J. HILLIS MILLER HEALTH CENTER • UNIVERSITY OF FLORIDA • GAINESVILLE
COLLEGE OF DENTISTRY
DEPARTMENT OF BASIC DENTAL SCIENCES
area code 904 392-2661
Box J-424
zip 32610

October 16, 1975

R. A. Mitsch
3M Company
3M Center
Bldg. 235-1N
St. Paul, Minn. 55101

Dear Dr. Mitsch:

Enclosed is the copy of the paper I promised to send you at the ACS meeting. If you or other interested parties at 3M have any ideas on how we can better characterize these fluorocompounds please let either Dr. Taves or me know.

Yours truly,

Warren Guy

Warren S. Guy, D.D.S., Ph.D.

WG:jb

cc: Dr. Taves

Organic Fluorocompounds in Human Plasma: Prevalence and Characterization

Guy, W.S. (University of Florida, Department of Basic Dental Sciences); Taves, D.R. (University of Rochester, Department of Pharmacology and Toxicology); Brey, W.S., Jr. (University of Florida, Department of Chemistry).

Taves discovered that samples of his own blood serum contained two distinct forms of fluoride (1-4). Only one of these was exchangeable with radioactive fluoride. The other, non-exchangeable form was detectable as fluoride only when sample preparation included ashing. This paper is concerned with three aspects of this newly discovered, non-exchangeable form: 1) its prevalence in human plasma, 2) how its presence in human plasma affects the validity of certain earlier conclusions about the metabolic handling of the exchangeable form of fluoride, and 3) its chemical nature.

Preliminary work in this laboratory suggested that the non-exchangeable form was widespread in human plasma but did not exist in the plasma of other animals. Ashing increased the amount of fluoride an average of 1.6 ± 0.25 SD μM (range 0.4 - 3.0) in samples of plasma from 35 blood donors in Rochester, N.Y. (5). No such fluoride was detectable (above 0.3 μM) in blood serum from eleven different species of animal including horse, cow, guinea pig, chicken, rabbit, sheep, pig, turkey, mule and two types of monkey (6).

Standard methods for analysis of exchangeable fluoride in serum have in the past included ashing as a step in sample preparation (7). Taves showed that the amount of fluoride in serum that would mix with radioactive fluoride was only about one-tenth the amount generally thought to be present based on analyses using these older methods (4). When plasma samples from individuals living in cities having between 0.15 and 2.5 ppm fluoride in their water supply were analysed by these older methods, no differences were found between the averages for the different cities. This led to the conclusion that "homeostasis of body fluid fluoride content results with intake of fluoride up to and including that obtained through the use of water with a fluoride content of 2.5 ppm" (8). If the non-exchangeable form of fluoride predominated in these samples, differences in the exchangeable fluoride concentration would probably not have been apparent, and it would be unnecessary to postulate such rigorous

homeostatic control mechanisms for fluoride.

In this study plasma samples were collected from a total of 106 individuals living in five different cities with between 0.1 and 5.6 ppm fluoride in their public water supply. These were analyzed for both forms of fluoride. In this way the relationship between exchangeable fluoride concentration in the plasma and the consumption of fluoride through drinking water was re-evaluated, and the prevalence of the non-exchangeable form was further studied.

With respect to the chemical nature of the non-exchangeable form of fluoride several lines of evidence suggested that it was some sort of organic fluorocompound of intermediate polarity, tightly bound to plasma albumin in the blood. It migrated with albumin during electrophoresis of serum at pH nine (3) and was not ultrafilterable from serum (2). Attempts at direct extraction from plasma with solvents of low polarity like heptane, petroleum ether and ethyl ether were generally unsuccessful. Treatment of albumin solution (prepared by electrophoresis of plasma) with charcoal at pH three did remove the bound fluorine fraction. And finally, when plasma proteins were precipitated with methanol at low pH the fluorine fraction originally bound to albumin appeared in the methanol-water supernatant in a form which still required ashing to release fluorine as inorganic fluoride (5). Based on these considerations the non-exchangeable form of fluoride in human plasma is referred to as "organic fluorine" throughout the rest of this paper.

In order to further characterize the organic fluorine fraction, it was purified from 20 liters of pooled human plasma and characterized by fluorine nmr.

Materials and Methods

Analytical Methods. Values for organic fluorine were calculated by taking the difference between the amount of inorganic fluoride in ashed and unashed portions of the same material.

The following procedure was used to prepare ashed samples: 1) samples (sample size for plasma was 3 ml) were placed in platinum crucibles and mixed with 0.6 mmoles of low fluoride $MgCl_2$ and 0.1 mmoles of NaOH, 2) these were dried on a hotplate and then ashed (platinum lids in place) for 2-4 hr at 600° C in a muffle furnace which had been modified so that the chamber received a flow of air from outside the building (room air increased the blank and made it more variable), and 3) ashed samples were dissolved in 2 ml of 2.5 N H_2SO_4 and transferred to polystyrene diffusion dishes using 2 rinses with 1.5 ml of water.

The following procedure was used for separation of fluoride from both ashed and unashed samples: 1) samples (sample size for unashed plasma was 2 ml) were placed in diffusion dishes (Organ Culture Dishes, Falcon Plastics, Oxnard, Calif., absorbent

removed, rinsed with water), acidified with 2 ml of 2.5 N H_2SO_4, and agitated with a gentle swirling action on a laboratory shaker for 30 min to remove CO_2; 2) for each sample the trapping solution (0.5 ml, 0.01 N NaOH + phenolthalein-p-nitrophenol indicator) was placed in a small polystyrene cup in the center-well of the diffusion dish, 1 drop of 10% Triton-X 100 was added to the sample to decrease surface tension and make the diffusion rate more uniform between samples containing plasma and those not, the lid with a small hole made near its lateral margin was sealed into place with petroleum jelly, 0.02 ml of 4% hexamethyldisiloxane (Dow Corning, Fluid 200, 0.65 cs, Midland, Mich.) in ethanol was injected through the hole in the lid into the sample, and the hole was sealed immediately with petroleum jelly and a strip of paraffin film; and 3) samples were diffused with gentle swirling for at least 6 hr, diffusion was terminated by breaking the seal, and trapping solutions were removed (the indicator color was checked at this point to insure that they were still alkaline) and dried in a vacuum oven (60° C, 26 in-Hg vacuum, in the presence of a NaOH desiccant).

Fluoride was determined by potentiometry with the fluoride electrode. The system used consisted of a fluoride electrode oriented in an inverted position (model 9409A, Orion Research Inc. Cambridge, Mass.), a calomel reference electrode (fiber type), a plastic vapor shield which just fitted over the bodies of both electrodes forming an enclosed sample chamber in which water-saturated tissue paper was placed above the sample to prevent evaporization of the sample, and a high impedence voltmeter (model 401, Orion).

Samples were prepared and read in the following way: 10 µl of 1 M HAc was drawn into a polyethylene micropipette (Beckman Micro Sampling Kit, Spinco Div., Beckman Inst. Co., Palo Alto, Calif.) and deposited into the cup containing the residue from the trapping solution after drying; the flexible tip of the micropipette was used to wash down the walls of the cup; and the solution was then transferred to the surface of the fluoride electrode and the reference electrode brought into position. Surfaces of the two electrodes were blotted dry between samples.

Samples were read in order of increasing expected concentration and sets of samples were read between bracketing calibration standards. These standards were used in two different ways during a run. First, they were flooded onto the electrode surfaces to equilibrate them to concentrations expected for samples and to make them uniform. This procedure permitted the analyst to take reasonably stable readings for samples within one minute. Secondly, they were used in 10 µl volumes for readings used in preparing the standard curve.

Values for identical samples (usually triplicates) were averaged and the average blank was subtracted from sample means. These were then divided by the average fractional recovery of

fluoride (usually 90 to 95%) in standards treated the same way as the sample set.

Plasticware (Falcon Plastics) was used for all analytical procedures to avoid contamination by fluoride from glass. Liquid volume measurements were made with 1, 5 and 10 ml polystyrene pipettes and a polycarbonate volumetric flask (100 ml).

Reagents were purified to insure uniformly low blanks. Water was redistilled and deionized. Acetic acid and ammonia were redistilled. Fluoride contamination in $MgCl_2$ (analytical grade) was reduced by preparing a 1 M solution containing HCl to pH 1 and scrubbing with hexamethyldisiloxane vapor in a column through which the solution was continuously recycled. Following scrubbing the solution was boiled to one third volume to remove any residual volatile silicones and then made just basic with NH_4OH. Fluoride contamination in H_2SO_4 was reduced by repeated extractions of a 6.7 N solution with hexamethyldisiloxane and then boiling to one third volume to remove the residual silicone.

Buffered calibration standards were made from the same NaOH and HAc stock solutions as for samples.

The blanks for ashed samples ranged between 0.2 and 1.5 nmoles fluoride and were typically about 0.5 nmoles. The blanks were smaller for unashed samples; these ranged between 0.05 and 0.2 nmoles fluoride and were typically about 0.1 nmoles.

Factors affecting recovery of fluoride during diffusion were investigated with $^{18}F^-$ tracer. Recovery during diffusion was 97% after 80 min from 5 ml containing 2 ml of plasma. Increasing the acidity of the sample up to 5 N, the volume of the sample up to 7.5 ml, the amount of cold F^- up to 1 µmole, the amount of fluoride complexors up to 1 µmole of $Th(NO_3)_4$ had no material effect on the rate of fluoride diffusion. The absence of both plasma and detergent in the sample compartment markedly slowed the rate of diffusion. Not shaking the sample also slowed the rate of diffusion. Increasing the alkalinity of the trapping solution to 0.1 N increased the rate of diffusion but the lower concentration, 0.01 N, was required here to permit a lower ionic strength in the sample reading solution.

Overall recovery of added cold fluoride was measured. In samples containing neither plasma nor detergent the recovery after 6 hr diffusion averaged 93% and 95% for ashed and unashed samples, respectively. In samples containing plasma the recovery was 95% after 3 hr diffusion.

The degree to which fluorine from organic fluorocompounds could be fixed as inorganic fluoride by ashing varied from less than 1% for volatile compounds like p-aminobenzotrifluoride, m-hydroxybenzotrifluoride, benzyl fluoride and benzotrifluoride to over 80% for less volatile compounds like 5-fluorouracil, fluoroacetate and p-fluorophenylalanine.

Methods used here for separation of fluoride (diffusion at rm. temp.) (9) and its quantitation (fluoride electrode) (10) are considered to be quite specific for fluoride. One potentially

important interference, however, was codiffusable organic acids which might partially neutralize the trapping solution and thus lower the pH of the buffered reading solution. Indeed, it was found that samples containing relatively large concentrations of acetic acid (e.g., fractions 2, 3 and 4 from step 4 in the purification system) completely neutralized the trap within a few hours. The significance of this problem in the analysis of fluoride in blood plasma was investigated in two ways. First, four samples of human plasma were allowed to diffuse for three weeks, and no change in the color of the phenolphthalein indicator in the trapping solution was observed. Secondly, samples containing the same plasma were diffused for different periods up to 158 hr and the apparent fluoride was determined. No changes were observed between samples which correlated with diffusion time.

The sensitivity of the analytical method was limited by the blank rather than the sensitivity of the instruments used. Reproducibility varied with the amount of fluoride being measured. The coefficient of variation averaged 55% in the low range (samples containing 0.25 to 0.75 nmoles F^-) and 6.6% in the high range (10-12 nmoles F^-).

Blood Plasma. Human plasma was obtained from blood banks in five cities. According to public records these cities had not changed the fluoride concentration of their public water supply for at least six years prior to obtaining the samples. Samples were received in individual polyethylene bags which were part of the Fenwall ACD blood collection system. In blood collection using this system 450 ml of blood is drawn into a bag containing 67.5 ml of anticoagulant acid citrate dextrose (ACD) solution. When the cells are removed the ACD solution remains in the plasma. Because of this dilution of plasma a correction factor of 1.3 was applied to values obtained here for the concentration of fluoride. The potential error in this factor was ± 0.1 because of variation between standard limits for hematocrit and minimum volume of the blood donation. Bovine blood was obtained at slaughter and mixed immediately with ACD solution in 1 liter polyethylene bottles.

Electrophoresis. A continuous flow electrophoretic separator (model FF-3, Brinkman Inst., Inc., Westbury, N.Y.) was employed. Sample flow rate was 2.3 ml/hr, buffer flow rate was 72 ml/hr, voltage was 0.67 kv, and current was 140 mamp. Separation took 19 hr. Plate separation was 1 mm and operating temperature was between 2 and 4° C. The buffer was 0.12% $(NH_4)_2CO_3$, made by bubbling CO_2 from dry ice into redistilled NH_4OH until the pH reached 9.0.

Purification System. Steps in the purification system are summarized in table I. In the first step one liter of plasma (pooled from 5-6 individuals) was dialysed in seamless cellulose tubing (1 in. diameter) against 20 liters of water at 4° C. The dialysate was changed twice at 24 hr intervals. In the second step dialysed plasma was freeze dried.

In the third step the dried powder from electrophoresis was extracted with methanol in a soxhelet extraction apparatus (model 6810 G, Ace Glass, Inc., Vineland, N.J.). Cellulose extraction thimbles (model 6812 G, Ace Glass) were soaked overnight in methanol. Operating conditions were 25° to 30° C under a vacuum of 24 in-Hg. Coolant for the condenser was 80% ethanol; inlet temperature was -10° to -20° C and outlet temperature was -10° to 0°. Two liters of methanol were refluxed through the apparatus for a period of 4 hr and approximately 400 ml were lost to evaporation during that period. Glass beads were placed in the flask to prevent bumping.

In the fourth step the residue from the methanol extract was fractionated according to the method described by Siakotos and Rouser (11) for separating lipid and non-lipid components. The method is based on liquid-liquid partition in a column containing a dextran gel (Sephadex G-25, coarse, beaded, Pharmacia Fine Chemicals, Inc., N.Y.). Four eluents are used: 1) 500 ml chloroform/methanol, 19/1, saturated with water, 2) 1000 ml of a mixture of 5 parts of chloroform/methanol, 19/1, and 1 part of glacial acetic acid, saturated with water, 3) 500 ml of a mixture of 5 parts chloroform/methanol, 19/1, and 1 part glacial acetic acid, saturated with water, and 4) 1000 ml of methanol/water, 1/1. Their method was modified for use here by increasing the column length to that attained by using a full 100 grams of dextran beads. Sample size corresponded to that from 2.5 liters of the original plasma.

In the fifth step the residues from eluents 2 and 3 from two runs of step four were combined, applied to a silicic acid column, and eluted by reverse flow with an exponential gradient of increasing amounts of methanol in chloroform. The column (model SR 25/45, 2.5 cm i.d. x 45 cm, Pharmacia) was filled to a height of 30 cm with silicic acid (Unisil, 100-200 mesh, Clarkson Chem. Co., Inc., Williamsport, Pa., heat activated at 110° C for 2 days) and was washed with a complete set of elution solvents before use. The gradient maker (model 5858, set 4, Ace Glass Co.) was filled with 1 liter of methanol in the upper chamber and 2 liters of chloroform in the lower. The flow rate was adjusted by the height of the solvent reservoirs to an average of 3 ml/min for the first liter of eluent. The sample had to be transferred to the column by repeated washings with chloroform because of its low solubility in this solvent. This usually required about 30 ml of chloroform total. Dead volume for the system as 90 ml. Fractions of 15 ml volume were collected in carefully cleaned glass tubes.

Tubing and fittings to the columns were polytetrafluoroethylene (supplied largely by Chromatronix, Inc., Berkeley, Calif.).

All solvents were redistilled. Methanol and chloroform were ACS certified (Fisher Scientific Co.) and acetic acid was analytical reagent grade, U.S.P. (Mallinckrodt Chemical Works, St. Louis). Solvents were removed from samples in a flash evaporator.

NMR. The nmr spectrum was obtained on a Varian XL-100 spectrometer with Nicolet Technology Fourier Transform accessory. The sample was dissolved in an approximately 1/1 mixture of CH_3OH and $CDCl_3$ and spectra were run in a 5 mm tube. External referencing to $CFCl_3$ was used for the chemical shifts, and these are expressed with positive numbers to lower field (i.e., higher frequency). External lock was used. Typical conditions were a pulse length of 15 microseconds, a delay time between pulse cycles of 2.5 sec, and a time constant of -1 sec for exponential processing.

Results

Values for inorganic fluoride (F^-) and organic fluorine (R-F) in 106 plasma samples from humans living in five cities are shown in table II. These data show that the average fluoride concentration in plasma is directly related to the fluoride concentration in the water supply, and that the average organic fluorine concentration in plasma is not. No relationship between fluoride in plasma and organic fluorine in plasma was apparent by inspection of values for individual samples. The distribution of the values within cities are shown in figures 1 and 2. In both cases the distributions appear to be log normally distributed with only 3 or 4 individuals surprisingly deviant. In the cases of the two individuals with little or no apparent organic fluorine (figure 2, Andrews group, left margin), the inorganic fluoride levels were both in excess of 7 μM, making the difference measurement for organic fluorine difficult. The overall mean value for organic fluorine was 1.35 ± 0.85 SD μM.

Plasma was electrophoresed in an attempt to reproduce the findings of Taves (3) using plasma from another individual. Results shown in figure 3 closely match those found earlier in that a predominant form of organic fluorine appeared to migrate with albumin at pH 9, and in that organic and inorganic forms were clearly separated.

The recovery, mass balance and purification factors for steps in the purification system listed in table I are recorded in table III. These data show that about one-third of the original amount of organic fluorine in plasma is recovered in the major peak from silicic acid chromatography. Another third is accounted for in other fractions and the rest is not accounted for, presumably because of adsorption to surfaces of containers in

which samples were placed.

The blank for the purification process was obtained by using bovine rather than human plasma. No organic fluorine was detectable in the original bovine sample but as a further check the sample was dialysed to remove inorganic fluoride to facilitate making the measurement for organic fluorine by difference. Some organic fluorine was apparent in dialysed bovine plasma: 0.13 ± 0.11 SD μM (n=6), a statistically significant though small difference. This trace amount of organic fluorine clearly was not found in the same silicic fractions as the dominant peak from human plasma as shown in figure 4.

Human plasma had been stored in polyethylene bags with ACD solution. Analysis of ACD solution from unused blood bags and analysis of blood plasma before and after placing it in the bags showed that not more than 5% of the organic fluorine in human plasma could have come from this source.

The distribution of organic fluorine in fractions from silicic acid chromatography are shown in figure 4 for four batches corresponding to 5 liters of the original plasma each. There is clearly one dominant peak lying in approximately the same elution position for each batch (the exact position varied with column use and the degree of hydration of the silicic acid adsorbant). There were always some smaller secondary peaks, but they varied in size and position relative to the major peak.

The sample used for characterization by nmr was obtained by combining the fractions containing the major peaks in each of the four batches. Much of the material from batches one and two had been used for other purposes prior to this combination. The combined sample was rechromatographed on silicic acid and a single sharp peak obtained. The final sample was taken from the central portion of that peak and contained 3.3 μmoles of organic fluorine.

Four sample runs were made on the nmr spectrometer with 15,000 to 17,000 scans each and with a sweep width of 15,151 Hz in all but one run, where it was 7,576. The results of all runs were consistent with the spectrum shown in figure 5 and the chemical shifts shown in table IV. A blank run on the solvent mixture showed no instrumental artifacts which might have contributed to the spectrum. Chemical shifts determined for perfluoro-octanoic acid are also included in table IV. Comparison of the shifts in the unknown with that of perfluoro-octanoic acid show that there is a constant difference in shifts of about 2 ppm except for the $-CF_2-$ peak next to the functional group (peak E) where the shift is about 6 ppm. Only the latter is enough to be considered a significant deviation since external referencing was used for each. The difference in shift for peak E is consistent with the presence of amide or ester derivatives, or possibly with the presence of a sulfonic acid derivative as the functional group. One explanation for the additional peaks in the spectrum is the presence of branched isomers, peaks A and B

representing $-CF_3$ groups at branch points, peak C the $-CF_3$ groups two carbons removed from the branch points, and peak H representing $-CF_2-$ next to the branch points.

The sample was reanalyzed for organic fluorine following characterization by nmr to check for contamination; no additional fluorine was apparent. The degree to which fluorine from perfluoro-octanoic acid is fixed as inorganic fluoride during ashing was found to be 21 ± 3 SD % (n=3).

Discussion

These findings suggest that there is widespread contamination of human tissues with trace amounts of organic fluorocompounds derived from commercial products. All available information on this subject is in accordance with this interpretation. A series of compounds having a structure consistent with that found here for the predominant form of organic fluorine in human plasma is widely used commercially for their potent surfactant properties. For example, they are used as water and oil repellents in the treatment of fabrics and leather. Other uses include the production of waxed paper and the formulation of floor waxes (12). The findings presented here that the concentration of organic fluorine was not related to the concentration of inorganic fluoride either in blood or in the public water supply, and the earlier finding that there was little or no organic fluorine in the blood of animals other than human (6) are all in keeping with environmental sources such as these.

The prevalence of organic fluorine in human plasma is probably quite high since 104 of the 106 plasma samples tested here and all 35 in an earlier study (5) had measurable quantities. The prevalence of the particular compounds isolated and characterized here, i.e., perfluoro fatty acid (C_6-C_8) derivatives, is not known since the starting material for each batch shown in figure 4 was pooled from between 25 and 30 individuals and since only about one third of the original organic fluorine content was accounted for in the fractions containing these compounds (see table III).

Peaks other than the one characterized by nmr appear in the chromatograms shown in figure 4 suggesting that human plasma contains other forms of organic fluorocompounds. They are probably not volatile compounds like freons since it is doubtful that these would be detected by the analytical methods used in this study. They correspond in solubility to very polar lipids since they appear in fractions two and three in the fourth purification step. According to the authors of the method used in that step the first eluent contains most fats, the second and third eluents contain very polar fats like gangliosides and certain bile acids in addition to compounds like urea, phenylalanine and tyrosine. The last fraction contains water soluble non-lipid compounds (11). Components of these other peaks are

less polar than the compounds in the predominant peaks in accordance with the methanol-in-chloroform gradient used to elute them in the fifth purification step. Other forms not seen in silicic acid fractions may also exist since only about half the original organic fluorine was recovered in these fractions.

The actual amounts of the perfluorinated fatty acid derivatives in human plasma is not known both because individual plasma samples were not assayed for these particular compounds and because the degree to which organic fluorine from these compounds is converted to inorganic fluoride during ashing is not known. Metal salts of perfluorinated fatty acids have been reported to decompose at 175 to 250° C forming CO_2, volatile perfluorinated olefins one carbon shorter, and one atom of fluoride per molecule (13). About 3 fluorine atoms per molecule of perfluoro-octanoic acid were fixed as inorganic fluoride by ashing methods used here. Thus, values reported here for fluoride after ashing fractions from the major peaks in figure 4 probably represent somewhere between one-third and one times the molar amount.

Little has been published about the metabolic handling and toxicology of perfluorinated fatty acid derivatives. Computer assisted literature searches using Medline, Toxline and Chemcon developed no information on these subjects. This was surprising with respect to the widespread commercial use of such compounds. It would appear from information presented here that rapid excretion of such compounds into urine is unlikely since they are bound to albumin in the blood. On this topic it can also be stated that other chemicals are usually not toxic in blood concentrations similar to those found here for organic fluorine.

The concentration of organic fluorine in human plasma may be changing with time. In 1960 Singer and Armstrong reported that the plasma of 70 individuals residing in communities with 1 ppm or less fluoride in their public water supply had an average concentration of fluoride of 8.8 µM (8). They prepared their samples by ashing them and then distilling fluoride from the ash acidified with perchloric acid (7). Thus, it seems likely that their values for "fluoride" would have included organic fluorine had it been present. Assuming that inorganic fluoride concentrations at that time were similar to those found in this study (see table II), the organic fluorine component would exceed 7 µM. In 1969 the same investigators using the same method reported an average fluoride concentration of 4.5 µM for 6 plasma samples each pooled from at least 3 individuals supposedly living in fluoridated communities (14). This corresponds to an organic fluorine component of only about 4 µM. Organic fluorine concentration presented here averages only 1.35 µM. Therefore, there may have been a decrease in the concentration of organic fluorine in human plasma since the late 1950's. An alternant explanation might be that differences in

the analytical methods or differences in the sample populations caused these values to vary.

Organic fluorine is the predominant form of fluorine in human blood except where the concentration of fluoride in drinking water is high (in which case fluoride predominates, see table II). This together with the finding reported here that there is no apparent relationship between the concentrations of organic fluorine and inorganic fluoride in plasma helps explain why in earlier studies (8) no relationship was found between plasma fluoride determined in ashed samples and the fluoride content of the public water supply. The data in table II show that when methods specific for inorganic fluoride are applied, a clear relationship between fluoride in plasma and fluoride in the public water supply (between 0.1 and 5.6 ppm) can be demonstrated. Thus, there is no need to postulate the existence of such rigorous homeostatic control mechanisms for plasma fluoride as suggested earlier (8). Average plasma fluoride concentrations for individuals living in the same city as reported here reflect the balance established between fluoride in blood and that in bone mineral over periods of years. These findings do not contradict a passive homeostatic control mechanism in which bone mineral damps swings in blood fluoride concentration over relatively shorter periods of time.

The values presented here for the average inorganic fluoride concentration of plasma from individuals living in a community having about 1 ppm fluoride in the water supply are consistent with recent findings of others using similar methods (14, 15).

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Q. I wonder if you tried to correlate within individuals the level of organic fluorine with age.

A. It would certainly be interesting to have this information but unfortunately we cannot supply it at this time. An expeditious approach might be to analyze chord blood from infants of mothers who had not received fluorine-containing anesthetics at childbirth. It would also be of interest to know whether individuals living in isolated regions have organic fluorine in their blood plasma.

Q. Did you say the sample analyzed by nmr contained methyl alcohol?
A. Yes, I did.

Q. Methyl alcohol will react very rapidly with fluorinated acids. The nmr spectrum may, therefore, represent that of methyl ester derivatives.

A. Methanol was also used in the last three steps of the purification system. The nmr spectrum is consistent with the presence of methyl ester derivatives of perfluorinated fatty acids (C_6-C_8) and their branched isomers.

Table I

PROCEDURE FOR PURIFICATION
OF FLUOROCOMPOUNDS FROM BLOOD PLASMA

Fraction Treated	Treatment	Fraction Removed
blood plasma	step 1: exhaustive dialysis against distilled water	smaller, water-soluble components
plasma proteins & protein-bound substances in water solution	step 2: lyophilization	water
plasma proteins & protein-bound substances	step 3: methanol extraction--soxhelet, 25°C, 24 in-Hg vacuum	plasma proteins
plasma lipids	step 4: column chromatography--liquid-liquid partition on Sephadex	lipids of low polarity and residual polar contaminants
polar lipids	step 5: column chromatography--adsorption on silicic acid	unknown: several yellow fractions

Table II

CONCENTRATION OF FLUORIDE (F^-) AND ORGANIC FLUORINE (R-F) IN BLOOD PLASMA SAMPLES FROM FIVE CITIES HAVING DIFFERENT FLUORIDE CONCENTRATIONS IN THEIR WATER SUPPLY

	[F^-] in Plasma[a], μM			[R-F] in Plasma[a,b], μM		
City ([F^-] in Water, ppm)	Mean± SD(n)	Range	Diff.[c] P<.05	Mean± SD(n)	Range	Diff.[c,d] P<.05
Albany, N.Y. (<.1)	0.38± 0.21 (30)	0.14- 1.1		1.2± 0.6 (30)	0.3- 2.6	
			sig,			n.s.
Rochester, N.Y.(1.0)	0.89± 0.75 (30)	0.35- 4.2		1.6± 1.2 (30)	0.5- 6.8	
			n.s.			n.s.
Corpus Christi, Tex.(0.9)	1.0± 0.35 (12)	0.60- 1.7		1.3± 0.9 (12)	0.4- 3.9	
			sig.			n.s.
Hillsboro, Tex.(2.1)	1.9± 0.9 (4)	0.60- 2.6		2.3± 0.6 (4)	1.5- 2.8	
			sig.			sig.
Andrews, Tex. (5.6)	4.3± 1.8 (30)	1.4- 8.7		1.1± 0.5 (30)	0.1- 2.3	

[a] Each value used in the computation was the average of at least three replicate analyses and was corrected for dilution by ACD solution by multiplying it by 1.3.

[b] taken to be the difference between the amount of inorganic fluoride measured in ashed and unashed aliquots of the same sample

[c] by t-test assuming equal variance in each group

[d] The difference between Rochester and Andrews is statistically significant.

Table III

MASS BALANCE, RECOVERY AND PURIFICATION
FACTOR FOR STEPS IN THE PURIFICATION SYSTEM

Fraction	Dry Wt. grams	Amt. R-F[a] nmoles	Recovery[b] %	Purification
human plasma (ACD, 2.5 liter batch)	200	1725 ±273(6)		
Methanol Extraction				
extract	10.1	1476 ±60(6)	85.6 ±14.0	17 X
residue	--	105 ±37(4)	6.1 ±1.0	--
Sephadex Column				
Fraction I	--	125 ±18(4)	7.3 ±1.6	--
Fractions II + III	1.29	1195 ±129(6)	69.3 ±13.3	108 X
Fraction IV	--	118 ±29(4)	6.8 ±1.2	--
Silicic Acid Column				
major peak	.03[c]	630[d]	36.5	2,440 X
other peaks combined	--	240[d]	13.9	--

[a] mean ± SD(n)
[b] percent of the amount of R-F in the original plasma sample, mean ± SD
[c] estimate based on weighing the contents of two tubes in the center of the major peak
[d] estimate based on area under peaks from graph

Table IV

RESULTS OF NMR SPECTROSCOPIC ANALYSIS

Peak Designation	Chemical Shift[a], ppm: Sample	Chemical Shift[a], ppm: Perfluoro-octanoic Acid	Suggested Assignments
A	-70.7		$-CF_3$ groups at branch points
B	-71.9		terminal $-CF_3$ in branched isomers
C	-80.0		terminal $-CF_3$ in straight chain
D	-81.0	-82.6	$-CF_2-$ next to X[b]
E	-114.3	-120.2	$-CF_2-$ in $-CF_2-CF_2-CF_2-$
F	-120.3	-123.1	$-CF_2-$ next to branch points
G	-121.5	-124.2	$-CF_2-$ next to terminal $-CF_3$
H	-122.3		
I	-126.0	-127.6	

[a] External referencing to $CFCl_3$ was used for the chemical shifts, and these are expressed with positive numbers to lower field (i.e., higher frequency).

[b] where X is likely to be -CO-Y

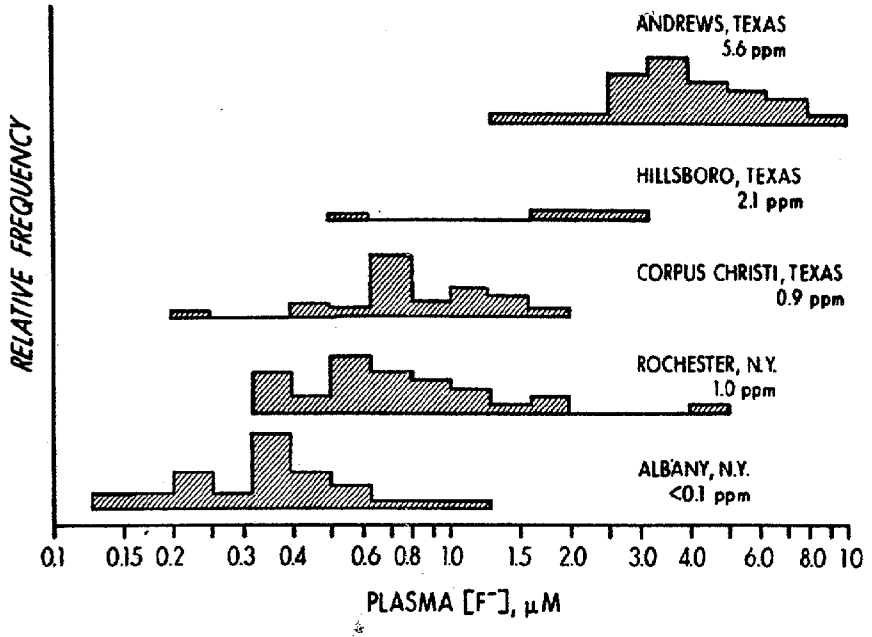

Figure 1. Relationship Between the Concentration of Fluoride in Human Plasma and the Concentration of Fluoride in the Drinking Water

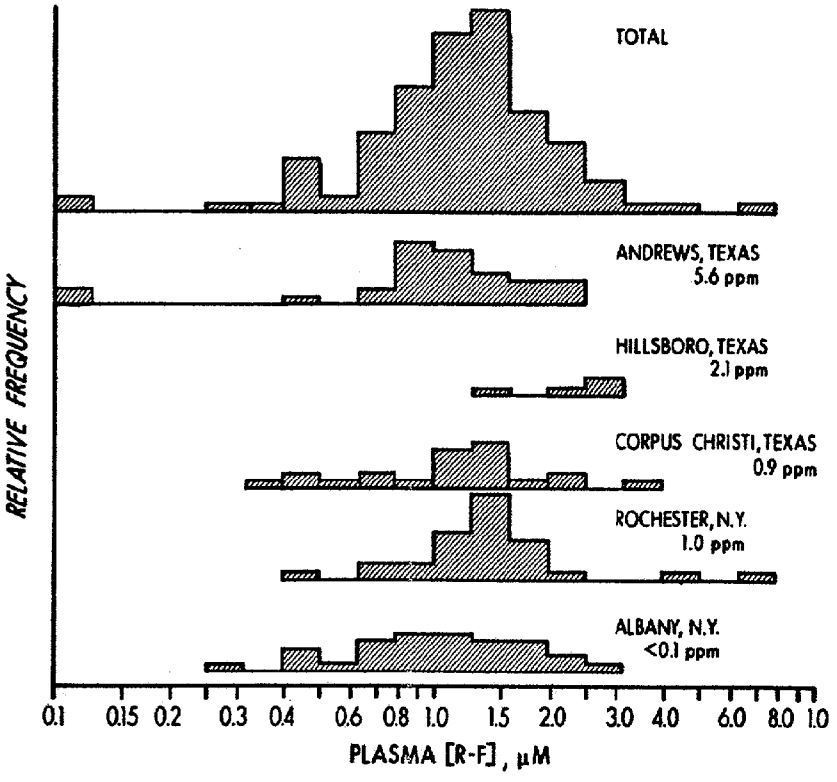

Figure 2. Relationship Between the Concentration of Organic Fluorine in Human Plasma and the Concentration of Fluoride in the Drinking Water

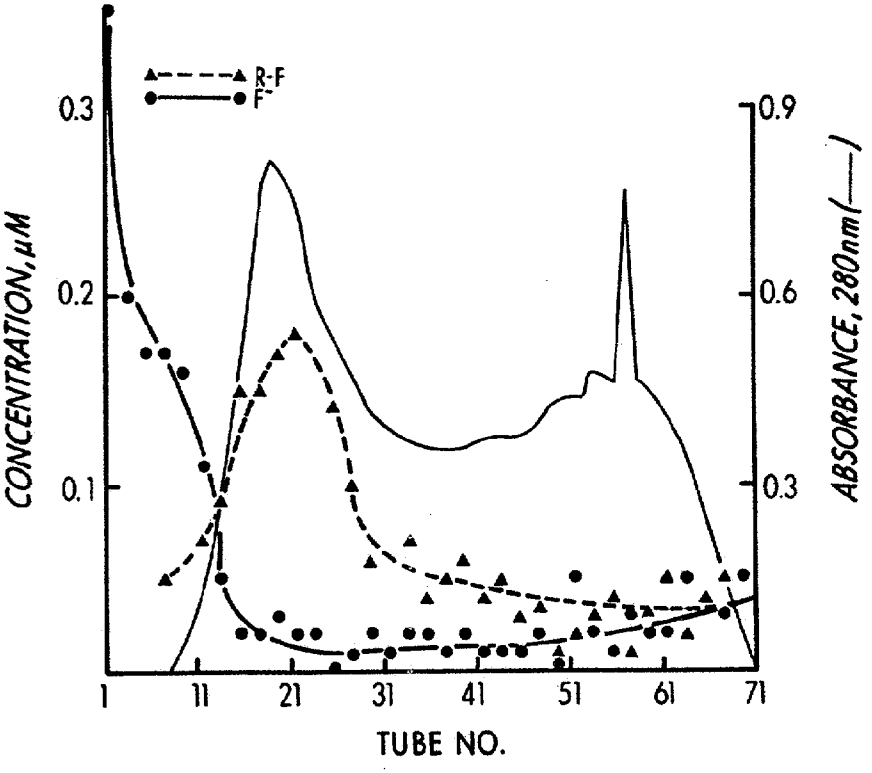

Figure 3. Separation of Fluoride and Organic Fluorine in Human Plasma by Electrophoresis

A sample (about 45 ml) of human plasma was electrophoresed in pH 9 buffer and fractions between the sampling port (near tube 72) and the positive pole (near tube 1) were analysed for the fluoride content of both ashed and unashed aliquots. Relative concentrations of proteins were estimated by absorbance at 280 nm.

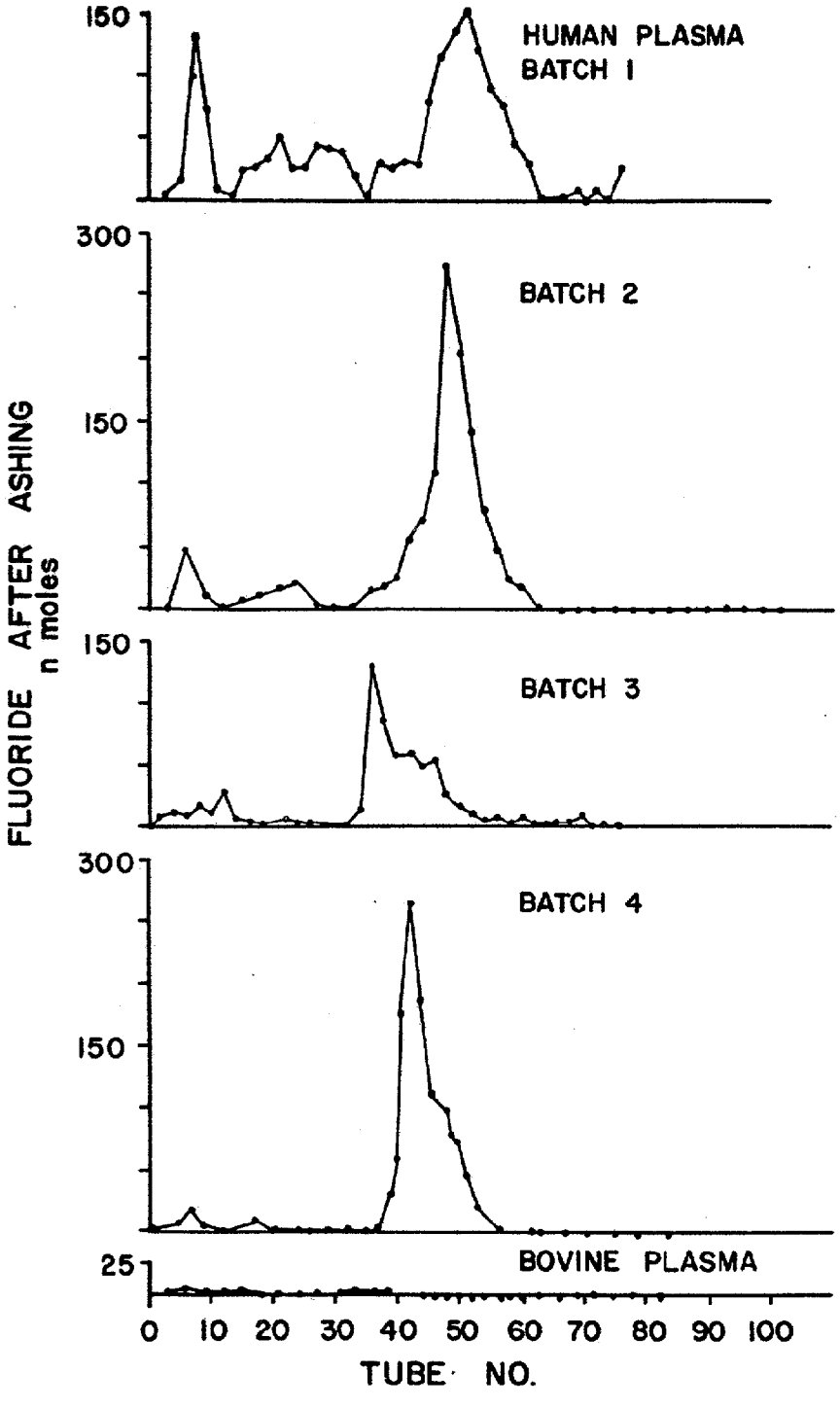

Figure 4. Distribution of Organic Fluorine from Human and Bovine Plasma in Fractions from Silicic Acid Chromatography

Figure 5. NMR Spectrum of Organic Fluorocompound(s) Isolated from Human Plasma