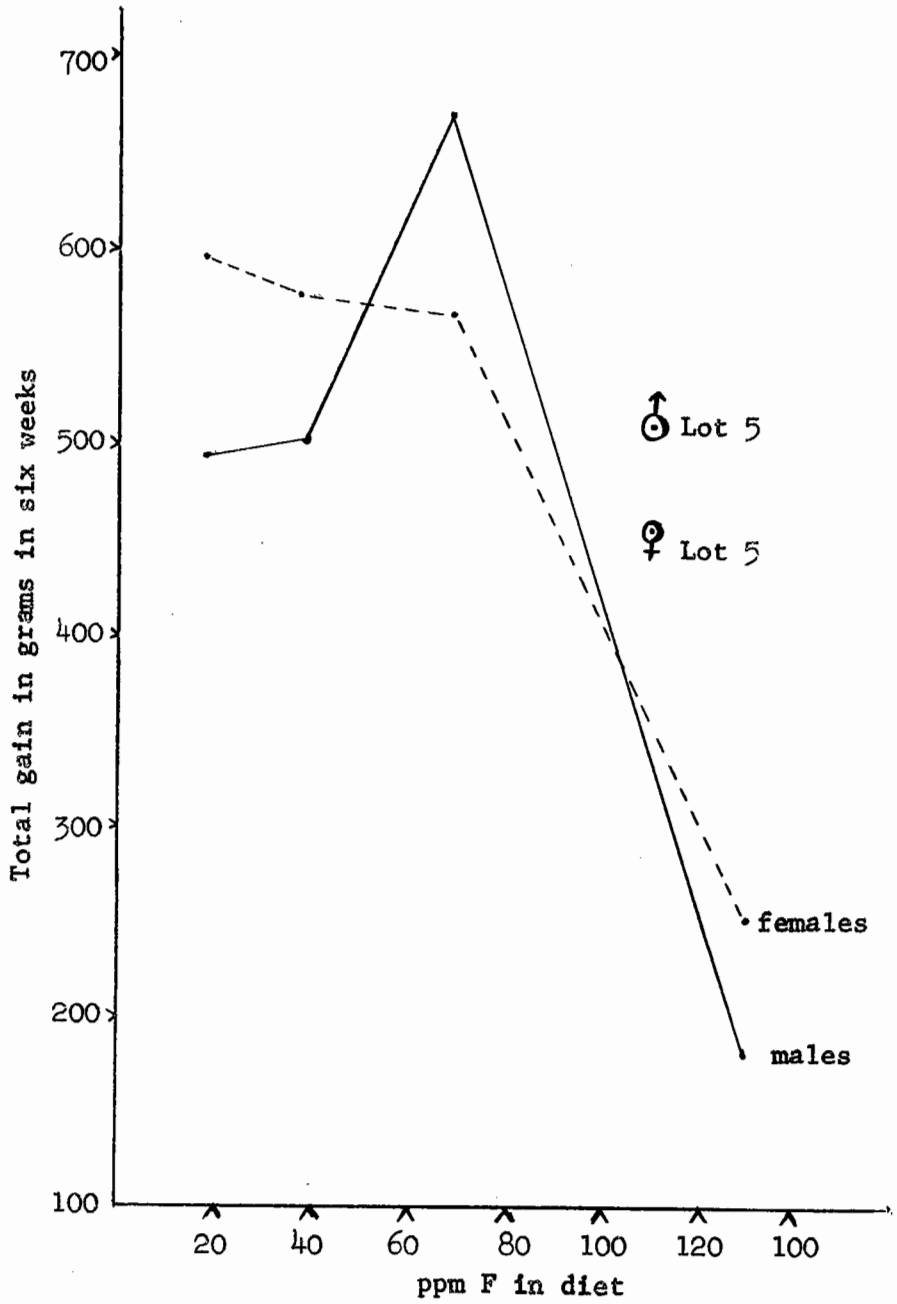
*Not included in analysis of variance since F present in diet as contaminated hay.

TABLE 7b. Analysis of variance of total weight gains.

Source	D/F	Variance	F Value		
			Obs.	5%	1%
Total	31				
Fluorine levels**	3	15,830	14.42	3.24	5.29
Ascorbic acid	1	2,048	1.86	4.49	8.53
Sexes	1	722	< 1		
F × AA	3	3,376	3.07	3.24	5.29
F × S	3	1,118	1.02	3.24	5.29
AA × S	1	1,201	1.09	4.49	8.53
F × AA × S	3	854	< 1		
Error	16	1,098			

** Highly significant (P=.01)

FIG.3. The effect of dietary fluorine on total weight gains of male and female Guinea Pigs.



Coefficients of Digestibility.

Table 8 summarizes the percent digestibility of the five experimental diets and their fluorine content,

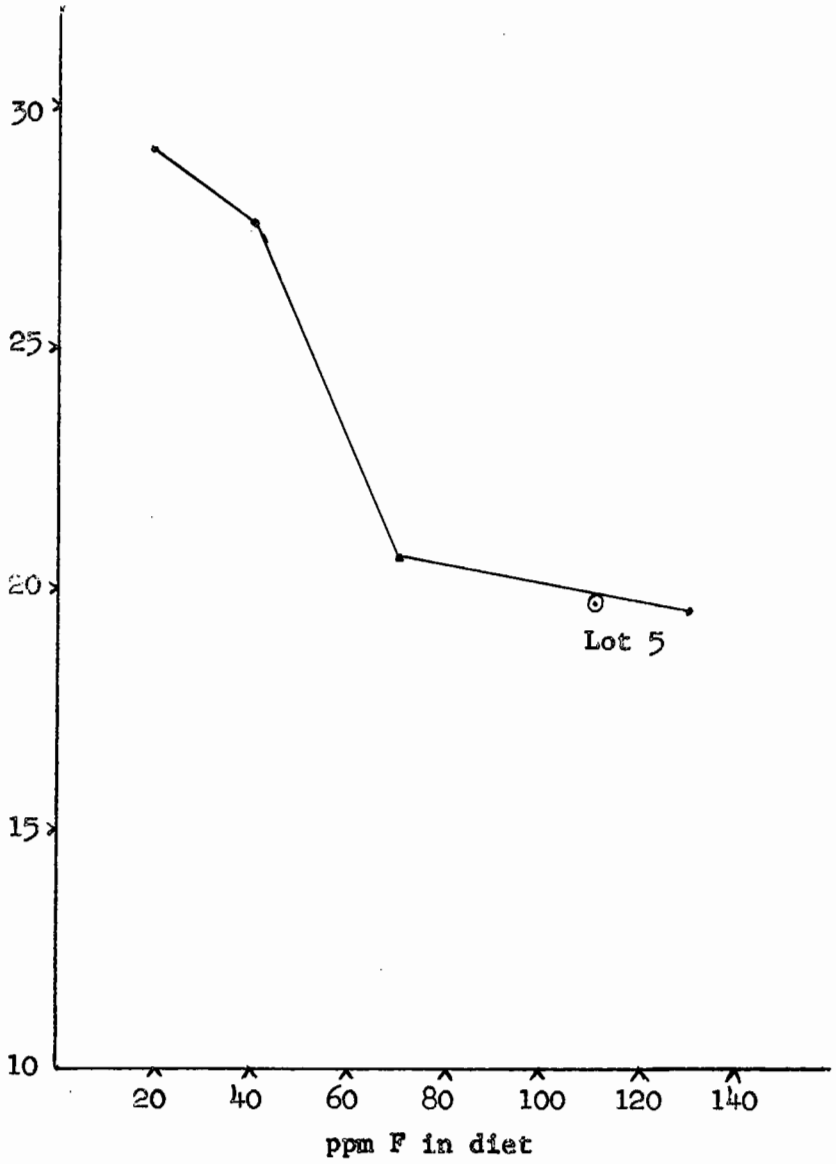
TABLE 8. Fluorine content and digestibility of diets.

Diet No.	p.p.m. F	Percent Digestibility					
		Dry matter	Crude protein	Ether extract	N.F.E.	Crude fiber	Calories
1	19	49	63.7	65.4	54.5	28.1	44.8
2	40	50	60.7	67.8	58.2	27.6	48.8
3	70	47	60.7	87.5	54.5	20.7	43.6
4	130	52	66.0	84.3	60.6	19.7	47.8
5	112	51	61.0	84.2	59.3	19.8	47.4

It will be noted that the chemical values in Table 8 disregard the two ascorbic acid levels. This was necessary because of the difficulty experienced in obtaining sufficient feces for analysis from only four animals.

Only the digestion of crude fiber appears to be correlated with the fluorine content of the diet (see Fig.4). The digestibility of the crude fiber for diet No.5 is shown separately on this graph.

FIG. 4 - Relationship between fluorine in the diet and digestibility of crude fiber



Fluorine Storage in Bones.

The femurs removed from the experimental animals at post mortem were submitted to the Aluminum Laboratories, Arvida, P.Q. for fluorine analysis. The four femurs from each lot were ground, ashed and pooled as one sample for the purpose of analysis. Table 9a shows the fluorine values obtained and Table 9b presents the analysis of variance for this data.

As might be anticipated from a perusal of the raw data, fluorine level in the diet had a highly significant effect on fluorine storage in the bone. Ascorbic acid levels did not influence this storage. The ascorbic acid levels were therefore averaged for each diet lot number to give a better estimate of the fluoride storage in the bones at each dietary fluorine level. Fig.5 illustrates graphically the relationship of dietary fluorine to bone storage of fluorine. The average fluorine content of the femurs of guinea pigs in dietary lot 5, wherein the fluorine in the feed was present as contaminated hay, is indicated on this graph separately. It will be noted that this value falls within the shaded area on the graph that represents the 95% confidence limits calculated from the observed data.

TABLE 9a. Fluorine in guinea pig femur ash.

Ascorbic acid	Diet Lot No.	1	2	3	4	5*
	F p.p.m.	19	40	70	130	118
0.5 mg/day		875	2250	3400	5700	4500
1.0 mg/day		850	2325	3575	5250	4500
Totals		1725	4575	6975	10950	9000

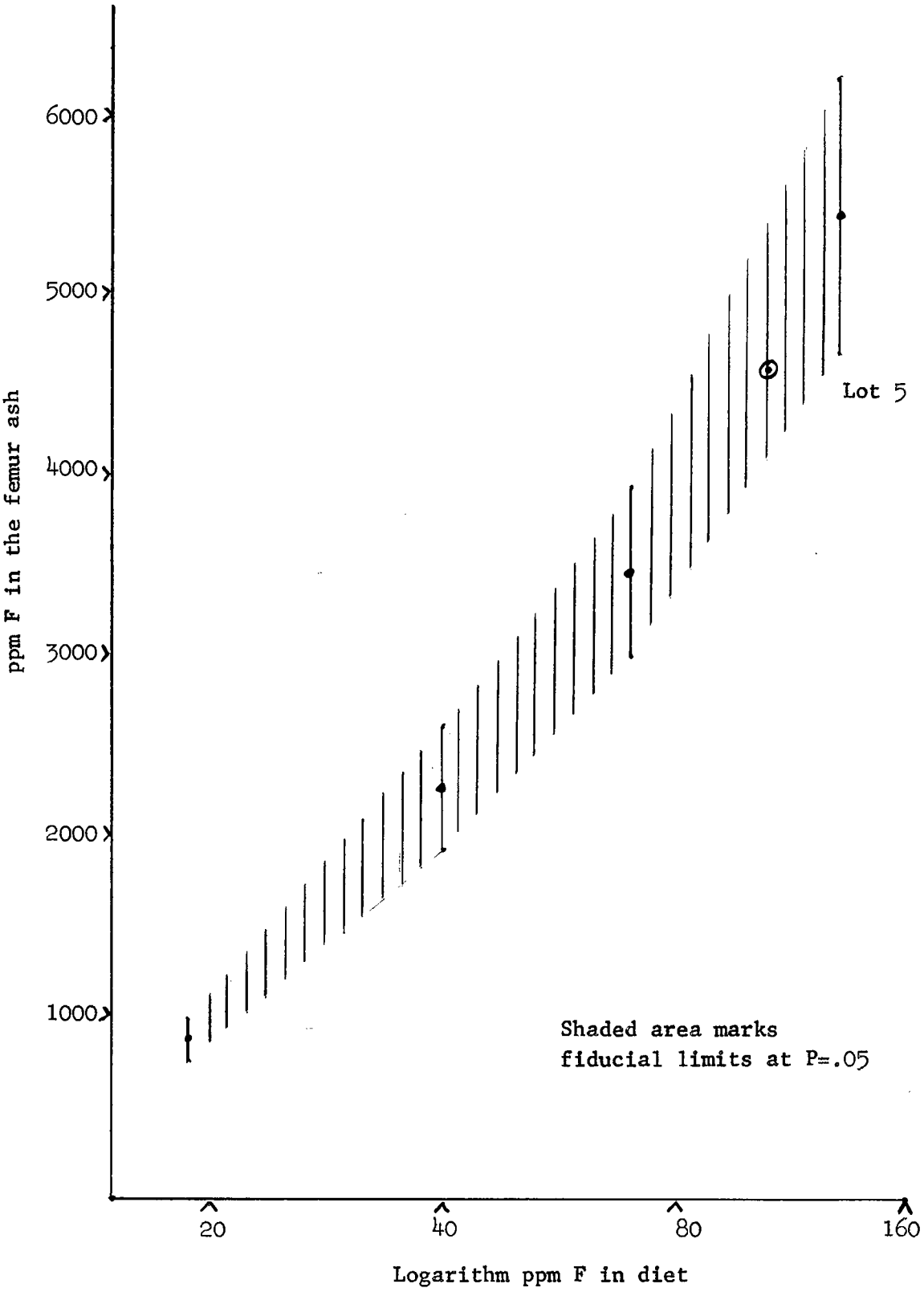
TABLE 9b. Analysis of variance of fluorine in bone ash data.

Source	D/F	Variance	F Value		
			Obs.	5%	1%
Total	7				
F levels**	3	7,624,453	201,774	9.28	29.46
Ascorbic acid	1	6,328	< 1	-	-
Error	3	37,787			

* Not included in analysis - F present as contaminated hay

** Highly significant P=.01.

FIG.5.- Fluorine in Guinea Pig Femur Ash in Relation to Fluorine in the Diet



Cell Measurements.

Histological sections were prepared and examined in the manner previously described in Phase II. In the case of the ameloblasts, the heights of the three highest cells were measured in the area previously designated as area No.5 for each incisor section. The numerical values were then averaged and converted to microns as the best estimate of the ameloblast height for that particular section. Table No.10a presents the data accumulated from these measurements with the analysis of variance of these data presented in Table 10b.

Fluorine levels in the diet had a highly significant effect on ameloblast heights thus confirming the results obtained in Phase II. Although ascorbic acid levels produced no significant effect on ameloblast heights under the conditions of this experiment, it will be noted that there is a barely significant effect on cell heights attributable to the interaction of fluorine and ascorbic acid levels in the diet.

Figure 6 depicts graphically the effect of ascorbic acid and fluorine levels in the diet on ameloblast heights. Examination of this graph would suggest that increased ascorbic acid in the diet tended to enhance the effect of fluorine when the element was present in the diet at levels above approximately 80 p.p.m. Reference to the data on body weight gains as presented in Table No.7a would suggest that some of the animals on diet lot 4 were suffering from severe fluorine toxicosis by the end of the feeding period.

TABLE 10a. Ameloblast heights of male and female guinea pigs fed graded amounts of fluorine at two ascorbic acid levels.

Diet Lot No.			1	2	3	4	5*
Animals/lot			8	8	8	8	8
Ascorbic acid	Sex	F p.p.m.	19	40	70	130	112
0.5 mg/day	M		29	29	23	23	21
			28	26	25	24	23
	F		25	29	27	24	25
			33	27	27	25	24
Sub-totals			115	111	102	96	93
1.0 mg/day	M		32	26	26	12	26
			27	27	26	21	26
	F		27	26	27	17	25
			30	30	26	18	23
Sub-totals			116	109	105	68	100
Total			231	220	207	164	193
Average Ameloblast heights			28.9	27.5	25.9	20.2	24.1

*Not included in analysis of variance since fluorine in diet was present as contaminated hay.

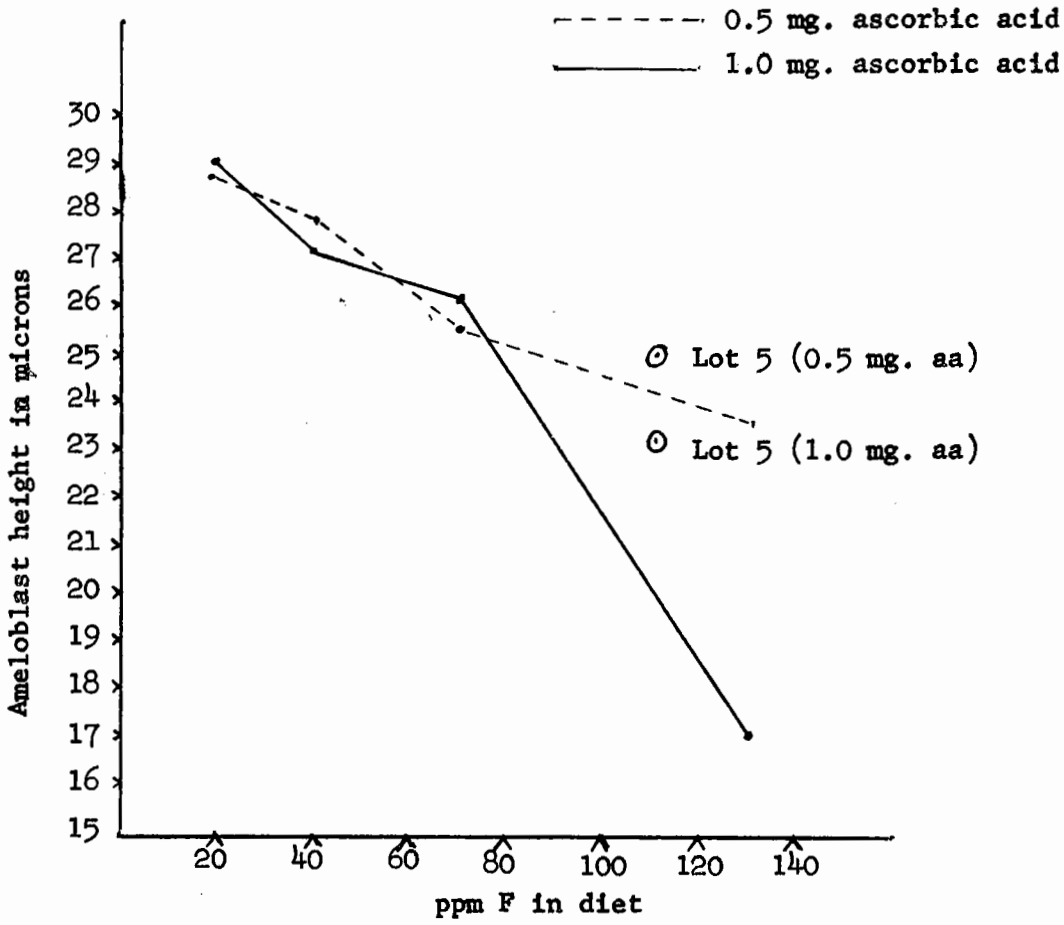
TABLE 10b. Analysis of variance of ameloblast heights.

Source	D/F	Variance	F Value		
			Obs.	5%	1%
Total	31				
Fluorine levels**	3	115	16.6	3.24	5.29
Ascorbic acid	1	18	2.6	4.49	8.53
Sexes	1	5	< 1	-	-
F × AA*	3	22.66	3.32	3.24	5.29
F × Sex	3	1	< 1		
AA × Sex	1	0	< 1		
F × AA × Sex	3	2	< 1		
Error	16	6.81			

* Significant P = .05

** Highly significant P = .01

FIG.6. The effect of fluorine and ascorbic acid in the diet on the height of Guinea Pig incisor ameloblasts



In this connection it is interesting to note that the animal most severely affected (as judged by a negative weight gain) also had the most severely depressed incisor ameloblasts (see Tables 7a and 10a). Animal lots that received dietary fluorine in the form of contaminated hay are shown separately in Fig.6. It will be noted that their ameloblast heights fall above the lines followed by the sodium fluoride-treated animals for both dietary levels of ascorbic acid.

The method of measurement of the odontoblast cells differed from that used in the previous trial. In this experiment the three highest cells seen in the section were measured and the average of the numerical values taken as the best estimate of the maximum odontoblast height for that particular animal. This method conforms more closely to that employed by other workers in this laboratory in the bioassay for vitamin C. Table No.11a shows the data accumulated from these measurements. Analysis of variance of these data is presented in Table 11b.

The effect of fluorine on odontoblast heights was highly significant statistically. Sex also exerted a statistically significant effect on the heights of these cells under the conditions of this trial. Fig.7 depicts graphically the effect of fluorine in the diet on the height of the odontoblast cells of male and female guinea pigs. The treatment lots that received fluorine in the form of contaminated hay are indicated separately on the graph. It will be noted that their odontoblast cell heights also fall above the line followed by the sodium fluoride treated animals.

TABLE 11a. Odontoblast heights of male and female guinea pigs fed graded amounts of fluorine at two ascorbic acid levels.

Ascorbic acid	Sex	Diet Lot No.	1	2	3	4	5*
		Animals/lot	8	8	8	8	8
		F p.p.m.	19	40	70	130	112
0.5 mg/day	M		35	43	36	25	30
			45	39	28	27	34
1.0 mg/day			35	32	30	24	37
			34	43	31	32	35
Sub-totals			149	157	125	108	136
0.5 mg/day	F		44	43	30	27	35
			53	41	36	37	34
1.0 mg/day			34	37	37	31	41
			38	41	38	36	39
Sub-totals			169	162	141	131	149
Total			318	319	266	239	285

* Not included in analysis of variance since fluorine in the diet was present as contaminated hay.

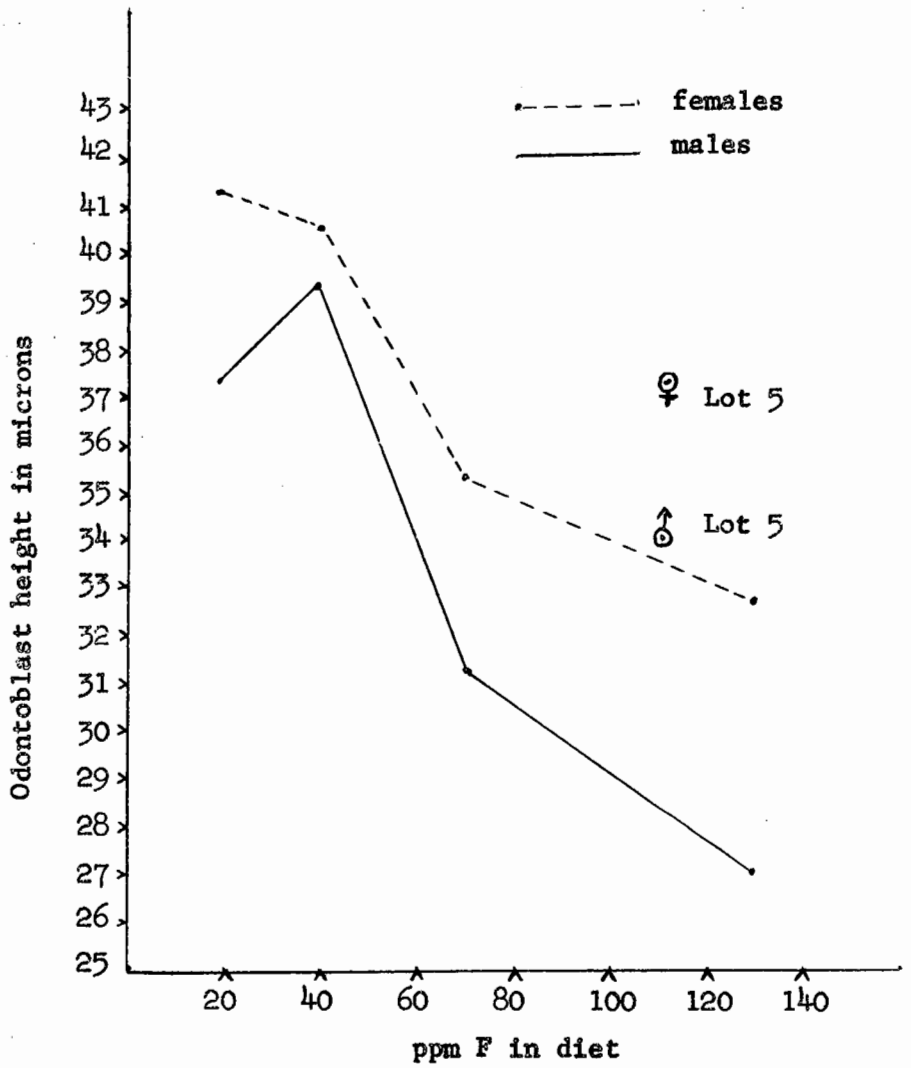
TABLE 11b. Analysis of variance of odontoblast heights.

Source	D/F	Variance	F Value		
			Obs.	5%	1%
Total	31				
Fluorine levels**	3	196.66	9.68	3.24	5.29
Ascorbic acid	1	41.	2.02	4.49	8.53
Sexes*	1	128.	6.30	4.49	8.53
F × AA	3	51.	2.51	3.24	5.29
F × Sex	3	7.66	< 1		
AA × Sex	1	0	< 1		
F × AA × Sex	3	14.33	< 1		
Error	16	20.31			

* Significant P = .05

** Highly significant P= .01

FIG.7. - The effect of fluorine in the diet on the height of the incisor odontoblasts of male and female Guinea Pigs.



RESULTS AND INTEGRATING DISCUSSION.

Feed Consumption and Body Weight Changes.

In the first fluorine feeding trial (Phase II) feed consumption and weight gains were not affected significantly by the levels of fluorine in the diet. The range of fluorine in these diets was from 24 to 86 p.p.m. In the second fluorine feeding trial (Phase III) a range of fluorine content in the diets of from 19 to 130 p.p.m. produced statistically significant differences in feed intake and resulting weight gains.

Many workers in the field of toxicology prefer to express the toxicity of cumulative poisons on the basis of milligrams intake per kilogram body weight per day. These values were calculated for the guinea pigs in Phase III. The mean weight achieved during the six-week period and the average feed consumption per day were computed for each animal on test. Expressed in terms of milligrams per kilogram body weight per day and as milligrams per unit metabolic size per day the fluorine intakes of guinea pigs on diets 1 to 5 are shown in Table 12.

TABLE 12. Fluorine intakes of guinea pigs on diets varying in fluorine content.

Diet Lot No.	p.p.m. F in diet	Mg. F/kg. B.W./day		Mg.F/ $(W_{kg}^{.75})^*$ per day (males and females)
		Males	Females	
1	19	1.7	1.9	1.4
2	40	3.6	3.6	2.8
3	70	6.8	6.3	5.1
4	130	10.9	11.0	8.3
5	112	10.0	10.7	8.0

* Unit metabolic size

It is interesting to note that the actual intake of fluorine per unit body weight is essentially the same for males and females in diet lots 1 to 4. This would suggest that where sex differences have occurred in this trial they are true effects and not merely due to the possibility of one sex receiving a more toxic dose of fluorine due to smaller size or greater feed intake per unit size.

Depression of weight gains in young growing guinea pigs might have been considered as the criterion for a fluorine bioassay. However reference to Fig.3 would indicate that weight gains are not affected markedly on diets containing less than 70 p.p.m. fluorine.

The original purpose of the development of a bioassay for fluorine was to detect reasonably small differences in fluorine content of contaminated forages. It would be desirable to be able to differentiate between levels of 10 p.p.m. over a range of forages bearing from 10 to 100 p.p.m. fluorine for example. Since body weights do not appear to be affected until a level of somewhere around 70 p.p.m. is reached it would not be feasible to establish a dosage response curve based on this criterion.

Digestibility of Nutrients.

The observed depression of crude fiber digestion that accompanied increasing fluoride content in the diet was interesting. Because of the small number of observations made in this experimental series no definite conclusions could be established. A possible explanation might be that fluorine exerts a depressant effect on the microflora in the caecum of the animal thus inhibiting the breakdown of cellulose.

Absorption of Fluorine.

Reference to Table 5 reveals that the fluorine content of the feces of animals consuming diet No.5 (fluorine content derived from contaminated hay) is almost double that present in the feces of animals consuming diet No.4 (fluorine content derived from added NaF). This would suggest either greater absorption of fluorine as sodium fluoride or that the fluorine present in contaminated hay is less available for absorption. Further support for this contention is afforded by reference to Figs. 6 and 7 which show that both ameloblast and odontoblast heights were depressed less by fluorine in the diet as contaminated hay than by an equivalent amount of fluorine in the diet as sodium fluoride.

Fluorine Storage in Bone.

The fluorine content of the guinea pig femur bone ash after a six-week feeding period appears to bear direct relationship to the level of fluorine in the diet. Even when allowance is made for error in the chemical analysis of bone for fluoride content, it should be possible to differentiate between 10 p.p.m. F increments in the diet if this criterion were employed in a bioassay of forages (see Fig.5). Unfortunately in this experiment it was not possible to ascertain whether or not a sex difference occurs in the matter of bone storage since bone samples from males and females had to be pooled to provide sufficient material for chemical analysis.

Ameloblast and Odontoblast Heights.

Cell measurements of odontoblasts and ameloblasts were disappointing in both trials as a potential criteria of fluorine intake. Reference to Appendix Fig.7 and also Fig.6 shows that at dietary fluorine intakes ranging from 20 p.p.m. to 80 p.p.m. it was only possible to demonstrate an ameloblast height depression of about 8 microns. Since this would be the potential useful range of the bioassay it is doubtful if a spread of 8 microns is sufficient for practical measurements to be made. The interaction of ascorbic acid and fluorine levels on ameloblast heights (see Table No.10b) is interesting but not readily explicable, especially since ascorbic acid alone apparently had no effect on the height of these cells. Since measurements of odontoblasts were not made in the same way in the two trials, results are not comparable, but based on Phase III the same objection can be raised against odontoblasts as a bioassay criterion of fluorine intake as was mentioned for ameloblasts. That is, the depression in cell heights is only about 8 microns over a spread in fluorine intake of from 20 p.p.m. to 80 p.p.m.

SUMMARY AND CONCLUSIONS.

1. Young growing guinea pigs were successfully maintained on a pelleted diet consisting of 75% ground hay for a period of six weeks. Thus this species might be a satisfactory test animal for a bioassay of fluorine in forage crops.

2. Fluorine in the diet of young guinea pigs depressed feed consumption and weight gains when the level in the complete diet approached 70 p.p.m. This level represented an average intake of approximately 6.5 mg. per kg. body weight per day during the six-week feeding period. In terms of metabolic size, 70 p.p.m. in the diet resulted in an intake of 5.1 mg/unit metabolic size/day.

3. The digestion of crude fiber was depressed by increasing increments of fluorine in the diet from a high of 28.1 percent for the basal diet that contained 19 p.p.m. fluorine to a low of 19.7 percent for the diet that contained 130 p.p.m. fluorine. It is theorized that this depression in crude fiber digestion may be due to inhibition by fluorine of the caecal microflora in this species.

4. The storage of fluorine in the bone was increased in linear relationship to the logarithm of the fluorine content of the diet. This criterion appeared to be the best of the several considered as far as reflection of fluorine intake was concerned.

5. The heights of ameloblast and odontoblast cells were progressively depressed by increasing increments of fluorine in the diets. In this regard however there did not appear to be a sufficiently great enough change in height per unit fluorine increment to warrant consideration of this phenomenom as a bioassay criterion for fluorine-containing forage crops.

6. A sex difference in fluorine tolerance was observed especially in so far as feed consumption and weight gains were concerned. In this regard male guinea pigs appeared to withstand higher dietary intakes of fluorine than did females. This would suggest that sex should be considered in fluorine feeding experiments where guinea pigs are the test animal.

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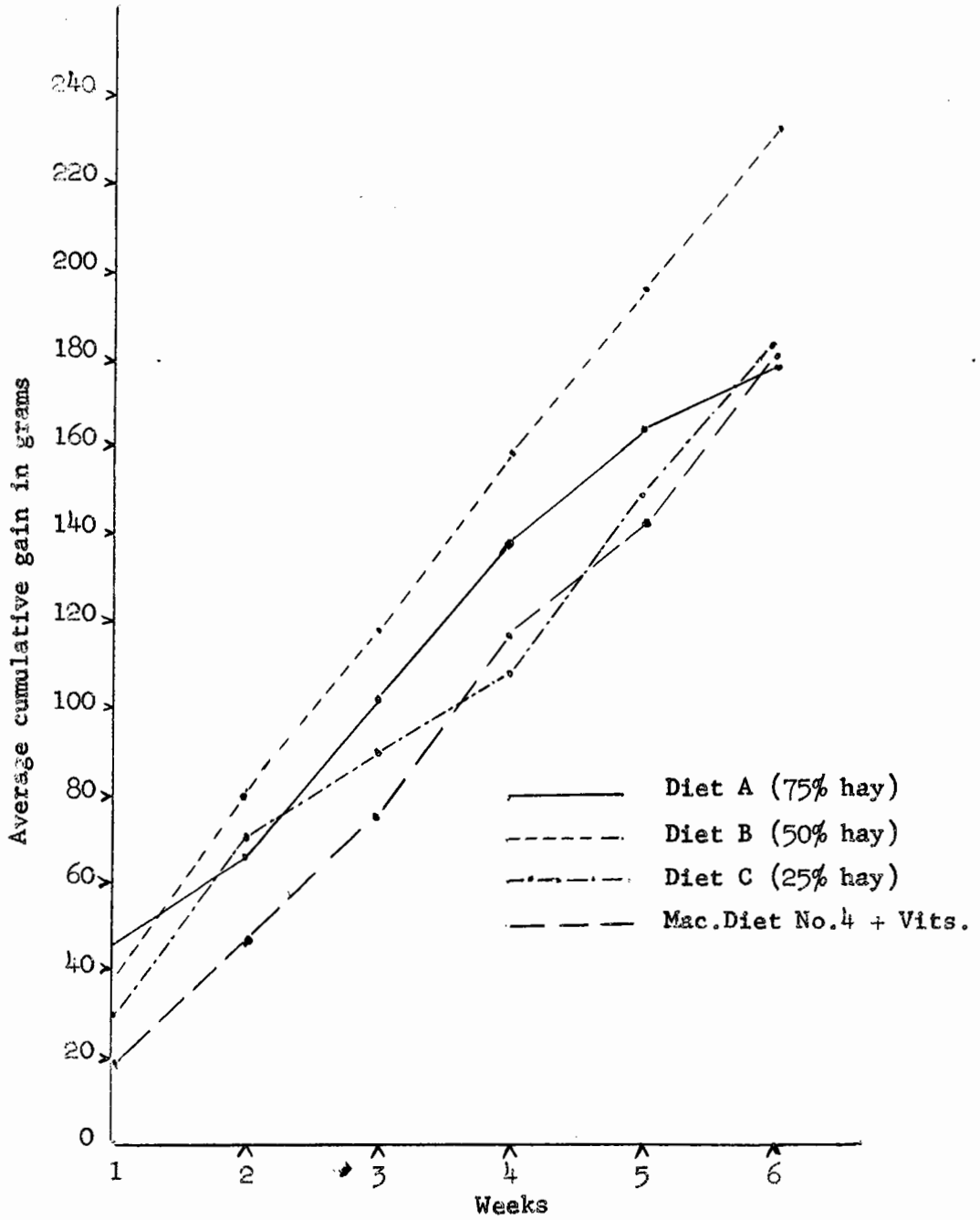
A P P E N D I C E S

APPENDIX FIG. 1. Cumulative weekly weight gains on diets of varying hay content.



HISTOLOGICAL TECHNIQUE.

Fixation.

After a minimum of 48 hours in 10% formalin the tissues were rinsed in water and transferred to 80% alcohol if prolonged storage was anticipated.

Decalcification.

(1) The exposed portion of the lower incisor was clipped off as was the portion of the ramus of the mandible posterior to the last molar.

(2) The specimen was then placed in a small glass container with a ground glass lid and covered with a 10% solution of commercial nitric acid (70% HNO_3) in water.

(3) After 48 hours the lateral surface of the mandible covering the molars was trimmed away with a scalpel and the molars were extracted. The acid solution was changed and the trimmed mandibles returned to the acid bath for an additional 48 hours.

(4) The individual specimens were then identified by attaching a small aluminum number tag, and were rinsed in water for a few minutes. They were then placed in 2% potassium alum solution for a 12-hour period to neutralize the acid.

(5) Specimens were then rinsed in running tap water for 30 minutes prior to dehydration.

Dehydration.

Specimens were moved progressively through 50, 70, 80, 90, 95, 100 percent alcohols allowing four or five hours in each alcohol concentration.

Clearing.

Cedar oil was used as the clearing agent, changing once after two hours and leaving overnight in the final bath.

Infiltration.

"Tissue mat" (Fisher) 60-63° was used for both infiltration and embedding. The specimens were passed through three lots of melted "tissue mat" in a constant temperature oven set at 65°C. The time interval in each lot was about one hour.

Embedding.

The jaw specimens were embedded in tissue mat with the lateral (trimmed) surface of the mandible down. The paraffin blocks containing the specimens were labelled with the appropriate lot number.

Sectioning.

(1) Blocks were trimmed and then mounted on wooden holders by heating the wooden block to the charring point on the coil of a hot plate, and then pressing the charred end into the block.

(2) The trimmed block was then inserted in a microtome and after orientation sagital sections were cut until the center of the incisor tooth was reached.

(3) Ribbon sections were then cut and saved for mounting.

Mounting.

(1) Two or three drops of Mayer's albumen fixative were placed on a previously acid-cleaned microscope slide.

(2) Five or six drops of tap water were flooded on the slide and mixed with the Mayer's fixative.

(3) Desirable sections were then transferred to the liquid on the slide. Usually four sections to a slide.

(4) The slide was then placed on the metal top of a serological bath (CENCO) with water temperature about 65°C.

(5) Slides were carefully watched at this stage as over-heating is undesirable. When the sections were flattened satisfactorily the excess fluid was drained off over the edge of the slide onto a paper towel.

(6) Sections were then placed on a wire mesh screen elevated one inch above the surface of the metal plate covering the water bath and allowed to dry.

Staining.

The sections were stained by the progressive hematoxylin-eosin method as follows:

Staining (continued).

Xylol I - 2 mins. (changed after every 15 slides).

Xylol II - 2 mins.

Alcohol - 2 mins. each - 95, 80, 70, 50, 30, 15 percent (changed after each 15 slides).

Tap water - 2 mins.

Hematoxylin - 4 mins.

Alkaline tap water - 1 min.

Alcohol - 2 mins. each 15, 30, 50, 70, 80, 95 percent.

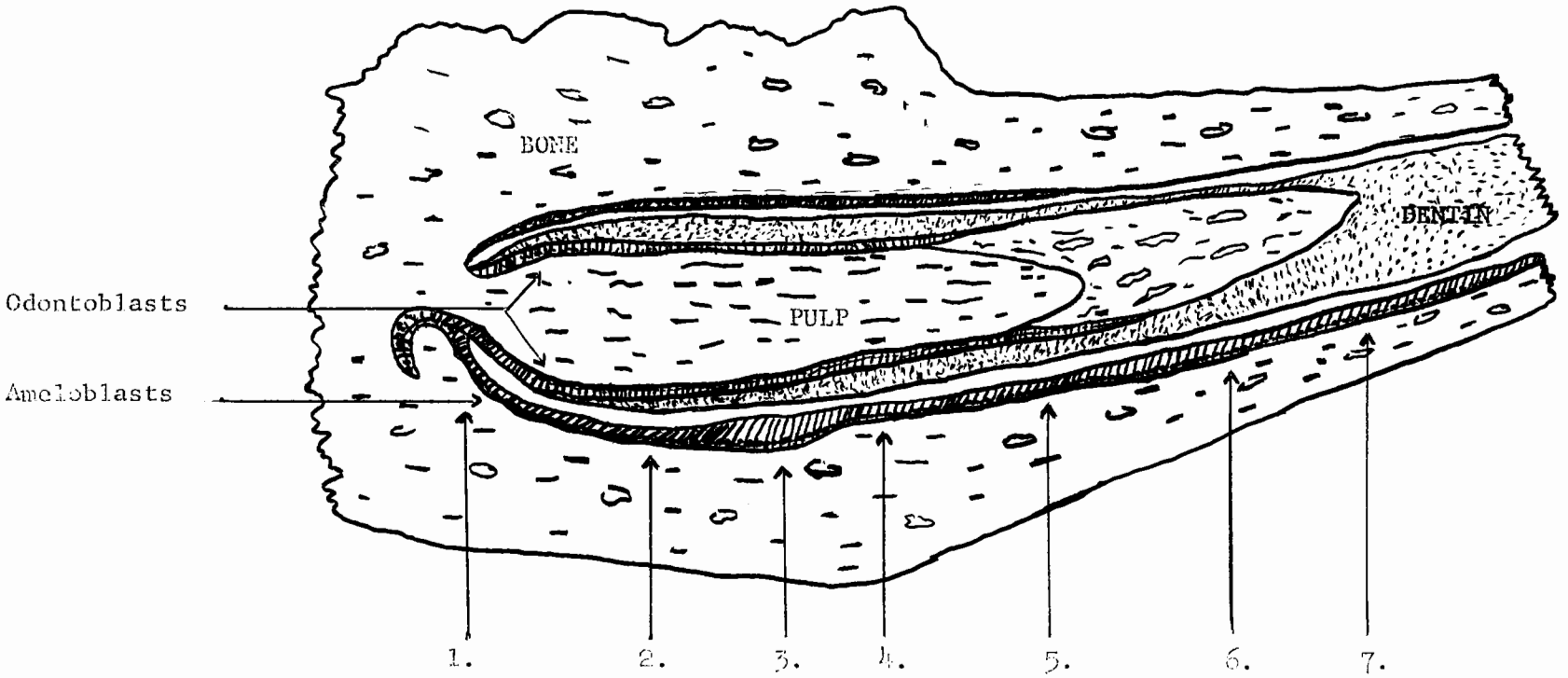
Eosin in absolute alcohol - 5 mins.

Carbol-Xylol - 2 mins.

Xylol - 2 mins.

Canada Balsam mounting.

APPENDIX FIG.2. Diagrammatic Illustration of a Lower Incisor Section.



Ameloblast Areas:

1. Area of alignment
2. Area of increasing slope
3. Area of maximum height
4. Area of minimum height
5. Area of uniform columnar cells often adhering to enamel matrix
6. Area of uniform columnar cells lightly stained
7. Area of uniform columnar cells lightly stained

APPENDIX TABLE 1. Analyis of Covariance of Gains (y) and Feed Intakes (x) of Guinea Pigs on Various Levels of Dietary Fluorine Intake.

Source of variation	D/F	$\Sigma(xy)$ Group totals	No.	Individuals	$\Sigma(x-\bar{x})(y-\bar{y})$	Covariance xy	rxy	b
Total	34			10,688,083	171,203			
7 Fluorine treatments	6	52,719,162	5	10,543,832	26,952			
Groups 1-5 vs. 6-7	1				29,506			
Remainder	27				114,745	4249.8	0.67	0.1042

Correction factor $\frac{5624 \times 65,450}{35} = 10,516,880$

Covariance xy $= \frac{(x-\bar{x})(y-\bar{y})}{D/F}$

rxy $\frac{\text{Covariance}}{sx \times sy} = \frac{4250}{31.4 \times 201.9} = \frac{4249.8}{6339.6} = 0.6703$

$b = \frac{\text{Covariance xy}}{\text{Variance x}} \quad \frac{4249.8}{40,784} = 0.1042$ grams gain/gram feed intake.

APPENDIX TABLE 2. Effect of Dietary Fluorine on Odontoblast Height (y).

ANALYSIS OF VARIANCE

Source	D/F	Σy^2 Group totals	No.	Σy^2 Individual	$\Sigma(y-\bar{y})^2$	s^2	s	F Value Obs.	Nec. .05	Nec. .01
Total	169			191,167	16,511					
Total Groups	48	180,485			5,829	121.44				
Between F levels	6	176,928			2,272	378.67		4.29	2.17	2.95
Between locations measured	6	176,256			1,600	266.67		3.02	2.17	2.95
Interaction	36				1,957	54.36		< 1		
Remainder	121				10,682	88.28	9.4			

Correction factor $\frac{5449^2}{170} = 174,656$

APPENDIX TABLE 2. Effect of Dietary Fluorine on Ameloblast Height (x).

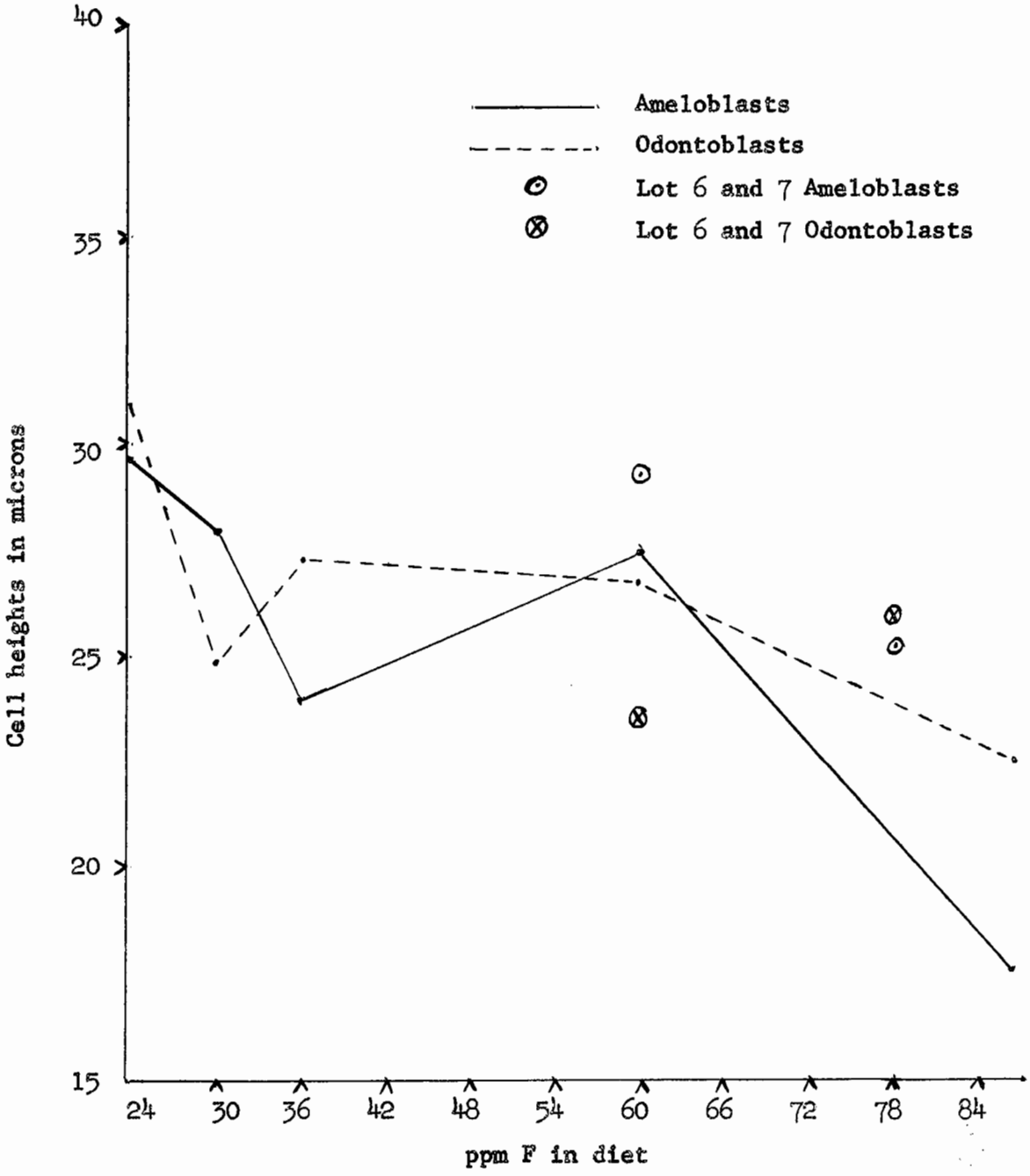
ANALYSIS OF VARIANCE

Source	D/F	Σx^2 Group totals	No.	Σx^2 Individual	$\Sigma(x-\bar{x})^2$	s^2	s	F Value Obs.	Nec. .05	Nec. .01
Total	201			174,750	22,262					
Total Groups	48	167,015			14,527	302.6				
Between F levels	6	153,499			1,011	168.5		3.33	2.16	2.92
Between locations measured	6	165,491			13,003	2167.2		42.87	2.16	2.92
Interaction	36				513	14.25	< 1			
Remainder	153				7,735	50.55	7.1			

Correction factor $\frac{5550^2}{202} = 152,480$

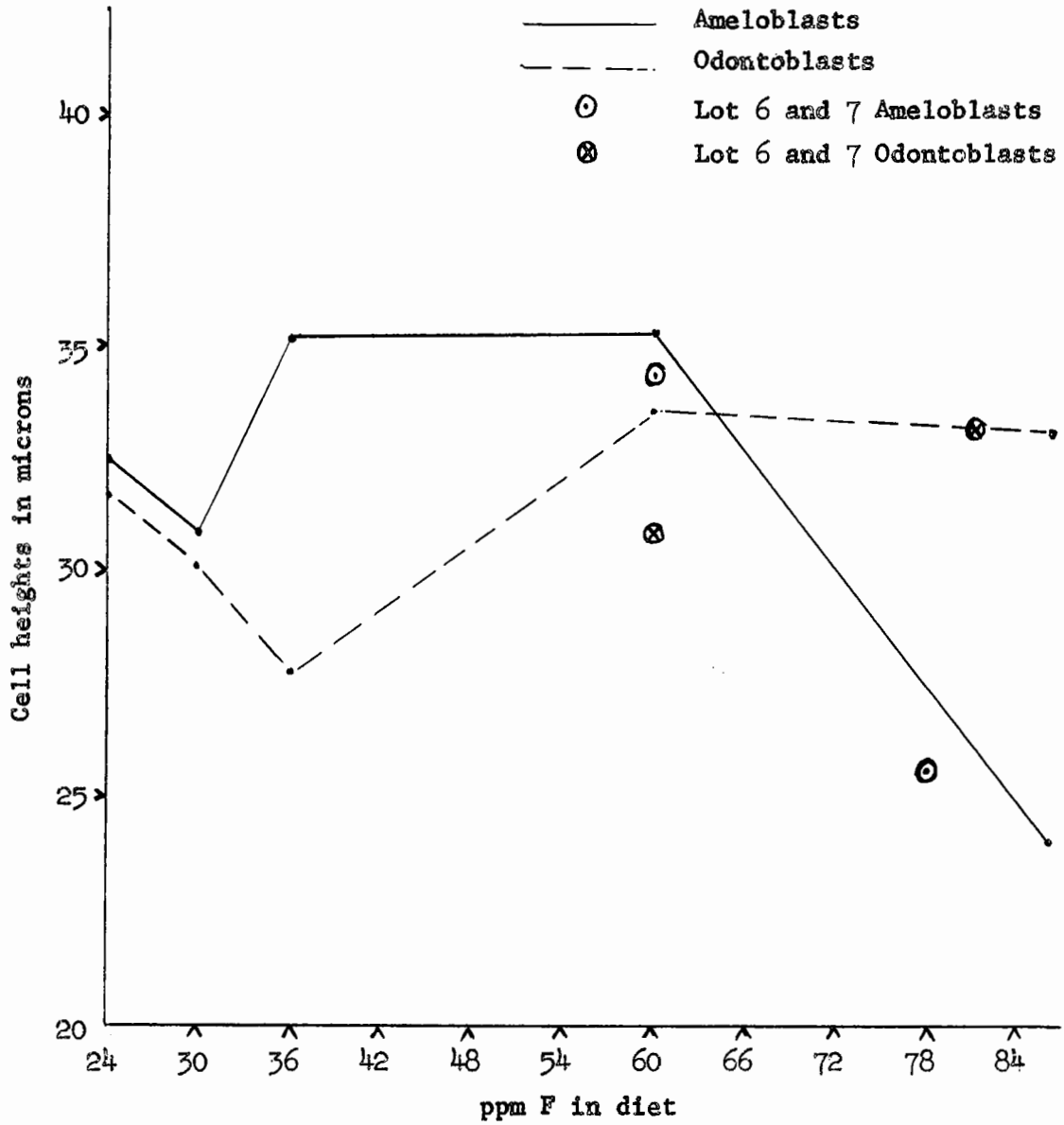
APPENDIX FIG. 3. - Ameloblast and odontoblast heights of Guinea Pigs on various dietary fluorine levels as measured at:

AREA NO.1



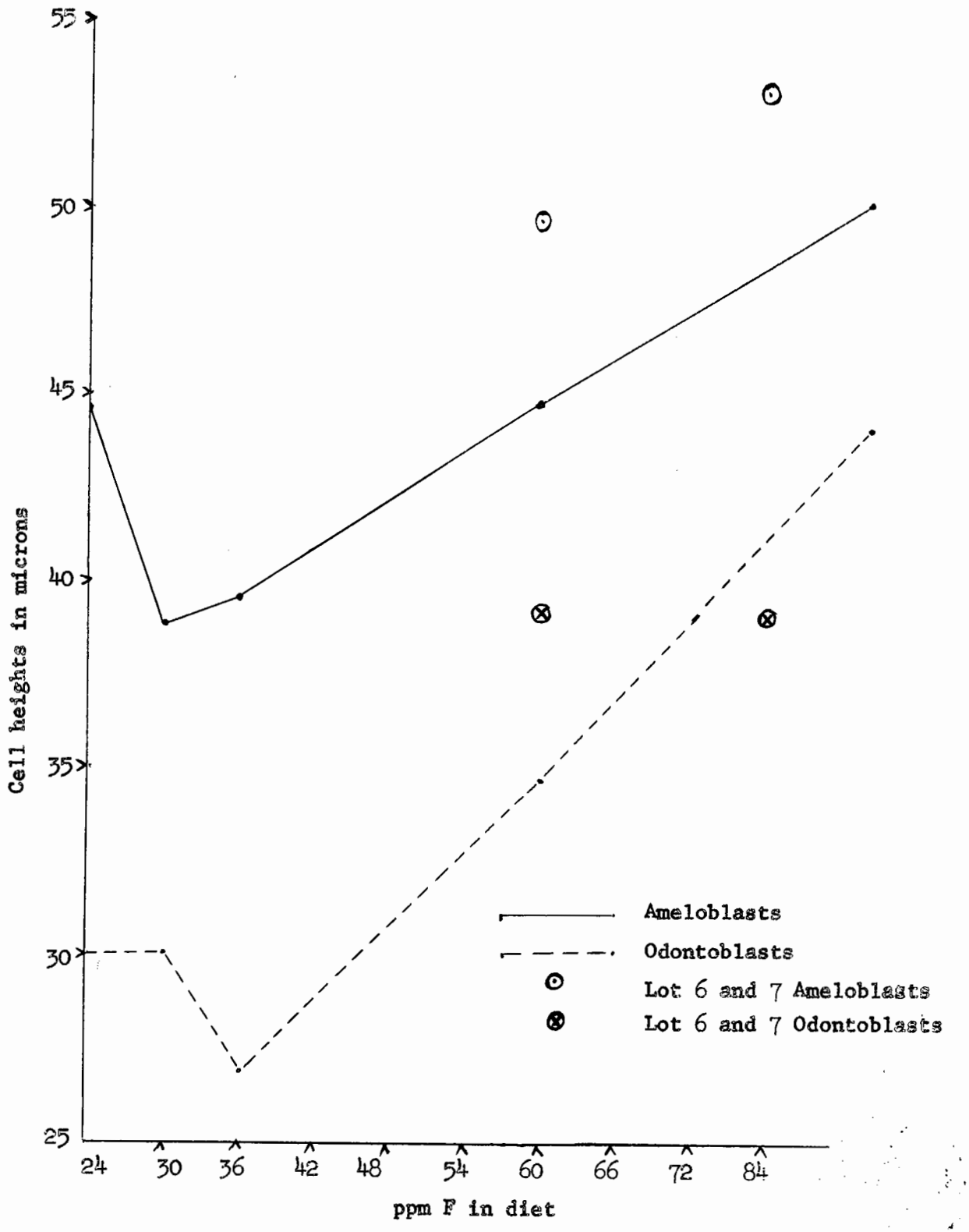
APPENDIX FIG.4. - Ameloblast and Odontoblast heights of Guinea Pigs on various dietary fluorine levels as measured at:

AREA NO.2



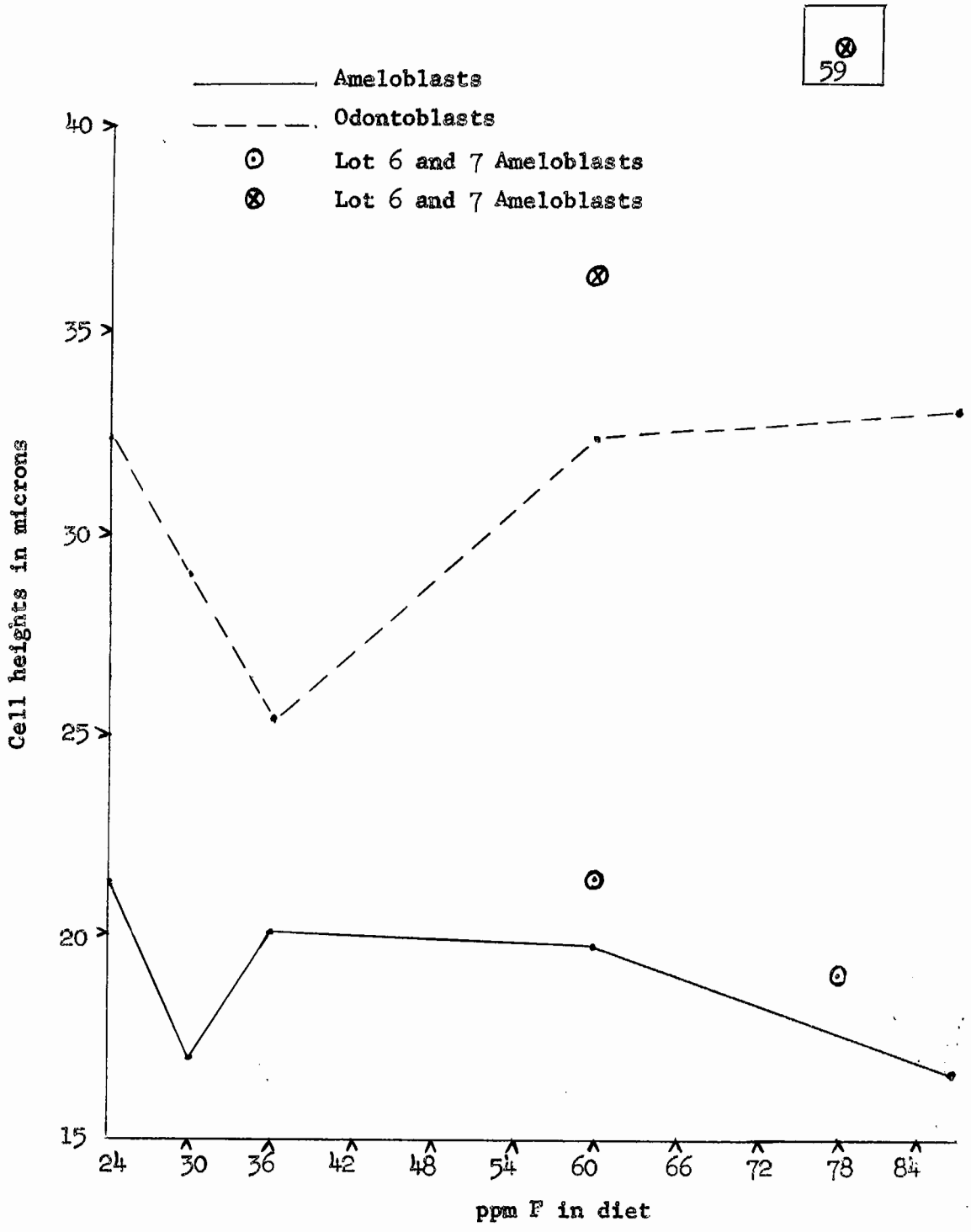
APPENDIX FIG.5. - Ameloblast and Odontoblast heights of Guinea Pigs on various dietary fluorine levels as measured at:

AREA NO.3



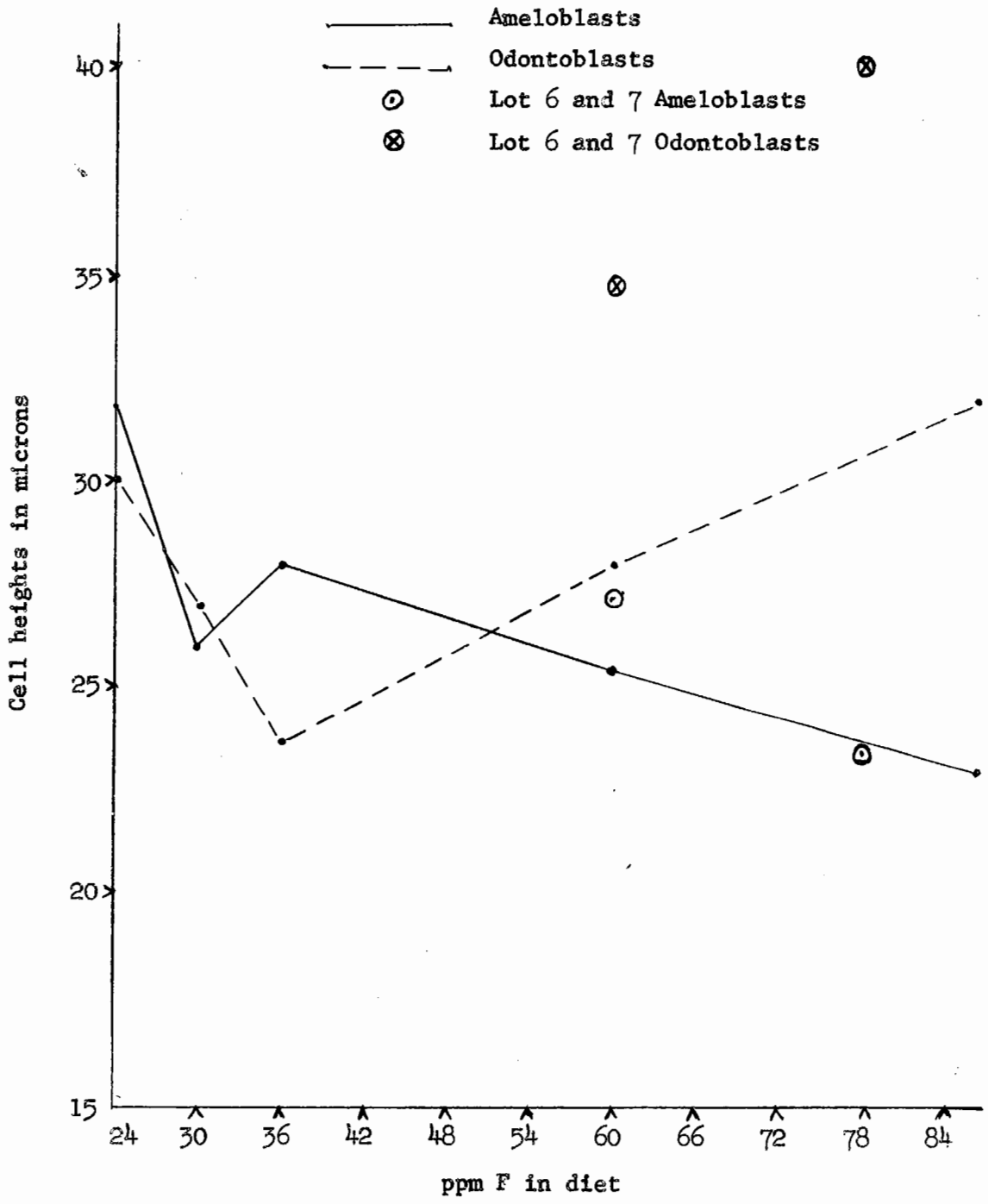
APPENDIX FIG.6. - Ameloblast and Odontoblast heights of Guinea Pigs on various dietary fluorine levels as measured at:

AREA NO.4



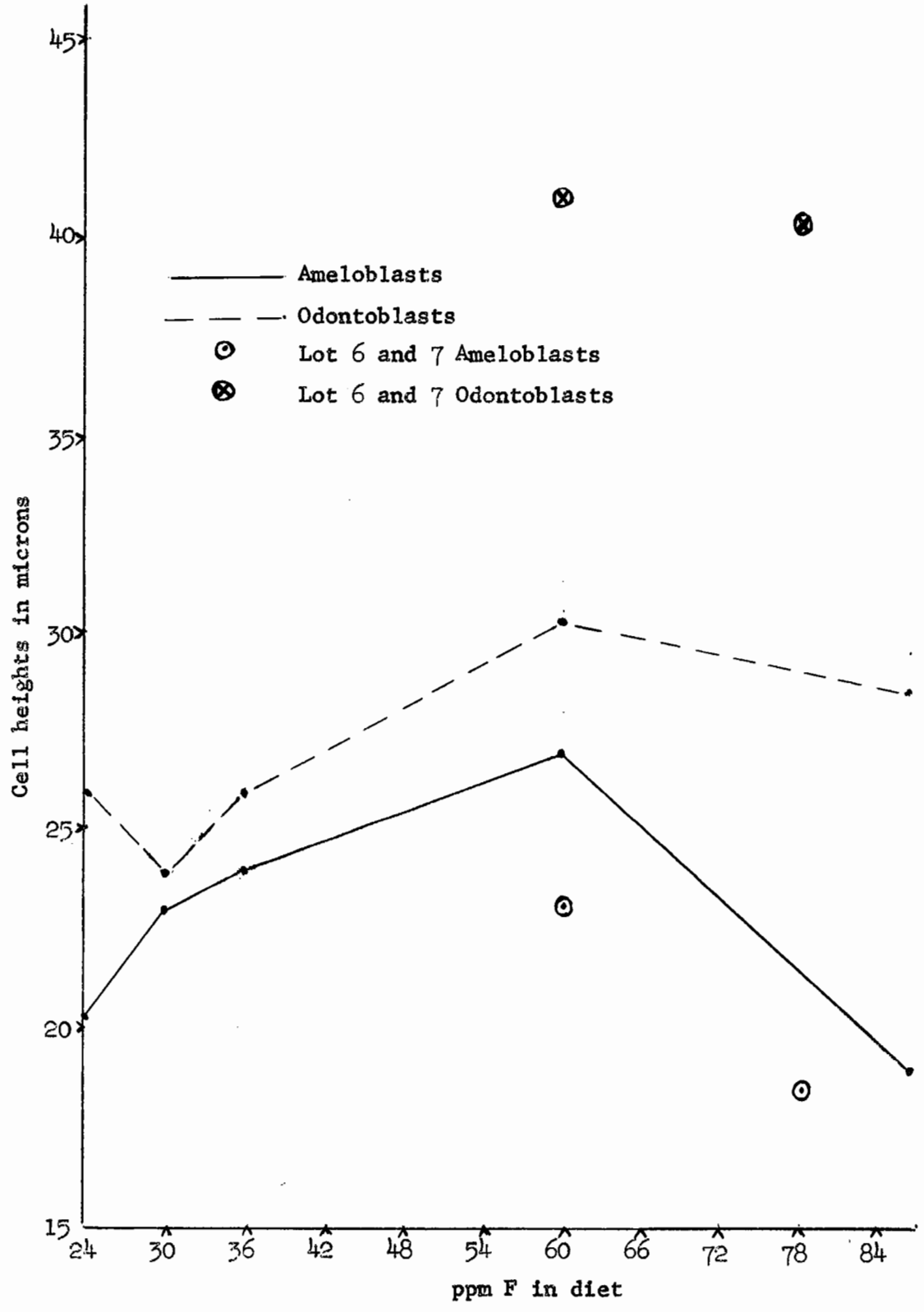
APPENDIX FIG. 7. - Ameloblast and Odontoblast height of Guinea Pigs on various dietary fluorine levels as measured at:

AREA NO.5



APPENDIX FIG.8. - Ameloblast and Odontoblast heights of Guinea Pigs on various dietary fluorine levels as measured at:

AREA NO.6



APPENDIX FIG.9. - Ameloblast and Odontoblast height of Guinea Pigs on various dietary fluorine levels as measured at:

AREA NO.7