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HYPOTHESIS

Mechanism of Perfluoroalkyl Halide Toxicity: Catalysis of Perfluoroalkylation by Reduced Forms of Cobalamin (Vitamin B_{12})

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Abstract—Perfluoroalkyl halides (PFHs) are synthetic products widely used in various fields. Perfluorooctyl bromide (PFB) is used in medicine as a component of blood substitutes and for artificial lung ventilation. In both cases, it is considered a completely inert compound acting as a solvent for oxygen. However, there are many reports of PFH-induced intoxication, including lethal cases. Mechanisms underlying toxic effects of this compound remain unknown. In this study, we demonstrate that the reduced form of cobalamin (vitamin B_{12}) typical for B_{12}-dependent enzymes can catalyze the reactions of perfluoroalkylation, aromatic substitution, or addition by double bonds. Synthesis of perfluoro derivatives from PFHs during catalysis by cob(I)alamin-like super nucleophiles is a new possible mechanism responsible for *in vivo* formation of highly toxic compounds from "chemically inert" substances widely used in medicine. Catalytic perfluoroalkylation might possibly contribute to nitric oxide depletion and modulation of activity of guanylate cyclase, cytochromes, NO-synthases, and other heme-containing proteins.

Key words: vitamin B_{12}, heme, guanylate cyclase, cobalamin, perfluorooctyl bromide, perfluoroalkyl halides, perfluorocarbons, Perftoran, cytochrome, Freon, cytotoxicity, NO-synthases, Perflubron

Perfluorocarbons (PFCs), their hetero-analogs, and perfluoroalkyl halides (PFHs) are hydrophobic, chemically inert, water insoluble compounds. Polymeric PFCs (Teflon) are widely used as protecting covers in various fields from conduits (in chemical industries) to frying-pans. They are components of various friction reducing lubricants and insulators in electric engineering. Lower gaseous PFHs (bromotrifluoromethane) are known as Freons; they are also employed in fire-extinguishers and automatic fire-prevention systems in military equipment and the Space Shuttle. Liquid PFCs and their hetero-analogs exhibit extremely high oxygen solubilizing capacity, and some of them are used as a base in blood substitutes with gas-transport function (e.g., Perftoran). Fine (<0.1 μm) emulsions stabilized by nonionic detergents are infused into blood vessels at large hemorrhages, and micro-droplets of PFC transfer oxygen like erythrocytes [1-3]. Subsequently, the infused emulsions are decomposed; detergents are rapidly (usually within a day) excreted and PFC may form new emulsions. They may also be subjected to phagocytosis. Alternatively, PFCs can be solubilized in lipids and remain in the body for months. PFCs are excreted with expired air or gradual skin evaporation [4-6]. So, the characteristic "lifetime" values for PFC and PFH (hours and months for CF_3Br and $C_8F_{17}Br$, respectively) in the body depend on vapor pressure and consequently are a function of molecular mass.

Artificial lung ventilation employing PFHs is a new method of treatment in pulmonology [7, 8]. Liquid PFH (usually about one liter) is administered into one lung of a patient together with a hose (equipped with a dispenser) for inhalation of air or oxygen. After the treatment the hose is removed and PFH remaining in the lung is gradually (weeks–months) excreted with expired air. Some proportion of PFH was also found in blood [9].

Until recently there was a notion that PFCs and their hetero-analogs share not only oxygen solubilizing capacity, but also "absolute inertness". Consequently, selection of perfluoro-organic compounds for development of

Abbreviations: GC) guanylate cyclase; PFB) perfluorooctyl bromide; PFC) perfluorocarbon; PFH) perfluoroalkyl halide; *CF*) perfluorocarbon-radical.

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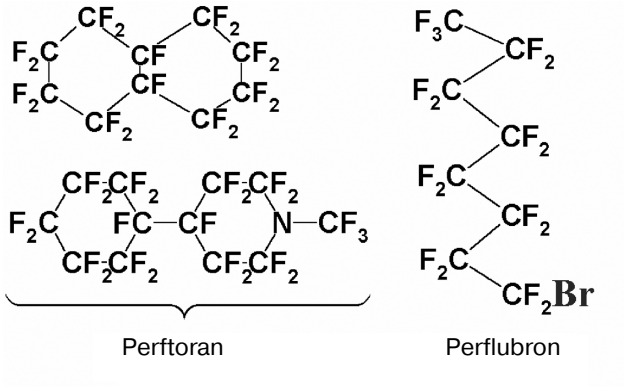
blood substitutes was mainly determined by the availability of the pure compounds and their vapor pressure. For example, the basis of Perftoran consists of a mixture of isomers of perfluorodecalin and perfluoro(1-methyl-4-cyclohexyl)piperidine. Perfluorooctyl bromide ($C_8F_{17}Br$, PFB) is employed in blood constitutes (Perflubron, Oxygent, etc.) and in artificial lung ventilation. Total number of volunteers and patients subjected to the effects of large doses of PFB in medical treatments and trials is unknown, but it definitely exceeds 10,000.



Many cases of poisonings (including lethal ones) after inhalation of gaseous PFHs have been reported [10-12]. However, many survivors who were exposed to very large doses of PFH did not suffer health problems later. For example, there is a case of poisoning of a battle tank crew by $CHBrF_2$ (a component of fire-preventive systems): a driver died, but a gunner did not have health problems [12]. NASA studied effects of low concentrations of $CBrF_3$ on health of potential astronauts (this compound is used in the Space Shuttle fire-preventive system and there was a model scenario of its leakage). The persons had to inhale air containing 1% $CBrF_3$ for 24 h. This treatment caused only "insignificant changes at the level of the central nervous system" [13, 14]. However, it is clear that under "earth" conditions (e.g., dry cleanings, etc.) people can be exposed to much higher concentrations of PFHs for longer periods. There are some cases when serious health problems (even death of the patient) appeared after a lag-period of PFH poisoning. There was a mysterious case report of death of a healthy man after a single inhalation of high concentration of $CHClF_2$: initially he complained of dyspnea, three weeks later "infectious bronchitis" was diagnosed, but one week later the patient died from myocardial infarction [15].

Some evidence exists that the physiological properties of various PFC/PFB based blood substitutes and their pure components are different [16, 17]. For example, PFB (but not perfluorodecalin) stimulated synthesis of reactive oxygen species by monocytes; PFB also stimulated CD11b expression in monocytes and granulocytes and increase of Syk phosphorylation in neutrophils. Reasons for these discrepancies remained unclear. If these effects of perfluoro-compounds were to be attributed to oxygen transport only, PFC and PFB would share these properties. Although there was some correlation between different solubility of these compounds in body lipids and physiological effects and *in vitro* experiments, peculiarities of PFB effects remained unclear [18].

Similarity between oxygen solubilizing capacity and other physicochemical parameters suggests the existence of some other reasons underlying these differences. There is indirect evidence for involvement of NO metabolism in the manifestation of these differences. We have tried to explain these differences by involvement of hydrophobic perfluoro-organic compounds in metabolism of nitric oxides. Environmental heterogeneity is a determining factor due to acceleration of NO oxidation during micellar catalysis [19-21] and shift of equilibrium systems of higher oxides in competitive reactions (1)-(3) in hydrophobic phases [22]:

$$2\,NO + O_2 = 2\,NO_2 \leftrightarrow N_2O_4\,, \quad (1)$$

$$NO_2 + NO \leftrightarrow N_2O_3\,, \quad (2)$$

$$N_2O_3 + HX = ONX + NO_2^- + H^+\,. \quad (3)$$

Like the solubility of oxygen, the solubility of NO is higher in liquid PFC and PFH than in water (partition coefficient $Q_{NO} >> 1$). It is clear that during administration of PFC or PFH as a separate phase or their solubilization in hydrophobic phases, parameters of micellar NO oxidation by oxygen will be changed due to increase of Q_{O_2} and especially Q_{NO}. Consequently, "poisoning" induced by chemically inert PFC or PFH (including gaseous, lipid soluble compounds) may be explained by a common mechanism of "NO-catastrophes", resulting is a loss of stability of the regulatory system of NO metabolism [19]. However, Q_{NO} values for PFB and perfluoro-compounds lacking bromide, including perfluorodecalin, the main Perftoran component, insignificantly differed. Thus, differences in physiological effects of PFC and PFH could not be attributed to differences in NO solvation only. So, we questioned a dogma on inertness of PFB *in vivo* and started to search for natural super nucleophiles capable of reacting with PFB at body temperature.

There are several strong nucleophiles typical for mammalian cells. These include superoxide (O_2^-), peroxynitrite ($OONO^-$) and its derivatives, and numerous metal complexes, often with "unusual" oxidation state (e.g., Fe^+ as in 4Fe,4S-clusters). Low-valence complexes of nickel and cobalt may catalyze reaction of perfluoroalkylation. For example, triphenylphosphine complex of nickel (0) can catalyze reactions of perfluoroalkyl chloride addition to unsaturated and aromatic compounds [23]. Glyoximate complex

of Co^{3+} and zinc as a reductant can catalyze perfluoroalkylation of electron-deficient alkenes by perfluoroalkyl bromides where Co^{+} complex acts as the actual catalyst [24].

Nickel is a cofactor for many bacterial enzymes (e.g., dehydrogenases [25-27]). In the human body, it can be found in bacteria of the gastrointestinal tract. Cobalt is a cofactor of many enzymes, especially bacterial and vertebrate hydrolases [28]. Cobalamin (vitamin B_{12}), the most known form of cobalt *in vivo*, acts as the cofactor of some transferases [29-31]. Although ingested (or administered as a drug) vitamin B_{12} contains Co^{3+}, the latter is reduced in active enzymes. Since cob(I)alamin is one of the strongest nucleophiles, we have investigated its effect on PFH activation.

MATERIALS AND METHODS

Perfluorooctyl bromide, perfluorodecalin (P&M, Russia), cyanocobalamin, hydroxocobalamin (Sigma, USA), high purity argon, and commercially available organic solvents were used without additional purification.

N^1-Acryloyl,N^4-(5-benzyloxycarbonyl-aminonaphthalene-1-sulfonyl)piperazine was obtained by acylation of piperazine by benzyloxycarbonyl-5-aminonaphthalenesulfonyl chloride and acryloyl chloride using conventional methods [32, 33].

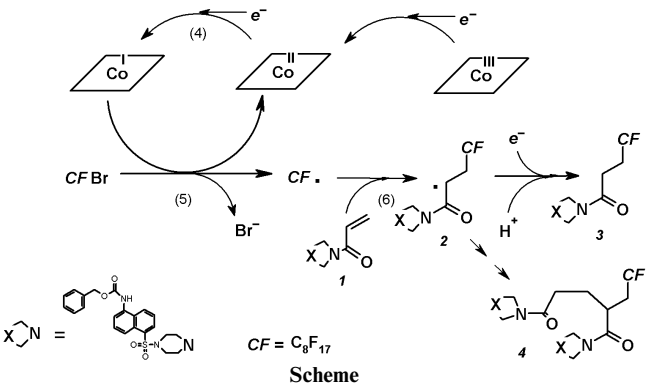
Thin layer chromatography (TLC) was carried out using Silufol plates and a mixture of ethyl acetate–hexane–acetic acid 20 : 40 : 1 as eluent. Fluorescent derivatives were detected using a Sorbphil (Russia) device for TLC and chromatogram irradiation at 254 and 365 nm.

Molecular masses of perfluoroalkylation products were determined using a PE/SCIEX API 3000 mass-spectrometer (nanoelectrospray, +70 V) calibrated using a series of synthetic peptides. The sample was injected into capillary (Protana, Denmark) in the mixture methanol–water–formic acid 50 : 50 : 1. Results were not corrected for ions with separated mass and charge centers, but these parameters were taken into consideration as possible source for increased calibration error.

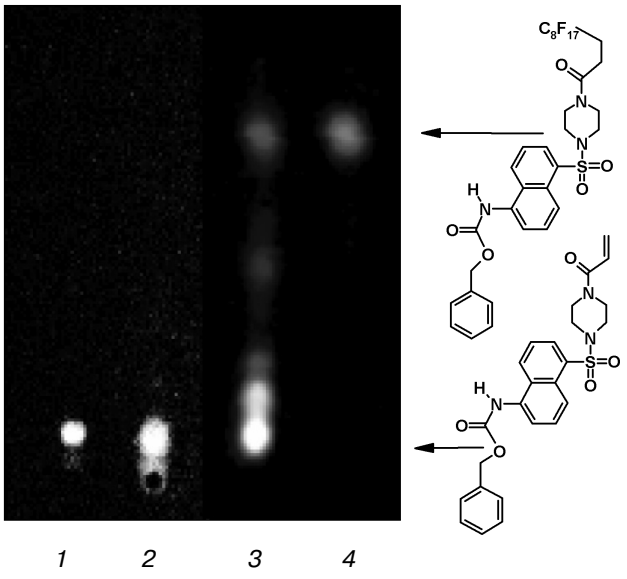
Perfluoroalkylation by perfluorooctyl bromide catalyzed by cob(I)alamin (typical protocol). Samples containing 5 ml of solution of 1.67 mg N^1-acryloyl,N^4-benzyloxycarbonyl-5-aminonaphthalenesulfonyl piperazine (substituted acrylamide with the fluorescent group) and 1.67 mg cyanocobalamin in isopropanol, perfluorooctyl bromide or perfluorodecalin (150 µl) and 200 µg pulverous zinc were mixed using a magnetic stirrer at 25°C under a stream of argon. Control samples did not contain cobalamin and/or zinc. After stirring for 30 min, samples containing zinc and cobalamin changed color from pink to yellow-green due to cobalt reduction (in cobalamin). Twelve hours later samples were filtered through a glass filter and mixed with 80 ml water and 10 ml ethyl acetate. The upper layer was separated, and aliquots (1 µl) were used for TLC. Perfluoroalkylation product (R_f = 0.85, detected by fluorescence) was found only in the sample containing zinc, cobalamin, and perfluorooctyl bromide. For purification of reaction product the ethyl acetate extract was evaporated to dryness under vacuum, dissolved in 500 µl ethyl acetate, and separated by preparative TLC using the same elution system as above. A zone containing the reaction product (detected by fluorescence) was cut from the TLC plate, the product was extracted with ethyl acetate–methanol mixture, filtered, and evaporated to dryness. For mass-spectrometry analysis the resultant sample was dissolved in methanol.

RESULTS AND DISCUSSION

The reduced form of cobalamin activates PFB via formation of *CF*-radical and cob(II)alamin; the mechanism is similar to that described for glyoximate complex (see the Scheme, reactions (4) and (5)). Since cob(II)alamin may be reduced to Co(I) again, in the presence of reducing agents cobalamin (vitamin B_{12}) can act as catalyst of perfluoroalkylation by PFB. The reaction was "visualized" using the fluorescent targets. Substituted acrylamide *1* contains the acylaminonaphthalene sulfamide chromophore group. Addition of perfluoroalkyl radical to activated double bond leads to formation of a new radical *2* and then fluorescent reaction product *3*, which is more hydrophobic than the initial reactant *1*. Although the yield of this product was rather low (about 10%, according to TLC analysis; see the figure), sharp increase of its hydrophobicity (and mobility during TLC separation) allowed it to be identified and isolated as a homogenous substance. Addition of $CF^{\bullet}$ at the double bond of the acrylamide fragment is confirmed by preserved fluorescence. Since perfluoroalkylation caused minimal changes in spectral characteristics, this suggests that the system of conjugated double bonds of the chromatophore remained unchanged, and *CF*-radical did not influence this system. (Introduction of electron-acceptor CF_3-group into aromatic systems is widely used for shift of absorbance maxima and emission during development of chromophores employed in biochemical analysis such as trifluoromethylcoumarin fluorogenic substrates.) The perfluoroalkylation reaction was confirmed by mass-spectrometry.



Scheme



Chromatogram of perfluoroalkylation reaction products (separation on Silufol plates in ethyl acetate–hexane–acetic acid, 20 : 40 : 1; detection by fluorescence at 365 nm): *1*) initial acrylamide *1* (see Scheme); *2*) reaction mixture before addition of a reductant (cobalamin is seen as a dark spot at the origin of the chromatogram); *3*) reaction mixture after termination of reaction (ethyl acetate extract); *4*) purified product of perfluoroalkylation *3* (see Scheme)

The presence of a molecular ion which did not undergo significant fragmentation suggested that M_r of the reaction product *3* increased by 418 ± 2 relative to that of initial reactant *1* (M_r of C_8F_{17} is 419). Since structures of reaction products obtained during cobaloxime-dependent acrylate perfluoroalkylation were confirmed by data of elemental analysis, NMR, and mass-spectrometry [24], we consider addition reaction followed by double bond reduction; however, mass-spectrum analysis does not exclude substitution with preservation of the double bond. Nevertheless, within the goals of the present study, demonstration of the absence of chemical inertness of perfluoroalkyl bromides in the presence of vitamin B_{12}, the identification of exact composition of reaction products by means of fluorogenic targets is not ultimately important. It is much more important that the reaction runs and it is accompanied by formation of new products containing perfluoalkyl radical and they are characterized by abnormally high hydrophobicity. Mass-spectrometry also revealed traces of product with *m/z* ratio ≈1370. It might by perfluorooctyl derivative *4* (two residues of substituted acrylamide), formed during reaction of perfluoroalkyl radical *2* with the second molecule of substituted acrylamide *1*. It should be noted that in acrylate–cobaloxime radical, formation of such products was not observed [24].

Previous study of perfluoroalkylation during cobaloxime catalysis revealed that "ordinary" cobalt salts are not catalysts, and the C–F-bond is stable [24]. Yield was about 70%, but this value decreased when spatially hampered targets was used. Hydroperfluoroalkanes, products of *CF*-radical reduction followed by C–H-bond formation, were identified as side products and their yield increased in the case of spatially hampered acrylates. Considering that we have used doubly substituted acrylamide as a target, it is possible that $C_8F_{17}H$ was a side product in our experiments. As in the case of cobaloxime, bond C–F was stable because we did not find perfluorodecalin-induced perfluoroalkylation.

The principal difference between PFCs and PFBs with respect to super nucleophiles (reduced form of cobalamin) may explain differences between blood substitutes based on PFC (e.g., Perftoran) and PFB. The latter is not chemically inert under physiological conditions. This discovery suggests involvement of catalytic perfluoroalkylation into mechanisms underlying poisonings by low molecular weight PFH including Freons. Due to high volatility and short life-time in the body (τ), this contribution would be less than in the case of higher homologs. However, high hydrophobicity of PFH generally prevents their reduction by hydrophilic cob(I)alamin: cyanocobalamin is readily soluble in water but almost insoluble in lipids and, consequently, reagents are in different phases and this is accompanied by a decrease in reaction rate (see discussion in [19]). Thus, toxicity should depend on partition coefficients of PFH (Q_{PFH}) between lipid and water phases. For lower homologs τ is shorter but Q_{PFH} is smaller, i.e., increase in molecular mass of PFH potentiates one effect and attenuates the other. It is possible that the so-called Stern region (the interphase region) enriched by detergents stabilizing emulsion makes significant contribution to the reaction of reduction of hydrophobic PFH in heterogeneous media *in vivo* [22, 34]. Sorption of lipids and detergents on PFC emulsions was studied in detail during development of Perftoran [35-37]. Generally, lipid composition at the interphase is rapidly changed and initially used emulsion stabilizer (Pluronic) is excreted much faster than PFC itself. It is possible that a similar process occurs in PFH emulsions. It is clear that cobalamin sorption on these emulsions and the rate of its reduction to Co^+ (reaction (4)) and the rate of subsequent PFH reduction to *CF*-radical (reaction (5)) will depend on compositions of lipid and interphase region. Thus, quantitative modeling of the contribution of PFH reduction to toxicity *in vivo* is possible only in very complex models including all varieties of body lipids.

The same speculations are applicable to perfluoroalkylation products: if PFH reduction has taken place the perfluoroalkyl radicals formed will remain in hydrophobic phases and react with other hydrophobic compounds of

these phases. We have demonstrated addition at double bonds and aromatic substitution (particularly, using dansyl group as the activated aromatic fluorophore, data not shown). However, lipophilic radical scavengers like tocopherol, dihydrolipoic acid, and also cysteine and tyrosine residues of proteins should interact first.

Possible role of NO in PFH toxicity. In the introductory part we have already discussed an NO-dependent mechanism underlying toxicity of chemically inert perfluoro-organic compounds. This mechanism includes modulation of micellar NO oxidation followed by subsequent conversions of higher nitrogen oxides. Catalytic reduction of PFH followed by formation of perfluoroalkyl radicals suggests existence of principally a different NO-dependent mechanism of toxicity, which is specific only for PFH (PFB), but not PFC.

NO is a physiological scavenger of free radicals. It also acts as an inhibitor of radical chain reactions. Taking into consideration its hydrophobic nature, the recombination

$$\boldsymbol{CF}\bullet\ (C_8F_{17}) + \bullet NO = \boldsymbol{CF}NO \qquad (7)$$

(here, the symbol *CF* is used to define all perfluoroalkyl radicals—both free $CF^{\bullet}$ radicals and also constituents of compounds) is one of the most probable conversions of perfluoroalkyl radicals *in vivo*. For reaction (7) in a gaseous phase at $CF^{\bullet} = CF_3 - C_4F_9$ absolute rate constants (about $1.6\cdot10^{-11}$ cm^3/molecule·sec = 10^{10} $M^{-1}\cdot sec^{-1}$) are known [38], i.e., reactions proceed in the diffusion controlled region. Reaction products, perfluoronitrosoalkanes (lower homologs are blue gases), are rather stable to oxidation by oxygen; trifluoronitrosomethane is purified (to remove contaminants) by concentrated NaOH solution (see [39-41] and their references).

We demonstrated that even for the couple B_{12}–$C_8F_{17}Br$ differences in hydrophobicity are not principally critical for the interaction, i.e., $C_8F_{17}^{\bullet}$ may be obviously formed *in vivo*, in spite of heterogeneity of the medium in the body. We did not find any information on $C_8F_{17}NO$ in the literature. From the general chemical viewpoint it should be more hydrophobic but chemically similar to CF_3NO and known lower homologs. Due to shift of electron density typical for *CF* (perfluoroalkyl radicals exhibit electrophilic properties) *CF*NO could form stable complexes with heme-containing proteins, which are characterized by well documented complex formation with NO. These include hemoglobin, guanylate cyclase, NO-synthases, and cytochromes (see "Discussion" and Table 1 of the review by Khatsenko on cytochrome P-450 inhibition [42]).

Complex formation with hemoglobin and its subsequent inactivation by *CF*NO is not fatally dangerous because of high quantities of this protein in the body. However, hemoglobin might act as a *CF*NO transporter from places of its formation (e.g., cells relatively enriched with reduced forms cobalamin and cobalamin-like components) to other cells and targets. This is quite possible if we take into consideration that Fe-NO hemoglobin transports NO [43]. Impossibility of rearrangement like Fe-NO → SNO and redox-conversions typical for nitrosohemoglobin [44, 45] would principally differ *CF*NO from Fe-NO hemoglobin.

NO is a short living activator of guanylate cyclase (GC) [46-49]. Since many regulatory systems including vasodilatation and hormone secretion employ NO-dependent GC activation [46, 50, 51], a study of *CF*NO interaction with GC has become one of the hottest subjects in toxicology.

In the case of cytochromes, *CF*NO–heme complex formation should result in their inhibition. This might cause impairments in respiratory chain functioning and accumulation of metabolites that are normally oxidized by these proteins (see "Discussion" in the review by Khatsenko [42]). In this connection, poisoning by PFH activation products (gaseous products like CF_3Br or PFB) might influence pharmacokinetics of drugs. It should be noted that some authors suggest involvement of cytochromes in the reduction of glyceryl trinitrate to NO [52]. NO-synthases (NOSs) are one of the most important heme-containing enzymes. Inhibition of NOSs by excess of NO is considered as physiological mechanism regulating these enzymes [53, 54]. If the complex *CF*NO–heme is rather stable in NOSs, these enzymes cannot effectively bind oxygen and activate it through arginine oxidation. This inevitably causes blockade of NO biosynthesis. Inhibition of cytochrome oxidase in the presence of NO was discussed as a possible mechanism of regulation of cell respiration [55]. If *CF*NO can exhibit similar action it will cause more potent and almost irreversible effect.

Under certain conditions (such as deficit of arginine), NOS can synthesize superoxide instead of NO [56, 57]. (This property is also typical for other proteins containing a system of heme reduction under aerobic conditions.) Recombination $CF^{\bullet} + {}^{\bullet}O_2^- = CFO_2^-$ will result in formation of perfluoro-analog hydroperoxide anion, which almost definitely will be decomposed *in vivo* with formation of fluoroanhydride of lower homolog *CF*COF and further *CF*COOH. Toxicity of various fluoroanhydrides is well documented and long chain perfluorocarbonic acids may exhibit mutagenic and generally toxic effect [58-61].

Thus, several groups of heme proteins directly or indirectly involved in nitric oxide metabolism might represent potential targets just for one of the probable products of catalytic activation of PFH. This uncovers the mystery of case reports on deaths from myocardial infarctions in persons subjected to PFH poisonings.

In general, the reaction of reductive perfluoroalkylation by perfluoroalkyl bromides catalyzed by vitamin

B_{12} might be a source of many potentially dangerous products and abnormal physiological processes during PFH penetration into the body. It is clear that low concentrations of tissue cobalamin make this reaction less noticeable, but not less unpleasant, especially at high half-life periods for excretion of higher homologs. It is also possible that besides B_{12} other metabolites can act as reductants of perfluoroalkyl halides. In this connection only experiments with PFH, particularly, with perfluorooctyl bromide may be potentially dangerous. Since our current knowledge does not allow evaluation of long-term consequences and risk factor, experiments on human beings employing PFH should be maximally limited and volunteers as well as their physicians should be informed about incomplete chemical inertness of PFH and PFB *in vivo*.

Simultaneously, the scientific community should develop biochemistry and methods of detoxification of PFH and their potential metabolites, because, in spite of ecologist warnings, PFH are still widely used in techniques and consumer services and accidental contacts with these substances will inevitably happen. It is possible that short term administration of stabilizing emulsions of chemically inert PFC (like Perftoran) followed by subsequent hemosorption together with "extracted" PFH will represent an effective method for excretion of poorly volatile PFH and products of their conversions.

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