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EFFECT OF VARIOUS METABOLIC INHIBITORS ON BIPHENYL METABOLISM IN ISOLATED RAT HEPATOCYTES

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Abstract—The effect of three inhibitors of mitochondrial function (menadione, rotenone and 2,4-dinitrophenol) on drug metabolism in isolated rat hepatocytes has been studied. Menadione (at 1.25×10^{-4} M) caused almost complete inhibition of biphenyl Phase I metabolism whereas rotenone (2×10^{-5} M) inhibited the same reaction only by 25 per cent although the subsequent conjugation of the Phase I metabolite was markedly depressed. Qualitatively similar findings were observed with hepatocytes isolated from phenobarbital-pretreated rats, and with liver microsomes isolated from control rats. 2,4-Dinitrophenol (2×10^{-4} M) caused a marked enhancement of biphenyl Phase I metabolism but a marked inhibition of subsequent conjugation. This enhancement of Phase I metabolism was not observed in control cells with other substrates (benzo[a]pyrene, 7-ethoxycoumarin), nor in biphenyl metabolism in "induced" cells or in liver microsomes isolated from control rats. It is tentatively suggested that products of 2,4-dinitrophenol metabolism may "activate" biphenyl metabolism in intact liver cells. Furthermore, it is suggested for all three inhibitors that direct effects on the drug metabolizing enzyme systems (Phase I and Phase II) are as important as their effects on mitochondrial function in explaining their inhibition of drug metabolism. It appears that Phase II metabolism of xenobiotics is more susceptible to inhibition by metabolic inhibitors than is Phase I metabolism, probably due to depletion of the cellular ATP levels.

The metabolism of xenobiotics occurs predominantly in the liver and is usually a two step process involving cytochrome P-450 linked mono-oxygenation (Phase I) and subsequent conjugation reactions (Phase II). During this process, the liver is exposed not only to the possible toxic effects of the xenobiotic itself but to those of its biologically active metabolites, and the concentration of active metabolites in hepatocytes depends on the balance between the rate of the reaction leading to their formation and that of their further metabolism. While Phase I reactions may or may not lead to the formation of active metabolites, Phase II reactions usually cause "detoxication" (although there are certain exceptions to this).

In order to test the involvement of active metabolites in cellular change, it is desirable to identify selective inhibitors for both Phase I and Phase II reactions. Classical cytochrome P-450 inhibitors are not necessarily applicable for such purposes. For example, SKF 525A effectively inhibits not only Phase I reactions in isolated rat hepatocytes but also Phase II reactions [1]. Since it is well known that the metabolism of xenobiotics may be profoundly influenced by mitochondrial control over cofactor levels involved in both the Phase I and Phase II reactions, we have investigated the effects of certain mitochondrial metabolic inhibitors, (rotenone, an inhibitor of NADH oxidation, menadione, an electron acceptor and the uncoupler, 2,4-dinitrophenol) on cytochrome P-450-linked xenobiotic mono-oxygenation and subsequent conjugation in isolated rat hepatocyte suspensions. The results of these studies are presented in this paper and these not only highlight the potential use of such inhibitors to assess the role of xenobiotic metabolism in chemical toxicity, but also provide information of a more fundamental nature, notably the control of cofactor supply and the relationship that exists between the Phase I and Phase II reactions in liver cells.

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MATERIALS AND METHODS

Animals used were male Wistar albino rats (weight 60–80 g) bred in the University animal house and allowed free access at all times to standard laboratory diet (Spillers No. 1, Spillers Ltd., Croydon, U.K.) and water. Treated rats were given a daily i.p. dose of sodium phenobarbitone in 0.9% NaCl (80 mg/kg body wt.) for 3 days. At least 20 hr were always allowed to elapse between the last injection and sacrifice of animals. Collagenase (Type II), hyaluronidase (Type II), sulphatase (Type HI), benzo[a]pyrene, saccharo-1, 4β-lactone, rotenone, menadione and 2,4-dinitrophenol were obtained from Sigma Ltd., Poole, Dorset, U.K. Pure β-glucuronidase ("Ketodase") was obtained from William Warner & Co. Ltd., Eastleigh, Hampshire, U.K. Biphenyl and its major primary metabolite, 4-hydroxybiphenyl were obtained from BDH, Poole, Dorset, U.K. and purified as described previously [1]. 7-Ethoxycoumarin was synthesised by the method of Ullrich and Weber [2] and 7-hydroxycoumarin was obtained from Fluka A.G., Buchs, Switzerland. All tissue culture media and supplements were obtained from Gibco-Biocult Ltd., Paisley, Scotland.

Cell isolation

Hepatocytes were isolated by collagenase–hyaluronidase digestion of liver slices as previously described [3] and were diluted in culture medium (L-15 medium containing 10% foetal calf serum and 10% tryptose phosphate broth) to a concentration of 2×10^6 viable cells/ml. Viability of the hepatocytes as judged by trypan blue dye exclusion was greater than 85 per cent.

Assay systems

Unless otherwise stated studies were performed on suspensions of freshly isolated hepatocytes (5 ml) contained in 50 ml conical flasks at a concentration of 2×10^6 viable hepatocytes/ml maintained at 37°, the basic protocol for the studies being a 10 min preincubation with inhibitor followed by incubation with the drug substrate.

Biphenyl. Cells were incubated with biphenyl (final conc. 70 μM) and the free and conjugated metabolites were measured as described previously [4].

Benzo[a]*pyrene and 7-ethoxycoumarin.* Cells were incubated with benzo[*a*]pyrene (80 μM) or ethoxycoumarin (70 μM) and the free and conjugated metabolites were measured as described previously [5].

4-Hydroxybiphenyl. Cells were incubated with 4-hydroxybiphenyl (70 μM). One millilitre samples of the cell suspension were extracted with *n*-heptane (3.5 ml) to remove unmetabolised 4-hydroxybiphenyl and the aqueous phase was hydrolysed using the modified deconjugation procedure described by Wiebkin *et al.* [4]. The resulting free 4-hydroxybiphenyl was then extracted and determined fluorimetrically at pH 6.0 using λex 275 nm and λem 310 nm [3].

7-Hydroxycoumarin. Cells were incubated with 7-hydroxycoumarin (70 μM). Following dilution (× 5) with a suitable isotonic solution (PBS'A'), 1 ml samples were extracted with diethyl ether (5 ml) to remove unmetabolised 7-hydroxycoumarin and the aqueous phase hydrolysed using the modified deconjugation procedure. The resulting free 7-hydroxycoumarin was extracted with diethyl ether and 1 ml of the ether phase was back extracted into 5 ml 0.2 M glycine/NaOH buffer pH 10.4. 7-Hydroxycoumarin was then measured fluorimetrically using λex 370 nm and λem 450 nm.

Oxygen uptake studies. Cells (2.5 ml) were equilibrated in a Clark oxygen electrode for 2–3 min before it was sealed, ensuring all air bubbles had been expelled. The control rate of oxygen uptake of isolated rat hepatocytes was obtained and, once linearity had been attained (usually after about 2 min), the effect of inhibitors and/or biphenyl on this rate was then monitored.

ATP estimation. The procedure adopted was based on the Boehringer kit for the estimation of ATP in blood (Cat. No. 123897) derived from the method of Adam [6]. The ATP content of the hepatocytes following incubation with substrate with and without inhibitor was estimated by measuring the change in fluorescence at λ 365 nm and λ 420 nm.

NADPH extraction and estimation. The method used was based on the suggested scheme of Klingenberg [7]. At the end of the incubation period with substrate with and without inhibitor, the NADPH content of the hepatocytes was estimated by the change in fluorescence at λex 365 nm and λ em 420 nm.

Microsomal studies. Liver microsomes were isolated at 0–4° using conventional differential ultracentrifugation techniques [8]. Incubations were carried out at 37° using 0.2 ml microsomal suspension (approx. 10 mg protein/ml) in the presence of a NADPH-regenerating system in 0.1 M phosphate buffer pH 7.6, consisting of NADP (1.2 μmoles) glucose-6-phosphate (15 μmoles) and glucose-6-phosphate dehydrogenase (1.5 units) in each incubation. After incubation with biphenyl (70 μM) with or without inhibitor, hydroxy metabolites were extracted and measured fluorimetrically as described previously. Protein was assayed by the method of Lowry *et al.* [9].

Anaylsis and presentation of results. Whenever three or more samples were assessed, the results are given as mean ± S.E.M. Statistical analysis was carried out using Student's *t*-test. Some of the data presented in the subsequent tables and figures describe single experiments; in these instances each study was performed at least twice with similar results in each experiment.

RESULTS

The effect of three metabolic inhibitors, rotenone, menadione and 2,4-dinitrophenol on the production of free and conjugated 4-hydroxybiphenyl by isolated viable hepatocytes from untreated rats incubated with 70 μM biphenyl for 25 min at 37° is summarised in Table 1. Treatment of the cells with rotenone (final concentration 2×10^{-5} M) resulted in a 25 per cent reduction in the overall hydroxylation step. The conjugation reaction was, however, much more severely affected, 4-hydroxybiphenyl sulphate and glucuronide formation being inhibited by 89 per cent and 64 per cent respectively, so resulting in much higher levels of free 4-hydroxybiphenyl being detected (740 per cent of control) in incubations in which rotenone was present.

Menadione (final concentration 1.25×10^{-4} M) was found to be a very potent inhibitor of enzyme activity, reducing the total 4-hydroxybiphenyl produced to less that 10 per cent of the activity present in the uninhibited hepatocytes.

Treatment of the cells with 2,4-dinitrophenol (final concentration 2×10^{-4} M) resulted in marked inhibition of sulphate and glucuronic acid conjugation of the free 4-hydroxybiphenyl produced by these cells (88 per cent and 50 per cent inhibition respectively). However such treatment yielded a significant increase in the overall hydroxylation rate (262 per cent of the rate in control hepatocytes).

Rotenone and menadione had similar effects on biphenyl metabolism when hepatocytes from phenobarbitone-treated rats were employed (Table 1). Menadione was again the more potent inhibitor, reducing total biphenyl 4-hydroxylase activity to 11 per cent of control value. Rotenone inhibited the 4-hydroxylation of biphenyl by 52 per cent, sulphate conjugation by 82 per cent and glucuronic acid for-

Table 1. Effect of metabolic inhibitors on biphenyl 4-hydroxylation and subsequent conjugation in suspensions of rat hepatocytes from untreated and phenobarbitone-pretreated animals

Treatment	4-Hydroxybiphenyl metabolites produced			
	Free	Sulphate	Glucuronide	Total
Control cells				
None	*0.1 ± 0.02 (100)	0.90 ± 0.06 (100)	0.22 ± 0.07 (100)	1.22 ± 0.08 (100)
Rotenone (2×10^{-5} M)	0.74 ± 0.06 (740)	0.10 ± 0.03 (11)	0.08 ± 0.003 m (36)	0.91 ± 0.03‡ (75)
Menadione (1.25×10^{-4} M)	0.02 ± 0.01 (20)	0.03 ± 0.02 (3)	0.03 ± 0.02 (14)	0.03 ± 0.01§ (7)
2,4-Dinitrophenol (2×10^{-4} M)	2.98 ± 0.22 (2980)	0.11 ± 0.08 (12)	0.11 ± 0.09 (50)	3.20 ± 0.19‖ (262)
Phenobarbitone cells				
None	†1.33 (100)	2.61 (100)	4.42 (100)	8.36 (100)
Rotenone (2×10^{-5} M)	2.98 (224)	0.48 (18)	0.33 (12)	3.99 (48)
Menadione (1.25×10^{-4})	0.51 (38)	0.12 (5)	0.25 (6)	0.88 (11)
2,4-Dinitrophenol (2×10^{-4} M)	7.05 (530)	0.48 (18)	1.06 (24)	8.59 (103)

Rat hepatocytes (10^7 cells in 5 ml vol) were preincubated with metabolic inhibitors for 10 min at 37°, then incubated for a further 25 min with biphenyl (70 μM). Free and conjugated 4-hydroxybiphenyl metabolites were extracted and measured as outlined in text.

* Values are mean of three separate experiments (± S.E.M.) expressed as nmoles/2 × 10^6 cells/25 min.

† Values expressed as nmoles/2 × 10^6cells/25 min; and are the means of two separate determinations differing by less than 10 per cent from each other.

Statistically different from control at ‡$P < 0.1$, §$P < 0.01$, ‖$P < 0.02$ levels. Numbers in parentheses are values expressed as a percentage of the appropriate control.

mation by 88 per cent. 2,4-Dinitrophenol (2×10^{-4} M) inhibited the conjugation of 4-hydroxybiphenyl (sulphate 82 per cent; glucuronide 76 per cent) but the total biphenyl hydroxylation rate was unaffected in contrast to the situation in hepatocytes from untreated rats.

The effects of 2,4-dinitrophenol on 7-ethoxycoumarin *O*-de-ethylation and benzo[*a*]pyrene 3-hydroxylation and subsequent conjugation of the Phase I metabolites produced by isolated hepatocytes were similar to those observed on biphenyl 4-hydroxylation in cells from phenobarbitone pretreated rats. In hepatocytes from untreated rats, a marked reduction was seen in the formation of conjugates of the Phase I metabolites with little effect on the initial Phase I reaction (Table 2).

To investigate the direct effect of rotenone and 2,4-dinitrophenol upon conjugation, studies were also carried out using 4-hydroxybiphenyl and 7-hydroxycoumarin (both at a final concentration of 70 μM) as substrates. Rotenone inhibited the sulphate and glucuronic acid conjugation of 4-hydroxybiphenyl markedly, the total conjugation rate being only 6 per cent that of the control. 2,4-Dinitrophenol was just as potent as rotenone, inhibiting the overall conjugation rate of 4-hydroxybiphenyl by 95 per cent

Table 2. Effect of 2,4-dinitrophenol of 7-ethoxycoumarin *O*-de-ethylation and benzo[*a*]pyrene 3-hydroxylation and subsequent conjugation in suspensions of rat hepatocytes from untreated animals

Substrate	Treatment	Metabolites produced			
		Free	Sulphate	Glucuronide	Total
7-Ethoxycoumarin	None	*0.9 (100)	4.6 (100)	5.2 (100)	10.7 (100)
	2,4 Dinitrophenol (2×10^{-4} M)	7.2 (800)	0.7 (15)	0.8 (15)	8.7 (81)
Benzo[*a*]pyrene	None	†4.1 (100)	14.6 (100)	14.3 (100)	33.0 (100)
	2,4 Dinitrophenol (2×10^{-4} M)	26.6 (649)	1.9 (13)	3.7 (26)	32.0 (97)

Rat hepatocytes (2 × 10^6 cells in 1 ml vol) were preincubated with 2,4-dinitrophenol for 10 min at 37°, then incubated for a further period with either 7-ethoxycoumarin (70 μM) or benzo[*a*]pyrene (80 μM). Free and conjugated metabolites were extracted and measured as described in the text.

* Values expressed as nmoles 7-hydroxycoumarin produced/2 × 10^6 cells/25 min.

‡ Values expressed as pmoles 3-hydroxybenzo[*a*]pyrene produced/2 × 10^6 cells/30 min.

Numbers in parentheses are values as percentage of the appropriate controls.

Table 3. Effect of metabolic inhibitors on the conjugation of 4-hydroxybiphenyl and 7-hydroxycoumarin in suspensions of rat hepatocytes from untreated animals

Substrate	Treatment	Conjugates produced		
		Sulphate	Glucuronide	Total
4-Hydroxybiphenyl	None	5.6 (100)	32.8 (100)	38.3 (100)
	Rotenone (2×10^{-5} M)	1.8 (32)	0.5 (2)	2.3 (6)
	2,4-Dinitrophenol (2×10^{-4} M)	0.0 (0)	1.8 (5)	1.8 (5)
7-Hydroxycoumarin	None	5.5 (100)	16.7 (100)	22.2 (100)
	2,4-Dinitrophenol (2×10^{-4} M)	1.8 (33)	1.8 (11)	3.5 (16)

Rat hepatocytes (2×10^6 cells in 1 ml vol) were preincubated for 10 min with the inhibitors at 37°, then incubated with either 4-hydroxybiphenyl (70 μM) or 7-hydroxycoumarin (70 μM) for a futher 25 min. Conjugated metabolites were extracted and measured as described in the text.

Values expressed as nmoles produced/2×10^6 cells/25 min and are the means of duplicate estimations differing by less that 10 per cent from each other.

Numbers in parentheses are values expressed as percentage of the appropriate control.

and 7-hydroxycoumarin conjugation by 84 per cent (Table 3).

When hepatocytes were pretreated with 2,4-dinitrophenol for 10 min prior to addition of biphenyl, significantly more free 4-hydroxybiphenyl was noted throughout the 45 min incubation period than in non-pretreated cells. 4-Hydroxybiphenyl sulphate formation was reduced more than that of 4-hydroxybiphenyl glucuronide. The total 4-hydroxybiphenyl produced by the 2,4-dinitrophenol preincubated cells was stimulated by approx. 100 per cent of that produced by control cells (Fig. 1). At lower concentrations of 2,4-dinitrophenol (2×10^{-5} M), no increase in total 4-hydroxybiphenyl production was observed but enhanced amounts of free 4-hydroxybiphenyl were found. At 2×10^{-5} M dinitrophenol, 4-hydroxybiphenyl sulphate formation was significantly inhibited, but there was no apparent effect on 4-hydroxybiphenyl glucuronide production. At a concentration of 1×10^{-3} M 2,4-dinitrophenol, 4-hydroxybiphenyl formation was much greater than that formed at the 2×10^{-5} M level but less than that produced at 2×10^{-4} M. Hardly any conjugates were formed at 2×10^{-3} M dinitrophenol (Table 4) and a slight cytotoxic cell response was detected, as judged by dye exclusion. This reduced stimulation when compared to 2×10^{-4} M level in the total and free 4-hydroxybiphenyl produced is likely to be due to 2,4-dinitrophenol toxicity at this 1×10^{-3} M level. No cytotoxic response was observed in the hepatocytes at either the 2×10^{-5} M or 2×10^{-4} M level of 2,4-dinitrophenol.

In contrast to its effect on hepatocytes 2,4-dinitrophenol (2×10^{-4} M) had no effect on biphenyl 4-hydroxylation in rat liver microsomes. Rotenone (2×10^{-5} M), however, inhibited activity by 38 per

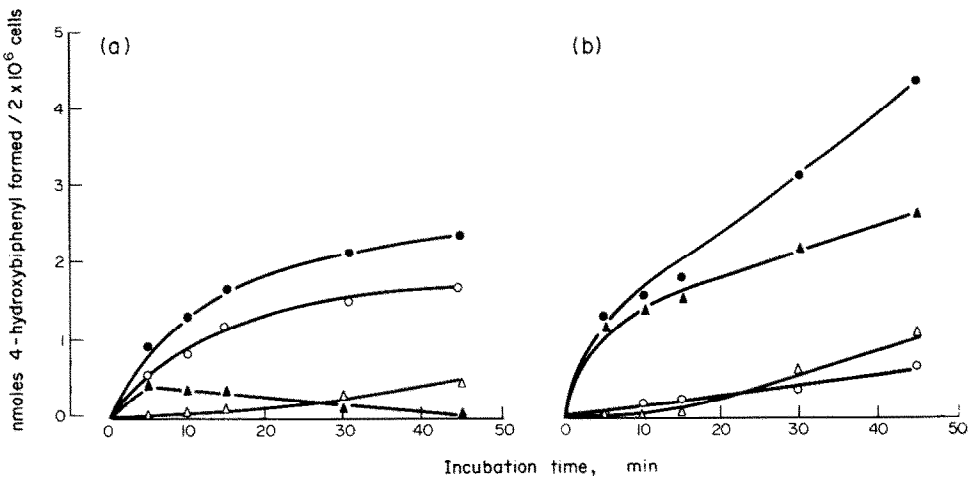

Fig. 1. Time courses of the appearance of free and conjugated 4-hydroxybiphenyl in suspensions of hepatocytes from untreated rats incubated with biphenyl (70 μM) (A) with preincubation for 10 min without 2×10^{-4} M 2,4-dinitrophenol, (B) with preincubation for 10 min with 2×10^{-4} M 2,4-dinitrophenol. Cells (2×10^6/ml), after preincubation with or without 2,4-dinitrophenol were incubated with 70 μM biphenyl for the times indicated at 37° and the free and conjugated 4-hydroxybiphenyl estimated fluorimetrically as described in the text. Values are the means of duplicate estimations differing by less than 10 per cent from each other. ▲——▲ Free 4-hydroxybiphenyl; △——△ 4-hydroxybiphenyl glucuronide; ○——○ 4-hydroxybiphenyl sulphate; ●——● Total 4-hydroxybiphenyl.

Table 4. Dose dependency of the effect of 2,4-dinitrophenol on biphenyl 4-hydroxylation and subsequent conjugation in suspensions of rat hepatocytes from untreated animals

Treatment	4-Hydroxybiphenyl metabolites produced			
	Free	Sulphate	Glucuronide	Total
None	*0.05 (100)	0.94 (100)	0.17 (100)	1.16 (100)
2,4-Dinitrophenol				
1×10^{-3} M	2.00 (4000)	— (0)	— (0)	2.00 (172)
2×10^{-4} M	2.24 (4480)	0.11 (12)	0.06 (35)	2.41 (208)
2×10^{-5} M	0.42 (840)	0.65 (69)	0.16 (94)	1.23 (106)

Rat hepatocytes (10^7 cells in 5 ml vol) were preincubated with various levels of 2,4-dinitrophenol for 10 min at 37°, then incubated for a futher 25 min with biphenyl (70 μM). Free and conjugated 4-hydroxybiphenyl metabolites were extracted and measured as outlined in text.

* Values expressed as nmoles/2×10^6 cells/25 min, and are the means of two separate determinations differing by less than 10 per cent from each other.

Numbers in parentheses are values expressed as a percentage of the appropriate control.

Table 5. Effect of metabolic inhibitors on biphenyl 4-hydroxylase activity in suspensions of rat liver microsomes from untreated animals

Treatment	Activity*
None	5.05 ± 0.24 (100)
Rotenone (2×10^{-5} M)	3.11 ± 0.09 (62)
Menadione (1.25×10^{-4} M)	0.02 ± 0.01 (0.4)
2,4-Dinitrophenol (2×10^{-4} M)	5.03 ± 0.18 (100)

Rat liver microsomes (1 mg protein/ml) were preincubated with metabolic inhibitors for 10 min at 37° in the presence of an NADPH regenerating system, then incubated for a futher 15 min with biphenyl (70 μM). 4-hydroxybiphenyl was extracted and measured as outlined in the text.

* Activities (mean ± S.E.M. of four animals) expressed as nmoles 4-hydroxybiphenyl produced/15 min/mg microsomal protein.

Numbers in parentheses are values expressed as percentage of control.

cent and menadione (1.25×10^{-4} M) caused almost complete inhibition (Table 5).

All three inhibitors caused a reduction in the ATP level within the cells; rotenone (2×10^{-5} M) produced a drop to 60 per cent of the control level, while menadione (1.25×10^{-4} M) and 2,4-dinitrophenol (2×10^{-4} M) resulted in the level being reduced to 40 per cent of the control value (Table 6). Rotenone and 2,4-dinitrophenol also caused the NADPH level to drop to 57 per cent and 41 per cent respectively, of the control value, whereas menadione preincubation resulted in an increase in the NADPH level within the hepatocytes to 136 per cent of the control level (Table 6). Rotenone (2×10^{-5} M) reduced the rate of endogenous oxygen uptake to 63 per cent of the control value, while menadione increased oxygen uptake (310 per cent) at 1.25×10^{-4} and 2,4-dinitrophenol also exhibited enhancement of endogenous oxygen uptake of the hepatocytes (213 per cent at 2×10^{-4} M; 150 per cent at 2×10^{-5} M). Biphenyl at the concentration used for these studies (70 μM) produced no measurable effect on oxygen uptake.

DISCUSSION

The effect of rotenone (an inhibitor of mitochondrial NADH oxidation [10]) in reducing the level

Table 6. Effect of metabolic inhibitors on the ATP content, NADPH content and oxygen uptake of suspensions of rat hepatocytes from untreated animals

Treatment	ATP content*	NADPH content*	Oxygen uptake (% of control rate)
None	9.77 (100)	5.81 (100)	100
Rotenone (2×10^{-5} M)	5.86 (60)	3.29 (57)	63.0 ± 8.1†
Menadione (1.25×10^{-4} M)	3.91 (40)	7.82 (136)	309.5 ± 66.6
2,4-Dinitrophenol (2×10^{-4} M)	3.91 (40)	2.38 (41)	213.1 ± 18.4
(2×10^{-5} M)	—	—	150.2 ± 2.0

Rat hepatocytes (2×10^6 cells/ml) were preincubated with inhibitors for 10 min at 37°, then incubated for a further 30 min with biphenyl (70 μM). The ATP content and NADPH content of the cells was estimated as described in the text. For the oxygen uptake studies, the cells were first incubated at 37° in a Clark electrode to obtain the control rate, then further incubated with metabolic inhibitors to monitor the change in rate.

*Values expressed as nmoles/10^6 cells after 30 min incubation with biphenyl and are the means of duplicate estimations differing by less that 10 per cent from each other.

† Values (± S.E.M.) are expressed as the percentage of the control uninhibited rate.

Numbers in parentheses are values expressed as percentage of the control.

of NADPH in isolated rat hepatocytes of untreated fed animals (Table 6) has been previously observed in hepatocytes isolated from starved untreated and phenobarbitone pretreated animals [11]. These reductions of NADPH levels (Table 6) were mirrored by reductions in biphenyl 4-hydroxylase activity in isolated hepatocytes from both untreated and phenobarbitone-pretreated rats (75 per cent and 48 per cent respectively of control values). In addition to its effects on NADPH levels rotenone may also inhibit biphenyl metabolism by directly competing with it for binding to cytochrome P-450. It has been reported [12, 13] that rotenone is hydroxylated to form rotenolones and that these reactions are brought about by the cytochrome linked monooxygenase P-450 system of the liver microsomes [14]. In hepatocytes derived from fed animals the direct effects of rotenone on the microsomal system are probably much more important than its mitochondrial interactions in causing inhibition of phase I xenobiotic metabolism. This view is supported by the finding that the effect of rotenone in inhibiting phase I reactions is greater in microsomes than in intact cells (Table 5, cf. Table 1). Also, 2,4-dinitrophenol does not inhibit Phase I metabolism although it produces a more significant reduction of NADPH levels than rotenone. The insensitivity of the hydroxylation rate to a lowering of the NADPH level suggests either that the NADPH concentration is not rate limiting in fed animals or that the NADPH for cytochrome P-450 reductase is compartmentalised and therefore the NADPH measurements made in the present study do not fairly reflect the levels available for drug metabolism.

Since it has been shown that the rotenone effect in hepatocytes is not directly on the conjugation enzymes (N.B., it has no effect on conjugation in liver microsomes [15]), it is probably decreased synthesis of the essential conjugating cofactors, 3′-phosphoadenosine 5′phosphosulphate (PAPS) and uridinediphosphoglucuronic acid (UDPGA) which is responsible for the decreased conjugation. This is a likely consequence of the decreased availability of ATP (see Table 6).

Menadione (1–50 μM) has been reported [16] to cause a marked stimulation in the oxidation of NADPH and a marked decrease in the demethylation of aminopyrine and *p*-chloro-*N*-methylaniline in rat liver microsomes; it also stimulates mitochondrial respiration [17]. In the present studies monooxygenase activity was found to be reduced by menadione to very low levels both in isolated hepatocytes (untreated and phenobarbitone induced) and in rat liver microsomes, but the reduction in the concentration of NADPH observed in microsomal suspensions [16] was not seen in isolated hepatocytes, rather, an increase was seen. As well as increasing the rate of NADPH oxidation it is likely that menadione also produces an increase in the rate of NADPH generation via the hexose monophosphate (HMP) shunt in the cells, in a like manner to the effect of phenazine methosulphate in stimulating reduced pyridine nucleotide generation via the HMP shunt [18]. Treatment of hepatocytes with menadione therefore has a dual cellular interaction, on the one hand increasing the amount of oxidised cofactor through an "uncoupling effect" on the transfer of electrons to cytochrome P-450 but on the other channelling oxidised cofactor equivalents into the HMP shunt pathway to increase NADPH generation. The uncoupling of the microsomal respiratory system rather than a mitochondrial effect is presumably responsible for the decrease in the P-450 mediated hydroxylation P-450 mediated hydroxylation seen in the present study, since in isolated microsomes menadione has a major inhibiting effect.

The reduction in the hepatocyte ATP content may be explained by the fact that menadione causes the electrons in the mitochondrial transport chain to bypass at least one of the phosphorylation sites present within the chain [19] and this may lead in turn to the reduction in UDPGA and PAPS levels with a concomitant lowering of conjugating ability.

The primary effect of 2,4-dinitrophenol in isolated hepatocytes and possibly the most significant in terms of the effect on drug metabolism activity is the inhibition of ATP synthesis, which is reflected in the reduction of cellular ATP levels. This is likely to result in a marked decrease in the synthesis of PAPS and UDPGA and hence the sulphotransferase and glucuronidation activity within the cells. This effect on the conjugation of added substrate is exacerbated by the fact that 2,4-dinitrophenol is itself a substrate for sulphate and glucuronic acid conjugation and therefore it is also likely to serve as a competitive inhibitor.

2,4-Dinitrophenol was without effect on cytochrome P-450-linked mono-oxygenase activity in rat liver microsomes. It did not influence benzo[*a*]pyrene hydroxylase or 7-ethoxycoumarin de-ethylase activity in intact hepatocytes but caused a stimulation of biphenyl 4-hydroxylation. This stimulating effect of 2,4-dinitrophenol on biphenyl 4-hydroxylase in hepatocytes but not microsomes from untreated rat is difficult to explain. 2,4-Dinitrophenol has been shown by us to be metabolised by isolated rat hepatocyte suspensions by route(s) other than conjugation (data not shown), possibly by reduction of one or other of the nitro groups on the molecule, to the appropriate aminonitrophenol or to the diaminophenol as has been shown both *in vivo* [20, 21] and *in vitro* [20, 22, 23]. It is conceivable therefore that a reduction product(s) of 2,4-dinitrophenol may "activate" in some way a particular form of cytochrome P-450 that is involved in biphenyl metabolism and not in 7-ethoxycoumarin and benzo[*a*]pyrene metabolism. On this argument the reason for the failure of isolated microsomes to display activation of biphenyl 4-hydroxylase is that dinitrophenol reduction occurs in the cytosol [22,23]. The increase in biphenyl 4-hydroxylation is also not seen in phenobarbitone-treated cells presumably because the "latent" P-450 species involved in activation has been changed by this treatment. It is noteworthy that whereas biphenyl 4-hydroxylation is primarily a cytochrome P-450-mediated reaction, 7-ethoxycoumarin *O*-de-ethylation and benzo-[*a*]pyrine hydroxylase activity is mediated primarily by another cytochrome P-450 species, cytochrome P-448.

It is possible that the changes in drug metabolising pattern mediated by the metabolic inhibitors might

be indicative of irreversible cell damage but there is no indication of such damage in these studies by viability measurements involving dye uptake. Experimentally, this possibility would be extremely difficult to test for as it would be difficult to effectively remove the inhibitor within the short time scale of the experiments.

The present studies with metabolic inhibitors in isolated hepatocyte suspensions have demonstrated that Phase II metabolism of xenobiotics is more dramatically affected than is Phase I metabolism. It is likely that in fed animals the NADPH required for P-450 mediated reactions is derived mainly from the HMP shunt, therefore mitochondrial inhibitors have a relatively minor effect on xenobiotic oxidation. In starvation, with a glycogen depleted liver in which the generation of reducing equivalents is almost entirely mitochondrial, mitochondrial inhibitors may be expected to have much greater influence on xenobiotic Phase I metabolism.

The increased amounts of free hydroxybiphenyl which escapes from the cells in the early phase of biphenyl metabolism [4] indicates that there is no immediate tight coupling of Phase I and Phase II reactions, rather the oxidation and conjugation reactions are probably largely independent of one another. This is at variance with studies carried out in microsomes which propose that Phase I reactions by the cytochrome P-450-mediated mono-oxygenase system and glucuronic acid conjugations are in some way coupled [24–26] but in agreement with studies which have demonstrated that glucuronic acid conjugation can be induced by disulfiram concomitant with inhibition of MFO activity [27] and studies in isolated rat hepatocytes [4] and perfused rat liver [28] which demonstrate that biphenyl 4-hydroxylation and subsequent glucuronic acid conjugation are not tightly coupled. The results with 2,4-dinitrophenol on the conjugation of the oxidation products of biphenyl, benzyrene and 7-ethoxycoumarin presented in this paper futher substantiate the proposition that, in intact cells, Phase I and Phase II xenobiotic reactions are not *de facto* tightly coupled.

The possible coupling between the microsomal mono-oxygenase system which generates arene oxides and the cytosol glutathione S-epoxide transferase which conjugates the Phase I generated oxide could not be studied using the system described in this paper since, although 4-hydroxybiphenyl is believed to be produced from biphenyl via an arene oxide [29], glutathione-derived conjugates represent only a small percentage of the total biphenyl metabolites excreted by the rat [30].

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