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On the possible *in vitro* use of perfluoro compounds as oxygen reservoir for the microsomal monooxygenase system

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The unusually large capacity of certain fluorocarbons (FC's) to dissolve gasses of biological significance has lead to much interest in these compounds in recent years. Emulsions of fluorocarbons in various media have been developed as O_2/CO_2 carriers for use as artificial blood, perfusion media and breathing mixtures [1–3]. Their ability to dissolve molecular oxygen could, therefore, make FC's a suitable oxygen carrier for *in vitro* investigations on hepatic drug oxidizing microsomes. A larger oxygen reservoir within the optical cuvette would be of special interest in kinetic experiments where often the physically dissolved oxygen in the incubation mixture becomes a limiting factor. This limitation in metabolite production could possibly be ameliorated by adding larger quantities of oxygen dissolved in FC's, provided that they have no effects of their own on the mixed function oxidase. One fluorocarbon, perfluoro-*n*-hexane, has been shown to be a dead-end inhibitor of the monooxygenase system in hepatic microsomes [4, 5]. This compound yields a type I binding spectrum, forms a stable enzyme–substrate complex, and stimulates the consumption of NADPH and oxygen whilst remaining unmetabolized [5, 6]. These findings have lead to concern that the use of other fluorocarbons in perfusion media could interfere with the normal metabolism of the perfused tissue [4, 5]. Isolated livers, perfused with a fluorocarbon containing medium, have been reported to show increased rates of gluconeogenesis and oxygen consumption, when compared to control organs perfused with an erythrocyte medium [7].

This communication reports investigations into the effects of a commercial perfusion emulsion of perfluorotributylamine (FC 43) and a detergent polymer (Pluronic 68) upon the drug metabolizing systems of rat liver microsomes.

Materials and Methods. The fluorocarbon emulsion ('Fluosol', Green Crasco Corporation, Osaka, Japan) composed of 35% w/v perfluorotributylamine (FC 43) and 4% w/v Pluronic 68 in distilled water, was a gift of Medac GmbH (Hamburg, Germany). Pluronic 68 flake was provided by BASF-Wyandotte Corp. (Michigan, U.S.A.). It is a block polymer of ethylene oxide and propylene oxide of a molecular weight of about 8000. Perfluorotributylamine (FC 43) was a kind gift of Professor Zander (Dept. Physiology, University of Mainz, Germany). Cytochrome *c*, glucose 6-phosphate dehydrogenase, glucose oxidase and pyridine nucleotides were obtained from Boehringer Mannheim GmbH (Mannheim, Germany). All other reagents and solvents were of analysis grade from E. Merck (Darmstadt, Germany). Female Sprague–Dawley rats from Zentralinstitut für Versuchstierzucht (Hannover, Germany) were used throughout the study. Animals were pre-treated with phenobarbitone (PB) or 3-methylcholanthrene (3MC), and pooled hepatic microsomes were prepared from 4 to 6 animals, as described elsewhere [8]. Microsomal protein concentrations were determined by the method of Lowry *et al.* [9] using bovine serum albumin as a standard.

The following enzyme activities were measured: NADPH-cytochrome *c*-reductase [10], *p*-nitroanisole-*O*-demethylase [11] and 7-ethoxycoumarin-*O*-deethylase by a modification [8] of the method of Ullrich and Weber [12]. Cytochrome b_5 and P-450 concentrations were measured by the method of Omura and Sato [13, 14]. Oxygen consumption was recorded using a gold microelectrode [15] and NADPH-concentrations were measured spectrophotometrically.

All assays were performed at 37° using 66 mM Tris/HCl buffer pH 7.4. The highest concentration of fluorocarbon emulsion normally used was 10% w/v which is equivalent to 52 mM perfluorotributylamine and 4 mg/ml Pluronic 68. The effect of Pluronic 68 alone was also measured but FC 43 alone was not normally included in assays since the compound is virtually insoluble in water [16].

Results. The effects of FC 43 emulsion and Pluronic 68 alone upon some parameters of the hepatic drug metabolizing system are shown in Table 1. At the concentrations used, neither FC 43 emulsion nor Pluronic 68 had any effect on either endogenous oxygen consumption or at the conversion of model substrates. Also the NADPH consumption did not seem to be affected. Both these parameters were measured in the absence of added substrates and reflect the endogenous rates. The stoichiometry between NADPH and oxygen consumption is in favor of the latter suggesting the occurrence of other oxygen reactions, e.g. a partial uncoupling from endogenous substrates. Obviously, in the absence of substrates they cannot correlate to substrate oxidation.

Pluronic 68, at a concentration of 10 mg/ml, reduced the rate of *p*NA-*O*-demethylation, but no effects on any of the parameters were observed at concentrations of 4 mg/ml or below. This shows that an inhibitory effect can be obtained only at concentrations higher than those used for oxygen supplementation.

7-Ethoxycoumarin deethylation was measured in methylcholanthrene-induced animals and also shows no influence of either Pluronic 68 or the FC 43 emulsion. No comparison is attempted with the data from the phenobarbital-induced microsomes. The point to be made is that also in MC-microsomes fluorocarbons and a polymeric detergent within certain concentration limits have no effect on monooxygenation. Also the reduction of cyto-chrome P 450 by NADPH via the NADPH–cytochrome *c*-reductase was not affected.

FC 43 emulsion and Pluronic 68 did not elicit a substrate induced binding spectrum upon addition to oxidized microsomes from control or pretreated animals. Similarly, no binding spectra were produced when up to 100 μl FC43 alone were added to 3 ml microsomal suspension (1 mg protein/ml) followed by thorough mixing. The fluorocarbon emulsion and Pluronic 68 also had no effect upon the rate of conversion of cytochrome P-450 to cytochrome P-420 (data not shown).

The inclusion of 10% v/v fluorocarbon emulsion in microsomal suspensions caused an increase in the reduced P-450-CO absorption maximum at 450 nm but there was no change in the wavelength of the spectral peak. This apparent increase in P-450 concentrations occurred regardless of the source of microsomes (Fig. 1) and was not produced by Pluronic 68 alone (Fig. 1). A similar increase in the reduced cytochrome b_5 absorption spectrum was also found when fluorocarbon emulsion was present in the assay medium (data not shown).

Discussion. The present study has shown that the fluorocarbon perfluorotributylamine (FC 43) fails to produce a substrate induced binding spectrum and does not stimulate microsomal NADPH or oxygen consumption, implying that the interference of FC 43 with the P-450 system is probably

Table 1. The influence of fluorocarbon emulsion on some microsomal parameters

Conditions of assay	NADPH consumption *‡ nmoles NADPH consumed/mg microsomal protein/min. endogenous rate (no substrate)	oxygen consumption *‡ nmoles O_2 consumed/mg microsomal protein/min endogenous rate (no substrate)	NADPH cystchrome *‡ reductase nmoles cytochrome reduced/mg microsomal protein/min	pNA-O-demethylase *‡ nmoles *p*-nitrophenol formed/mg microsomal protein/min	7-ethoxycoumarin-O-deethylase†§ nmoles 7-hydroxy-coumarin formed/mg microsomal protein/min
Control	10.57 ± 0.31 (4)	16.75 ± 1.92 (4)	253.0 ± 13.6 (8)	2.53 ± 0.06 (4)	6.58
Plus 10% v/v Fluorocarbon emulsion	9.74 ± 0.23 (4)	13.47 ± 2.83 (4)	252.4 ± 17.9 (8)	2.76 ± 0.14 (3)	7.53
Plus 4 mg/ml Pluronic 68	9.92 ± 0.18 (4)	17.45 ± 3.75 (4)	246.1 ± 17.0 (6)	2.64 ± 0.18 (3)	7.19

* Expressed as mean ± S.E.M. (n) of determinations on *n* preparations of pooled microsomes.
† Expressed as the mean of determinations on 2 preparations of pooled microsomes.
‡ Microsomes prepared from PB pretreated animals.
§ Microsomes prepared from 3MC pretreated animals.

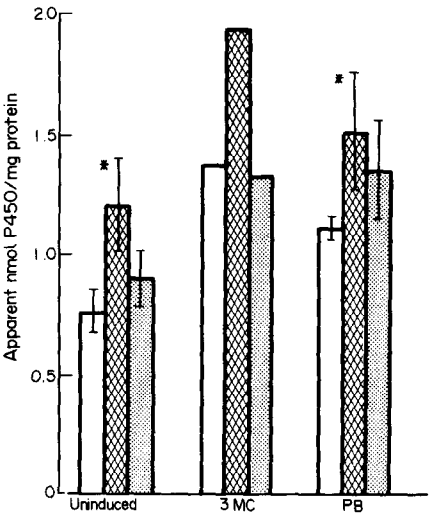

Fig. 1. The enhancement of 'apparent' cytochrome P 450 concentrations by FC 43 emulsion. The concentrations were: FC 43 emulsion 10% (v/v); Pluronic 68, 4 mg/ml. Data is presented as mean ± S.E.M. for determinations on 3–5 preparations of pooled microsomes for uninduced and PB pretreated animals and as the mean of determinations on 2 preparations for 3MC pretreated animals. *Indicates significantly different from control determinations, $P < 0.05$. Open bars: control; cross hatched: + 10% (v/v) FC 43 emulsions; stippled bars: + 4 mg/ml Pluronic 68.

minimal. In support of this view is the finding that the microsomal metabolism of *p*-nitroanisole and 7-ethoxycoumarin and the activity of NADPH cytochrome *c*-reductase were also unaffected by FC 43 emulsion. It seems, then, that the perfluorinated tertiary amine FC 43 is inert, at least with regard to the parameters examined here, and that, unlike perfluoro-*n*-hexane, FC 43 can be included in perfusion media without fear of inhibiting the monooxygenase systems. Since isolated rat livers perfused with a FC 43 containing medium can remain viable with no significant increase in oxygen consumption over that in an erythrocyte medium [17, 18], the use of an FC 43 perfusion fluid may be valid for many drug metabolism studies in perfused organs.

The increase in the reduced P-450–CO absorption spectra of microsomes in the presence of FC 43 emulsion is unlikely to reflect a true increase in the concentration of the P-450–CO complex. Assay conditions are such that the sample cuvette is saturated with CO and thus gas concentration is not a limiting factor. Any increase in CO concentration due to presence of the fluorocarbon would not be expected to increase the amount of complex formed. If the fluorocarbon were uncovering buried or latent forms of the cytochrome, then this would probably be reflected in changes in enzyme activities in the presence of fluorocarbon. Furthermore induction might be expected to markedly modify this spectral enhancement. Rather, the observed effect is probably due to the increased turbidity of the samples with 10% (v/v) FC 43 emulsion, which is a milky liquid. Solutions of Pluronic 68 alone are clear and no spectral enhancement was found when it was added to the sample. FC 43 emulsion is produced by sonication [19] and Franklin [20] has reported that other emulsions, produced by sonication of aqueous insoluble compounds, also cause enhancement of the reduced cytochrome P-450–CO absorption complex.

The spectral enhancement with increasing turbidity does not affect chromophores in solution as witnessed by the failure of the emulsion to affect the absorption characteristics of NADPH, cytochrome *c* or *p*-nitrophenol. Thus, the particulate nature of microsomes in suspension must play a role in the enhancement.

In summary, the reported results show that addition of a perfluorotributylamine containing fluorocarbon emulsion to rat microsomes does not severely affect a number of parameters that are related to microsomal drug oxidation. This is also true when the polymeric detergent Pluronic 68 alone is employed in concentrations not surpassing 4 mg/ml. In spite of their relative inertness the use of fluorocarbon emulsions as oxygen reservoirs for artificially prolonged reaction periods is very limited by the fact that within reasonable proportions they cannot carry sufficient oxygen to maintain metabolic formation clearly longer and more linear with time than under normal incubation conditions.

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Dose dependent formation of zinc-thionein in livers and kidneys of rats and mice by zinc injection

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The induction of zinc-thionein, a metallothionein, in the livers of rats exposed either parenterally or perorally to zinc is well documented, as are some distinct properties that distinguish zinc-thionein from cadmium-containing metallothioneins, such as short half-lives of the apoprotein and the metal [1–10]. Zinc-thionein is also reported to be induced in the livers of rats by physiological alterations caused by starvation [1], infection [11], and stress [12].

Although the induction of zinc-thionein in the intestine of rats by zinc loading is also reported along with liver zinc-thionein [4, 13–15], there are conflicting reports concerning the induction of zinc-thionein in the kidneys; one research group reported that zinc-thionein was induced in the kidneys by feeding with excess zinc [2, 3, 6], while another group reported that zinc-thionein was not induced in the kidneys of rats injected with zinc [4, 16, 17], although both groups reported induction in the livers.

The reports concerning the induction of zinc-thionein have been restricted only to rat, and they contrast with the reports for the induction of cadmium-containing metallothioneins in other kinds of animal.

In this study the following four questions were examined; (i) whether or not zinc-thionein is induced in the kidneys by intraperitoneal injection of zinc, (ii) whether or not there are any dose–response relationships for the amount of induced zinc-thionein, (iii) whether or not there are any effects of zinc loading on the copper content and distribution, especially in the kidneys, as observed by cadmium loading [18, 19], and (iv) whether or not zinc-thionein is induced by zinc injection in liver and kidney of the mouse.

Experimental. Female rats of the Wistar strain (9-weeks-old, mean body weight ± S.D.; 196 ± 8.4 g) and female mice of the ICR strain (8-weeks-old, mean body weight ± S.D.; 27.3 ± 1.5 g)(Clea Japan, Tokyo) were fed standard laboratory chow (Clea, Japan) and distilled water *ad lib.* Zinc acetate (purest grade, Wako Pure & Chemical Industries,