On the interactions of perfluorochemical emulsions with liver microsomal membranes

V. V. Obraztsov

Russian Academy of Sciences, Institute of Cell Biophysics, 142292 Pushchino (Russian Federation)

A. S. Kabalnov, K. N. Makarov

Nesmeyanov Institute of Organo-Element Compounds, Vavilov St. 28, 117813 Moscow (Russian Federation)

U. Gross*, W. Radeck and S. Rüdiger

Project Group Fluorine Chemistry, Geb. 4.1, Rudower Chaussee 5, O-1199 Berlin (Germany)

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Abstract

The interactions of rat liver microsomal membranes with various emulsified perfluorochemicals (PFCs) have been studied. The percentage of microsomal cytochrome P_{450} forming a complex with a PFC decreased with increasing critical solution temperature (CST) of the PFC. The complex was apparently not formed with lipophobic PFCs whose CSTs were higher than 46 °C, e.g. perfluorotributylamine. The complex was destroyed by an excess of a lipophobic PFC emulsion. The rate of complex formation depended on the solubility of the PFC in water and on the dispersity of the emulsion. The results obtained have been used for an analysis of liver cytochrome P_{450} induction after intravenous administration of fluorocarbon emulsions to rats. It is suggested that the membrane-related biological activities of PFCs depend mainly on the solubility of PFCs in lipids and water.

Introduction

Although chemically inert perfluorocarbons (PFCs) are not metabolized in living organisms, they exhibit some types of biological activity. Firstly, by their ability to dissolve large volumes of gases, including O_2 and CO_2, fluorocarbons can act as gas transporters in living systems [1, 2]. Secondly, since they are insoluble in water, fluorocarbons provide a hydrophobic surface on which proteins, lipids and other surface-active components of cells can adsorb. As a result, various modifications of bio-organic molecules, e.g. protein denaturation or lipid oxidation, may be effected [3, 4]. Thirdly, by the interaction of the numerous submicron particles of PFC emulsions with specialized cells, it is possible to affect the reticuloendothelial and immune

*To whom correspondence should be addressed.

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system functions [5]. Finally, PFCs can dissolve in the hydrophobic regions of biological membranes leading to biological alterations, as well.

The latter phenomenon has only been discovered recently [6–9] and is under extensive study at present. More advanced investigations are concentrating on the interactions of PFCs with the endoplasmic reticular membranes of liver cell microsomes. These membranes contain the enzymes of the cytochrome P_{450}-dependent monooxygenase system, which is capable of effecting the oxidative modification of various organic molecules [10]. Over 20 years ago it was shown that perfluorohexane (PFH) forms a complex with cytochrome P_{450} [11]. The interaction of PFH or other PFCs with microsomal cytochrome P_{450} results in changes in the activity of the monooxygenase system [12, 13] without any effect on the structure of the PFC molecule. It has been suggested [14] that the formation of a PFC–cytochrome P_{450} complex is the molecular basis for various alterations in animal organisms after PFC administration. These include intensive synthesis of liver cytochrome P_{450} whereby the total cytochrome content increases 3–4-fold [15], and the activation of phase II xenobiotic biotransformation enzymes, i.e. UDP-glucuronosyltransferase and glutathione *S*-transferase [16]. The induction of these specific liver enzymes was followed by the activation of the liver detoxification function and consequently the blood clearance of some drugs [17, 18] and animal resistance to some poisons [19] increased. The physicochemical properties of fluorocarbon inducers of liver cytochrome P_{450} [14] and the composition of the cytochrome P_{450} isoform induced by PFCs [20] have also been studied.

Obviously, investigation of PFC–microsome interaction is of great importance for the evaluation of the side-effects of fluorocarbon blood substitutes [9]. Furthermore, fluorocarbon inducers of cytochrome P_{450} are of interest as drugs for the activation of the liver detoxification function.

However, experimental data relating to PFC–microsome interaction and induction of the cytochrome P_{450} isoform by PFCs seem to be controversial. Here we report new results arising from equilibrium and kinetic studies of the interactions of submicron PFC emulsions with rat liver microsomal membranes. These results have been used to analyze the observed cytochrome P_{450} induction in rat liver after the intravenous administration of PFC emulsion.

Experimental

The following perfluorinated compounds were studied and their purity (percentage in brackets) checked by GLC methods: perfluoroindane, PFI (99.5%); perfluorodimethylcyclohexane, PFDMCH (99.5%); perfluoromethylpropylcyclohexane, PFMDCH (97.4%); perfluorohexane, PFH (99.2%); perfluorodecalin, PFD (98.5%); perfluoroacenaphthene, PFAN (99.1%); perfluorooctane, PFO (99.8%); perfluorocyclohexylmethylmorpholine, PFCHMM (99.0%); perfluorodibutylmethylamine, PFDBMA (99.1%); perfluorotripropylamine, PFTPA (99.2%); perfluoromethylcyclohexylpropyl ether, PFPMC

(99.2%); perfluoromethylcyclohexylpentyl ether, PFAMC (99.0%); perfluoro-2-ethoxybicyclo[4.4.0]decane, PFED (99.0%); perfluorotributylamine, PFTBA (98.5%); and perfluoro-octyl bromide, PFOB (99.3%).

Submicron emulsions of each separate fluorocarbon in 4% Proxanol 268 (equivalent to Pluronic F68) solution with a 0.1 volume fraction of the dispersed phase were prepared ultrasonically using an UZDN-1 disperser at 10 °C and flushing with N_2 gas. The droplet diameters differed between 90–360 nm depending on the fluorocarbon employed. As the degree of biological interactions of PFC emulsions reported herein depends on their droplet size, it was necessary to relate the experimental results to a normalized droplet size of 250 nm (Table 1) in order to compare them quantitatively. Sonication of PFC emulsions can also produce F^- ions and in these experiments it was shown that the generation of fluoride ion was at a concentration level of 5×10^{-5} mol l^{-1}. Separate tests have shown that amounts of this order do not interfere with the biochemical parameters examined in this study.

The particle size of the emulsions was measured by means of a Coulter N-4 (Coultronics) photon correlation spectrometer. Hence it was found, for example, that PFTBA forms an emulsion with a narrow particle distribution having a mean diameter of 90 nm, whereas PFD shows a broader dispersity. The mean droplet diameters and the dispersity of the emulsions also depend on the energy input during emulsification. By changing the energy uptake, it was possible to prepare individual emulsions with mean droplet sizes in the range 180–400 nm (see Fig. 7 below).

In order to study cytochrome induction, PFC emulsions (10 ml kg^{-1}) were administered by a single intravenous injection 3 d before sacrificing to a group of five male Wistar rats weighing 150–200 g. For induction of the phenobarbital-type liver cytochrome P_{450}, the animals received i.p. injections of phenobarbital (PB) (80 mg kg^{-1}) for 4 d and were sacrificed

TABLE 1

Relation between PFC, water solubility and rate of complex formation with microsomal cytochrome P_{450}

PFC[a]	Water solubility[b] at 25 °C (mol l^{-1})	Initial rate of complex formation (mmol min^{-1})	
		As measured	Normalized to 250 nm diameter
PFD	9.9×10^{-9}	1.6×10^{-6}	1.8×10^{-6}
PFO	3.8×10^{-9}	1.8×10^{-7}	2.2×10^{-7}
PCH	2.2×10^{-9}	4.6×10^{-7}	1.3×10^{-7}
PFAN	6.2×10^{-10}	1.7×10^{-7}	9.9×10^{-8}
PFTPA	2.8×10^{-10}	8.4×10^{-8}	5.9×10^{-8}
PFCHMM	1.3×10^{-10}	2.9×10^{-8}	3.6×10^{-8}
PFMCP	8.0×10^{-11}	1.4×10^{-8}	7.6×10^{-9}

[a]PFC emulsion, PFC conc. = 0.66×10^{-4} v/v%.

[b]Calculated according to the method reported previously [22].

24 h after the last injection. Liver microsomes were obtained by a conventional method involving differential centrifugation. The cytochrome P_{450} content in the microsomes was measured using the method of Omura and Sato [21]. For statistical comparisons, arithmetic means, variances and standard deviations were calculated for each group of experiment [14, 20]. Comparisons between groups were analyzed by a student's test method.

The concentration of PFC complexes found with microsomal cytochrome P_{450} was determined spectrophotometrically. Thus, to 1.5 ml of the microsome suspension (0.05 M phosphate buffer, pH 7.0) in 1-cm length cuvettes in a Specord M40 spectrophotometer was added PFC emulsion (sample) or the same volume of a 4% Proxanol solution (reference). Complex formation was indicated by spectral changes having a maximum at 390 nm and a minimum at 420 nm. The concentration of the complex was determined on the basis of the molar extinction coefficient $\epsilon_{390-420} = 68\ mM^{-1}$ [12].

Results and discussion

The addition of PFC emulsion to microsomal membranes results in spectral changes similar to those observed after the interaction of cytochrome P_{450} with type I substrates [12]. The amplitude of the spectral changes, $\Delta A_{390-420}$ was found to depend on the concentration of the PFC emulsions, as shown in Fig. 1 for PFD. As further shown in Fig. 1, the cytochrome P_{450} in the microsomes of the PB-treated rats has a much greater affinity towards PFD (curve B) than that of the untreated rats (curve A). The

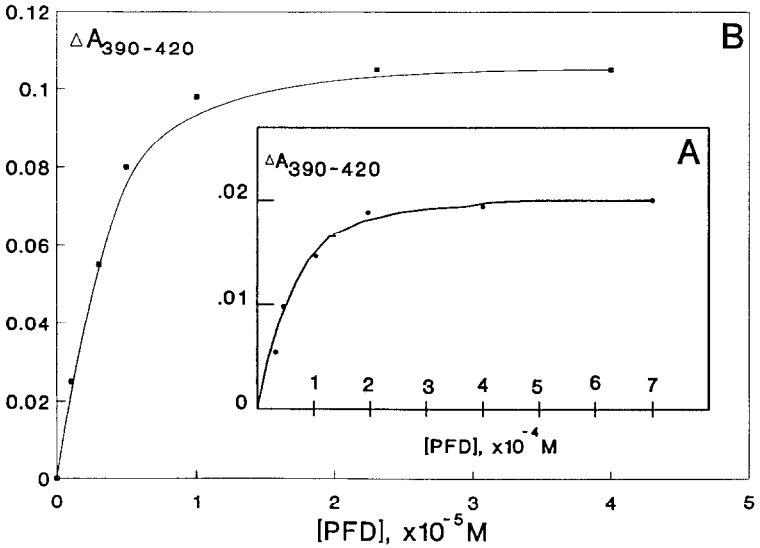

Fig. 1. Formation of PFD–cytochrome P_{450} complexes by the addition of PFD emulsion to microsomes. (A) Microsomes from untreated rats; protein concentration in cuvettes = 8 mg ml^{-1}; cytochrome P_{450} content = 0.85 nmol mg^{-1} of protein. (B) Microsomes from PB-treated rats; protein concentration = 2 mg ml^{-1}; cytochrome P_{450} content = 2.05 nmol mg^{-1} of protein.

association constants K_a (PFD concentration for $\Delta A_{390-420} = \frac{1}{2}\Delta A_{390-420(max.)}$) were 2.3×10^{-6} M and 5.0×10^{-5} M, respectively. The degree of saturation of microsomal cytochrome P_{450} by PFD (the ratio of cytochrome bound to PFD to the total amount of cytochrome) was 35% and 4% for curves B and A, respectively.

The degree of saturation depends on the kind of fluorocarbon employed (Fig. 2). Lipophilic PFCs with low CSTs (critical solution temperatures in n-hexane) readily form an enzyme–substrate complex with the cytochrome, whereas PFCs with high CSTs, e.g. PFTBA, do not undergo complex formation. It should be stressed that PFC–cytochrome interaction is neither influenced by the presence of heteroatoms in the PFC molecules nor by their structure. The lack of interaction between microsomal cytochrome P_{450} and some PFCs [23], in particular PFTBA, as well as the inertness of PFTBA with respect to monooxygenase enzymes [24] has been established previously.

The properties of PFC emulsions obtained from mixtures of PFCs are additive with respect to the individual PFCs as far as their specific interactions with microsomal membranes are concerned. Figure 3 depicts a group of isotherms which demonstrate the influence of PFMCP on the interaction of PFD with microsomal cytochrome P_{450}. Even a preformed PFC–cytochrome P_{450} complex can be destroyed by an excess of a PFC emulsion with a high CST, e.g. PFTBA (Fig. 4).

We suggest that the decline in PFC–cytochrome interaction of PFD/PFMCP mixtures with increasing PFMCP content (Fig. 3) as well as destruction of the PFD–cytochrome complex by addition of an excess of PFTBA emulsion

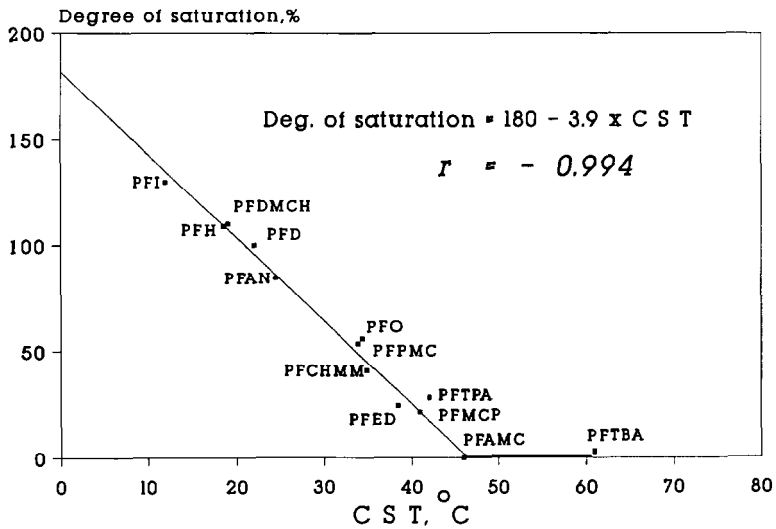

Fig. 2. Saturation of microsomal cytochrome P_{450} by different PFCs. An excess of PFC emulsion (1.3×10^{-3} %vol/vol) was added to microsomes from PB-treated rats; the protein and cytochrome content was as described in Fig. 1(B). Saturation of cytochrome P_{450} by PFD has been taken as 100%.

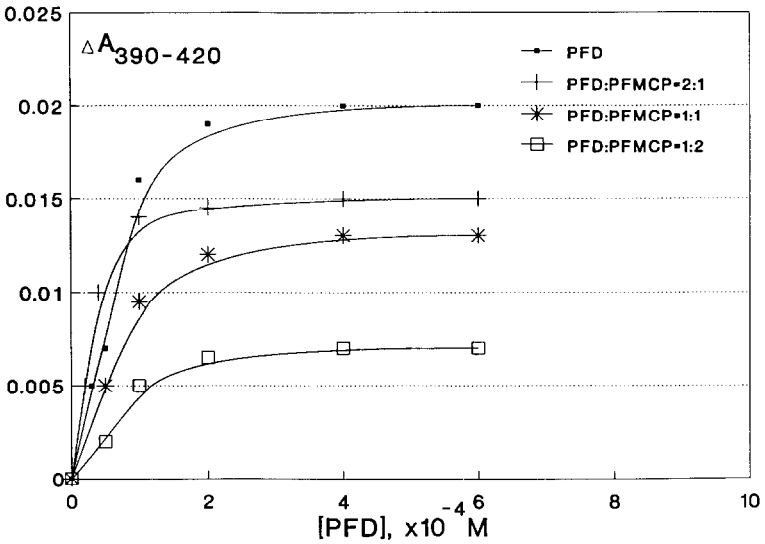

Fig. 3. Formation of the PFD–cytochrome P_{450} complex by the addition of emulsions containing a PFD/PFMCP mixture in various ratios. The microsomes of untreated rats were used; the protein and cytochrome content was as described in Fig. 1(A).

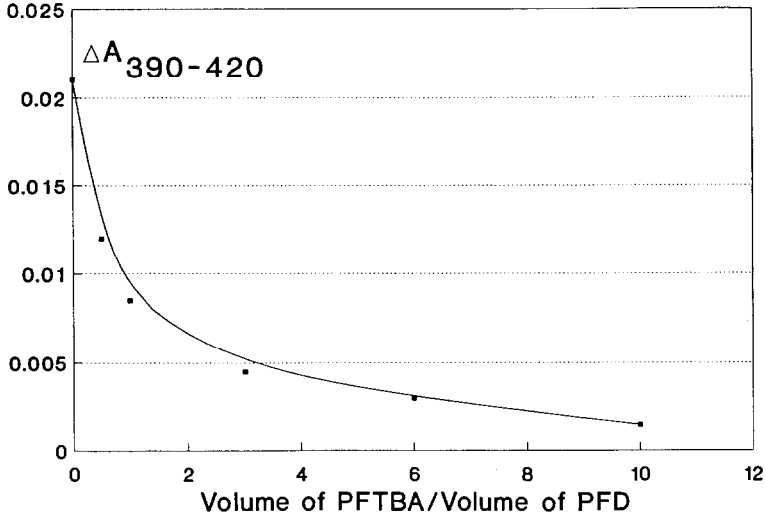

Fig. 4. Destruction of a PFD–cytochrome P_{450} complex by the addition of PFTBA emulsion. The microsomes of untreated rats were used; the protein and cytochrome content was as described in Fig. 1(A). The PFD–cytochrome complex was subject to preliminary formation by the incubation of microsomes with PFD emulsion at a concentration of 2×10^{-4} M.

(Fig. 4) can be explained by re-extraction of PFD from the hydrophobic lipid region of microsomal membranes into a membrane-insoluble PFC. Thus, more lipophobic PFCs reduce the action of lipophilic PFCs.

It has been shown earlier that the CST of PFC mixtures is additive [25]. This enables the prediction not only of the complex-forming behaviour of individual PFCs but also of their mixtures. This phenomenon is of physiological significance since most clinically tested emulsions are constructed from mixtures of two fluorocarbons with different lipophilicity [1, 2]. Furthermore, in this respect it is of interest that the ratio of PFCs in the blood changes after administration of the emulsions into animals [26].

The time-dependent formation of the enzyme–substrate complex obtained after mixing a fluorocarbon emulsion with microsomes, as indicated by the spectral change $\Delta A_{390-420}$, is depicted in Fig. 5. The initial rate of complex formation depends on the concentration of the fluorocarbon emulsion (Fig. 6), on the particle size of the emulsion (Fig. 7) and on the type of PFC employed (Table 1).

The results obtained show that most of the biological effects are caused by emulsified PFCs. Such behaviour can be observed by investigating both the equilibrium and kinetic parameters of complex formation. Thus, unlike PFC solutions in organic solvents [11–13], PFC emulsions exhibit a sharp band in their complex formation curve which attains a horizontal plateau at higher PFC concentrations. Obviously, since the curves are not linear in Scatchard coordinates, K_a exhibits a variable character. Such a trend in the complex formation curve might be connected with the limited solubility of PFCs in the lipid phase of the microsomal membranes. This suggestion is supported by the results obtained for the interaction of microsomal cytochrome P_{450} with various PFCs (Fig. 2).

The formation of PFC–cytochrome P_{450} complexes by the interaction of microsomes and fluorocarbon emulsions occurs via multistage process.

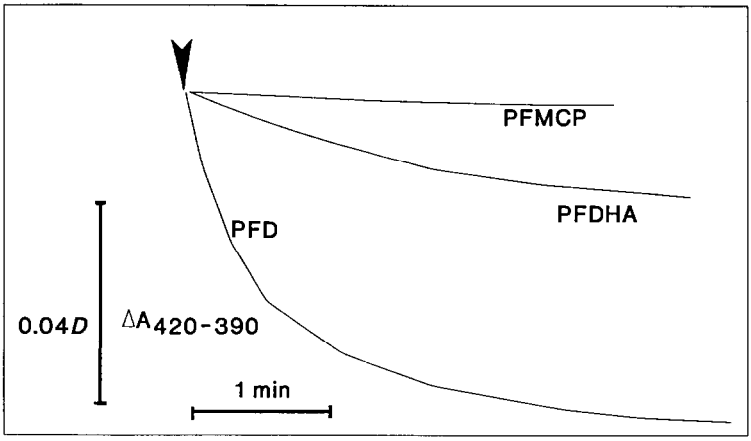

Fig. 5. Original record of changes in the optical spectrum, $\Delta A_{420-390}$, as revealed immediately after mixing a microsome suspension with a fluorocarbon emulsion. Microsomes from PB-treated rats; protein concentration = 3.7 mg ml^{-1}; cytochrome P_{450} content = 1.94 nmol mg^{-1} of protein. Fluorocarbon concentration in cuvette = 0.66×10^{-4} %vol/vol. Arrowhead shows the moment of mixing.

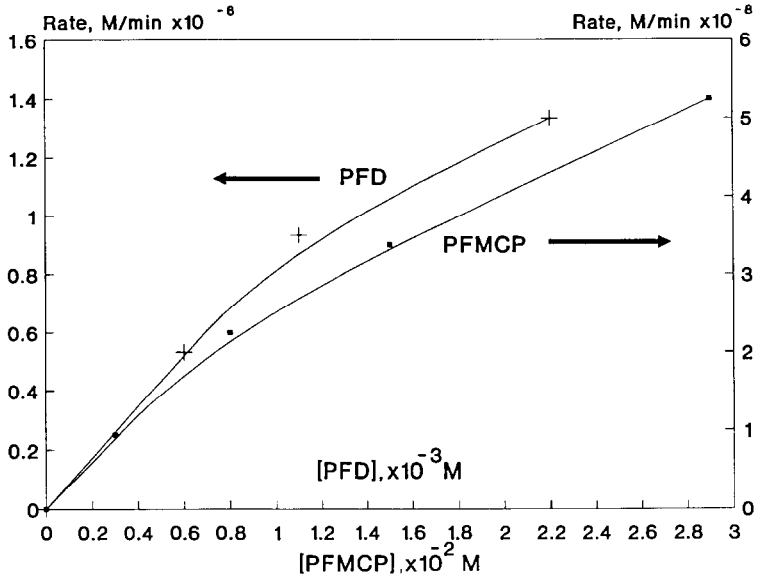

Fig. 6. Influence of the fluorocarbon concentration on the initial rate of complex formation. Conditions as described for Fig. 5.

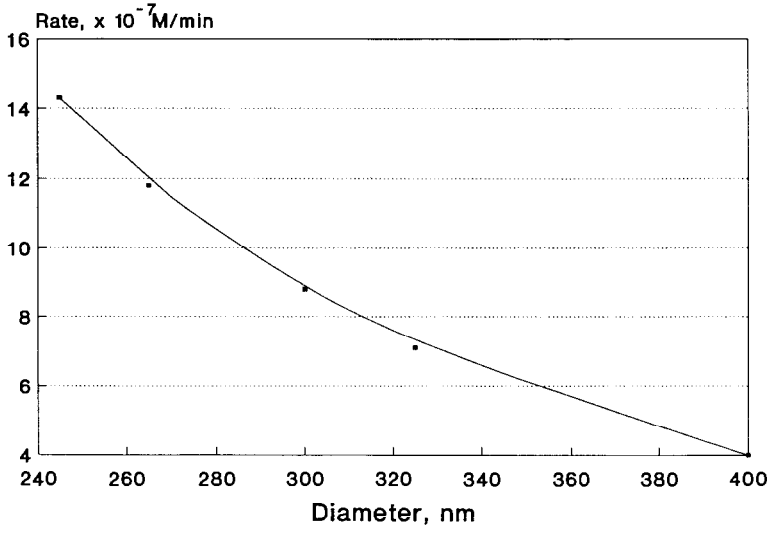

Fig. 7. Influence of the mean particle diameter of the PFD emulsion on the initial rate of complex formation. Conditions as described for Fig. 5.

Since the rate of complex formation between microsomal cytochrome P_{450} and water-soluble substrates is high [27], in the case of PFC emulsions it can be suggested that the limiting stage in the whole process is molecular diffusion of the PFC from emulsion droplets to the microsomal membranes.

The characteristic time (τ) for the mass transfer of a fluorocarbon from an emulsion droplet to a microsome can be expressed by the following equation (at a standard concentration of emulsion):

$$\tau = \frac{r^2}{D \times S} \quad (1)$$

where r is the mean radius of the emulsion particle, D the molecular diffusivity of the fluorocarbon in water and S the solubility of the fluorocarbon in water.

Quantitative evaluation of τ for PFC emulsions ranging from PFD to PFTBA ($S = 10^{-8}$ to 10^{-11} ml ml^{-1}; $D = 10^{-5}$ cm^2 s^{-1}; $r = 100$ nm) shows that the whole process lasts from a few minutes to several days. The rate of cytochrome–PFC interaction decreases with increasing emulsion particle diameter (Fig. 7) in accordance with theoretical considerations. Extrapolation of the plot of the interaction rate against the reciprocal diameter to zero rate suggests that a fluorocarbon emulsion with a droplet diameter greater than 630 nm would not interact with the microsomes. However, the biological significance of such an extrapolation is doubtful.

The results presented in Table 1 show that complex formation depends on the solubility of the PFC in water, in accordance with eqn. (1). However, both the normalization of the rate to a standard emulsion droplet diameter (250 nm) and the uncertainty associated with the determination of PFC solubility in water make this dependence qualitative rather than quantitative.

However, the results obtained by investigation of PFC–microsome interaction *in vitro* can be useful employed for an analysis of the experimental values of liver cytochrome P_{450} induction after administration of fluorocarbon emulsions to Wistar rats (Table 2). The results listed in Table 2 are statistically significant (95%).

Thus only lipophilic PFCs which are capable of effective interaction with microsomal cytochrome P_{450} give a stable induction of cytochrome P_{450} in animal liver. The rate of microsome–fluorocarbon interaction which depends mainly on the solubility of the fluorocarbon in water appears to be important as far as the inductive effect of fluorocarbons is concerned. This may explain

TABLE 2

Classification of fluorocarbons in accordance with their liver cytochrome induction ability

Inducers	
PFI, PFD, PFAN, PFTPA	Liver cytochrome P_{450} level increased 2–4-times after intravenous administration to rats
Non-inducers	
PFMCP, PFAMC, PFTBA	Liver cytochrome P_{450} level did not differ significantly from that of the control
PFDBMA, PFCHMM, PFED, PFPMC	Irreproducible induction of cytochrome P_{450}
PFH, PFO	Death of rats as a result of gas/vapour embolism

why PFTPA is a cytochrome P_{450} inducer in contrast to the more lipid-soluble but less water-soluble PFMCP. It should be noted that some fluorocarbons which possess intermediate solubility values in lipids and water are incapable of reproducible induction; at present this phenomenon cannot be explained.

Conclusions

Although this study deals with the interaction of liver microsomes and fluorocarbon emulsions, the results obtained possess a methodological significance in the analysis of the biological activity of PFCs. Thus, if the observed biological effect of PFCs is connected with their membrane action, then the effect must be critically dependent on the solubility of the fluorocarbon in lipids and in water. In some cases, the final magnitude of the biological effect may well be determined by the solubility of the fluorocarbon in lipids, but the initial rate of the effect will be dependent on the solubility of the fluorocarbon in water. Obviously, biological effects connected with the gas transport, adsorption and corpuscular properties of fluorocarbon emulsions will not be dependent on the kind of PFC in such a manner.

At present, any clinical application of fluorocarbon emulsions demands a deep and extensive investigation of the interaction of PFCs with various biological entities, in particular biological membranes.

Comparable results to those reported here have been found with the more lipophilic perfluoro–octyl bromide (PFOBr). PFOBr is obviously decomposed by microsomal cytochrome P_{450} as shown by the release of bromide ion. The pathway of the corresponding C_8F_{17} radical produced is still unknown. Perfluoro-octyl bromide does not seem to be an inducer of cytochrome P_{450} but is likely to be a substrate in monooxygenase reactions [28].

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