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Title: THE FATE OF FLUORIDE AND THE EFFECT OF FLUORIDE UPON GLUCOSE METABOLISM IN INTACT RATS

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The inhibition of the enolase enzyme system by fluoride has been established for many years in *in vitro* experiments; however, this inhibitory action has never been demonstrated in an intact mammal. The present work was designed to examine the effect of fluoride administered by intravenous infusion in rats, under the assumption that enolase inhibition would be reflected in depressed glucose catabolism. Companion experiments were also carried out using ^{18}F as a tracer to examine the time course of fluoride distribution in rats, thereby providing a better understanding of the over-all toxic action of fluoride.

Radiorespirometric studies were carried out using ^{14}C-labeled glucose to investigate the effect of fluoride upon glucose catabolism in intact rats. The results demonstrate that, when the concentration of administered fluoride builds up to 2×10^{-4} M on the basis of even distribution of fluoride in body fluid, significant

inhibition of glucose catabolism occurs.

Use was also made of ^{18}F as a radiotracer of stable fluoride in the tissues of rats intravenously infused with various concentrations of fluoride. When fluoride was infused at a concentration of 0.126 M (2 mg fluoride per hour) the amount of fluoride accumulated in soft tissue was proportional to the cumulative amount of fluoride infused. The concentration of fluoride in soft tissue ranged from 10^{-4} to 10^{-3} M at a time which corresponded to the maximum fluoride inhibition of glucose catabolism seen in the radiorespirometric studies.

The concentration of fluoride causing significant inhibitory effect on enolase *in vitro*, as reported in the literature, is on the order of 10^{-3} M. Two independent methods in the present study show that, when administered fluoride reaches a concentration of 10^{-4} to 10^{-3} M in blood and soft tissues, severe inhibition of glucose catabolism occurs in intact rats. Therefore, there is every reason to believe that one of the major toxic actions of fluoride in rats is due to the inhibition of the enolase system, a key step of glucose catabolism.

Data obtained in ^{18}F experiments in which 0.126 M fluoride was administered indicate that the fluoride in blood and soft tissues was promptly deposited in bone. Upon termination of fluoride administration, the major route of depletion of fluoride from blood and

soft tissues was by way of renal excretion, while fluoride incorporated into the bone remained at the same level without noticeable depletion over the period of these experiments. When ^{18}F-labeled NaF was administered to rats at concentrations lower than 0.126 M, fluoride build-up in blood and soft tissues appeared to follow a similar pattern as that observed in 0.126 M fluoride experiments. In the ^{18}F experiments it was also noted that, as the cumulative dose of fluoride administered by way of continuous infusion increased, there existed a defined ceiling in the capacity for fluoride removal by bone deposition or renal excretion. This resulted in excessive accumulation of fluoride in blood and soft tissues.

It is is believed that the findings in the present work add much to the current understanding of fluoride toxicology in intact mammals. It is also noteworthy that a detailed description for preparation of ^{18}F is presented in the present work, including several important revisions of previously published procedures.

The Fate of Fluoride and the Effect of Fluoride upon Glucose Metabolism in Intact Rats

by

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THE FATE OF FLUORIDE AND THE EFFECT OF FLUORIDE UPON GLUCOSE METABOLISM IN INTACT RATS

I. INTRODUCTION

Fluoride is a commonly occurring element known to have beneficial effects at very low levels and harmful, toxic effects in certain chemical forms upon various biological species at high dose. It has been established beyond reasonable doubt that human populations consuming water with inorganic fluoride at concentrations of 1 to 2 ppm suffer no deleterious effects (17, 18, 21, 32). In fact the demonstrated beneficial role of fluoride in the development and maintenance of sound bones and teeth has caused some researchers to suggest that fluoride is one of the essential elements (19, 22, 23, 26). On the other hand, the toxicity of inorganic fluorides at high dose to man and animal is well documented and has been most recently reviewed by Eager (9). It is true that the toxicology of fluoride has been studied by numerous investigators; however, there is yet much to be learned. The increasing use of fluorinated compounds in many industrial processes and the increasing discharge of fluorides from industrial operations into the environment makes it essential to improve our understanding of fluoride toxicology.

Classical investigations into fluoride toxicology were characterized by an emphasis on symptomatic manifestations. According to

Roholm (30) the first detailed records of investigations into acute and chronic experimental fluoride poisonings were published in 1890. The work that followed until World War I centered around disease in man and domestic animals associated with the fluoride compounds in waste products of industrial operations.

The investigations on acute and chronic fluoride intoxication in mammals have continued to the present time. Major findings with regard to acute lethal toxicity of NaF are summarized in Table 1, on the basis of information from reviews by Hodge and Smith (13) and by Largent (15). Among these studies only two series of investigations involved continuous intravenous infusion. Leone, et al. (16) observed lethal effects of NaF when infused into dogs over periods of several minutes. Lu, et al. (20) infused NaF solutions intravenously into monkeys and rats at a rate of 1 mg of fluoride per kg per minute until death occurred. Since a portion of the infused fluoride accumulated in the body before death actually occurred, it may be assumed that the lethal doses were lower than the observed doses in both of these experiments. Table 2 summarizes findings for chronic lethal toxicity of NaF in man and rats and of HF in rats. This information is taken from a review paper of Hodge and Smith (13). The data on HF toxicity (33) in rats via inhalation are included in Table 2 to illustrate that inhalation is an important, direct route of administration or exposure of an intoxicant to an animal. In inhalation

Table 1. Acute Lethal Toxicity of NaF in Selected Mammals*

Animal	Route of Administration	Dose mgF/Kg body wt	Effect	Ref.
Rat	Subcutaneous	110	LD_{100}	(11)
Rat	Subcutaneous	55	Minimum for lethality	(25)
Rat	Oral	45-90	Lethal (?)	(15)
Mouse	Oral	45	LD_{50}/24 hr	(14, 16)
Rat	Intraperitoneal	23-45	LD_{100}	(8)
Rat	Intraperitoneal	23.5	LD_{50}/24 hr	(8)
Rat	Intravenous	40	Minimum for lethality	(15)
Rat	Intravenous infusion	60	Lethal in minutes	(20)
Dog	Intravenous infusion	36	Lethal in 7 min.	(16)
Dog	Intravenous infusion	20	LD_{50}	(16)
Man	Oral	30	Minimum for lethality	(13, 30)
Man	Oral	32-63	LD_{100}/24 hr	(13, 30)

*Data modified from Hodge and Smith (13) and Largent (15).

Table 2. Chronic Lethal Toxicity of NaF in Man and Rat and Chronic Lethal Toxicity of HF in Rat*

Animal	Route of Administration	Dose mgF/Kg body wt/da	Duration days	Effect	Ref.
Rat	Water	22.6	28-56	LD_{100}	(13)
Rat	Diet	78-84	42	Complete Inhib. growth	(28)
Rat	Injection	68	(?)	Minimum for Lethality	(13)
Rat	Inhalation (HF)	(25 ppm in air)	14	LD_{100}	(33)
Man	Environment (Occupational)	0.3-1.2	(10-20 yrs)	Crippling fluorosis	(13, 30)
Man	Intravenous Inj.	11	9	None	(13)

*Data Modified from Hodge and Smith (13).

studies estimation of the amount of intoxicant actually absorbed is often a difficult task (7).

To gain insight into the mechanism underlying the toxicity of fluoride, information on the distribution of administered fluoride in experimental animals was needed. Research in this area was severely limited by the lack of accurate micro-methods for fluoride analysis (15, p. 57; 24; 26, p. 100; 30). The early 1930's saw the breakthrough of simple, reliable and relatively sensitive methods of fluoride estimation that made possible studies of the distribution of fluoride in soft tissues (including blood) of whole organisms (26, p. 101). Work in this area has continued ever since (7) by modifying and automating these procedures (26, p. 101).

A new dimension of measuring fluoride in soft tissue was introduced when Wallace and Wallace-Durbin (35, 36) used radiofluorine (^{18}F) as a tracer. Wallace-Durbin administered ^{18}F via single-dose injection with and without stable carrier fluoride to determine concentrations of administered fluoride in soft tissues of intact rats. Similar studies in lambs (27) and cattle (3) have also been made.

Two considerations become evident when one compares chemical and radiotracer techniques for fluoride determination in soft tissue. First, chemical methods provide the sum of both bound and unbound fluoride in tissue which may lead to erroneous conclusions about distribution of endogenous and exogenous fluoride. Second,

Dost, *et al.* (7) found substantial variation in amounts of fluoride among control groups of rats assayed several months apart, as well as within each control group, especially in the soft tissues. This makes it very difficult to assess the net incorporation of administered fluoride in test animals. The use of ^{18}F overcomes these two problems, since the radiotracer technique reveals only the fate of exogenous fluoride.

Regardless of the use of stable fluoride or ^{18}F-labeled fluoride compounds in studies of the mechanism underlying fluoride intoxication, the route and method of substrate administration are of paramount importance. Of all the route options an investigator has open to him, clearly the most direct would be intravenous injection, since this route would place a known quantity of intoxicant directly into the circulatory system. The method of substrate administration adopted by most investigators has been single-dose administration which results in a transient increase of fluoride in the animal's body with a continuously changing effective concentration. This type of experiment does not provide meaningful kinetic information which is essential in obtaining a correct understanding of the fate of fluoride in intact animals.

In recent years considerable effort has also been devoted by many investigators to study the interference of fluoride on enzymic activities. The bulk of these experiments has involved the use of

in vitro systems with isolated preparations of tissues from a variety of biological systems. Table 3, taken from Hodge and Smith (13, p. 183), summarizes the effects of fluoride on various enzyme systems. From this information it appears that the enzyme enolase, which is part of the key enzymic system of energy metabolism, deserves attention. The sensitivity of enolase to fluoride was first demonstrated by Warburg and Christian (41). They proposed that in the presence of fluoride and phosphate, an inactive magnesium fluorophosphate complex was reversibly bound to the enzyme displacing the active magnesium, thereby inhibiting the action of the enzyme. This contention still remains plausible, although other hypotheses for fluoride action are continually being published as more work is being done. However, as pointed out by Hamilton (10), these recent hypotheses have not been verified by *in vivo* experiments.

In order to better understand the mechanism underlying fluoride intoxication in mammals it appears that the well demonstrated inhibition of enolase is playing an important role in intact animals. If so, does such inhibition occur at approximately the same fluoride concentration in blood and soft tissues as compared to that observed in *in vitro* studies?

In the present work radiorespirometric methods were employed to study glucose catabolism in normal rats and in rats continuously infused with fluoride solutions at various levels. Emphasis was also

Table 3. Effects of Fluoride on Enzyme Systems*

Enzyme	Molar conc. of F^- for effective inhibition	Effect
Enolase	10^{-3}	Accumulation of phos-phoglyceric acid
Acid phosphatase	10^{-2}	Increase blood organic phosphate (?) Lower inorganic phosphorus
Alkaline phosphatase	10^{-2}	As above
Cytochrome c	10^{-3}	Cellular anoxia
Esterases	10^{-6}	Accumulation of lower fatty acids
Amylase	10^{-2}	Accumulation of salivary carbohydrates
Carboxylase	10^{-2}	Accumulation of pyruvate
Lipase	10^{-5}	Accumulation of higher fatty acids

*Taken from Hodge and Smith (13, p. 183).

placed on the recovery of rat metabolism to the normal state upon termination of fluoride infusion. The concentrations of exogenous fluoride in blood and soft tissues were studied by the use of ^{18}F-labeled fluoride continuously infused in experiments patterned after the conditions in glucose catabolism studies. The depletion of fluoride from blood and soft tissues of rats upon termination of fluoride infusion was also studied.

II. MATERIALS

Experimental Animals

Male Sprague Dawley rats obtained from Bio Science Animal Laboratories, Oakland, California were used in all phases of this work. The average weight of the animals upon receipt was approximately 210 g. They were maintained on Purina Laboratory Chow and water, *ad libitum*, until the desired weight was attained.

Radiochemicals

Glucoses -1-, -2-, -3-, -3(4)- and -6-^{14}C used in radiorespirometric experiments were purchased from New England Nuclear Corporation, Boston, Massachusetts. The ^{125}I (radio-iodinated serum albumin) for blood volume determination was purchased from Abbott Laboratories, North Chicago, Illinois.

The toluene-^{14}C used as a primary standard in liquid scintillation counting was purchased from Packard Instrument Company, Downers Grove, Illinois.

Chemicals

Reagent grade chemicals were used in all operations.

The 95% enriched lithium-6 carbonate was purchased from the Atomic Energy Research Establishment, Harwell, England.

Other Materials

Precision bore quartz tubing used to encapsulate $^{6}Li_2CO_3$ for neutron irradiation was purchased from Ace Glass Incorporated, Vineland, New Jersey. TRIGA high flux aluminum irradiation tubes were obtained from Gulf Energy and Environmental Systems, Incorporated, San Diego, California.

III. METHODS

Animal Preparation

Animals were maintained on a fasting-feeding schedule of 24 hours feeding *ad libitum* of Purina Laboratory Chow alternated with 24 hours without feed. Water was available constantly. This procedure is a variation of that used by Potter, *et al.* (29) and permits greater reproducibility in intact animals (40) by stabilizing sharp fluctuations in enzyme levels of animals subjected to fasting for the first time. This procedure has the additional benefit of holding the weight range of rats to 300-350 g.

Rats were anesthetized intraperitoneally with sodium nembutal (Abbott Laboratories, North Chicago, Illinois) at 50 mg per kg body weight. An indwelling cannula was placed in the anterior vena cava through the right jugular vein according to the technique of Weeks and Davis (42). The polyethylene-silicone rubber cannulae were made by Mr. J. J. Krake of the Upjohn Research Laboratory, Kalamazoo, Michigan, using the method described by Heatley and Weeks (12). All animals were allowed to recover from the operation with full feeding for at least six days, or until the presurgical weight of 300-350 g was regained. Animals ready for experimentation, but not immediately used, were put on the fasting-feeding schedule described above.

Cannulated rats were prepared for continuous infusion in the following steps. On the morning following scheduled removal of food, rats were given 1.5 g of glucose in 3 ml of water by stomach intubation. Six hours later the rats were considered to be in a post-absorptive state (39, 40) with substantial liver glycogen storage. At this time each animal was placed in a restrainer cage (Aerospace Industries, Model 1 R350, Garnerville, New York) and the cannula was connected to a substrate infusion syringe. The syringe had previously been filled with substrate solution and pumped sufficiently to purge the connecting line of all air bubbles (Figure 1).

Radiorespirometric Studies of the Effect of Fluoride on Glucose Catabolism

Substrate Preparation and Administration

Carbon-14 specifically labeled glucose substrate solution at a prescribed concentration was administered by continuous intravenous infusion. The rate of infusion for all experiments was 0.84 ml per hour. The glucose concentration was adjusted to provide 150 mg of glucose, containing 0.010 to 0.015 μCi ^{14}C, to the rat per hour. In all but exploratory experiments the infusion of labeled glucose was allowed to continue for 16 hours and the measurement of respiratory $^{14}CO_2$ was continued at least four hours after the termination of infusion. By this time the radioactivity in respiratory CO_2 was

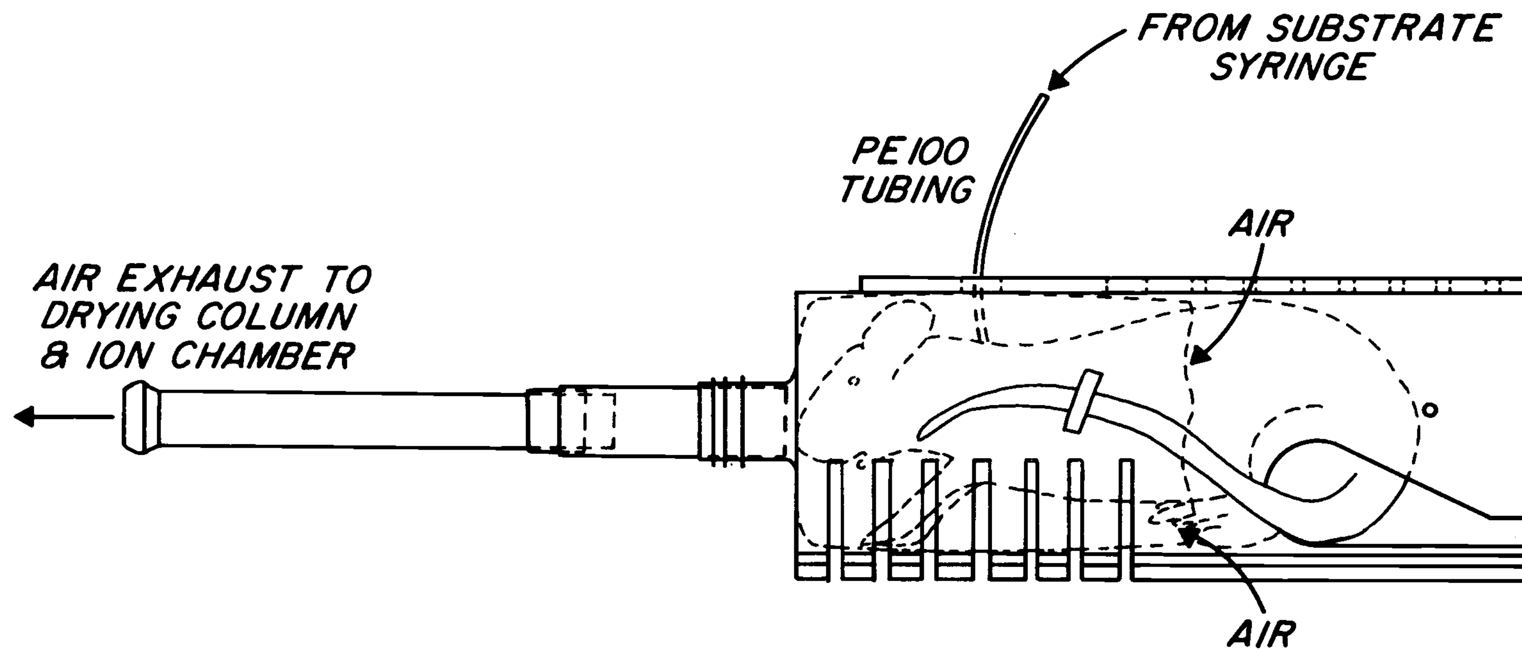


Figure 1. Restrainer cage designed for experiments involving substrate administration by means of continuous intravenous infusion.

negligible.

Studies on the effect of fluoride ion on glucose catabolism, during the prescribed time interval in the infusion experiment, required the addition of fluoride in the form of NaF to the ^{14}C-labeled glucose substrate solutions. The addition of fluoride to glucose substrate solutions was done in such a way that the concentration of glucose in the substrate solution remained the same throughout the infusion time. The concentration of fluoride (0.126 M) in the substrate solution was determined in a series of preliminary experiments and was such that it elicited a prompt, reproducible, physiological response in the rat as evidenced by a significant change in the rate of $^{14}CO_2$ production. When infused at 0.84 ml per hour, fluoride is delivered at a rate of two mg fluoride ion per hour to the rat. Fluoride infused at this concentration allowed complete recovery of the rat to normal levels of $^{14}CO_2$ production several hours after termination of the fluoride administration, but with the continuance of ^{14}C-labeled glucose substrate solution.

Substrate solutions were administered with 10 ml plastic disposable syringes (Plasti-pak, Becton Dickinson Company, East Rutherford, New Jersey) which were attached to indwelling cannulae of the rats by means of PE 100 tubing (Clay-Adams, Incorporated, New York) two feet in length. The loaded syringes were driven by a Harvard Model 600-2-200 syringe pump (Harvard Apparatus

Company, Dover, Massachusetts).

Substrate Calibration

All ^{14}C-specifically labeled glucose substrates were calibrated by adding 30 μl of the substrate solution to a conventional glass counting vial which contained 5 ml of 1:2 ethanolamine-absolute ethanol solution and 10 ml of toluene scintillation solution containing 0.3% p-terphenyl and 0.003% POPOP-1,4-bis-2-(5-phenyloxazolyl)-benzene as primary and secondary phosphors, respectively. The radioactivity of the solution was then determined by means of a liquid scintillation counter (Packard Tricarb Model 314EX). Counting efficiency was determined by use of calibrated toluene-^{14}C as an internal standard (38, p. 131).

Radiorespirometer

Respiratory $^{14}CO_2$ of rats was measured continuously by means of a radiorespirometric system described in detail by Wang (37) and Dost, Reed and Wang (6). Air was drawn past the sides of the restrained animal in a plastic cage at a rate of 500 ml per minute, carrying the respiratory $^{14}CO_2$ through a drying column and then to a one liter flow ion chamber. Beta radiation from the radioactive decay of $^{14}CO_2$ produces electrons which are collected on the anode of the ion chamber. The current produced by these electrons is

directly proportional to the radioactivity present in the chamber and is measured by a vibrating reed electrometer. The analog signal from the electrometer is converted to digital form by a voltage-to-frequency converter. The digital pulses are accumulated on a decade scaler and printed out at pre-set time intervals upon receipt of command from a programmer and timer, which also controls simultaneously the operation of three other identical systems. The calibration of gaseous ^{14}C measurement in the ion chamber has been previously described (37, p. 343).

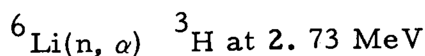
Metabolic Fate of Fluoride in Rats

To better analyze the effect of fluoride on catabolism of glucose it was necessary to learn the metabolic fate of the fluoride as it was administered to rats in the above stated manner. Use was made of ^{18}F as a radioactive tracer.

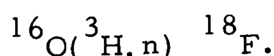
Fluorine-18 Production

Due to the relatively short half-life of ^{18}F (110 minutes) it was necessary that it be produced locally. Fluorine-18 was produced at the Oregon State University TRIGA nuclear reactor facility (General Atomic Mark II TRIGA Reactor, San Diego, California). Reports in the literature (31, 34) do not provide adequate details on the methodology of ^{18}F production and distillation. Therefore, the procedures devised for the present work warrant detailed description.

Fluorine-18 was produced by irradiating 500 mg samples of pure, enriched lithium carbonate (95% ^{6}Li) in a fused quartz capsule (65 mm long by outside diameter 8 mm, wall thickness 1 mm). The wall thickness of the capsule was thinned slightly in the center to facilitate breaking just prior to the distillation procedure to be described later. The quartz work was carried out by Mr. Mario Boschetto, Oregon State University glass blower. The prepared capsule was placed in a standard aluminum TRIGA irradiation tube (3/4 in by 4 in) previously weighted by a small 20 g piece of lead. The added weight was used to insure correct positioning of the TRIGA tube in the central core since samples were lowered into the reactor by a line through a water filled tube. The TRIGA tube was drilled with a sufficient number of holes to allow water circulation to keep the capsule cool during irradiation. Irradiations were carried out for 1.25 hours in the central core of the nuclear reactor at the 500 kW power level which provides a neutron flux of approximately 10^{13} neutrons per cm^2 per second. Enriched lithium carbonate was used to take advantage of the high thermal neutron cross section (950 barns) of ^{6}Li to provide the following sequential reactions:



$$^{6}Li(n, \alpha)\ ^{3}H \text{ at } 2.73 \text{ MeV}$$



$$^{16}O(^{3}H, n)\ ^{18}F.$$

Upon completion of irradiation, the aluminum TRIGA tube, containing the quartz capsule, was pulled out of the flux and allowed to decay. This was necessary because the aluminum of the TRIGA tube contained several impurities which upon neutron activation created high levels of radiation. In addition, the aluminum container upon neutron irradiation produced considerable ^{24}Na ($t_{1/2}$= 15 hour) via the neutron, alpha reaction: $^{27}_{13}Al\ (n, \alpha)\ ^{24}_{11}Na$.

The capsule, one hour after irradiation, was transferred to a micro-distillation apparatus (Figure 2) designed in this laboratory. Care was taken in the design of the distillation apparatus that, in the event there was a sudden surge of positive or negative air pressure during the operation, solutions in either the distillation or the receiving flasks could not escape over side arms or be drawn into the pumping equipment.

The capsule was dropped vertically down a piece of 11/16 inch OD tygon tubing 2 1/2 feet in length with the lower end clamped off by a large pair of artery forceps. The tubing contained 15 ml of 18N H_2SO_4. After insertion of the capsule, the top of the tygon tubing was securely clamped off. The capsule, now floating in acid, was crushed externally with long handled adjustable pliers, the teeth of which had been covered with short pieces of tygon tubing. After breaking the capsule the dissolution process took approximately five

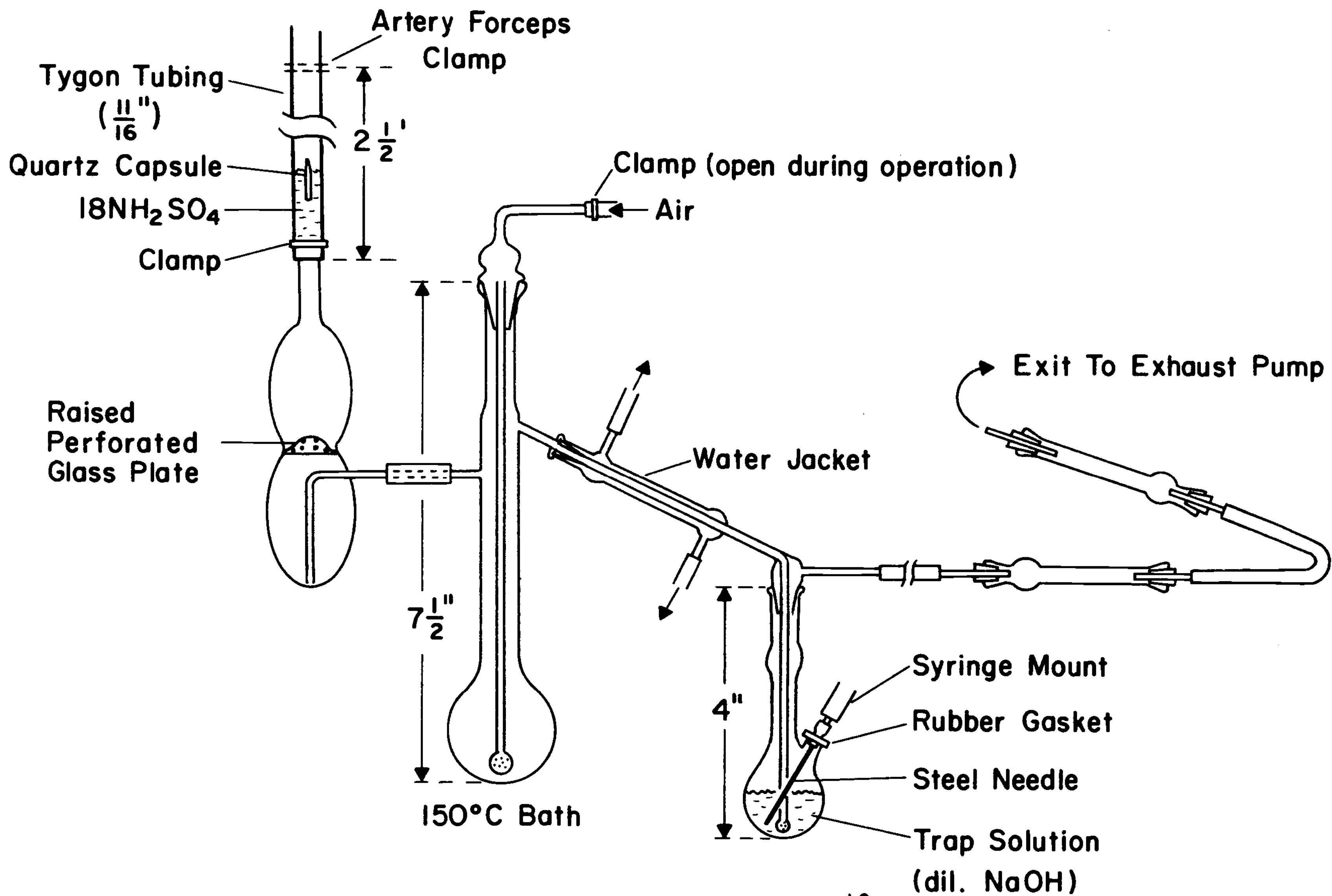


Figure 2. Distillation apparatus designed for preparation of $Na^{18}F$. Air-flow scheme is shown.

minutes, provided the $^{6}Li_2CO_3$ was not caked or packed in the bottom of the broken end of the capsule. As the $^{6}Li_2CO_3$ dissolved in the H_2SO_4 the tygon tubing became noticeably distended. Upon release of the lower clamp the positive pressure in the clamped tubing was used to drive the acid-carbonate solution over into the distillation flask which had a bulb capacity of about 30 ml and had previously been placed in an ethylene glycol bath at 150° C. If this pressure was not great enough to accomplish complete transfer, or a rinse of acid was desired, the glassware below the tygon tube was designed so that a slight negative pressure from the air pump would gently pull all of the acid-carbonate solution into the distillation flask when both clamps were removed from the tygon tubing. When all the solution had been drained into the distillation flask the top clamp to the tygon tubing was closed. All procedures were observed from behind lead brick shielding by the use of mirrors.

A gentle negative pressure applied above the trapping solution of the distillation apparatus (see Figure 2) admitted air into the distillation flask by way of a small perforated glass bulb placed directly into the acid-carbonate solution. The air stream, carrying the volatile ^{18}F-labeled HF and SiF_4 was distilled into the trap solution through a perforated glass bulb. The trapping solution was made up of about 5 to 7 ml of a dilute NaOH solution and was adjusted so that upon completion of distillation the final volume could be easily

neutralized with a necessary amount of HCl to render the final solution neutral in pH and isotonic with blood. By the use of a shielded Geiger counter, the build-up of radioactivity in the receiving flask was monitored to ensure that the distillation of ^{18}F-labeled compounds was complete. The distillate, containing carrier-free $Na^{18}F$ was made up to a desired volume by addition of physiological saline or distilled water. The yield of ^{18}F for experimental purposes was calculated for five experiments to be 1.4 ± 0.2 mCi at the beginning of ^{18}F substrate infusion. Assuming 70% distillation efficiency estimated by previous workers, the yield at the end of the neutron irradiation was 4.0 ± 0.4 mCi of ^{18}F. This yield is in good agreement with those reported by previous workers (2, 31, 34).

Fluoride Metabolism

Two series of experiments were conducted to determine the distribution or fate of administered fluoride in intact rats using ^{18}F as a tracer of infused stable fluoride. The rates of intravenous infusion, with regard to volume and glucose concentration in the substrate solution, were the same as those used in radiorespirometric studies. In the first series of experiments NaF was added to carrier-free $Na^{18}F$ solutions prepared above (along with glucose) in the amount equivalent to 100% (0.126 M) of the fluoride concentration previously used in radiorespirometric studies. In this series

of experiments blood samples were collected during and after the $Na^{18}F$ infusion to determine both the build-up and depletion of administered fluoride in blood. In the second series of experiments NaF was added to carrier-free $Na^{18}F$ solutions (along with glucose) in amounts equivalent to 15% (0.019 M), 30% (0.038 M), and 60% (0.076 M) of the concentrations of fluoride solutions used in radiorespirometric studies. During infusion of these fluoride substrate solutions, blood samples were frequently taken in order to determine blood fluoride concentration during the infusion (build-up) phase. To determine the fate of fluoride in tissues other than blood, animals from both of the above series of experiments were sacrificed at prescribed times and all major organs and representative samples of major tissues were taken for fluoride determination by means of scintillation-counting of ^{18}F radioactivity.

Sampling Technique

Urine was periodically collected from plastic troughs situated beneath each restrained animal and rinsed into two dram polyethylene counting vials for ^{18}F analysis. By tail clipping, blood samples were frequently collected during, and/or after, the ^{18}F-fluoride-glucose infusion. Collection of blood samples was in heparinized capillary tubes of 0.077 ml capacity (Red Tip, 75 mm by 1.4 mm, Clay-Adams, Incorporated, New York). The entire capillary tube was

drained and placed in a two dram polyethylene vial containing 3 ml of water for consistent counting geometry. Just prior to termination of the experiment for an individual rat, two separate blood samples were taken, as before, and the animal was sacrificed. As soon as possible all the major organs and representative samples of major tissues were removed and placed in polyethylene vials for ^{18}F determination.

Blood volume of experimental rats was measured according to the standard technique of Abbott Laboratories (1) by introducing radio-iodinated (^{125}I) serum albumin into the blood stream via the indwelling cannula.

Fluorine-18 Analysis

All samples and standards were placed in two dram polyethylene vials for counting by means of a NaI(Tl) (3 by 3 in) scintillation detector with well (Harshaw Chemical Company, Cleveland, Ohio). The mode of decay for ^{18}F is by way of positron emission. For detection purposes use was made of the 0.51 MeV gamma ray spectral peak associated with the two back-to-back gamma rays created upon the annihilation of the positron. Only samples with a counting rate inflicting less than 15% dead time loss were counted in the well position of the NaI detector. Samples with a counting rate inflicting greater than 15% dead time loss were counted in a fixed geometry 12 cm above the NaI well detector. Impulses from the NaI detector were

amplified and fed into a TMC 400 multichannel gamma ray analyzer (Technical Measurements Corporation, North Haven, Connecticut). The data were printed out by a TMC Model 500P printer. The data were processed using Covell's method for background correction (4, p. 55). In most instances a one minute count was sufficiently long to obtain more than 2000 net counts per minute (corrected for background only) to provide a relative standard deviation of data at ±2%. In those infrequent cases where less than 2000 net cpm were obtained the count time was lengthened appropriately to obtain over 2000 net counts. For correction of radioactive decay all counts were calculated back to the zero time, i.e., the beginning of the infusion of ^{18}F substrate solutions.

Standards for decay correction were prepared by adding 0.05 ml of ^{18}F substrate solution to 0.95 ml distilled water in a polyethylene vial. The solution was then diluted prior to counting. The data so obtained were applied to all calculations in the ^{18}F experiments.

To determine the possible presence of long lived radioactive contaminants which may have occurred during the production of ^{18}F, all unused ^{18}F substrate solution was kept for gamma ray analysis 24 hours later. No significant contaminants were found.

IV. RESULTS AND DISCUSSION

The primary objective of this work was to gain information on the biochemical mechanism underlying the toxic action of fluoride ion in rats. Glucose catabolism was chosen as a test system in view of the known inhibitory action of fluoride upon several key enzymic systems involved in the oxidation of glucose in rats. Experiments were designed to study the fate of fluoride administered to rats and the catabolism of glucose by means of radiorespirometric methods under *in vivo* conditions. All radiorespirometric data presented had a deviation of less than ±5% and represent at least three replicate experiments except in the case of glucose-3-^{14}C labeled substrate where the data are derived from two replicate experiments.

Glucose Catabolism in Intact Rats

In Figure 3 is given the radiorespirometric pattern for normal rats metabolizing ^{14}C-specifically labeled glucose substrates. The rates for conversion of carbon atoms of glucose to respiratory CO_2 follow an ascending slope during the first few hours of glucose infusion and reach relatively constant levels. Upon termination of substrate infusion the rates for conversion of carbon atoms to respiratory CO_2 decline sharply and reach insignificant levels in approximately four hours. These results confirm the findings of Wang, *et al.*

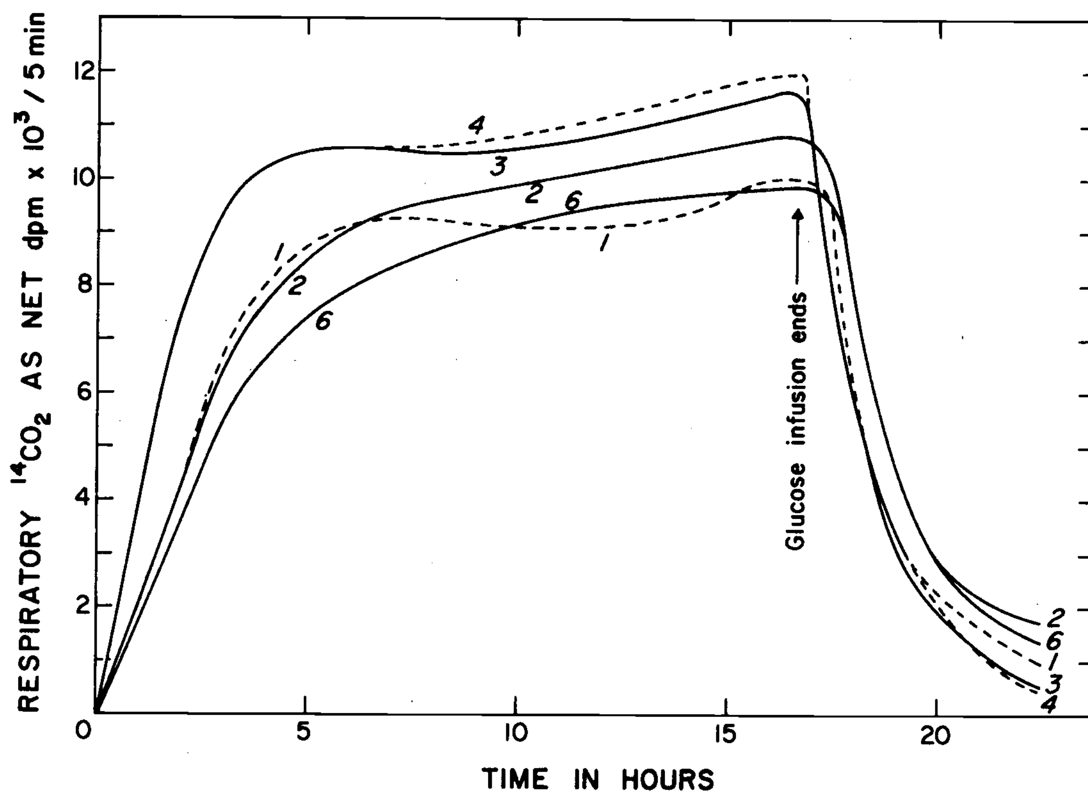


Figure 3. The radiorespirometric pattern for glucose catabolism by rats metabolizing ^{14}C-specifically labeled glucoses administered by means of continuous intravenous infusion. (Numerals refer to the labeled positions in glucose molecule.)
Substrate: Glucose solution at a concentration of 179 mg/ml, delivered at a rate of 0.84 ml/hr, equivalent to 150 mg glucose administered/hr.
Specific activity of ^{14}C-labeled glucoses was 0.1 μCi/mM glucose.

(40) wherein rats were infused with ^{14}C-specifically labeled glucose substrates under the same conditions described in this work.

The extensive and prompt conversion of C-3 and C-4 of glucose to respiratory CO_2, exceeding that of other carbon atoms of glucose, reveals that the Embden-Meyerhof-Parnas (EMP)-pyruvate decarboxylation pathway is playing a predominant role in rat catabolism. The observation that the conversion of C-1 to respiratory CO_2 is slightly more prompt than C-2 indicates that the pentose phosphate (PP) pathway is operative in rats. The conversion of C-6 to respiratory CO_2 is lower than that of C-2. However, as pointed out by Wang, *et al.* (39), there are other indications that the glucuronic acid (GA) pathway is also operative in rats. Catabolism of pyruvate, the key intermediate of glucose via various pathways is presumably by way of the tricarboxyclic acid (TCA) cycle judging from the abundant evidence in the literature. It is interesting to note that upon termination of substrate infusions, production of $^{14}CO_2$ from the various carbon atoms of glucose fell off drastically. This points out that accumulation of substrate glucose in the endogenous pool of glucose is not extensive.

The information collected on glucose catabolism in normal rats permits one to analyze the effect of fluoride ion on glucose catabolism in rats. It should be noted that the use of intravenous infusion technique permits the study of the kinetic aspect of glucose

catabolism in both normal and in fluoride intoxicated rats. Data obtained in this type of experiment could provide much information on the relationship of fluoride concentration in blood to possible inhibitory action on glucose catabolism.

In order to determine the optimal concentration of fluoride ion that can exert a significant inhibitory effect, if present, upon glucose catabolism in rats, a series of radiorespirometric experiments was carried out. Data obtained with glucose-3(4)-^{14}C labeled substrate are given in Figure 4. Glucose-3(4)-^{14}C was used as a test substrate since it is known that the EMP-pyruvate decarboxylation pathway is the major catabolic route in rats and, hence, the conversion of C-3 or C-4 of glucose to CO_2 is a prompt process. Any effect of fluoride on respiratory mechanisms in rat would be promptly reflected in the rate of conversion of these two carbon atoms of glucose to respiratory CO_2.

In order to ascertain whether there was any effect on glucose catabolism due to the presence of a non-toxic salt in the substrate solution, infusion experiments with NaCl as test compound were first carried out. Glucose-3(4)-^{14}C labeled substrate solutions containing 0.252 M chloride (7.5 mg chloride per hour) and 0.000 M chloride were infused into rats for three hours in the same manner as fluoride was administered. The results from these infusion experiments did not significantly differ from control experiments at 0.126 M chloride

(3.8 mg chloride per hour).

It was next important to find out the level of fluoride which, if infused with glucose into rats, will cause a defined, highly reproducible inhibitory effect, if present, upon glucose catabolism, and yet will not cause death. Figure 4 shows that animals infused with fluoride solution up to 0.032 M concentration (0.5 mg fluoride per hour or a total dose of 1.5 mg) exhibit relatively no inhibitory effect on glucose catabolism. At the 0.063 M fluoride level (1 mg fluoride per hour or a total dose of 3 mg), some inhibitory effect of infused fluoride upon glucose catabolism is noted. Evidently, during infusion at the fluoride concentration of 0.063 M, sufficient fluoride was introduced into the blood and tissues after a period of two to three hours to build up to a fluoride level high enough to inflict inhibition. However, upon termination of administration of fluoride solution, continuous excretion reduced the effective fluoride concentration in blood gradually as evidenced by the continuous reduction of the inhibitory effect. Two and one-half hours after termination of fluoride infusion, glucose catabolism had returned to the normal state at which time the effective fluoride concentration evidently had been reduced to a very low level.

When the concentration of infused fluoride solution was 0.126 M (2 mg fluoride per hour or a total dose of 6 mg) inhibition of glucose catabolism reached the maximum level two hours after the

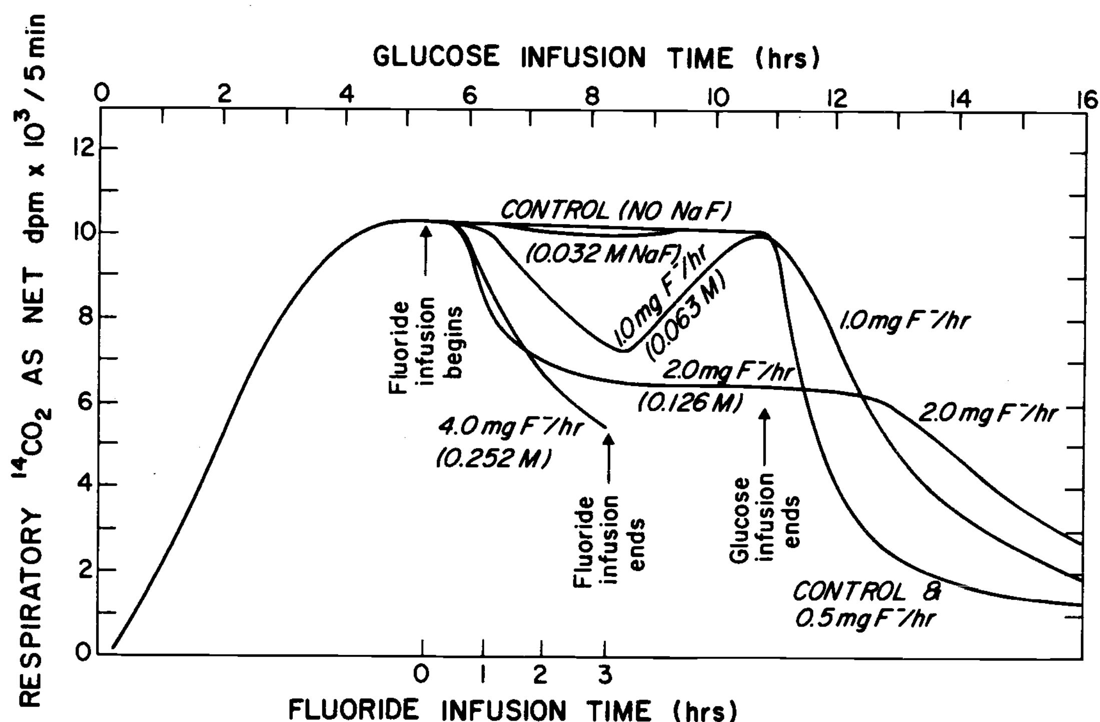


Figure 4. The effect of fluoride ion upon glucose catabolism in rats using the radiorespirometric method with glucose-3(4)-^{14}C as the tracing substrate.

Substrate: Glucose solution at a concentration of 179 mg/ml, delivered at a rate of 0.84 ml/hr, equivalent to 150 mg glucose administered/hr.

Specific activity of ^{14}C-labeled glucose was 0.1 μCi/mM glucose.

Fluoride concentration in glucose substrate solutions:

control - no NaF
0.032 M NaF for a total dose of 1.5 mg F^-/rat
0.063 M NaF for a total dose of 3.0 mg F^-/rat
0.126 M NaF for a total dose of 6.0 mg F^-/rat
0.252 M NaF for a total dose of 12.0 mg F^-/rat

beginning of fluoride infusion, and persisted. This fact indicates that at this concentration the build-up of fluoride ion in blood and tissue exceeds the rate of removal and excretion. However, the experimental animals did survive.

When the fluoride solution was infused at a concentration of 0.254 M (4 mg fluoride per hour or a total dose of 12 mg) not only was glucose catabolism severely inhibited as exhibited by continuous decline of $^{14}CO_2$ production, but this concentration also led to death within a few hours after termination of fluoride administration.

The results reported in Figure 4 allow one to make rough calculations for the range of effective fluoride concentration in blood and presumably in soft tissues of rat that can exert a noticeable inhibitory effect on glucose catabolism. First, when a fluoride solution at 0.032 M was infused into rats for three hours, the cumulative amount of fluoride administered was approximately 1500 µg. As shown in Figure 4, there was no significant inhibitory effect at this dose. Presumably, the administered fluoride was promptly removed from the blood and soft tissues. Second, fluoride infused at a concentration of 0.063 M delivered a dose of approximately 3000 µg fluoride per rat in three hours and caused a maximum of 30% inhibition on the rate of respiratory $^{14}CO_2$ production. The maximum inhibition coincided with the termination of fluoride infusion. If one assumes that the maximal removal rate of fluoride from blood of rats is 1500 µg

fluoride in three hours, it can be concluded that the animals infused with 3000 μg fluoride will result in an effective dose of 1500 μg fluoride in rats which causes 30% inhibition of glucose catabolism. As a very conservative estimate, assuming that all of the unremoved fluoride (1500 μg) remains exclusively in the blood, the effective fluoride concentration in blood would then be 3×10^{-3} M. If one assumes that all the excess fluoride is distributed perfectly throughout the animal, the effective fluoride concentration in the body of the rat would be 2×10^{-4} M. One will note that these estimated concentrations are in the same order of magnitude as reported in the literature (13, 41) and given in Table 3 for observed fluoride concentration that result in inhibition of the enzyme enolase in _in vitro_ experiments.

Figure 4 also provides information to calculate an LD_{100} dose for rats undergoing continuous intravenous infusion. Although only four animals were infused at a concentration of 0.256 M fluoride (equivalent to 4 mg per hour) for a period of three hours, all animals died within four hours after termination of fluoride infusion. This would be, therefore, an LD_{100} dose at 36 mg fluoride per kg body weight which compares favorably with LD_{100} doses observed for rat and dog (Table 1).

Therefore, it appears that for the stated objective, the optimal level of fluoride infusion to obtain steady glucose catabolism inhibition is 0.126 M. Experiments were then carried out to investigate the

inhibitory effect of fluoride upon specific pathways of glucose. The results for experiments with ^{14}C-glucose labeled at the C-1, C-2, and C-6 positions are shown in Figure 5 and the relative extents of inhibition for all labels are shown in Table 4. In contrast to data presented in Figure 4 (p. 30), the glucose infusion time in this experiment was extended from 10.5 hours to 16.7 hours. It is noted that when fluoride was infused at 0.126 M for three hours, glucose catabolism was first inhibited and began to recover at 12 hours and finally resumed a near normal state at 17 hours. A striking feature seen in Table 4 is the prompt, extensive inhibition by fluoride on the conversion of C-3 and C-4 of glucose to respiratory CO_2. Maximum inhibitory effect is observed at 1.7 hours after termination of fluoride infusion (or 4.7 hours after beginning of fluoride infusion). At this time the rates for the conversion of all carbon atoms to respiratory CO_2 are reduced by approximately the same extent, due to fluoride inhibition of the EMP pathway. However, in the case of C-6 the inhibition is slightly greater in extent. This finding appears to indicate that the PP and GA pathways, known to be operative in the rat (29, 43), are not interfered with by fluoride ion to any significant degree.

The Fate of Administered Fluoride in Rats

It has been demonstrated in radiorespirometric studies that, indeed, fluoride can inhibit glucose catabolism, and further, it appears that the site of inhibitory action is the enzyme enolase of the EMP pathway. Having infused fluoride at the prescribed rate and having correlated this administered fluoride dose with maximal inhibition of glucose catabolism, it was possible to roughly estimate the

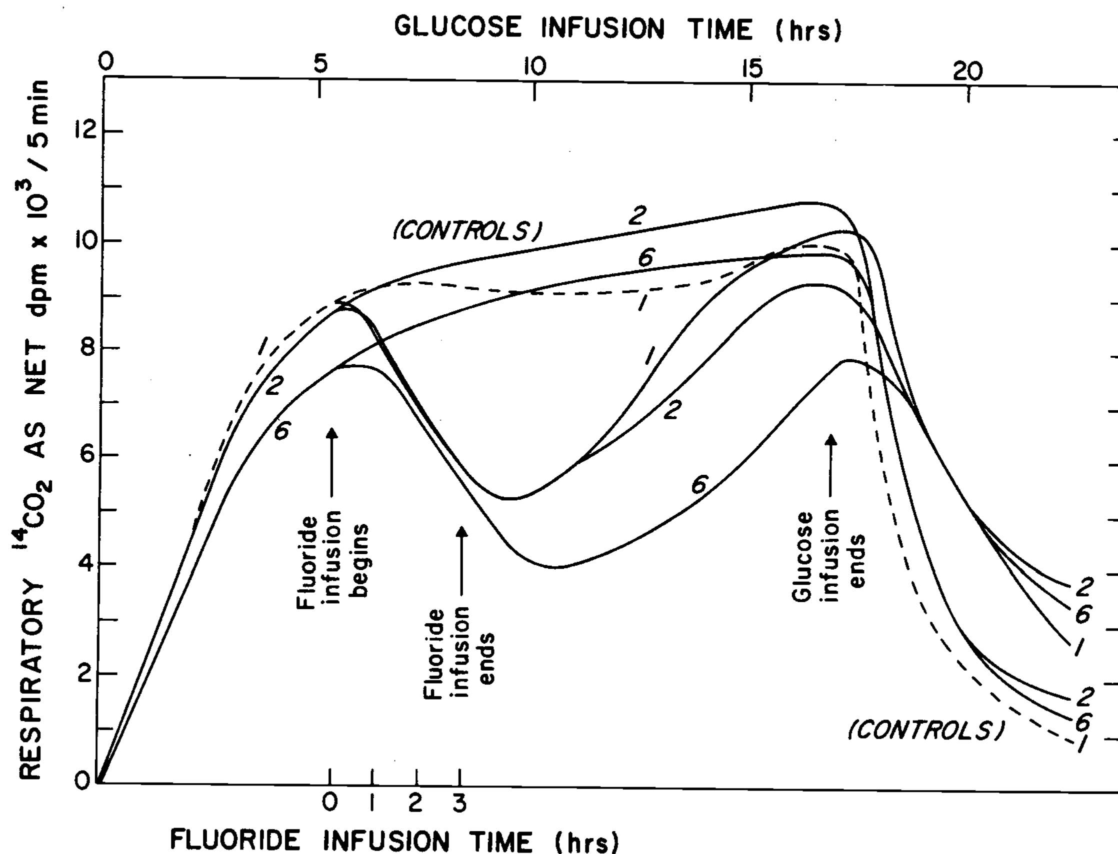


Figure 5. The effect of fluoride ion upon glucose catabolism in rats using radiorespirometric method with glucose -1-, -2-, -6-^{14}C as tracing substrates. (Numerals refer to the labeled positions in glucose molecule.)
Substrate: Glucose solutions at a concentration of 179 mg/ml, delivered at a rate of 0.84 ml/hr, equivalent to 150 mg glucose administered/hr.
Specific activity of ^{14}C-labeled glucoses was 0.1 μCi/mM glucose.
Fluoride concentration in the glucose substrate solutions: 0.126 M NaF delivered at a rate of 2 mgF/hr for three hours or a total dose of 6 mg F^-/rat

Table 4. Fluoride Inhibition of Glucose Catabolism for Each Labeled Glucose Substrate

		Percent Inhibition of $^{14}CO_2$ Production Carbon-14 Glucose Label				
Phase:	Time (hr)	Glu-1	Glu-2	Glu-3	Glu-4*	Glu-6
Begin F^- Infusion	0	0	0	0	0	0
	1	11%	10%	10%	10%	6%
	2	25	26	41	43	23
End F^- Infusion	3	37	40	47	51	43
Low Point	4.7	42	46	48	58	56

*Data for glucose-4 was obtained by difference, making use of data obtained in glucose-3(4)-^{14}C and glucose-3-^{14}C experiments.

range of fluoride concentration in blood or in metabolically-involved body fluid that can affect significant inhibition. The estimate made for the effective fluoride concentration to cause maximum inhibition was in the same order of magnitude as in in vitro experiments on fluoride inhibition of enolase.

It appears, therefore, that if one used ^{18}F as a tracer for administered fluoride, more precise information on the kinetic fate of fluoride could be obtained. For instance, the range estimate of fluoride concentrations causing inhibition of glucose catabolism mentioned above could be readily verified with much more precision. In addition, one could obtain information on the kinetics of build-up and depletion of administered fluoride in various types of tissue, especially blood. Results given in Table 5, derived from ^{18}F tracer experiments, show the fluoride distribution in rats continuously infused with ^{18}F-labeled fluoride at 0.126 M along with unlabeled glucose. To obtain this set of data experimental rats were sacrificed at different time intervals during three hours of infusion.

A rapid initial build-up of fluoride in soft tissues is seen in the data presented in Table 5. As the infusion of fluoride proceeds, fluoride accumulation in soft tissues continues; the rate of accumulation appearing to be dependent on the cumulative amount administered. In the case of blood and liver, the amount of fluoride also increases with increased cumulative dose. However, it is noted that percent

Table 5. Distribution of Administered Fluoride in Rat Tissues During Infusion Phase

	Infusion Phase[1]					
Time	1.9 hr		2.3 hr		3 hr	
Total F^- Infused, µg	3860		4570		6000	
Tissue	µgF	%[2]	µgF	%	µgF	%
Blood	112.0	2.9	127.0	2.8	145.0	2.4
Liver	74.0	1.9	80.0	1.8	100.0	1.7
Kidney	19.0	0.5	24.0	0.5	33.0	0.6
Spleen	3.2	0.1	4.2	0.1	6.8	0.1
Lung	29.0	0.8	36.4	0.8	39.6	0.7
Heart	12.2	0.3	17.5	0.4	--	--
Brain	2.8	0.1	4.1	0.1	3.4	0.1
Testes	3.4	0.1	3.8	0.1	5.0	0.1
Fat Pads	3.7	0.1	4.5	0.1	6.9	0.1
Stomach	6.7	0.2	13.4	0.3	15.4	0.3
Sm. Intestine	29.0	0.8	46.5	1.0	58.0	1.0
Caecum	3.9	0.1	7.3	0.2	6.7	0.1
Lg. Intestine	6.6	0.2	15.1	0.3	14.1	0.2
Muscle (est.)[3]	390	11	715	16	791	13
Bone (est.)[3]	715	19	1250	27	2790	46
Urine	139	4	53	1	379	6

[1] Rats were continuously infused with a 0.126 M fluoride-containing glucose substrate solution up to the time that they were sacrificed for fluoride analysis.

[2] Figure given is percent of total fluoride infused which was incorporated into the tissue.

[3] Estimates were calculated assuming the lean body mass of rat to be 45% body weight and bone to be 9% wet weight.

incorporation in these two tissues based on cumulative dose follows a slightly declining trend. This means that the capacity of fluoride incorporation into these two types of tissues has a defined limit which is presumably relative to the rate of fluoride removal. The latter appears to be proportional to the apparent concentration of fluoride in these two tissues. Bone tissue and urine appear to be the major sites for removal of fluoride from the blood and soft tissues.

In order to facilitate understanding of the data in Table 5, specific concentrations (μg fluoride per gram of tissue) of administered fluoride in various rat tissues are calculated and given in Table 6. Data presented in this manner eliminate concern over slight weight differences of various types of tissues in experimental animals. Moreover, the data on muscle and bone are more meaningful since the calculated values given in Table 5 involve crude estimates of total weight of muscle and bone of the experimental rat.

It is noted that specific concentrations of fluoride given in Table 6 for soft tissues are not uniform by any means, particularly in the case of lung and heart. The specific concentrations in all cases are proportional to the cumulative infused dose, but in the case of bone the trend is much more acute, showing preferential deposition in bone. This is not surprising, since it is a well known fact that bone acts as a fluoride ion scavenger.

Table 6. Specific Concentration of Administered Fluoride in Rat Tissues During Infusion Phase

	Specific Concentration:	Infusion Phase*	
Time	1.9 hr	2.3 hr	3 hr
Total F^- Infused, μg	3860	4570	6000
Tissue	μgF/g	μgF/g	μgF/g
Blood	4.8	5.4	6.1
Liver	6.5	6.0	9.0
Kidney	6.4	8.2	11.0
Spleen	4.5	6.5	9.2
Lung	20.8	20.6	22.6
Heart	11.5	16.4	--
Brain	1.5	2.2	1.7
Testes	2.0	2.7	2.9
Fat Pads	1.3	1.7	2.2
Stomach	4.2	7.0	7.0
Sm. Intestine	4.0	6.4	7.1
Caecum	3.8	5.9	6.9
Lg. Intestine	3.4	8.7	8.0
Muscle	2.6	4.7	5.3
Bone	21.3	36.3	89.7

*Rats were continuously infused with a 0.126 M fluoride-containing glucose substrate solution up to the time they were sacrificed for fluoride analysis.

Table 6 gives one a basis for estimating the fluoride concentrations found in various soft tissues of rats during the latter part of the fluoride infusion process. These estimates are 3×10^{-3} M fluoride in blood and 10^{-4} M to 10^{-3} M fluoride in other soft tissues. Estimates of the range of effective fluoride concentration that caused significant impairment of glucose catabolism in rats (2×10^{-4} M to 3×10^{-3} M) made in connection with the radiorespirometric experiments correlate well with the estimates made on the basis of data in Table 6. In turn, both the estimates based on radiorespirometric data and the more accurate determinations of exogenous fluoride concentration in rat tissue as given in Table 6 are to be compared with the concentration of fluoride cited in the literature for inhibition of enolase in _in vitro_ experiments, i.e. 10^{-3} M.

Having determined the dynamic build-up of fluoride in rat tissue, the next logical step was to examine the depletion of administered fluoride from tissues of rats upon termination of fluoride infusion. Rats were infused with ^{18}F-labeled 0.126 M fluoride-containing glucose substrate solution, as before, for three hours followed by continuous infusion of glucose substrate solution until the time they were sacrificed and tissues assessed for fluoride content. The results from these depletion experiments are shown in Tables 7 and 8. For ready comparison each of these two tables includes a column giving the amount of fluoride accumulated during three hours of

Table 7. Distribution of Administered Fluoride in Rat Tissues During Depletion Phase

			Depletion Phase[1]					
Elapsed Time	0 hr		1.1 hr		1.4 hr		3.1 hr	
Total F^- Infused, μg	6000		6000		6000		6000	
Tissue	μgF	%[2]	μgF	%	μgF	%	μgF	%
Blood	145.0	2.4	95.0	1.6	74.0	1.1	19.0	0.3
Liver	100.0	1.7	36.1	0.6	20.0	0.3	5.2	0.1
Kidney	33.0	0.6	17.4	0.3	15.3	0.3	3.1	0.1
Spleen	6.8	0.1	2.8	0.1	1.2	NS	0.3	NS
Lung	39.6	0.7	9.0	0.2	7.3	0.1	12.3	0.2
Heart	--	--	4.0	0.1	1.4	NS	0.3	NS
Brain	3.4	0.1	2.0	NS	1.4	NS	1.1	NS
Testes	5.0	0.1	2.7	NS	1.9	NS	0.4	NS
Fat Pads	6.9	0.1	4.8	0.1	4.3	0.1	2.1	NS
Stomach	15.4	0.3	3.6	0.1	2.2	NS	0.4	NS
Sm. Intest.	58.0	1.0	17.8	0.3	9.2	0.2	2.5	NS
Caecum	6.7	0.1	1.5	NS	1.7	NS	1.7	NS
Lg. Intest.	14.1	0.2	9.3	0.2	7.2	0.1	3.4	0.1
Muscle (est.)[3]	791	13	456	8	200	5	140	2
Bone (est.)[3]	2790	46	2780	46	2620	44	2980	50
Urine	379	6	552	9	1650	28	2427	41

[1] Rats had been previously infused intravenously for three hours with 0.126 M fluoride-containing glucose substrate solution. The time given indicates hours sacrificed after cessation of the fluoride infusion. During the post fluoride infusion time, infusion of normal glucose substrate solution was continued.

[2] Figure given is percent of total fluoride infused which was incorporated into the tissue. NS is not significant.

[3] Estimates were calculated assuming the lean body mass of rat to be 45% body weight and bone to be 9% wet weight.

Table 8. Specific Concentration of Administered Fluoride in Rat Tissues During Depletion Phase

	Specific Concentration: Depletion Phase*			
Elapsed Time	0 hr	1.1 hr	1.4 hr	3.1 hr
Total F^- Infused, μg	6000	6000	6000	6000
Tissue	μgF/g	μgF/g	μgF/g	μgF/g
Blood	6.1	3.7	2.9	0.9
Liver	9.0	3.4	1.9	0.4
Kidney	11.0	7.0	6.3	1.0
Spleen	9.2	3.0	1.7	trace
Lung	22.6	5.2	3.0	5.9
Heart	--	3.6	1.0	0.3
Brain	1.7	1.1	0.8	0.6
Testes	2.9	1.6	1.2	0.3
Fat Pads	2.2	1.5	1.4	0.8
Stomach	7.0	3.0	0.9	0.3
Sm. Intestine	7.1	2.3	1.5	0.3
Caecum	6.9	1.2	1.2	1.3
Lg. Intestine	8.0	3.6	3.8	1.9
Muscle	5.3	3.0	1.3	0.9
Bone	89.7	80.0	78.0	87.5

*Rats had been previously infused intravenously for three hours with 0.126 M fluoride-containing glucose substrate solution. The time given indicates the hour at which the animals were sacrificed after cessation of the fluoride infusion. During the post fluoride infusion time, infusion of normal glucose substrate solution was continued.

fluoride infusion and, hence, zero hour of depletion.

Depletion of administered fluoride from soft tissue in the rat appears to fall into three general categories. 1) Fast depletion is observed in liver, spleen, stomach and small intestine where depletion occurred over three hours by factors ranging from 20 to 30. 2) Moderate depletion is observed in kidney, heart, testes, blood and muscle where depletion occurred by factors ranging from about seven to 12. 3) Slow depletion is observed in lung, brain, fat pads and lower intestine where depletion occurred by factors of between three and four. Of special note, blood is more slowly depleted of administered fluoride than other tissues except brain, fat pads and lower intestine. This fact indicates that the fluoride concentrations in various soft tissues were those truly associated with the tissues and were not overshadowed by residual blood in the tissues.

A striking observation in Table 7 is that after three hours of depletion, fluoride in bone and urine accounts for 90% of the infused fluoride dose. It is noted that once fluoride was incorporated into bone, it remained undepleted throughout the depletion phase. In contrast, ever increasing amounts of fluoride were found in urine during depletion. Evidently once fluoride administration was terminated, the removal of fluoride from soft tissue was exclusively by way of renal excretion.

Wallace-Durbin (36) administered ^{18}F as single-doses, with and without stable carrier fluoride, orally and intravenously to rats. The percent of total fluoride dose for various tissues given in Table 7 generally agrees with Wallace-Durbin's findings with data observed at

least one hour after cessation of the fluoride infusion. Wallace-Durbin concluded that there was no significant deposition of fluoride in any of the soft tissues of the rat resulting from single-dose administration of stable fluoride ranging from 0.5 μg per kg body weight to 10 mg per kg body weight.

The findings in Table 7 also support the work of Dost, et al. (7), wherein 4.5 mg fluoride was administered to each rat by way of a single intraperitoneal injection. Since Dost, et al. did not use ^{18}F in their work, their incorporation data include contributions from endogenous fluoride. By subtracting fluoride values they found for untreated control animals from fluoride values observed for experimental animals administered with fluoride, the difference would presumably represent net incorporation of administered fluoride. If one were to observe the data for one or more hours after the cessation of fluoride administration in both types of experiments, the values of Dost, et al. agree fairly well with the data presented in Table 7.

It appears then, that the long-range depletion of fluoride from various soft tissues in the rat is independent of the method of administration (infusion or single injection), route of administration (oral, intraperitoneal, intravenous injection or infusion) and amount administered (0.5 μg to 18 mg fluoride per kg body weight).

Two other studies involving the fate of intravenous single-dose administration of ^{18}F to large domestic animals appear in the literature. Bell, Merriman and Greenwood (3) carried out such studies with beef cattle. However, the results are not comparable to the

present finding with rats. In another study using single-dose administration of ^{18}F, Perkinson, _et al_. (27) experimented with lambs and cattle. Their findings compare well with the data on percent fluoride incorporation in blood given in Table 7. It should be noted that Perkinson, _et al_. injected lambs with fluoride at the levels of 1.2 and 2.0 mg fluoride per kg body weight and the present work involved a dose of 18 mg fluoride per kg body weight in rat. Other lambs receiving single oral doses are not comparable to the present study.

Up to this point data have been presented on the effect of fluoride infused into rats at a relatively high dose. The high dose was necessary to facilitate the study of fluoride effect on glucose catabolism and the associated fate of fluoride in rat. It appeared then, that much could be learned from examining the fate of fluoride at lower doses. From determinations made in the present work and from reports in the literature, one would not expect to find anything new with regard to fluoride depletion after cessation of fluoride administration. However, the distribution of administered fluoride at various dose levels during the infusion process is not known and is of prime importance to the understanding of chronic toxicology of fluoride. Therefore, a series of experiments was performed in order to understand the toxic action of fluoride in animals subjected to prolonged exposure at low dose.

Rats were infused, as before, for three hours at fluoride

concentrations of 0.019 M, 0.038 M, and 0.076 M which are equivalent to 15%, 30%, and 60% of the concentration of fluoride solution used in earlier studies. Several small blood samples (0.077 ml) were taken from animals during the infusion process. The results are presented in Figure 6 and compared to blood data observed earlier at the fluoride concentration of 0.126 M.

It is seen that with all concentration levels of fluoride tested, the build-up of fluoride ion in the blood initially follows an acute ascending slope and gradually levels off approximately two to three hours after the beginning of infusion. Of more interest is the finding that fluoride concentrations in the blood appear to be almost directly proportional to the concentration of fluoride in infused solutions. This implies that the rate of depletion in soft tissues is also proportional to the fluoride concentration in blood.

The concentration of fluoride in blood is expressed in units of molarity on the right-hand axis of Figure 6. If one examines fluoride concentrations in blood at the leveling off phase, one has an additional basis to account for earlier observations that glucose catabolism is not significantly inhibited until the concentration of administered fluoride approaches the range of 0.063 M to 0.126 M, if enolase is the key enzyme involved in fluoride intoxication.

To examine the behavior of fluoride incorporation into tissues when fluoride was administered at low concentrations, the animals in

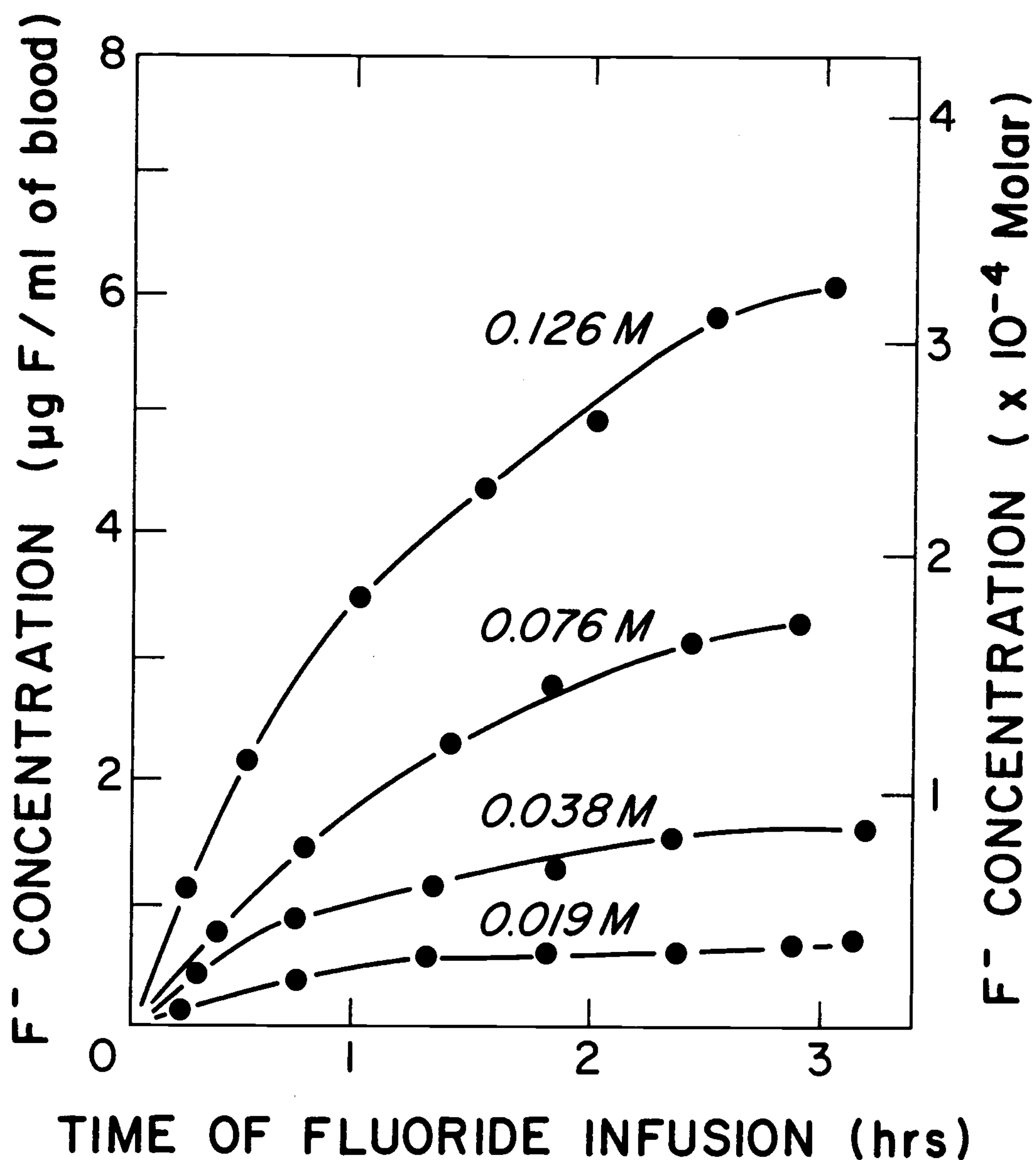


Figure 6. Accumulation of fluoride in blood when $Na^{18}F$-containing glucose substrate solutions are administered to rats by continuous intravenous infusion at various concentrations. Substrate: glucose solution at a concentration of 179 mg/ml, delivered at a rate of 0.84 ml/hr, equivalent to 150 mg glucose administered/hr.
Fluoride concentration in the glucose substrate solutions:
0.019 M NaF delivers a total dose of 0.9 mgF^-/rat
0.038 M NaF delivers a total dose of 1.8 mgF^-/rat
0.076 M NaF delivers a total dose of 3.6 mgF^-/rat
0.126 M NaF delivers a total dose of 6.0 mgF^-/rat

this series of experiments were sacrificed after three hours of fluoride infusion. The tissues were removed for fluoride analysis and the results are presented in Tables 9 and 10. For the purpose of comparison each table contains a column providing earlier findings observed at the fluoride concentration of 0.126 M at a corresponding time.

The results given in Tables 9 and 10 show that fluoride is accumulated in blood at a rate relatively higher than other soft tissues except lung. It is notable that at the lower infusion doses (0.019 M to 0.076 M fluoride), soft tissue shows a constant extent of fluoride incorporation calculated on the basis of total fluoride administered. This is not to be confused with the case of lung, which incorporated fluoride in an erratic manner. The incorporation of administered fluoride into bone when fluoride is infused at concentrations of 0.038 M and above shows a remarkable consistency in percent of uptake of total administered fluoride. However, at the 0.019 M fluoride infusion level bone uptake of fluoride is much greater. There is evidence in the literature (5, 27) indicating that experiments involving the use of tracer amounts of fluoride, calcium and phosphorous lead to problems in interpretation of data on the fate of these elements because of their affinity to bone.

It is also of interest to compare specific concentrations of fluoride in soft and skeletal tissues in the experimental animals. As seen in the data given in Table 10, a direct relationship exists

Table 9. Distribution of Fluoride in Rat Tissues Following Administration of NaF at Different Concentrations[1]

Conc. of Admin. F^-	0.019 M		0.038 M		0.076 M		0.126 M	
Total F^- Infused, µg (in 3 hrs)	900		1800		3600		6000	
Tissue	µgF	%[2]	µgF	%	µgF	%	µgF	%
Blood	17.2	1.9	38.7	2.2	77.0	2.1	145.0	2.4
Liver	4.0	0.5	7.7	0.4	23.1	0.6	100.0	1.7
Kidney	2.9	0.3	6.9	0.4	9.4	0.3	33.0	0.6
Spleen	0.2	NS[3]	0.6	NS	1.0	NS	6.8	0.1
Lung	6.2	0.7	25.0	1.4	6.9	0.2	39.6	0.7
Heart	0.4	0.1	0.5	NS	1.1	NS	--	--
Brain	0.2	NS	0.4	NS	1.0	NS	3.4	0.1
Testes	0.6	0.1	1.1	0.1	2.5	0.1	5.0	0.1
Fat Pads	0.8	0.1	1.6	0.1	4.6	0.1	6.9	0.1
Stomach	0.5	0.1	0.9	0.1	2.3	0.1	15.4	0.3
Sm. Intestine	2.7	0.3	5.9	0.3	11.0	0.3	58.0	1.0
Caecum	0.2	NS	0.5	NS	1.6	NS	6.7	0.1
Lg. Intestine	0.5	0.1	1.0	0.1	3.4	0.1	14.1	0.2
Muscle (est.)[4]	65	7	69	4	148	4	791	13
Bone (est.)[4]	645	74	820	46	1530	43	2790	46
Urine	47	5	217	12	494	14	379	6

[1] Rats were sacrificed upon completion of three hours of continuous intravenous infusion.

[2] Figure given is percent of total fluoride infused which was incorporated into the tissue.

[3] N S - not significant.

[4] Estimates were calculated assuming the lean body mass of rat to be 45% body weight and bone to be 9% wet weight.

Table 10. Specific Concentration of Fluoride in Rat Tissues Following Administration of NaF at Different Concentrations*

	Specific Concentration			
Conc. of Admin. F^-	0.019 M	0.038 M	0.076 M	0.126 M
Total F^- Infused, μg (in 3 hr)	900	1800	3600	6000
Tissue	μgF/g	μgF/g	μgF/g	μgF/g
Blood	0.8	1.7	3.4	6.1
Liver	0.4	0.8	2.4	9.0
Kidney	1.1	2.7	3.5	11.0
Spleen	0.3	0.8	1.6	9.2
Lung	3.1	9.7	2.7	22.6
Heart	0.4	0.5	0.9	--
Brain	0.1	0.2	0.6	1.7
Testes	0.6	0.8	1.7	2.9
Fat Pads	0.3	0.5	1.3	2.2
Stomach	0.2	0.4	1.1	7.0
Sm. Intestine	0.3	0.8	1.5	7.1
Caecum	0.2	0.3	1.2	6.9
Lg. Intestine	0.2	0.7	2.2	8.0
Muscle	0.5	0.5	1.0	5.3
Bone	20.5	25.2	46.5	89.7

*Rats were sacrificed upon completion of three hours of continuous intravenous infusion.

between cumulative administered dose and specific concentration of fluoride in these tissues. The observed fluoride concentrations in blood and soft tissues (Figure 6; Tables 9 and 10) also point to the conclusion that the rate of removal of fluoride by way of excretion to urine and deposition to skeletal tissues has a defined ceiling. Thus, when the concentration of administered fluoride is high, i. e. at 0.126 M, one finds that fluoride concentrations in blood and soft tissues are considerably higher than that estimated by linear extrapolation on the basis of administered doses. This observation implies that, at the stated high dose, additional accumulation of fluoride occurs in blood and soft tissue resulting from limited capacity of the animal to dispose of fluoride into urine and bone.

Finally, in order to graphically correlate the findings in experiments on the fate of fluoride with those on fluoride inhibition of glucose catabolism, results from both types of experiments are presented in the comparable time courses in Figure 7. It is noted that there exists a direct relationship between the extent of inhibition and concentration of fluoride ion in blood. The blood data are presented here for illustrative purposes with the understanding that the blood profile is directly related to that of soft tissue as shown by the present work.

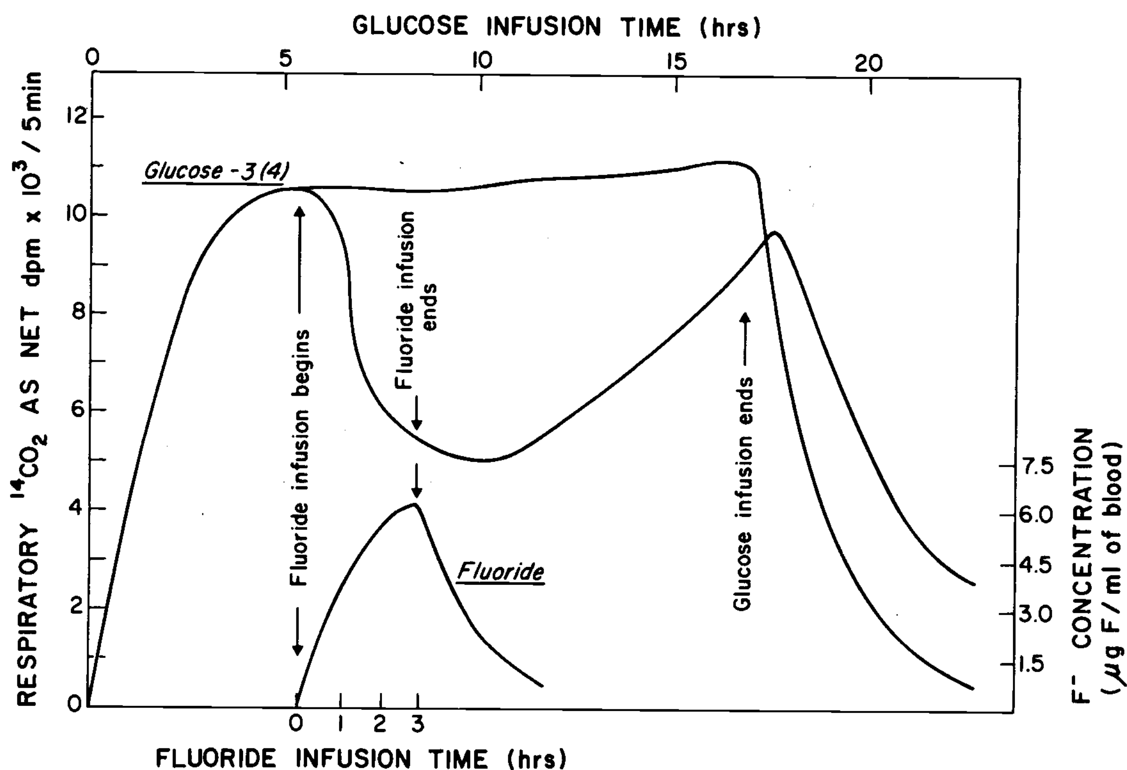


Figure 7. A correlation of the effect of fluoride ion upon glucose catabolism in rats as observed in radiorespirometric experiments with fluoride concentration in blood as detected in tracer experiments using $Na^{18}F$.
Data on effect of F^- upon glucose catabolism as observed in radiorespirometric experiments using glucose-3(4)-^{14}C as a tracing substrate was obtained from experiments reported in Table 4.
Data on fluoride concentration in blood was taken from experiments reported in Tables 6 and 8.

V. SUMMARY

The catabolism of glucose in rats was severely inhibited by fluoride ion when it was administered by way of intravenous infusion at concentrations ranging from 0.063 M to 0.126 M (1 to 2 mg fluoride per hour) for a period of several hours. Radiorespirometric data obtained in experiments with ^{14}C-labeled glucose reveal that the mode of fluoride inhibition is concerned with the enzyme systems associated with the Embden-Meyerhof-Parnas (EMP)-decarboxylation pathway. On the basis of even distribution of the administered fluoride in body fluid, it can be calculated that the concentration of fluoride accompanying a noticeable inhibitory effect is approximately 2×10^{-4} M. This is to be compared with a fluoride ion concentration of 10^{-3} M, reported in the literature, for significant inhibition of the enolase system in *in vitro* experiments.

The fate of infused fluoride ion in rat was conveniently traced by the use of ^{18}F. At a concentration of 0.126 M, the administered fluoride during the infusion process was found to be accumulated in all tissues at a rate dependent on the cumulative amount administered. It was demonstrated that bone is the major site of fluoride removal from blood and soft tissues. Upon termination of fluoride administration, renal excretion played the major role in fluoride removal. After depletion of several hours, the amount

of exogenous fluoride in the bone remained constant. These findings indicate that bone is important in immediate disposal of the administered fluoride from blood and that the kidney is important in eventual depletion of fluoride from blood and soft tissues.

Fluoride concentration in blood and soft tissues was determined directly to be 10^{-4} M to 10^{-3} M when ^{18}F-labeled fluoride was administered at 0.126 M for three hours or a total dose of six mg fluoride. This finding verifies the estimate, based on radiorespirometric data, of the fluoride concentration in body fluid which caused significant inhibition of glucose catabolism.

When fluoride solutions were infused into rats at lower concentrations (0.019 M to 0.076 M), the pattern for fluoride build-up in blood and soft tissues remained similar to those observed in the experiments with a higher concentration (0.126 M) of fluoride. The cumulative concentrations of fluoride in soft tissues at the end of the infusion process were found to be proportional to the respective concentrations of infused fluoride solutions.

It appears from the present work that one of the major toxic manifestations of fluoride in intact rats is the inhibition of the enolase enzyme system, a key step of glucose catabolism. This inhibitory effect can be observed when fluoride concentration in blood and soft tissues is built up to 10^{-4} M to 10^{-3} M, and can be readily reversed

when fluoride administration is terminated indicating rapid removal of fluoride from soft tissues.

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