

Changes of Fluoride Content in Bone:

An Index of Drug Defluorination in Vivo

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Increases of fluoride content in the skeletons of small animals were determined as a measure of the extent to which drugs are degraded *in vivo* to fluoride. Fluorinated drugs which are not metabolized to fluoride, such as halothane, Forane, and FX-50, did not change fluoride concentrations in the bones of mice and rats. Skeletal fluoride concentrations increased significantly after enflurane administration; large increases of fluoride in bone (to 50–100 per cent above normal) were observed after anesthetic doses of methoxyflurane. Defluorination of methoxyflurane and enflurane was increased following induction of microsomal enzymes by phenobarbital sodium, and was inhibited during ethanol intoxication. SKF-525 A and chronic pretreatment with ethanol did not affect the extent of defluorination. A significant amount of fluoride is released by biodegradation and deposited in the skeleton, remaining there for a long time. Therefore, both urinary excretion and skeletal deposition must be considered in the estimation of the extent of drug defluorination. (Key words: Defluorination, anesthetics *in vivo*; Fluoride deposition, skeleton; Microsomal enzyme induction, inhibition; Methoxyflurane; Enflurane; Forane; Halothane; FX-50; Fluorine determination, bones.)

FLUORINE is a trace element in the human body. Its level varies with age and environment.¹ The balance between fluoride uptake and fluoride excretion is of great importance. Acute toxicity of fluoride depends upon its level in the blood and soft tissues. Damage results mainly from the inhibition of certain enzymes, such as enolase, phosphatases, or dehydrogenases.² Fluoride disappears from the blood rapidly ($t_{1/2} = 4$ hours) by excretion in

urine (50 per cent) and by deposition in the skeleton (50 per cent).³ Thus, the skeleton provides, to some extent, a detoxication mechanism, by incorporating fluoride into the hydroxyapatite crystals of the mineral matrix of bone. Fluoride is released from bone slowly. During chronic exposure fluoride accumulates, damaging bone (fluorosis).¹

The purpose of this study was to determine *in vivo* the extent of biodegradation of some fluorinated drugs, especially anesthetics, to fluoride ion, by determining changes of bone fluoride content. Factors influencing defluorination and skeletal deposition of fluoride were investigated.

Methods

Fluoride deposition was studied in black C-57 mice, white Swiss mice (CFW), and Wistar rats. All animals were bred in our laboratory and maintained on the same diet. The concentration of fluoride in their drinking water was 0.4 ppm. Rats or mice from the same litter were divided equally into control and experimental groups. When the animals were 4–5 weeks old, they were exposed to one of the treatments described below. They were sacrificed 48 hours after treatment. The femurs, tibias, and rib cages were stripped of flesh, dried for 24 hours at 105 C, weighed, and immersed for a week in 20 ml of a 5 per cent water solution of sodium ethylenediamine tetraacetate (EDTA) at pH 6.8. The fluoride concentration in the eluate was measured with a specific fluoride-ion electrode (Orion). The elution was repeated once or twice, until the fluoride reading was negative. To confirm that all fluoride had been extracted, $MgSO_4$ was added to some samples after extraction, and the bones were combusted.⁴ The ash was dissolved in a 5 per cent solution of sodium EDTA and examined for fluoride content with a fluoride-ion electrode. No fluoride was detected in the ash. The fluoride concentrations

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TABLE 1. Deposition of NaF in the Mouse Skeleton*

Species	Dose F (mg/kg)	Pretreatment	$\mu\text{g F/g}$ of Dry Bone		Per Cent of Dose Administered
			Control	Treated	
Black mouse	57	None	335 (310-360)	612 (550-700)	48.6
			388 (365-432)	507 (429-645)	
White mouse	25	None	370 (328-435)	484 (420-603)	47.6
	25	Phenobarbital			45.6

* Figures in parentheses are ranges for four mice.

TABLE 2. Deposition of Fluoride in Mouse Bones after Administration of Polyfluorinated Hydrocarbons

	Dose (g/kg)	Number of Injections	Total Dose F (g/kg)	Increase of F in Bone	
				$\mu\text{g/g}$	Per Cent of Dose
Methoxyflurane	0.7	4	0.64	329*	5.1
	0.7	5	0.81	382*	4.7
	0.5	5	0.57	385*	6.7
	1.5	1	0.35	195*	5.6
Enflurance	4.1	2	4.3	64*	0.15
	2.8	6	8.5	154*	0.18
Forane	2.8	5	7.1	24	0.03
Halothane	2.0	5	2.9	14	0.05
FX-SO	35.0	2	53.2	13	0.007

* Significant increase from value in untreated mice, $P < 0.01$ by t test.

($\mu\text{g F/g}$ dry bone) in tibias, femurs, and rib cages were the same, varying in the error range of the method (± 3 per cent).

The methodology was tested in four female black mice, which received sodium fluoride (0.125 g/kg in water solution 0.0572 g F/kg) in five intraperitoneal injections during one week. Four female mice from the same litter served as controls. When the mice were sacrificed 48 hours later, the fluoride levels in the treated animals were higher than those in the control group (table 1). The experiment was repeated with 16 female white mice, but eight of these mice were pretreated with five doses of phenobarbital sodium (40 mg/kg) at 12-hour intervals. Six hours after administration of the last dose, 0.0275 g/kg of sodium fluoride was administered intraperitoneally to four pretreated and four untreated animals. The same dose of NaF was repeated 20 hours later. The

animals were sacrificed 48 hours after the last treatment, and the bones were analyzed for fluoride content (table 1). There was no difference between fluoride levels in pretreated and untreated animals.

Based on estimates that dry skeleton comprises 10 per cent of total body weight, the differences between the fluoride contents of skeletons of the experimental and control groups were equal to approximately 47 per cent of the administered dose. These experiments established that the techniques used, and the estimated skeletal weights, were correct, since the results were in agreement with results of similar experiments described previously.¹ They also proved that sodium phenobarbital pretreatment does not affect the distribution of fluoride in the rodent body.

Four studies were performed.

TABLE 3. Percentages of Organofluorines Deposited in the Rodent Skeleton as Fluoride

	Methoxyflurane		Enflurane	
	Mice	Rats	Mice	Rats
	1.5 μ /kg	0.75 μ /kg	1.5 μ /kg	3.6 μ /kg
Pretreatment				
None	5.6	8.7	0.15	0.18
With the same drug*	5.5		0.18	
Phenobarbital, 40 mg/kg (5x)	11.5†	13.2†	0.26†	0.19
Ethanol, two weeks	6.9	8.6	0.24	
Ethanol, 1.5 g/kg iv	2.9†	7.5†	0.04‡	0.18
Ethanol, 2 g/kg p.o.	4.1‡			
SKF-525-A, 50 mg/kg	6.0	6.5	0.11	

* Five repeated doses of methoxyflurane or enflurane.

† Deposition significantly different from values in the non-pretreated group, $P < 0.01$ by t test.‡ $P < 0.05$ by t test.

TABLE 4. Defluorination of Methoxyflurane in a Mouse Population with High Daily Fluoride Uptake

	Dose of F in Water (g/kg)	0.4 ppm				10 ppm			
		Survival/Death	F (μ g/g Bone)	Increase in F (μ g/g)	Per Cent of Dose	Survival/Death	F (μ g/g Bone)	Increase in F (μ g/g)	Per Cent of Dose
Methoxyflurane 0	0	12/0	219 189-281			12/0	554 487-642		
1.72 g/kg	0.39	6/0	524 505-563	305	7.8	6/0	814 716-938	260	6.7
2.18 g/kg	0.50	3/3	538 496-600	329	6.6	4/2	935 881-981	381	7.6
2.75 g/kg	0.63	1/5	361	142	2.3	2/4	688 640-736	134	2.1
3.50 g/kg	0.80	1/5	317	98	1.2	0/6			
LD ₅₀			2.3 g/kg (2.16-2.46)				2.5 g/kg (2.32-2.67)		

STUDY 1

Skeletal deposition of fluoride after administration of fluorinated drugs to untreated mice was studied. Halothane, methoxyflurane, enflurane, and Forane † were administered to black mice, males and females, intraperitoneally in olive oil (0.1 ml/kg), in single or repeated doses in the amounts indicated in table 2. The interval between injections was three days. Each drug was tested in five animals.

† 1-Chloro, 2,2,2-trifluoroethyl-difluoromethyl ether.

The usual sleeping times were one to two hours. In another group of five mice, perfluorobutylperfluorotetrahydrofuran (FX-80) was injected undiluted into the peritoneal cavity. No change in the behavior of the animals was observed. To prove that FX-80 is absorbed from the peritoneum, the brains of all mice were analyzed for FX-80 by gas chromatography.⁹

STUDY 2

Skeletal deposition of fluoride in mice and rats following pretreatment with microsomal

enzyme inducers and inhibitors was studied. There were six groups of each species, and in each group five animals survived. The control group received either no treatment or pretreatment with drugs affecting microsomal enzymic activity only (table 3). All experimental mice received methoxyflurane, 1.5 g/kg, in a single dose, or enflurane, 8.2 g/kg, in intraperitoneal injections administered 24 hours apart. One group of animals was pretreated with five doses of sodium phenobarbital (40 mg/kg in saline solution intraperitoneally) at 12-hour intervals. The anesthetics were administered six hours after the last injection of phenobarbital. A second group received ethanol for two weeks in drinking water in a 5 per cent concentration. A third group received ethanol intravenously (1.5 g/kg in 20 per cent solution), and a fourth group was given ethanol orally (2 g/kg) 10 minutes before administration of the anesthetic. A fifth group received SKF-525 A, † 50 mg/kg in water solution, intraperitoneally 45 minutes before anesthetic administration. A sixth group served as control. Similar sets of experiments were done in rats, but only half of the doses of anesthetics were used, since methoxyflurane and enflurane are more toxic to rats than to mice.

STUDY 3

The effect of high fluoride intake on skeletal deposition of fluoride following exposure to methoxyflurane was determined. Fourteen pregnant white mice were separated into two groups on the twelfth day of pregnancy. One group continued to receive a standard diet with 0.4 ppm of fluoride in the drinking water. The other group received a standard diet, but the fluoride concentration in the drinking water was increased to 10 ppm by adding NaF. The offspring continued to receive 0.4 ppm or 10 ppm fluoride in water for the remainder of their lifetimes. The addition of fluoride to the drinking water had no effect on growth or body weight. A month after delivery, the combined offspring of both groups (36 mice) were separated into six groups of six mice each. The animals in the first cage received

no further treatment. The animals in the second cage received 0.2 ml of olive oil per 10 g body weight intraperitoneally. Since there was no difference between fluoride levels in the bones of animals in these two cages, the animals from both cages were taken as controls. The animals in the other four cages were given methoxyflurane intraperitoneally in four doses close to the LD₅₀ (table 4).

STUDY 4

Skeletal deposition and release of fluoride following exposures to methoxyflurane were studied in 3-week-old white mice (100 males and 100 females). The mice were separated into six groups: two control groups (male and female); two groups (male and female) which received methoxyflurane, 1.5 g/kg, in a single injection; and two groups (male or female) which received five doses of methoxyflurane, 1 g/kg, during one week. Two animals from each group were sacrificed at different intervals after completion of treatment, and the fluoride contents in femurs and tibias were determined (fig. 1).

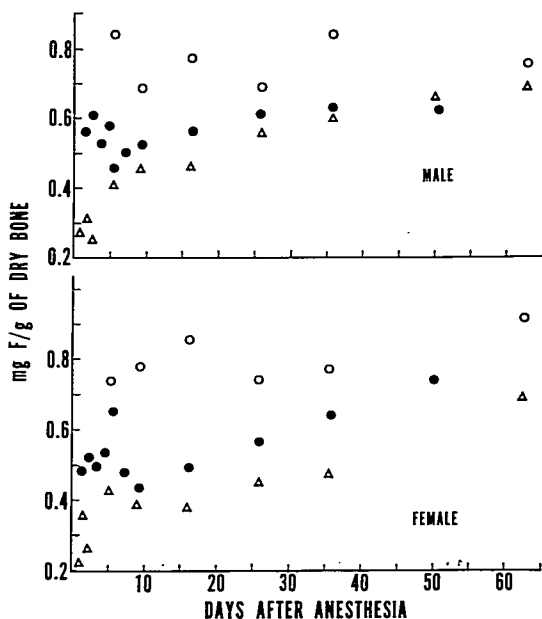
Results

Following administration of halothane, Forane, or FX-80, there was no change in fluoride content of bones, compared with bones of untreated animals (table 2). The concentration of FX-80 in brain tissue averaged approximately 50 µg per gram of wet tissue, indicating that FX-80 had been absorbed from the peritoneal cavity. Following methoxyflurane or enflurane anesthesia, the fluoride levels in bones increased significantly. The increases in fluoride in the skeletons (difference between experimental and control groups) represented 5.6 per cent of the fluoride administered as methoxyflurane and 0.17 per cent of that administered as enflurane.

The amounts of fluoride deposited in the skeletons of mice and rats exposed to methoxyflurane or enflurane almost doubled when the animals were pretreated with phenobarbital (table 3). The amount of fluoride deposited in bone did not change when the animals were pretreated with SKF-525-A or were exposed chronically to a small dose of ethanol. Animals receiving methoxyflurane, being acutely intoxicated with ethanol, showed significantly

† β-Diethylaminoethyl-diphenyl-propyl acetate, supplied courtesy of Smith, Kline, and French Laboratories.

FIG. 1. Fluoride concentrations in mouse bones following administration of methoxyflurane. Each point represents pooled results from two animals: Δ , untreated animals; \bullet , single injection of methoxyflurane (1.5 g/kg); \circ , repeated injection of methoxyflurane (5 g/kg total). Details are described in text, study 4.



less deposition of fluoride in bone than sober animals. Defluorination of enflurane was influenced by drug pretreatment in a similar way, but the difference was less significant than in experiments with methoxyflurane.

The toxicity of methoxyflurane (measured as the LD_{50}), was the same in both groups of animals, regardless of fluoride content in the drinking water (table 4). The highest mortality occurred during the first two hours after injection. The bones of the animals which died during the first two hours showed no increase of fluoride concentration compared with control values. All animals which survived 48 hours had increased fluoride levels in bones. The fractions of fluorine released from methoxyflurane and deposited in the skeletons of surviving animals were the same in the group raised on 0.4 ppm fluoride in drinking water and in the group receiving 10 ppm fluoride in drinking water.

Skeletal uptake and release of fluorine following methoxyflurane anesthesia are illustrated in figure 1. Fluoride levels in the bones of the control animals increased linearly with age. Fluoride uptake by the skeletons of the mice in the control groups is described by the following equations:

$$C_{\sigma} = 332 + 6.63 \text{ d};$$

$$C_{\eta} = 295 + 7.07 \text{ d},$$

where C is the concentration of fluoride in the bone ($\mu\text{g/g}$), and d is the age of the animals in days. Fluoride concentrations in the bones of animals one hour after administration of methoxyflurane were the same as those in the control group, but two hours later the fluoride levels in the bones of treated animals were significantly higher than in the control group (from 242 $\mu\text{g/g}$ to 362 $\mu\text{g/g}$, $P < 0.01$). The greatest increase in fluoride levels in bones was found from the second to the fourth day after

TABLE 5. LD₅₀'s of Methoxyflurane and Enflurane with Regard to Pretreatment*

Premedication	Methoxyflurane	Enflurane
None	2.30 (21.6-2.46)	4.00 (3.74-4.27)
Phenobarbital sodium	2.34 (2.20-2.49)	3.77 (3.56-3.99)
SKF 525-A	2.25 (2.11-2.40)	3.83 (3.73-3.99)
Sodium fluoride, 10 ppm	2.50 (2.32-2.67)	

* Each LD₅₀ was determined from values for 24 female white mice, separated into four groups using the tables published by C. S. Wells.¹⁰

anesthesia. The difference between the fluoride concentrations in bones of the experimental and the control groups obviously diminished during the first week, but it was two months before fluoride levels in the experimental and control groups were the same.

Discussion

Fluoride is a metabolic product of certain polyfluorinated compounds. The extent of defluorination of anesthetics in humans has been estimated from the amount of fluoride excreted in the urine.^{5,6} Testing the extent of biotransformation of new anesthetics, however, presents the problem of collecting urine from small animals. The determination of fluoride in bones was preferred for this study. The fluoride concentrations in tibiae, femurs, and ribs were the same (expressed as $\mu\text{g}/\text{kg}$ dry bone). Since femurs and tibiae are easiest to clean, they were used most often for sampling. Animals were sacrificed 48 hours after drug administration, when the maximal increase of fluoride concentration in bones occurred.

As established by the fourth experiment (fig. 1), the fluoride levels in bones are greatest between two and four days after anesthesia, and the washout of fluoride incorporated in the mineral matrix of the bone is very slow, comparable to the fate of fluorine absorbed from the environment.^{1,2}

Five fluorinated drugs, known to be metabolized in different ways and to variable extents, were used in this study. The disposition of these drugs estimated by fluoride increases in bones agreed with the findings based on urinary excretion.⁵⁻⁹

The deposition of fluoride in the skeletons of our experimental animals was much greater after methoxyflurane administration than after enflurane administration. This result is in agreement with findings in studies of man,

which demonstrated that renal fluoride excretion is much greater following methoxyflurane administration⁵ than following enflurane administration.⁶ Increased fluoride levels were not found in the bones of experimental animals following administration of fluorinated compounds which are not metabolized (such as Forane⁷ or the perfluorinated hydrocarbon FX-80⁸), or those which are extensively metabolized, but which do not have fluoride as a metabolic product (such as halothane⁶).

Since the fate of fluoride released by the biodegradation of drugs is similar to the fate of exogenous fluoride, only half the amount of fluoride released by biodegradation is excreted in the urine shortly after exposure (during one week).^{5,6} The remaining fluoride, in man as well as in animals, is incorporated into the skeleton,³ and is washed out so slowly that it does not perceptibly change the normal renal fluoride excretion. To account for the extent of drug defluorination, both urinary excretion and skeletal deposition must be considered. Since 6.5 per cent of methoxyflurane and 0.5 per cent of enflurane were found to be excreted in human urine as fluoride, we can estimate that 13 per cent of methoxyflurane and 1 per cent of enflurane are defluorinated by patients following anesthesia.

It has been shown that pretreatment with certain drugs (and even with the tested drug itself) quantitatively changes the metabolism and excretion of the tested drug. In our study, sodium phenobarbital, SKF-525 A, and ethanol were used for pretreatment. Pretreatment with these drugs does not have any effect on the distribution of fluoride in the body, so that enhanced or depressed increases of fluoride levels in bones indicate stimulated or inhibited metabolism of anesthetics.

The induction of microsomal enzymes by phenobarbital enhances the o-demethylation^{10,11,12} which precedes the defluorination of methoxyflurane and enflurane. As expected, mice and rats pretreated with phenobarbital had significantly higher concentrations of fluoride in bones than control groups treated with methoxyflurane or enflurane only.

Ethanol is a dose-dependent competitive inhibitor of microsomal mixed-function oxidase.¹³⁻¹⁵ Accordingly, chronic ethanol pre-

treatment has no effect on anesthetic defluorination, but when ethanol levels in the tissues are high, competitive inhibition of microsomal mixed-function oxidase diminishes the defluorination of methoxyflurane or enflurane. Similar effects of ethanol on the metabolism of other drugs have been reported.^{13, 15, 16}

Contrary to the reports of Berman and co-workers,¹² we did not find that SKF 525-A affected the defluorination of methoxyflurane or enflurane. According to reports of studies with other drugs, the inhibitory effect of SKF 525-A in the body lasts at least ten hours.^{10, 17} Considering the excretion rate constants of these anesthetics, the majority of methoxyflurane or enflurane should be exhaled during this period.^{5, 6} We expected, therefore, that the percentage of the dose defluorinated in the body would be smaller than in the control group. Since this was not shown in our experiment, we concluded that, *in vivo*, either the inhibition of the defluorination by SKF 525-A is small, or the effect of the inhibition is compensated by enhanced microsomal enzymic activity observed 23 hours after administration of SKF 525-A.^{10, 17}

We were not able to demonstrate induction of microsomal enzymes by the anesthetics themselves, since the fractions of the doses defluorinated in the body were the same whether the anesthetics were administered in single doses or by repeated injections.

Fluoride is toxic, and may contribute significantly to the toxicity of extensively defluorinated anesthetics. We investigated the possibility that increased fluoride levels in the body, provoked either by microsomal enzymic induction or by high environmental exposure to fluoride, had an effect on methoxyflurane or enflurane toxicity (table 5). We did not find increased mortality in pretreated animals. This suggests that fluoride is not responsible for deaths following overdoses of anesthesia.

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