Biochem. J. (1969) **115**, 547
Printed in Great Britain

The Effects of Unsaturated Fatty Acids on Hepatic Microsomal Drug Metabolism and Cytochrome *P*-450

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(*Received* 10 *June* 1969)

1. The effects of unsaturated fatty acids on drug-metabolizing enzymes *in vitro* were measured by using rat and rabbit hepatic 9000*g* supernatant fractions. 2. Unsaturated fatty acids inhibited the hepatic microsomal metabolism of 'type I' drugs with inhibition increasing with unsaturation: arachidonic acid > linolenic acid > linoleic acid > oleic acid. Inhibition was independent of lipid peroxidation. Linoleic acid competitively inhibited the microsomal *O*-demethylation of *p*-nitroanisole and the *N*-demethylation of (+)-benzphetamine. 3. The hepatic microsomal metabolism of 'type II' substrates, aniline and (−)-amphetamine, was not affected by unsaturated fatty acids. 4. The rate of reduction of *p*-nitrobenzoic acid and Neoprontosil was accelerated by unsaturated fatty acids. 5. Linoleic acid up to 3·5mM did not decelerate the generation of NADPH by rat liver soluble fraction, nor the activity of NADPH–cytochrome *c* reductase of rat liver microsomes. Hepatic microsomal NADPH oxidase activity was slightly enhanced by added linoleic acid. 6. No measurable disappearance of exogenously added linoleic acid occurred when this fatty acid was incubated with rat liver microsomes and an NADPH source. 7. The unsaturated fatty acids used in this study produced type I spectra when added to rat liver microsomes, and affected several microsomal enzyme activities in a manner characteristic of type I ligands.

The hydroxylation of drugs, carcinogenic dyes and a number of lipids can be catalysed by an enzyme system requiring NADPH and molecular oxygen (Gillette, 1966). This mixed-function oxidase system probably employs a flavoenzyme (NADPH–cytochrome *c* reductase) and a haemoprotein, cytochrome *P*-450. Kuntzman, Lawrence & Conney (1965) found that the apparent K_m value for hepatic microsomal hydroxylation of testosterone was less than that found for various drugs, and suggested that some lipid substances might be the preferential or physiological substrates in this hydroxylation scheme. Tephly & Mannering (1968), using hepatic microsomes from rats, found that a number of steroid hormones inhibited competitively the oxidation of ethylmorphine and hexobarbitone. Das, Orrenius & Ernster (1968) suggested that the oxidation of drugs and fatty acids might also be catalysed by a common NADPH-dependent mixed-function oxidase system involving cytochrome *P*-450. Lu & Coon (1968) have shown that a solubilized enzyme system from rabbit liver microsomes could function as a fatty acid ω-hydroxylase in the presence of molecular oxygen and NADPH. Cytochrome *P*-450, NADPH–cytochrome *c* reductase and a heat-stable factor were identified as being required components for the conversion of laurate into ω-hydroxylaurate.

Unsaturated fatty acids might also undergo ω-hydroxylation by microsomal NADPH-dependent enzymes. Preiss & Bloch (1964) showed that the 10000*g* supernatant fraction of rat liver contained NADPH-dependent enzymes that can catalyse the ω-hydroxylation of oleic acid as well as saturated straight-chain fatty acids. More recently, May & McCay (1968*a*, *b*) have found that the oxidation *in vitro* of polyunsaturated fatty acids, especially arachidonic acid, in phospholipids of microsomal membranes is dependent on the presence of NADPH. During this reaction NADPH was oxidized and a considerable amount of oxygen consumed. Although no lipid metabolites were identified, the altered phospholipids were more polar than normal microsomal phospholipids, but still susceptible to phospholipase A.

In this paper we report the effects of several unsaturated fatty acids on the hepatic microsomal metabolism of a number of selected drug substrates

and on components of the electron-transport system in microsomes.

MATERIALS AND METHODS

Male Long–Evans rats weighing 200–300g. and male Dutch rabbits weighing about 2·0kg. were used. The animals were killed by cervical dislocation, and the livers removed and immersed in 0·15M-KCl solution, pH7·0. All subsequent operations were carried out at 0–4°. Livers were cut into small pieces, washed and homogenized in 2 vol. of the KCl solution in a Potter–Elvehjem-type homogenizer equipped with a Teflon pestle.

The homogenates were centrifuged for 20min. at 9000*g* in a Sorvall RC2-B centrifuge. The 9000*g* supernatant was carefully withdrawn by a syringe. The waxy layer at the surface was avoided during removal of the supernatant since this layer was later shown to inhibit the metabolism of some drug substrates. For the isolation of microsomes the 9000*g* supernatant fraction was centrifuged at 60000*g* for 60min. in a Beckman Spinco model L preparative ultracentrifuge. The microsomes were resuspended in the iso-osmotic KCl solution and resedimented. Microsomal protein content was measured by the method of Lowry, Rosebrough, Farr & Randall (1951), with crystalline bovine serum albumin as the standard.

The basal incubation mixtures for drug metabolism experiments contained the 9000*g* supernatant equivalent to 0·33g. wet wt. of liver, 0·45mM-$NADP^+$, 4mM-glucose 6-phosphate, 5mM-$MgSO_4$, 4mM-nicotinamide and 50mM-potassium phosphate buffer, pH7·35, in a total volume of 5·0ml. A marble was added to each beaker to ensure vigorous agitation (Fouts & Waters, 1969). All incubations were done in duplicate in a Dubnoff shaker (100 oscillations/min.) at 37° and the gas phase was oxygen for oxidative metabolisms or nitrogen for reductases. The drug pathways studied, methods of assay used and the usual substrate concentrations were: *O*-demethylation of *p*-nitroanisole (Netter & Seidel, 1964), 2mM; side-chain oxidation of sodium hexobarbitone (Roberts & Plaa, 1966), 2·4mM; *N*-demethylation of aminopyrine (Cochin & Axelrod, 1959), 4·0mM; *N*-demethylation of (+)-benzphetamine hydrochloride or (+)-*N*-benzyl-*N*α-dimethylphenethylamine hydrochloride (Cochin & Axelrod, 1959), 2·5mM; *N*-demethylation of *p*-chloro-*N*-methylaniline (Kupfer & Bruggeman, 1966), 1·5mM; *p*-hydroxylation of aniline hydrochloride (Gram, Rogers & Fouts, 1967), 4·0mM; deamination of (−)-amphetamine sulphate (Axelrod, 1954), 0·13mM; reductive cleavage of the azo group of Neoprontosil or disodium 7-acetamido-1-hydroxy-2-(4-sulphamoylphenylazo)-naphthalene-3,6-disulphonate (Fouts & Brodie, 1957), 1·5mM; reduction of the nitro group of *p*-nitrobenzoic acid (Fouts, Kamm & Brodie, 1957), 2·4M. Lipids, at the concentrations used in these studies, did not interfere with any of the assay procedures employed for measuring drug metabolism.

Lipid oils were added to the incubation media by a calibrated micropipette. Buffer pH value was unchanged in the media after admixture of all lipid materials studied.

All lipids, except where otherwise stated, were purchased from the Hormel Institute, Austin, Minn., U.S.A., and stored under nitrogen at −20°. Lipid peroxidation *in vitro* was studied by using the basal incubation system described above and measuring the production of the 2-thiobarbituric acid chromogen, by the method used by Hunter, Gebicki, Hoffsten, Weinstein & Scott (1963). (+)-α-Tocopherol acetate was purchased from Sigma Chemical Co., St Louis, Mo., U.S.A.

NADPH generation, NADPH oxidase and NADPH–cytochrome *c* reductase assays were performed with a Shimadzu MPS-50L recording spectrophotometer. The asays were conducted at room temperature in 10mm.× 10mm. stoppered Pyrex cuvettes. The final volume in sample and reference cuvettes was 3ml. The initial linear phase was used to estimate the rates of each reaction. Linoleic acid was added as the oil to both cuvettes and shaken thoroughly with the cuvette contents. Bovine serum albumin was added to the phosphate buffer to effect homogeneous suspension of linoleic acid and to deter microsomal agglutination. However, it was not necessary to add extra protein, as albumin, in studies with rat 9000*g* soluble fraction (NADPH generation). Addition of linoleic acid as a solution in acetone (<0·2ml.) did not appear to improve dispersion of the acid in the reaction mixture compared with adding the linoleic acid as the oil. The soluble fraction obtained by ultracentrifugation of the 9000*g* supernatant fraction was used to study NADPH generation. Sample and reference cuvettes contained, in a 3ml. final volume, 50mM-potassium phosphate buffer, 4·1mM-$MgSO_4$, 3·3mM-glucose 6-phosphate and 0·5ml. of the soluble fraction. The extinction at 340nm. was then set to zero on the chart recorder. After the addition of 0·2ml. of water to the reference cuvette, 0·2ml. of 2·4mM-$NADP^+$ was added to the sample cuvette. A molar extinction of 6200 was used to calculate the amount of NADPH generated in 1min. NADPH oxidase activity was determined essentially by the method described by Gillette, Brodie & LaDu (1957). The reaction was carried out in 50mM-potassium phosphate buffer containing bovine serum albumin (0·5%). The microsomal protein concentration was 2mg./ml. After the addition of 0·21mM-NADPH, the decrease in extinction at 340nm. was measured. NADPH–cytochrome *c* reductase was determined by a modification of the method of Phillips & Langdon (1962). The assay system contained 150μg. of microsomal protein/ml., 0·1mM-NADPH, 0·3mM-KCN, 0·05mM-cytochrome *c* (type VI; Sigma Chemical Co.) and the phosphate–albumin buffer described above. The reduction of cytochrome *c* was followed at 550nm. The molar extinction of cytochrome *c* used in calculations was 18500.

For the measurement of residual linoleic acid remaining after incubation of microsomal enzymic systems, the reaction components were extracted with 20vol. of chloroform–methanol (2:1, v/v) by the procedure of Folch, Lees & Sloane-Stanley (1957). The chloroform layer was collected, filtered to remove tissue residue and evaporated *in vacuo* with a rotary evaporator. The extracts were taken up in a small volume of chloroform and kept in sealed tubes under nitrogen at −20° until analyses could be made. The amount of linoleic acid in each extract sample was determined by g.l.c. analysis of the derived methyl ester. Esterification was accomplished by evaporating the chloroform and heating the remaining lipid material in a Pyrex screw-cap culture tube (Teflon-lined cap) with 1ml. of 14% (w/v) boron trifluoride in methanol in a boiling-water bath for 15min. (Morrison & Smith, 1964). The fatty acid esters formed were extracted with 2ml. of *n*-hexane and analysed on a Packard 803 gas chromatograph with a flame-ionization detector.

The recoveries of exogenously added linoleic acid by g.l.c. determination were within 95–98%. The glass column (6ft. × ¼in. outer diam.) contained 10% ethylene glycol succinate on Chromosorb W (AW) (80–100 mesh). The operating temperature was 180° with nitrogen as a carrier at a flow rate of 60–70ml./min. Methyl linoleate was identified by co-chromatography with standard fatty acid methyl esters purchased from Mann Research Laboratories, New York, N.Y., U.S.A.

RESULTS

Oleic acid, linoleic acid, linolenic acid and arachidonic acid produced inhibition of the *O*-demethylation of *p*-nitroanisole by rat liver 9000*g* supernatant fractions. As is shown by the time-course experiments in Fig. 1, *p*-nitroanisole metabolism was most strongly inhibited by arachidonic acid, the most unsaturated of the fatty acids studied.

The relative inhibitory strength of unsaturated acids at 3·5mM was in decreasing order: arachidonic ($C_{20:4}$) acid > linolenic ($C_{18:3}$) acid > linoleic ($C_{18:2}$) acid > oleic ($C_{18:1}$) acid, indicating a possible correlation between inhibition and the extent of unsaturation of the fatty acid. A concentration-dependent inhibition of substrate metabolism was observed when linoleic acid was varied from 2·0 to 20mM.

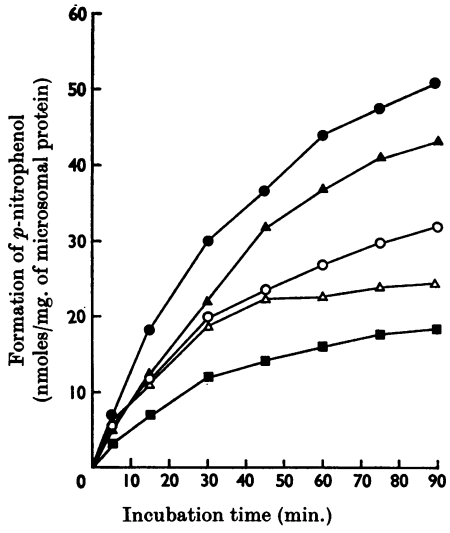

Fig. 1. Effects of unsaturated fatty acids on the time-course of *p*-nitroanisole *O*-demethylase activity of 9000*g* supernatant fractions prepared from adult male rat livers. The assay conditions are described in the Materials and Methods section. The final concentration of each acid was 3·5mM. ●, Control; ▲, oleic ($C_{18:1}$) acid; ○, linoleic ($C_{18:2}$) acid; △, linolenic ($C_{18:3}$) acid; ■, arachidonic ($C_{20:4}$) acid.

Similar results were obtained with hexobarbitone as a substrate of the hepatic microsomal enzymes from rat liver, with the same relative order of inhibition occurring among the unsaturated fatty acids. The metabolism of *p*-nitroanisole and hexobarbitone by rabbit liver 9000*g* supernatant fractions was also suppressed by unsaturated fatty acids. This showed that inhibition was not a species-specific (rat only) phenomenon.

A linear relationship was obtained in a double-reciprocal Lineweaver–Burk plot for *p*-nitroanisole *O*-demethylation by rat liver 9000*g* supernatant fractions (Fig. 2*a*). In the presence of 3·5mM-linoleic acid the same reciprocal plot was curvilinear at substrate concentrations less than 2mM. The plot defined by points at higher substrate concentrations was linear and consistent with a mechanism of competitive inhibition of demethylation by linoleic acid. The anomalous contour of the *p*-nitroanisole *O*-demethylase reciprocal plot in the presence of linoleic acid was observed repeatedly (five experiments). Trilinolein, the triglyceride of linoleic acid, at 3·5mM produced an uncompetitive type of inhibition in the same reciprocal plot of *O*-demethylase activity (Fig. 2*a*). The Lineweaver–Burk plots for linoleic acid inhibition of (+)-benzphetamine hydrochloride demethylation were consistent with a competitive-type inhibition, with neither described lines departing from linearity (Fig. 2*b*). There was no measurable inhibition of (+)-benzphetamine metabolism by trilinolein.

Inhibition by unsaturated fatty acids (final concentration 3·5mM) was also apparent for the *N*-demethylation of aminopyrine and *p*-chloro-*N*-methylaniline, but not for all oxidative pathways of drug metabolism by rat liver 9000*g* supernatant fraction. As shown in Fig. 3(*a*), arachidonic acid failed to inhibit rat liver aniline hydroxylase. This same observation was made for the oxidative deamination of (−)-amphetamine by rabbit liver 9000*g* supernatant fraction (Fig. 3*b*). None of the unsaturated fatty acids used in this investigation, up to a final concentration of 10mM, was able to produce inhibition of these two pathways.

A third type of response of drug-metabolizing enzyme activity to unsaturated fatty acids was shown in reductive reactions. The rate of anaerobic reductive cleavage of the azo linkage of Neoprontosil and the rate of reduction of the nitro group of *p*-nitrobenzoic acid by rat liver 9000*g* supernatant fractions was appreciably enhanced, as depicted in Fig. 4. The lower rate of *p*-nitrobenzoic acid reduction observed at early time-intervals has been attributed by Gram & Fouts (1966) to residual air dissolved in the reaction mixture. Table 1 summarizes the three effects exerted by unsaturated fatty acids on the hepatic microsomal drug-metabolizing pathways investigated. The omission

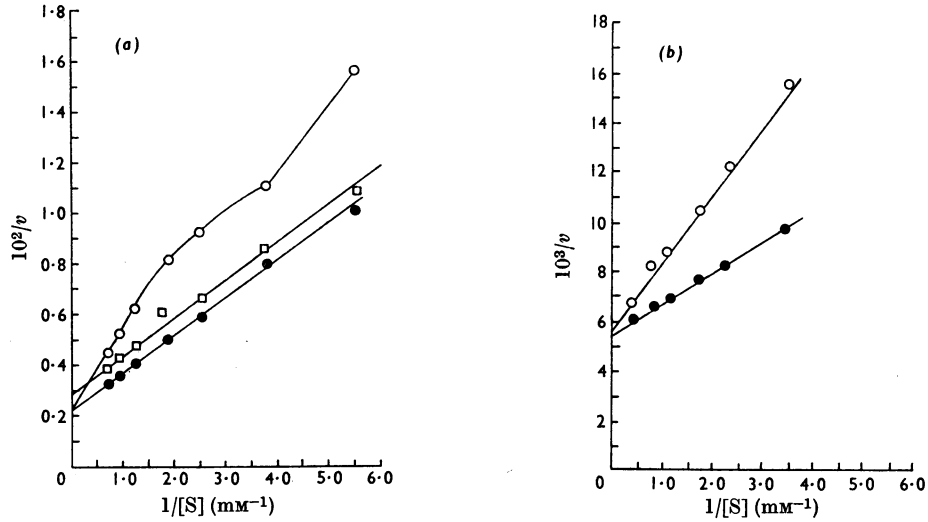

Fig. 2. Double-reciprocal plots for *p*-nitroanisole *O*-demethylase and (+)-benzphetamine *N*-demethylase by rat liver 9000*g* supernatant fractions. (*a*) Enzyme activity (*v*) for *p*-nitroanisole *O*-demethylase is expressed as μmoles of *p*-nitrophenol produced/30min./mg. of microsomal protein. S was *p*-nitroanisole. ●, *p*-Nitroanisole; ○, *p*-nitroanisole+linoleic acid (3·5mM); □, *p*-nitroanisole+trilinolein (3·5mM). (*b*) Enzyme activity (*v*) for (+)-benzphetamine *N*-demethylase is expressed as μmoles of formaldehyde produced/15min./mg. of microsomal protein. S was (+)-benzphetamine hydrochloride. ●, (+)-Benzphetamine; ○, (+)-benzphetamine+linoleic acid (3·5mM). Assay conditions are described in the Materials and Methods section.

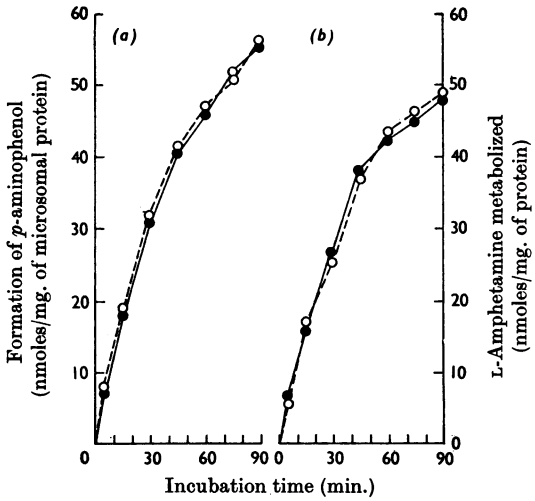

Fig. 3. Effect of arachidonic acid on the time-course of aniline hydroxylase (*a*) and (−)-amphetamine deaminase (*b*) by rat and rabbit liver 9000*g* supernatant fractions respectively. ●, Control; ○, arachidonic acid (3·5mM). Assay conditions are described in the Materials and Methods section.

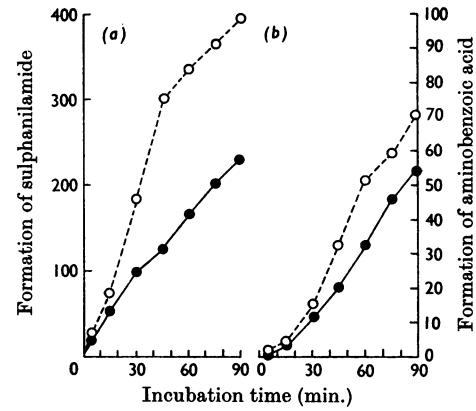

Fig. 4. Effect of arachidonic acid on the time-course of azo reduction of Neoprontosil (*a*) and nitro reduction of *p*-nitrobenzoic acid (*b*) (expressed as nmoles of aminobenzoic acid formed/mg. of microsomal protein) by rat liver 9000*g* supernatant fraction. ●, Control; ○, arachidonic acid (3·5mM). Neoprontosil metabolism was measured as the amount of diazotizable amino groups formed and is expressed as sulphanilamide (nmoles/mg. of microsomal protein). Assay conditions are described in the Materials and Methods section.

of 4mM-nicotinamide from the incubation media did not change qualitatively the results as reported in Fig. 1. However, without nicotinamide, substrate metabolism was generally decreased, especially when extended incubation periods (more than 20min.) were used. The reciprocal plots shown in

Figs. 2(*a*) and 2(*b*) were essentially the same with or without added nicotinamide.

To establish that the inhibition *in vitro* by these straight-chain unsaturated fatty acids on hepatic microsomal drug metabolism was not due to some secondary peroxidative product of the fatty acid, experiments were performed to show the time-course for the development of lipid peroxides as measured by the 2-thiobarbituric acid chromogen. It was understood that the chromogen formation does not always reflect the extent of peroxidation, but it is at least a relative measure of this reaction. Linoleic acid was used for this study because it did not interfere with the spectrophotometric assay of the thiobarbituric acid chromogen, as did linolenic acid and arachidonic acid. The thiobarbituric acid-reacting material (E_{532}) was depressed in the presence of *p*-nitroanisole and appeared to be even more depressed when both *p*-nitroanisole and linoleic acid were added to the reaction mixture (Fig. 5*a*). As shown in Fig. 5, (+)-α-tocopherol acetate (vitamin E), homogenized with the rat 9000*g* supernatant fraction, nearly completely prevented formation of chromogen (Fig. 5*a*), but did not change the extent of inhibition of *p*-nitroanisole *O*-demethylase by 3·5mM-linoleic acid (Fig. 5*b*). Thus linoleic acid can significantly affect the metabolism of drugs *in vitro* by rat liver microsomes without producing increased lipid peroxidation products, e.g. malondialdehyde, compared with control samples. Any effects on drug metabolism produced by unsaturated fatty acids were apparently independent of lipid peroxides or peroxidation.

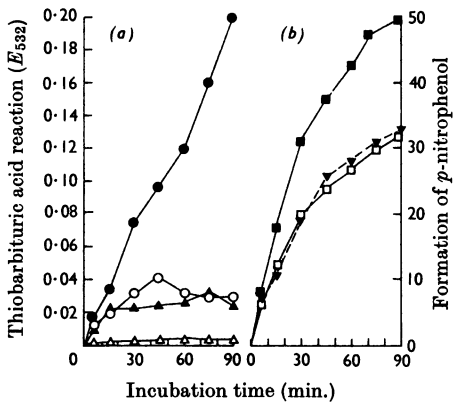

Fig. 5. Relationship of lipid peroxidation to linoleic acid inhibition of *p*-nitroanisole *O*-demethylase in rat liver 9000*g* supernatant fraction. (*a*) Lipid peroxidation was measured by the development of chromogen with thiobarbituric acid as described in the Materials and Methods section. ●, Control; ○, *p*-nitroanisole (2mM): ▲, *p*-nitroanisole (2mM)+linoleic acid (3·5mM); Δ, (+)-α-tocopherol acetate. (*b*) Effect of linoleic acid on the time-course curve of *p*-nitroanisole metabolism by rat liver 9000*g* supernatant fraction in the presence and absence of tocopherol, expressed as nmoles of *p*-nitrophenol formed/mg. of microsomal protein. ■, *p*-Nitroanisole (2mM); □, *p*-nitroanisole (2mM)+linoleic acid (3·5mM); ▼, *p*-nitroanisole (2mM)+linoleic acid (3·5mM)+(+)-α-tocopherol acetate. In both (*a*) and (*b*) the tocopherol oil was homogenized with the 9000*g* supernatant fraction such that there would be 15mg./g. wet wt. of liver. Conditions for incubation and assay methods are described in the Materials and Methods section.

The requirement by microsomal drug oxidase enzymes for NADPH would make inhibition of reduction of $NADP^+$ a possible mechanism for fatty acid inhibition of drug metabolism *in vitro*. However, as shown in Table 1, linoleic acid did not inhibit NADPH generation by the rat liver postmicrosomal (soluble) fraction. Microsomal NADPH–cytochrome *c* reductase was also uninhibited by linoleic acid (Table 1). As shown in Table 1, the rate of oxidation of NADPH by microsomal enzymes in the absence of exogenous electron acceptors was increased by 3·5mM-linoleic acid but generally unaffected at 0·5mM. It appears, then, that at the usual concentration of unsaturated fatty acid studied, 3·5mM, the steady-state concentration of NADPH, or the NADPH/$NADP^+$ concentration ratio, have not been changed to any significant degree.

Attempts were made to show that, under the conditions of this study, unsaturated fatty acids

Table 1. *Effect of linoleic acid on rat liver soluble-fraction NADPH generation, microsomal NADPH oxidation and microsomal NADPH–cytochrome* c *reduction*

Conditions and assay methods are described in the Materials and Methods section. Values represent means ± S.E.M. for at least four animals.

	NADPH generation (nmoles/min./0·5ml. soluble fraction)	NADPH oxidase (nmoles/min./mg. of microsomal protein)	NADPH–cytochrome *c* reductase (nmoles/min./mg. of microsomal protein)
Control	358±29	14·1±0·3	27·8±1·9
Linoleic acid (0·5mM) added	355±23	14·3±0·3	27·6±2·1
Linoleic acid (3·5mM) added	340±19	17·6±0·5*	26·4±1·6

* $P<0·05$.

Table 2. *Variation of incubation parameters and their effects on linoleic acid and (+)-benzphetamine metabolism by rat liver*

Basal incubation systems consisted of 1·0 ml. of microsomes (approx. 5 mg. of protein) suspended in 0·15 M-KCl–4 mM-glucose 6-phosphate–5 mM-$MgSO_4$–50 mM-potassium phosphate buffer, pH 7·35. Incubations were carried out in 5 ml. volumes under O_2 at 37°. Bovine serum albumin (5 mg./ml.), in addition to deterring microsomal agglutination by linoleic acid, facilitated suspension of the acid. (+)-Benzphetamine and linoleic acid metabolism were measured as described in the Materials and Methods section with the same microsomal pool. Linoleic acid and (+)-benzphetamine were 3·5 and 2·5 mM respectively. The NADPH-generating system contained the basal incubation system described above, 0·6 mM-$NADP^+$ and 3·0 units of purified glucose 6-phosphate dehydrogenase (Sigma type XII; 1 unit will reduce 1·0 μmole of $NADP^+$/min. at pH 7·4 at 25°).

Expt. 1	Substrate	Albumin	Metabolism (nmoles/30 min./mg. of microsomal protein)
Reductant: 0·3 mM-NADPH	Linoleic acid	−	0
	Linoleic acid	+	0
	(+)-Benzphetamine	−	69·1
	(+)-Benzphetamine	+	70·0
	Linoleic acid	−	0
Expt. 2			
Reductant: NADPH-generating system	Linoleic acid	+	0
	(+)-Benzphetamine	−	168·2
	(+)-Benzphetamine	+	170·2

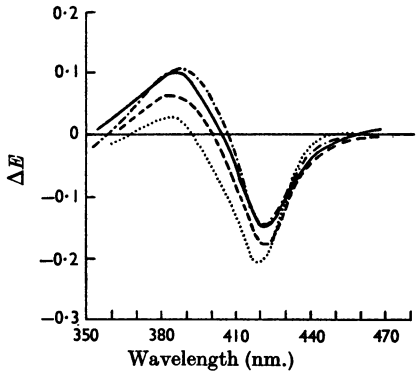

Fig. 6. Difference spectra produced by the interaction of unsaturated fatty acids with male rat liver microsomes. Unsaturated fatty acids were added as oils by micropipette to one of a pair of cuvettes containing 3·0 mg. of microsomal protein/ml. of cuvette contents. The washed microsomes were suspended in 50 mM-potassium phosphate buffer, pH 7·35, containing bovine serum albumin (0·5%). The cuvettes were shaken thoroughly and the difference spectra recorded. Fatty acid in combination with only the albumin–phosphate buffer did not produce difference spectra. ——, Oleic acid (20 mM): – – – –, linoleic acid (20 mM); –·–·–·, linolenic acid (20 mM); ·····, arachidonic acid (5 mM). The addition of 20 mM-arachidonic acid obscured the peak at 385 nm, preventing an accurate estimation of $E_{385}-E_{420}$. Changes in extinction were determined with a Shimadzu MPS-50L split-beam recording spectrophotometer at room temperature.

and drug substrates might be utilizing a common mixed-function oxidase pathway localized in rat liver microsomes. Linoleic acid or (+)-benzphetamine was incubated with microsomes and NADPH, or an NADPH-generating system, under oxygen. The results, given in Table 2, show that there was metabolism of (+)-benzphetamine with either NADPH source, but there was no disappearance of linoleic acid after incubation of the reaction mixtures. No additional peaks, which might suggest metabolic products of linoleic acid, e.g. ω-hydroxylinoleic acid, were detected during the g.l.c. analyses of the derived methyl esters from the chloroform extracts of linoleic acid-containing mixtures. Under similar reaction conditions, with 0·3 mM-NADPH as a reductant, May & McCay (1968*a*,*b*) showed a decrease in microsomal phospholipid linoleic acid, as well as other endogenously present unsaturated fatty acids.

Other workers have demonstrated that various drugs, substrates and inhibitors of mixed-function oxidases can combine with aerobic hepatic microsomal cytochrome to give one of two (type I or type II) characteristic spectra (Remmer *et al.* 1966; Imai & Sato, 1966; Schenkman, Remmer & Estabrook, 1967). The type I spectral change is characterized by a trough at 420 nm. and an absorption peak at 385–390 nm. The type II spectral change is characterized by an absorption peak at about 430 nm. and a trough at about 390 nm. The unsaturated fatty acids added to an albumin–phosphate buffer suspension of rat liver microsomes produced an absorption maximum (peak) at about 385 nm. and minimum (trough) at about 420 nm., indicative of type I spectral changes (Fig. 6). The spectral dissociation constant, K_s, as defined by Schenkman *et al.* (1967), was found for linoleic acid to be 2·5 mM, which is considerably greater than the K_s values of 0·33 mM and 0·08 mM respectively obtained by these authors for the type I substrates

aminopyrine and hexobarbitone. Unlike some of the ligands studied, the strong protein binding of unsaturated fatty acids, as well as their water-insolubility, probably imposes non-Michaelis conditions, so that the calculated K_s values are likely to be much greater than the true K_s values. The peak-to-trough ΔE for linoleic acid at saturating concentrations was 0·24 with a microsomal protein concentration of 3mg./ml. Values of this magnitude were obtained by McLean (1967) for the lipid solvents *n*-hexane and chloroform, which are also type I ligands. Neither arachidic (eicosanoic) acid, a straight-chain saturated C_{20} monocarboxylic acid, added in acetone solution or as the powder, nor trilinolein, added as the oil, produced spectral changes with rat liver microsomes.

DISCUSSION

Fatty acids bind to proteins with great affinity and can displace drugs from their binding sites on proteins (Boyer, Ballou & Luck, 1947; Solomon, Schrogie & Williams, 1968). In a study of inhibition of the metabolism of one polyunsaturated fatty acid by another *in vivo*, Holman & Mohrhauer (1963) concluded that enzyme–substrate (fatty acid) affinities increase with unsaturation. When linolenic acid was present in the substrate pool, its conversion into higher unsaturated fatty acids took precedence over the metabolism of linoleate. The π-electron character of double bonds would allow greater electron delocalization with interacting protein. This would account for the tenacious binding of unsaturated fatty acids to proteins, in addition to the electrostatic attraction of the fatty acid carboxylic anionic charge.

If we assume that the unsaturated fatty acids in this present study were bound to some (apo)enzyme component or active site required for the operation of a microsomal mixed-function oxidase system, then it is understandable why enzymic oxidation of a number of drug substrates was impaired and that inhibition was competitive and increased with increasing unsaturation of the fatty acid. The decreased lipid solubility generally occurring with increased unsaturation of lipids (Bloor, 1943) would not favour greater inhibition of drug oxidase activity being due to greater lipid solubility.

The kinetic interpretation of the uncompetitive inhibition of *p*-nitroanisole *O*-demethylase by trilinolein may indicate a combination of trilinolein with the *O*-demethylase–*p*-nitroanisole complex (Webb, 1963). It is not unreasonable to expect that the water-insouble *p*-nitroanisole might be in 'solution' in, or somehow in the form of a complex with, the lipid trilinolein. The interaction of *p*-nitroanisole or the *O*-demethylase–*p*-nitroanisole complex with lipid linoleic acid might account for the non-linear reciprocal plot for linoleic acid inhibition of *p*-nitroanisole *O*-demethylase. An uncompetitive-type and a competitive type-inhibition might be occurring simultaneously. As used as the hydrochloride salt, (+)-benzphetamine would be more water-soluble and less likely to interact with or partition into linoleic acid or trilinolein than *p*-nitroanisole. Thus the reciprocal plots of linoleic acid inhibition of (+)-benzphetamine *N*-demethylase were always linear.

The binding or interaction of substrates with microsomal cytochrome *P*-450 may be evidence of formation of an enzyme–substrate complex preceding substrate oxidation. The type I spectrum and competitive inhibition of microsomal drug metabolism produced by linoleic acid would be consistent with the formation of an enzyme–inhibitor or enzyme–alternative substrate complex of microsomes and fatty acids.

It was noteworthy that most drug substrates whose metabolism by hepatic microsomes was inhibited by fatty acids gave a type I spectral change with microsomal cytochrome (Schenkman *et al.* 1967); none of the substrates for the pathways inhibited has been shown to give a type II spectral change. We have found that *p*-nitroanisole, *p*-nitrobenzoic acid and Neoprontosil gave intrinsic extinctions in the Soret region, so that ligand–cytochrome-induced spectral changes with these substrates were not measured. The metabolism of aniline and (−)-amphetamine by rat and rabbit 9000*g* supernatant fractions respectively was refractory to inhibition by unsaturated fatty acids. Both rat and rabbit microsomes interact with these amines to give type II spectra (D. S. Hewick, personal communication). Therefore drugs whose metabolism was inhibited by fatty acids were of the same class (type I spectrum when added to microsomes) as the fatty acids.

These unsaturated fatty acids probably bind to a number of proteins in hepatic microsomes and in the postmicrosomal supernatant (soluble fraction), but inhibition of microsomal drug-oxidase activity, when observed, was unlikely to have been due to inhibition of enzymes responsible for the generation of NADPH (Table 1) or the reduction of cytochrome *P*-450. If this were the case, the rate of aniline hydroxylation, or the deamination of (−)-amphetamine, should have been diminished, since it is assumed that type I and II ligands are reduced by a common electron-transport chain. Moreover, we have made preliminary studies of rat microsomal cytochrome *P*-450 reductase, as studied by Gigon, Gram & Gillette (1969), and have shown that linoleic acid had no effect on the rate of reduction of cytochrome *P*-450, except perhaps to increase it. This would be in agreement with the findings of Gigon *et al.* (1969), who showed that type I com-

pounds enhance, whereas type II compounds decrease, the rate of reduction of cytochrome *P*-450 by NADPH. These latter workers have also shown that type I compounds generally do not alter NADPH–cytochrome *c* reductase activity but do stimulate NADPH oxidation in rat liver microsomes, as was shown for linoleic acid in the present study (Table 1). The addition of type II compounds to microsomal suspensions either did not affect or decreased NADPH–cytochrome *c* reductase and NADPH oxidase activities (Gigon *et al.* 1969).

The enhancement of nitro reductase and azo reductase activities in rat liver microsomes by unsaturated fatty acids was not unexpected. Recent findings (Sasame & Gillette, 1969) revealed that microsomal nitro reductase activity is generally unaffected or increased by compounds that give a type I spectral change with microsomes, and decreased by compounds giving type II spectra.

Although Castro, Sasame & Gillette (1967) have reported that fatty acids (about 1mM) converted cytochrome *P*-450 into its inactive form, cytochrome *P*-420, we have not observed this spectral shift in microsomes incubated with linoleic acid (3·5mM) and cofactors.

The lack of metabolism of linoleic acid added *in vitro* to rat hepatic microsomes, coupled with the binding capacity of this fatty acid to proteins, make it reasonable that a stable microsomal apoenzyme–fatty acid complex could exist, preventing the reaction of other type I substrates. The fact that the metabolism of type II substrates was not inhibited by unsaturated fatty acids could support different binding loci for type I and II compounds, or possibly the existence of more than one cytochrome for microsomal oxidations. The paucity of type II substrates for drug oxidases in microsomes makes it difficult to ascertain that the microsomal metabolism of any type II substrate will not be inhibited *in vitro* by unsaturated fatty acids.

The technical assistance of Mrs Gladys Rios, Miss Judith Fitzgibbon, Mrs Roberta Pohl and Mr Lysle Waters is gratefully acknowledged. This work was supported by U.S. Public Health Service Grants GM-12675 and 5TI-TM-00141 from the National Institute of General Medical Sciences, National Institutes of Health.

REFERENCES

Axelrod, J. (1954). *J. Pharmacol.* **110**, 315.

Bloor, W. R. (1943). *Biochemistry of the Fatty Acids*, p. 5. New York: Reinhold Publishing Corp.

Boyer, P. D., Ballou, G. A. & Luck, J. M. (1947). *J. biol. Chem.* **167**, 407.

Castro, J. A., Sasame, H. & Gillette, J. R. (1967). *Pharmacologist*, **9**, 203.

Cochin, J. & Axelrod, J. (1959). *J. Pharmacol.* **125**, 105.

Das, M. L., Orrenius, S. & Ernster, L. (1968). *Europ. J. Biochem.* **4**, 519.

Folch, J., Lees, M. & Sloane-Stanley, G. H. (1957). *J. biol. Chem.* **226**, 497.

Fouts, J. R. & Brodie, B. B. (1957). *J. Pharmacol.* **119**, 197.

Fouts, J. R., Kamm, J. J. & Brodie, B. B. (1957). *J. Pharmacol.* **120**, 291.

Fouts, J. R. & Waters, L. R. (1969). *Toxicol. appl. Pharmacol.* (in the Press).

Gigon, P. L., Gram, T. E. & Gillette, J. R. (1969). *Molec. Pharmacol.* **5**, 109.

Gillette, J. R. (1966). *Advanc. Pharmacol.* **4**, 245.

Gillette, J. R., Brodie, B. B. & LaDu, B. N. (1957). *J. Pharmacol.* **119**, 532.

Gram, T. E. & Fouts, J. R. (1966). *J. Pharmacol.* **152**, 363.

Gram, T. E., Rogers, L. A. & Fouts, J. R. (1967). *J. Pharmacol.* **155**, 479.

Holman, R. T. & Mohrhauer, H. (1963). *Acta chem. scand.* **17**, S84.

Hunter, F. E., Gebicki, J. M., Hoffsten, P. E., Weinstein, J. & Scott, A. (1963). *J. biol. Chem.* **238**, 828.

Imai, Y. & Sato, R. (1966). *Biochem. biophys. Res. Commun.* **22**, 620.

Kuntzman, R., Lawrence, D. & Conney, A. H. (1965). *Molec. Pharmacol.* **1**, 163.

Kupfer, D. & Bruggeman, L. L. (1966). *Analyt. Biochem.* **17**, 502.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.

Lu, A. Y. & Coon, M. J. (1968). *J. biol. Chem.* **243**, 1331.

McLean, A. E. M. (1967). *Biochem. Pharmacol.* **16**, 2030.

May, H. E. & McCay, P. B. (1968*a*). *J. biol. Chem.* **243**, 2288.

May, H. E. & McCay, P. B. (1968*b*). *J. biol. Chem.* **243**, 2296.

Morrison, W. R. & Smith, L. M. (1964). *J. Lipid Res.* **4**, 600.

Netter, K. J. & Seidel, G. (1964). *J. Pharmacol.* **146**, 61.

Phillips, A. H. & Langdon, R. G. (1962). *J. biol. Chem.* **237**, 2652.

Preiss, B. & Bloch, K. (1964). *J. biol. Chem.* **239**, 85.

Remmer, H., Schenkman, J., Estabrook, R. W., Sasame, H., Gillette, J. R., Narasimuhulu, S., Cooper, D. Y. & Rosenthal, O. (1966). *Molec. Pharmacol.* **2**, 187.

Roberts, R. J. & Plaa, G. L. (1966). *Biochem. Pharmacol.* **15**, 333.

Sasame, H. & Gillette, J. R. (1969). *Molec. Pharmacol.* **5**, 123.

Schenkman, J. B., Remmer, H. & Estabrook, R. W. (1967). *Molec. Pharmacol.* **3**, 113.

Solomon, H. M., Schrogie, J. J. & Williams, D. (1968). *Biochem. Pharmacol.* **17**, 143.

Tephly, T. R. & Mannering, G. J. (1968). *Molec. Pharmacol.* **4**, 10.

Webb, J. L. (1963). *Enzyme and Metabolic Inhibitors*, vol. 1, p. 160. New York: Academic Press Inc.