the whole oxidative metabolism might also suggest a direct interference with the signalling mechanism, either by directly interfering with the link of PMA with its receptor or by counteracting the depolarising effect of PMA on the plasma membrane [23]. In fact an FDP-induced membrane stabilizing effect could have resulted from the changes in ion permeability, with K^+ uptake, brought about by FDP binding to the cell surface [2].

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REFERENCES

1. M. E. Kirtley and M. McKay, *Mol. Cell Biochem.* **18**, 141 (1977).
2. M. P. Rigobello, M. Bianchi, R. Deana and L. Galzigna, *Aggressologie* **23**, 63 (1982).
3. M. L. Schinetti, R. Greco, G. Lazzarino and A. Bertelli, *Int. J. Tiss. Reac.* **V**, 55 (1983).
4. M. L. Schinetti, R. Greco, G. Lazzarino, G. Soldani and A. Bertelli, *Arzneim. Forsch./Drug Res.* **33**, 834 (1983).
5. R. Tarsi, L. Mulieri, F. Venturi and M. Simonetti, *J. Leukocyte Biol.* **37**, 72 (1985).
6. S. J. Weiss, in *Immunology of Inflammation*, (Ed. Peter Wards) p. 37, Elsevier, Amsterdam (1983).
7. M. A. Trush, M. E. Wilson and K. Van-Dyke, *Meth. Enzymol.* **LVII**, 462 (1978).
8. H. J. Cohen and M. E. Chovaniec, *J. clin. Invest.* **61**, 1088 (1978).
9. J. A. Badwey and M. L. Karnovsky, *J. biol. Chem.* **254**, 11530 (1979).
10. A. Ferrante and Y. N. Thong, *J. Immunol. Meth.* **24**, 389 (1978).
11. I. Fridovich, *J. biol. Chem.* **245**, 4053 (1970).
12. K. Cheung, A. C. Archibald and M. F. Robinson, *J. Immunol.* **130**, 2324 (1983).
13. L. C. McPhail, L. R. DeChatelet and R. B. Johnston, Jr., *J. clin. Invest.* **63**, 648 (1979).
14. T. W. Kensler and M. A. Trush, *Biochem. Pharmac.* **32**, 3485 (1983).
15. C. Clouverakis, *Metabolism* **17**, 703 (1968).
16. L. Galzigna, M. P. Rigobello, G. Scutari and L. Cattani, *Experientia*, in press.
17. H. Lagast, P. D. Lew and F. A. Waldvogel, *J. clin. Invest.* **73**, 107 (1983).
18. F. Di Virgilio, D. P. Lew and T. Pozzan, *Nature, Lond.* **310**, 691 (1984).
19. H. Lagast, T. Pozzan, F. A. Waldvogel and P. D. Lew, *J. clin. Invest.* **73**, 878 (1984).
20. N. Okamura, M. Uchida, T. Ohtsuka, M. Kawanishi and S. Ishibashi, *Biochem. biophys. Res. Comm.* **130**, 939 (1985).
21. G. Lazzarino, L. Cattani, R. Costrini, L. Mulieri, A. Candiani and L. Galzigna, *Clin. Biochem.* **17**, 42 (1984).
22. B. M. Babior and W. A. Peters, *J. biol. Chem.* **256**, 2321 (1981).
23. J. C. Whitin, C. E. Chapman, E. R. Simons, M. E. Chovaniec and H. J. Cohen, *J. biol. Chem.* **255**, 1874 (1980).

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The incorporation of palmitic acid into lipids in the rat after treatment with oleylanilide

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The potential toxicity of fatty acyl anilides in man was first raised in 1981 in connection with the Toxic Oil Syndrome [1]. Acyl anilides were found to be major contaminants of cooking oils, consumption of which was associated with mass poisoning in Spain. Suitable animal models for study of the syndrome have not, however, been generally established and there are conflicting reports on the toxicity of anilides [2]. Our studies in the rat have shown extensively hydrolysis of anilides after intragastric administration prior to or during absorption and we have been unable to detect anilides in body tissues [3]. Because of the toxicological importance of the general problem, we were therefore concerned by the detailed study of Casals *et al.* [4], showing significant disturbances in lipid metabolism in lung and adipose tissue after oral administration of oleylanilide to rats. We have repeated their dosing schedule and studied the incorporation of label into lipid fractions of lung, liver and epididymal fat following intravenous administration of labelled palmitic acid. This communication reports briefly on our failure to observe any significant disturbance in fatty acid incorporation into lipids following repeated oral dosing of oleylanilide.

Materials and methods

Chemicals. (1-14C) palmitic acid was obtained from the Radiochemical Centre (Amersham, U.K.). Oleic acid and aniline were obtained from the Sigma Chemical Co. (Poole, Dorset, U.K.). Oleylanilide was prepared by heating oleic acid (1 g) and aniline (2 g) at 150° for 24 hr, followed by acid and bicarbonate washes and recrystallization from methanol. Purity (98%) was checked by capillary gas chromatography with a flame ionization detector and the product identified by mass spectrometry [3].

Rats and dosage. Male Porton-derived Wistar rats, weighing 149–175 g were used. Four rats received daily intragastric doses of 5 mg oleylanilide in olive oil (0.5 ml) for 13 days. Dosing was then stopped and the experiments carried out on day 18 [4]. Four control rats received olive oil alone. All rats had free access to food and water.

Experiment. Each rat received an injection via a tail vein of about 1.6 μCi (1-14C)-palmitic acid in rat serum (0.2 ml), prepared as described by Cunningham [5]. After 10 min the animals were decapitated and samples of lung, liver and of perirenal and epididymal fat were frozen in liquid nitrogen for subsequent lipid extraction.

Extraction of lipids. Samples of tissue were homogenized in chloroform/methanol (2:1 v:v) using an Ultra-Turrex homogenizer, filtered and the filtrate washed with chloroform/methanol. The total extract was washed by layering on an equal volume of water and left overnight to give final extraction volumes of about 15 ml/g lung and liver and 30 ml/g fat tissue. Samples (5–10 ml) of the

extracts were applied to small silicic acid columns (240 mg silicic acid, 120 mg celite). The columns were washed with 20 ml chloroform to give a neutral lipid extract and with 15 ml methanol to give a phospholipid extract. Samples of the extract and of the column eluates were blown down under nitrogen for scintillation counting. Neutral lipids were separated further by thin layer chromatography on Silica Gel G with hexane : diethyl ether : glacial acetic acid (75 : 25 : 1, v : v : v). Spots were visualized in iodine vapour, allowed to fade, and the bands scraped off into scintillation fluid for counting.

Results

There were no significant differences in body weight gain between treated and control groups over the dosing period or over the subsequent period prior to the experiment (Table 1). There was no difference in the weight of epididymal fat bodies between the two groups. Perirenal fat was marginally greater in the treated group (Table 1), but it is unlikely that this is of any toxicological significance.

The recovery of radioactivity in extracts of lung, liver and fat tissue, after intravenous injection of labelled palmitic acid, is shown in Table 2 together with the percentage of this radioactivity appearing in the neutral and phospholipid subfractions. Again no differences were apparent between the two groups. Finally, separation of neutral lipid extracts of lung and liver by thin layer chromatography gave similar distributions of radioactivity in treated and control groups (results not shown).

Discussion

Casals *et al.* [4] have reported significant disturbances in lung and adipose tissue lipid metabolism following treatment of rats with oleylanilide on the dosing schedule used in the present work. Studies on microsomal preparations from these tissues showed decreased fatty acid incorporation into triacylglycerides and phospholipids. The synthesis *de novo* of fatty acids in a preparation of adipose tissue was diminished as was acyltransferase activity in lung and adipose tissue. On the basis of these results, a marked reduction in the incorporation of plasma free fatty acids into adipose and lung lipids would be expected to follow dosing of rats with oleylanilide. We have obtained no indication that this occurs. Furthermore, Casals *et al.* [4] report a high loss of adipose-tissue stores which we have again failed to find.

The reason for this disparity is not clear as we have used the same strain of rats and dosing schedule. A major difference however between the two studies relates to the synthesis of the anilide. In the present work only oleic acid and aniline were present in the initial reaction, and the purity of the final product was checked by GCMS. Casals *et al.* [4] included dimethylaminopropylethyl carbodi-imide in the initial incubation. We would suggest that the marked effects on lipid metabolism which they report may have been caused by a toxic impurity in the oleylanilide preparation.

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Table 1. Body and selected fat depot weights after treatment of rats with oleylanilide

	Control	Treated
Initial body weight ranges	149 g–164 g	159 g–175 g
Body weight at day 13 as percentage of initial weight	150 ± 2	148 ± 4
Body weight at day 18 as percentage of initial weight	169 ± 2	166 ± 2
Epididymal fat (g/100 g BW)	0.49 ± 0.04	0.60 ± 0.05 N.S.
Perirenal fat (g/100 g BW)	0.42 ± 0.05	0.57 ± 0.03 $P < 0.05$

Treated rats received oleylanilide intragastrically at a dose of 5 mg/day for 13 days. Tissue weights refer to a single epididymal fat body or to the perirenal fat from around one kidney. Results are given as the mean ± S.E.M. of four rats in each group.

Table 2. Recovery of label in lipid fractions of tissues following intravenous injection of labelled palmitic acid

		Total lipid extract (10^{-3} × dpm/g tissue)	Percentage of total extract in neutral fraction	Percentage of total extract in phospholipid fraction
Lung	Control	22.6 ± 1.5	46.3 ± 2.0	48.7 ± 1.2
	Treated	18.0 ± 0.9	47.5 ± 1.1	48.2 ± 1.7
Epididymal fat	Control	1.87 ± 0.17	81.2 ± 2.8	21.3 ± 4.6
	Treated	2.16 ± 0.32	75.2 ± 2.9	24.7 ± 3.7
Liver	Control	37.4 ± 2.05	56.4 ± 5.1	30.4 ± 1.6
	Treated	34.8 ± 2.07	61.5 ± 4.5	30.3 ± 0.6

Experiments were carried out 18 days after the start of dosing with oleylanilide. Rats received an intravenous injection of [1-14C] palmitic acid in rat serum and were decapitated after 10 min. Tissues were extracted as described under Materials and Methods. Results are normalised to a dose of 1.5×10^6 dpm per 100 g body weight, and are given as the mean ± S.E.M. from 4 rats in each group.

REFERENCES

1. P. Grandjean and S. Tarkowski (Eds.), *Toxic Oil Syndrome, Mass Food Poisoning in Spain*, by World Health Organization (1984).
2. W. N. Aldridge, *Human Toxicol.* **4**, 231 (1985).
3. V. J. Cunningham, S. P. Tucker and E. Bailey, *Archs Toxicol.* **54**, 157 (1983).
4. C. Casals, P. Garcia-Barreno and A. M. Municio, *Biochem. J.* **212**, 339 (1983).
5. V. J. Cunningham, *Biochem. J.* **136**, 545 (1973).

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Sexually dimorphic response of rat hepatic monooxygenases to low-dose phenobarbital

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It has been clearly established that the activities of most hepatic monooxygenases are greater in male rats than in female rats. Many studies have shown that this sexual dimorphism is due to the inductive effects of testicular androgens [1–3]. In contrast, there are far fewer studies comparing the inductive effects in male and female rats of the much more commonly used inducers, i.e. phenobarbital, methylcholanthrene, benzopyrene, etc. In this regard, while female rats appear to be more responsive to the inductive effect of phenobarbital, in that the same dose of the barbiturate will induce a greater percent increase in the activities of the hepatic monooxygenases in females when compared to males, a sexual difference remains, as induced levels of enzymes are still greater in the males [4, 5]. Unfortunately, studies comparing the responsiveness of hepatic drug-metabolizing enzymes in male and female rats have been confined to the use of only a single, maximally effective dose of the inducing agent. Thus, any alteration in the responsiveness of hepatic monooxygenases to submaximal, or even "subtle" levels of inducers, which are more likely encountered in the environment, is unknown. Although there have been dose–response studies using various inducing agents, they usually have been conducted on a single sex, i.e. male rats [6, 7].

In the present study, we have compared the inductive effects of low doses of phenobarbital on the kinetic parameters of cytochrome P-450-dependent hepatic microsomal hexobarbital hydroxylase and aminopyrine *N*-demethylase in male and female rats. Phenobarbital was chosen as the inducing agent because it is probably used more frequently than any other compound to investigate the mechanism(s) of monooxygenase induction.

Materials and methods

Sprague–Dawley [Crl:CD(SD)BR] rats born and raised in our own animal facilities were housed on hardwood bedding in plastic cages. Animals were given water and commercial rat diet *ad lib.* and were kept in air-conditioned quarters, 20–23°, with a photoperiod of 12 hr light/12 hr dark. At approximately 4 months of age, male and female rats were injected, daily, by the intraperitoneal route, with 1, 3 or 20 mg/kg of sodium phenobarbital (J. T. Baker Chemical Co., Phillipsburg, NJ) or an equivalent amount (2 ml/kg) of the 0.9% NaCl diluent, pH 9.0, for 6 consecutive days and killed on the following day.

Hepatic microsomes were prepared from the 100,000 *g* pellets as previously described [8]. Hepatic microsomal hexobarbital hydroxylase was assayed by our modification [8] of the radioenzyme procedure of Kupfer and Rosenfeld [9]. Basically, the assay measures the rate of microsomal conversion of radioactive hexobarbital, 5-[2-^{14}C]-cyclohexenyl-3,5-dimethylbarbituric acid (14.4 mCi/mmole; NEN Research Products, Boston, MA), to 3-hydroxyhexobarbital. Linear kinetic data for the enzyme were obtained from eight different hexobarbital concentrations (0.033 to 0.33 mM). Hepatic microsomal aminopyrine *N*-demethylase was determined by our modification [10] of the radioassay of Poland and Nebert [11] which measures the production of [^{14}C]formaldehyde from the radioactive aminopyrine substrate [N-*methyl*-^{14}C]antipyrine (112.2 mCi/mmole; NEN Research Products). Kinetic data for aminopyrine *N*-demethylase were obtained with fourteen different aminopyrine concentrations (0.05 to 2 mM). Microsomal protein content was determined by a modification [12] of the method of Bradford [13].

Michaelis constants (K_m) and maximal velocities (V_{max}) were determined from linear regression models of the data using the method of Hofstee [14]. The correlation coefficients for all Hofstee plots were positive, exceeded 0.95, and were found to be statistically significant ($P < 0.01$).

Experimental groups were compared for statistically significant differences by analysis of variance and Student's *t*-test.

Results and discussion

In agreement with previous studies [2–4], we have found a highly significant sexual difference in the kinetics of hepatic microsomal hexobarbital hydroxylase and aminopyrine *N*-demethylase in rats. Basically, the Michaelis constants were lower and the maximal velocities were higher for both hepatic monooxygenases in the males (Tables 1 and 2). Also, in agreement with more recent reports, particularly those using the sensitive radioenzyme assays [10, 11, 15], we have found that aminopyrine *N*-demethylase exhibited biexponential (non-linear) kinetics.

In this study, we proposed to determine if the sex of the animal was a determining factor affecting the ability of phenobarbital to induce hepatic monooxygenases at submaximally effective doses. Following a treatment schedule similar to ours, it has been reported that the maximally effective dose of phenobarbital to induce rat hepatic microsomal monooxygenases is at least 75 mg/kg [7, 16]. In fact, this is probably the most commonly used dose in induction studies. Thus, when we administered phenobarbital at 1, 3 and 20 mg/kg, we were actually administering about 1, 4 and 27% respectively, of the maximally effective dose of the barbiturate.

Our results indicate that the livers of the adult male rats were considerably more sensitive to the inductive effects of low doses of phenobarbital than were the livers of the female rats. As low a dose as 1 mg/kg of the barbiturate produced a significant elevation in the maximal velocities of hepatic hexobarbital hydroxylase, aminopyrine *N*-demethylase, the K_m of hexobarbital hydroxylase, and a decline in the K_{m_1} of aminopyrine *N*-demethylase for the male rats (Tables 1 and 2). In general, the maximal velocities of the enzymes in the males exhibited a dose–response relationship. The 3 mg/kg dose of phenobarbital