Biochemical Pharmacology, Vol. 27, pp. 1899–1907

0006-2952/78/0801-1899$02.00/0

BIPHENYL METABOLISM IN ISOLATED RAT HEPATOCYTES: EFFECT OF INDUCTION AND NATURE OF THE CONJUGATES

PHILIP WIEBKIN, JEFFREY R. FRY, CAROL A. JONES, RAYMOND K. LOWING and JAMES W. BRIDGES

Department of Biochemistry, University of Surrey, Guildford, Surrey, GU2 5XH, England

(*Received* 1 *September* 1977; *accepted* 13 *January* 1978)

Abstract—Biphenyl is metabolised by untreated isolated rat hepatocytes via hydroxylation at the 4-position followed by conjugation with sulphate and glucuronic acid. At a substrate concentration of 70 μM, the major metabolite produced is 4-hydroxybiphenyl sulphate. Hydroxylation at the 2- and 3-positions also occurs but to a much smaller extent. Induction *in vivo* by phenobarbitone or 3-methylcholanthrene results in a marked increase in the rate of the initial hydroxylation and a change in the pattern of the conjugates with 70 μM biphenyl. In the induced state 4-hydroxybiphenyl glucuronide is the dominant metabolite, 4-hydroxybiphenyl sulphate production being similar to controls. Two- and 3-hydroxylation of biphenyl in isolated rat hepatocytes is markedly increased by 3-methylcholanthrene pretreatment, but is unaffected by phenobarbitone pretreatment. Studies on the metabolism of two of the primary metabolites of biphenyl, 2- and 4-hydroxybiphenyl, in untreated isolated rat hepatocyte suspensions revealed that whereas at low (7 μM) concentrations of both metabolites, sulphate conjugation was predominant, with increasing substrate concentration the contribution of the glucuronidation pathway was elevated to such an extent that it became the predominant conjugating pathway at the highest concentrations used (140 μM). The reasons for the lag between the 'initial hydroxylation' and the onset of glucuronic acid conjugation of 4-hydroxybiphenyl were investigated. Attempts were made to eliminate this lag by prior incubations of the cells with 4-methylumbelliferone in an effort to activate the conjugating enzymes responsible. Glucuronidation of 4-hydroxybiphenyl is activated significantly by this treatment, whereas sulphation of 4-hydroxybiphenyl is not.

In a previous paper [1] we drew attention to the value that isolated suspensions possess as an *in vitro* system for total cellular drug metabolism. Using biphenyl as substrate we spotlighted some of the aspects of xenobiotic metabolism which do not readily lend themselves to study using conventional microsomal techniques. These include the toxicity of the substrate and products, further metabolism of primary metabolities and the relationship between the various metabolic pathways.

Certain questions were however left unresolved including the nature of the conjugates produced, the effect of drug metabolism inducing agents and the reasons for the lag in the onset of conjugation of 4-hydroxybiphenyl. We now report on the precise nature and extent of the conjugates produced with biphenyl and two of its primary metabolites, 2- and 4-hydroxybiphenyl, and the influence of *in vivo* pretreatment with phenobarbitone and 3-methylcholanthrene on the Phase I and Phase II metabolism of biphenyl by isolated rat hepatocytes. The 'lag' in the onset of conjugation of the major primary metabolite 4-hydroxybiphenyl [1] has also been further investigated.

MATERIALS AND METHODS

Cells, viability as judged by dye exclusion, were isolated [2], diluted to 2×10^6 viable cells/ml in L-15 medium supplemented with 10% foetal calf serum and incubated with biphenyl, final concentration 70 μM as described before [1]. The biphenyl hydroxylated products were determined fluorimetrically in the whole cell suspensions as described in [3]. The 'deconjugation' procedure has been modified in order that the extent of sulphation and glucuronidation can be determined separately. One-ml aliquots of the aqueous phase remaining after the heptane extraction of the free metabolites were treated with either ketodase, which was found to be free of aryl sulphatase activity, (2500 units), (William Warner & Co. Ltd., Eastleigh, Hampshire) or aryl sulphatase (375 units), (Type HI. Sigma, London). Saccharo-1-4β-lactone (Sigma, London) at a final concentration of 20 mM was incorporated into the aryl sulphatase in order to inhibit the contaminating β-glucuronidase activity that is present in the aryl sulphatase preparation used. The aryl sulphatase and saccharo-1-4β-lactone were both dissolved in 0.2 M acetate buffer pH 4.5; the ketodase was purchased as a solution in the same buffer. Tubes were incubated overnight in a shaking water bath at 37° and the biphenyl hydroxylated products released by the enzyme treatments determined as before. In experiments in which preincubation with 1-methylumbelliferone (Sigma, London) was carried out, the cells were centrifuged at 50 *g* for 1 min after 60 min treatment at 37°, the medium removed, replaced with the same volume of fresh medium and then incubated with biphenyl.

High pressure liquid chromatographic analysis of hydroxylated biphenyl products from hepatocytes incubated with biphenyl was carried out as described in [4].

The sources and purification of biphenyl and its metabolites were as described previously [1].

Pretreatment of animals. Treated rats were given a daily i.p. dose of either sodium phenobarbitone in 0.9% NaCl, 80 mg/kg body wt for 3 days or 3-methylcholanthrene in corn oil, 30 mg/kg body wt for 2 days. At least 20 hr were always allowed to elapse between the last injection and sacrifice of animals.

Although the data presented in the various Tables and Figures are mainly derived from single experiments, each study was performed at least twice with similar results in each experiment. Each value quoted is the mean of either two or three separate estimations.

RESULTS

We previously reported that after 45 min incubation of isolated rat hepatocytes with 70 μM biphenyl almost all the detectable 4-hydroxybiphenyl produced is conjugated [1]. However, the precise nature of the conjugates produced was uncertain since the β-glucuronidase preparation used contained 10% sulphatase activity. Using the modified 'deconjugation' procedure the time course for the production of free and conjugated 4-hydroxybiphenyl has been determined (see Fig. 1). It can be seen that 4-hydroxybiphenyl sulphate is the major conjugated metabolite formed by isolated rat hepatocytes from untreated rats. 4-Hydroxybiphenyl glucuronide is formed, but to a far lesser extent. It is interesting to note that there is an initial lag in the glucuronide conjugation of the newly produced free 4-hydroxybiphenyl, glucuronide formation being barely detectable over the first 10–15 min of incubation with biphenyl. Sulphate conjugation of the free 4-hydroxybiphenyl does not exhibit this 'lag' effect.

This time course differs slightly to the one previously reported [1]. This is due in our opinion to the crude β-glucuronidase preparation used in that particular study. The use of the more sophisticated 'deconjugation' procedure in the present study in which pure enzyme preparations were employed separately makes it possible to obtain results which more closely reflect the true profile of the time course.

The effect of pretreatment of animals with pheno-

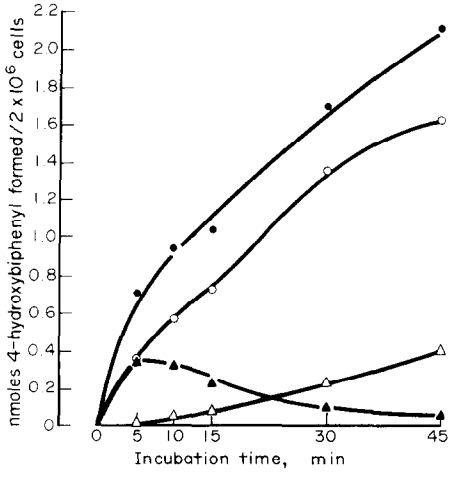

Fig. 1. Time course of the appearance of free and conjugated 4-hydroxybiphenyl in suspensions of hepatocytes from untreated rats. Cells (2×10^6 per ml) were incubated with 70 μM biphenyl for the times indicated at 37° and the free and conjugated 4-hydroxybiphenyl estimated fluorimetrically as described in Methods. Values are 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.

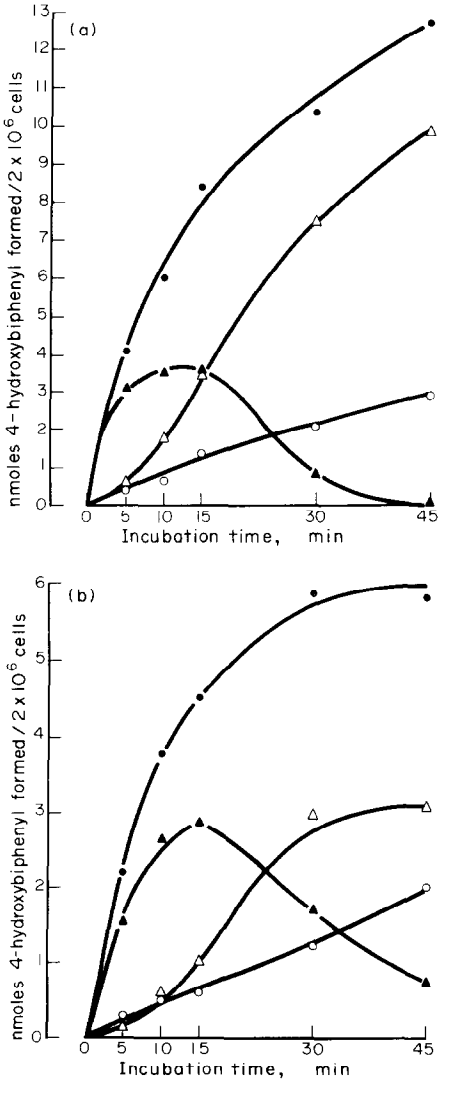

Fig. 2. Time course of the appearance of free and conjugated 4-hydroxybiphenyl in suspensions of hepatocytes from phenobarbitone or 3-methylcholanthrene pretreated rats. Cells (2×10^6 per ml) were incubated with 70 μM biphenyl for the times indicated at 37° and the free and conjugated 4-hydroxybiphenyl estimated fluorimetrically as described in Methods. Values are means of duplicate estimations differing by less than 10 per cent from each other. (a) Cells from phenobarbitone-pretreated rat; (b) cells from 3-methylcholanthrene-pretreated rat. ▲—▲ Free 4-hydroxybiphenyl; △—△ 4-hydroxybiphenyl glucuronide; ○—○ 4-hydroxybiphenyl sulphate; ●—● Total 4-hydroxybiphenyl.

Table 1. Effect of induction on the formation of 4-hydroxybiphenyl and its subsequent conjugation in suspensions of rat hepatocytes incubated with biphenyl

	Treatment		
	Control	Phenobarbitone	3-Methylcholanthrene
Apparent initial rate* of total 4-hydroxybiphenyl produced	0.14	0.80 (470)	0.44 (214)
Total† 4-hydroxybiphenyl produced	2.1	12.8 (510)	5.8 (176)
Apparent initial rate* of 4-hydroxybiphenyl produced	0.07	0.08 (14)	0.05 (—)
4-hydroxybiphenyl† sulphate produced	1.6	2.9 (80)	2.0 (25)
Apparent initial rate* of 4-hydroxybiphenyl glucuronide produced	0.002	0.120 (5900)	0.030 (1400)
4-hydroxybiphenyl† glucuronide produced	0.4	9.9 (2375)	3.1 (675)

* Rate expressed as nmoles produced/min/2 × 10^6 cells over the initial 5 min incubation period.
† Amounts expressed as nmoles produced/2 × 10^6 cells in 45 min.
Numbers in parentheses are values as a percentage increase of control.
Values are means of duplicate estimations differing by less than 10 per cent from each other.

barbitone and 3-methylcholanthrene on biphenyl metabolism in isolated rat hepatocytes is shown by the time courses in Figs 2a and b. The yields and viabilities of the hepatocytes isolated from the livers of the treated animals were similar to those obtained from the untreated animals.

The amount of 4-hydroxybiphenyl produced is markedly increased and the pattern of 4-hydroxybiphenyl conjugates is altered quite significantly by either treatment. The major conjugate produced by both phenobarbitone and 3-methylcholanthrene-treated cells is 4-hydroxybiphenyl glucuronide. The lag in the onset of conjugation of free 4-hydroxybiphenyl is even more marked in the treated than in untreated cells. There is a 470 per cent increase in the initial rate of total 4-hydroxybiphenyl production by phenobarbitone-treated hepatocytes and a 200 per cent increase by 3-methylcholanthrene-treated hepatocytes when compared to untreated cells (Table 1). Whereas the total production of 4-hydroxybiphenyl sulphate after 45 min incubation with biphenyl is increased only to a small degree in both phenobarbitone and 3-methylcholanthrene cells, there is a massive increase in the production of 4-hydroxybiphenyl glucuronide, 2375 per cent, in phenobarbitone-treated cells and a 675 per cent stimulation in 3-methylcholanthrene-treated cells when compared to untreated cells (Table 1).

Fluorimetric analysis of the hydroxylated products of biphenyl metabolism does not resolve 2- and 3-hydroxybiphenyl, and these can only be treated as a single metabolite. High pressure liquid chromatography can resolve these metabolites and studies reveal that 3-hydroxybiphenyl is a minor metabolite in untreated cells but increases to quite significant levels in 3-methylcholanthrene-induced cells (Table 2).

Two- and 3-hydroxylation of biphenyl by isolated rat hepatocytes occurs at very low levels and the time course of the production of free and conjugated 2- and 3-hydroxybiphenyl by untreated cells is shown in Fig. 3a. The picture is very similar to that of the 4-hydroxylation of biphenyl, free 2- and 3-hydroxybiphenyl production reaching a maximum after 10 min incubation and decreasing to undetectable levels by 45 min. A lag in the onset of conjugation of the free 2- and 3-hydroxybiphenyl also exists but by 45 min all the 2- and 3-hydroxybiphenyl

Table 2. Effect of 3-methylcholanthrene induction on biphenyl metabolism in suspensions of rat hepatocytes incubated with biphenyl using high pressure liquid chromatographic analysis

Treatment	Metabolites produced	2-hydroxybiphenyl produced	3-hydroxybiphenyl produced	4-hydroxybiphenyl produced
Control	Free	—‡	—	0.006 (0.01)
	Sulphate	—	0.050 (0.07)	0.720 (1.00)
	Glucuronide	0.007* (0.01)†	—	0.054 (0.07)
3-Methylcholanthrene	Free	0.021 (0.03)	0.016 (0.02)	0.234 (0.32)
	Sulphate	0.081 (0.11)	0.272 (0.38)	1.306 (1.81)
	Glucuronide	0.178 (0.25)	0.135 (0.19)	2.082 (2.89)

* Amounts expressed as peak heights (cm).
† Figures in brackets indicate approximate values expressed as nmoles product/25 min/2 × 10^6 cells. Various technical difficulties limited accurate quantitation of the peak height values.
‡ Hydroxylated biphenyl products were not detected.

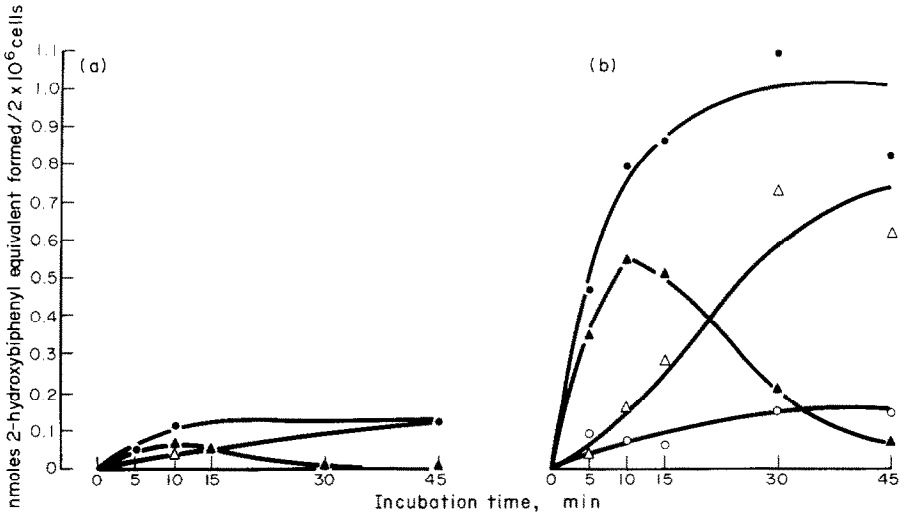

Fig. 3. Time course of the appearance of free and conjugated 2- and 3-hydroxybiphenyl in suspensions of rat hepatocytes. Cells were incubated with 70 μM biphenyl for the times indicated at 37° and the free and conjugated 2- and 3-hydroxybiphenyl estimated fluorimetrically as described in Methods. Values are means of duplicate estimations differing by less than 10 per cent from each other. (a) Cells from untreated rat; (b) cells from 3-methylcholanthrene-pretreated rat. ▲—▲ Free 2- and 3-hydroxybiphenyl; △—△ 2- and 3-hydroxybiphenyl glucuronide; ○—○ 2- and 3-hydroxybiphenyl sulphate; ●—● Total 2- and 3-hydroxybiphenyl.

is in the conjugated form. In contrast to the 4-hydroxybiphenyl conjugation situation, however, no 2-hydroxybiphenyl sulphate could be detected with the untreated cells; all the 2-hydroxybiphenyl conjugate present being in the form of the glucuronide. 3-Hydroxybiphenyl, in contrast, did form a significant amount of the sulphate conjugate (Table 2). Phenobarbitone pretreatment of cells did not affect the level or pattern of 2-hydroxylation of biphenyl. However, 3-methylcholanthrene pretreatment of cells resulted in a dramatic increase in the amounts of both free and conjugated 2- and 3-hydroxybiphenyl produced after 45 min incubation with biphenyl (Fig. 3b). As with the untreated cells the amount of free 2- and 3-hydroxybiphenyl present is at a maximum at 10 min and returns to low levels by 45 min of incubation concomitant with a rise in conjugated products. The lag in conjugation is more marked in cells from 3-methylcholanthrene-treated animals than in cells obtained either from control or phenobarbitone-pretreated animals. Both 2-hydroxybiphenyl sulphate and glucuronide are formed, the glucuronide being the major metabolite. Three-hydroxybiphenyl sulphate and glucuronide conjugates are also formed; the major metabolite is the sulphate (Table 2).

To investigate the possibility that the lag phase represents activation of the conjugating enzymes by the Phase I metabolite, hepatocytes were incubated with an alternative substrate, prior to the addition of 70 μM biphenyl. In an attempt to eliminate the lag phase 4-methylumbelliferone was used as this particular substrate has been shown by us (unpublished data) to be rapidly metabolised to the glucuronide and sulphate and then eliminated from the cells. 4-Methylumbelliferone, 0.7 μM, was used as this level did not result in any significant drop in hepatocyte viability (less than 10 per cent after 60 min at 37° compared to the appropriate controls). Furthermore, removal of 4-methylumbelliferone

Table 3. Effect of preincubation with and without 0.7 μM 4-methylumbelliferone

Route of conjugation	Experiment number	Rate of 4-hydroxybiphenyl conjugation: Control no preincubation	Control 60 min preincubation	4-methylumbelliferone 60 min preincubation	Per cent increase of 4-methylumbelliferone rate over 60 min control
Sulphate	1	0.040*	0.020	0.020	0
	2	0.070	0.040	0.060	50
Glucuronide	1	0.002	0.004	0.012	200
	2	0.000	0.008	0.020	150

* All values are nmoles 4-hydroxybiphenyl conjugate formed/min/2 × 10^6 cells measured over the first 10 min of incubation with biphenyl.

Values are the means of duplicate estimations differing by less than 10 per cent from each other.

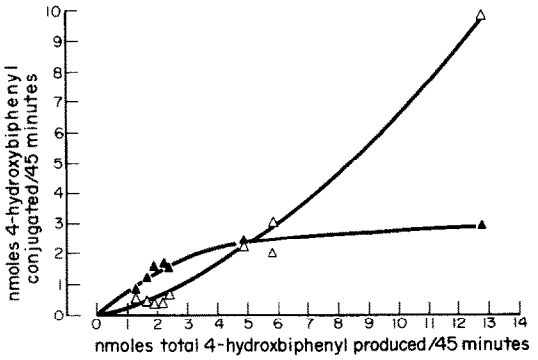

Fig. 4. Relationship between the amount of 4-hydroxybiphenyl conjugated and the total 4-hydroxybiphenyl produced in suspensions of rat hepatocytes. Cells (2×10^6/ml) from untreated and phenobarbitone or 3-methylcholanthrene pretreated rats were incubated with 70 μM biphenyl for 45 min at 37° and the free and conjugated 4-hydroxybiphenyl estimated fluorimetrically as described in Methods. Values are means of duplicate estimations differing by less than 10 per cent from each other. ▲—▲ 4-Hydroxybiphenyl sulphate; △—△ 4-hydroxybiphenyl glucuronide.

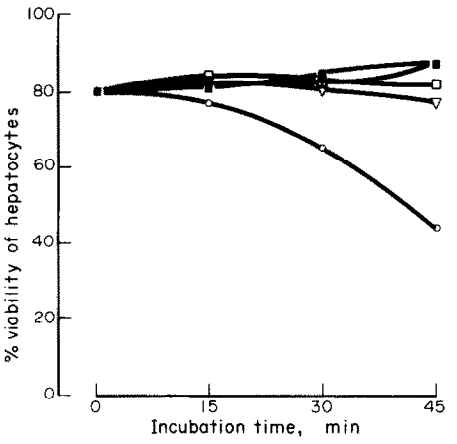

Fig. 5. Effect of 4-hydroxybiphenyl on viability of isolated rat hepatocytes from untreated animals. Cells (2×10^6/ml) were incubated with various concentrations of 4-hydroxybiphenyl for various time periods and samples were removed and assessed for viability as judged by dye exclusion. All suspensions contained 0.2% v/v dimethylformamide. Four-hydroxybiphenyl concentrations: ■—■ none; □—□ 7 μM; ▼—▼ 70 μM; ▽—▽ 140 μM; ○—○ 700 μM. Values are means of duplicate estimations differing by less than 10 per cent from each other.

from the cells as conjugates was complete after 60 min so that competition between this substrate and biphenyl is unlikely in these experiments. This preincubation of the hepatocytes with 0.7 μM 4-methylumbelliferone (see Table 3) increased the initial rate of the glucuronidation of 4-hydroxybiphenyl quite considerably over the appropriate control (175 per cent) while the initial rate of the sulphation of 4-hydroxybiphenyl was relatively unaffected by this treatment (25 per cent). It is also worthy of note that even controls preincubated for 60 min at 37° without any addition of substrate for conjugation showed a significant increase in the initial rate of 4-hydroxybiphenyl glucuronide formed together with a significant decrease in the formation of 4-hydroxybiphenyl sulphate when biphenyl was subsequently added (Table 3).

When the amount of total 4-hydroxybiphenyl produced after 45 min incubation of isolated rat hepatocytes with biphenyl is increased (as after induction), there is a marked change in the pattern of the 4-hydroxybiphenyl conjugates produced (see above). At low rates of production of total 4-hydroxybiphenyl, as with cells from non-induced animals, the sulphate conjugate predominates, while generation of large amounts of 4-hydroxybiphenyl, as with cells from phenobarbitone- or 3-methylcholanthrene-induced animals, leads to the glucuronide conjugate becoming the major metabolite (Fig. 4). There is a 'cross-over' (i.e. when the extent of sulphate and glucuronide produced is equivalent), which occurs when both conjugates reach a value of about 2.5 nmoles produced/45 min/2×10^6 cells.

To investigate this aspect further, incubations were carried out with isolated rat hepatocytes over a range of concentrations (7–700 μM) of the major primary metabolite, 4-hydroxybiphenyl. The upper concentration range of 4-hydroxybiphenyl which could be studied was limited by the toxicity of this compound to the cells; thus when cells were incubated at a concentration of 700 μM with isolated hepatocytes for 45 min a great decrease in cell viability was noted when compared with the dimethyl formamide control (Fig. 5) as judged by Trypan Blue dye exclusion. In the concentration range 7–140 μM, 4-hydroxybiphenyl induced no detectable toxic response from the cells (Fig. 5). Accordingly, concentrations of 4-hydroxybiphenyl in this range were used. The effect of 4-hydroxybiphenyl concentration on the nature and extent of the conjugates produced after 45 min incubations with isolated rat hepatocytes is summarised in Fig. 6. At low concentrations of 4-hydroxybiphenyl the major metabolite is 4-hydroxybiphenyl sulphate, the glucuronide being formed to a far lesser degree. However, with increase in substrate concentration glucuronidation quickly takes over as the major route of conjugation. The 'cross-over' point occurs when both conjugation rates reach a value of approximately 3 nmoles produced/45 min/2×10^6 cells (Fig. 6b), i.e. at a 4-hydroxybiphenyl concentration of about 20 μM (Figs 6a and b). In the 4-hydroxybiphenyl concentration range studied (7–140 μM) the total amount of sulphate and glucuronide conjugates both increase with increasing substrate concentration (Fig. 6b), i.e. total saturation of the sulphate conjugation mechanism is not obtained. However, the increase in the rate of glucuronic acid conjugation is approximately four times greater than that of sulphate conjugation.

Incubations were also performed with 2-hydroxybiphenyl, a minor primary metabolite of biphenyl metabolism in isolated rat hepatocytes over the concentration range (7–700 μM). As with 4-hydroxybiphenyl, 700 μM 2-hydroxybiphenyl was found to be toxic to the cells after 45 min incubation whereas 7–140 μM concentrations of 2-hydroxybiphenyl were not. At low concentrations of 2-hydroxybiphenyl the sulphate conjugate was again produced

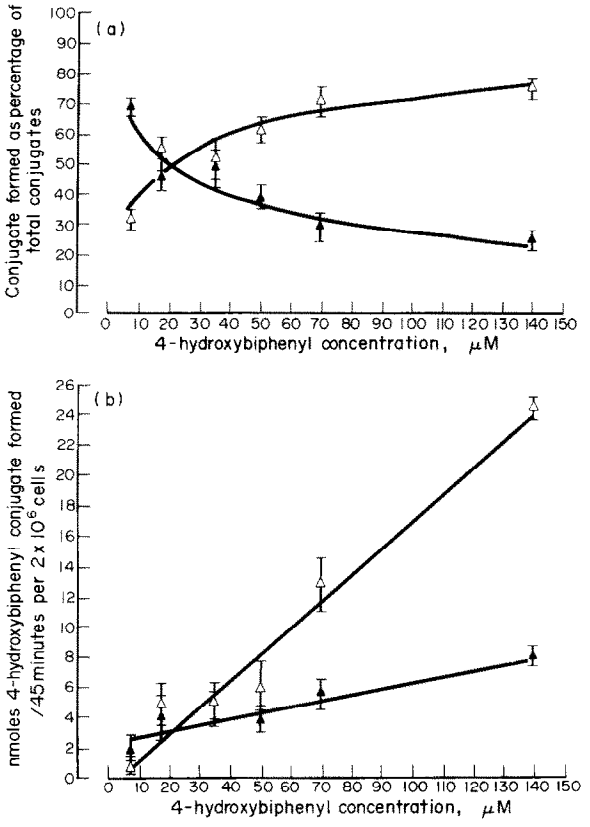

Fig. 6. Effect of 4-hydroxybiphenyl concentration on the formation of 4-hydroxybiphenyl sulphate and glucuronide in suspensions of rat hepatocytes from untreated animals. Cells (2×10^6/ml) were incubated with 7–140 μM 4-hydroxybiphenyl at 37° for 45 min. Conjugated 4-hydroxybiphenyl was estimated fluorimetrically as described in Methods. Each point is the mean of three separate experiments. (a) Relative proportion of conjugates formed; (b) amounts of conjugates produced. Bars are S.E.M. ▲—▲ 4-Hydroxybiphenyl sulphate; △—△ 4-hydroxybiphenyl glucuronide.

to a greater extent than the glucuronide but with increasing substrate concentration the glucuronide quickly takes over as the major route of conjugation (Fig. 7a). The 'cross-over' point in 2-hydroxybiphenyl metabolism in isolated rat hepatocytes occurs at a value of approximately 0.5 nmoles conjugates produced/45 min/2×10^6 cells (Fig. 7b), i.e. at a 2-hydroxybiphenyl concentration of about 8 μM (Figs 7a and b); this figure is much lower than that obtained for 4-hydroxybiphenyl.

The total extent of conjugation of 2-hydroxybiphenyl over the concentration range studied (7–140 μM) is thus the same as occurs with 4-hydroxybiphenyl, but there is a marked reduction in the sulphate conjugates, together with a concomitant increase in the glucuronide conjugates of 2-hydroxybiphenyl to that produced at an equivalent 4-hydroxybiphenyl concentration. Total saturation of the sulphate conjugation mechanism is again not obtained with 2-hydroxybiphenyl in the concentration range studied (7–140 μM) as the amount of sulphate and glucuronide conjugate both increase with increasing substrate concentration (Fig. 7b). The increase in the rate of glucuronic acid conjugation with increase in 2-hydroxybiphenyl is thirty times greater than that of sulphate conjugation.

DISCUSSION

Although many biochemical investigations have been carried out using viable hepatocytes isolated by enzymic methods [2, 5–8] it is only fairly recently that these studies have been extended to the Phase I metabolism of xenobiotic compounds [1, 2, 9–15]. The importance of the subsequent conjugation of the Phase I metabolites produced by isolated rat liver cells has been largely neglected. Since this appears to be an integral part of the mechanism by which the intact cell eliminates potentially toxic metabolites, we have investigated the nature and control of conjugation in isolated viable hepatocytes using 2- and 4-hydroxybiphenyl as model substrates.

Effects of induction on biphenyl metabolism. In a previous paper [1] we reported on the metabolism of biphenyl by hepatocytes isolated from control untreated rats and concluded that the major metabolite produced was 4-hydroxybiphenyl, present largely as a conjugate. A minor metabolite (and its conjugate) was also produced and this metabolite was attributed to 2-hydroxybiphenyl on fluorimetric analysis. These findings have been confirmed in the present study and, in addition, a number of new aspects have emerged: (1) The major conjugate pro-

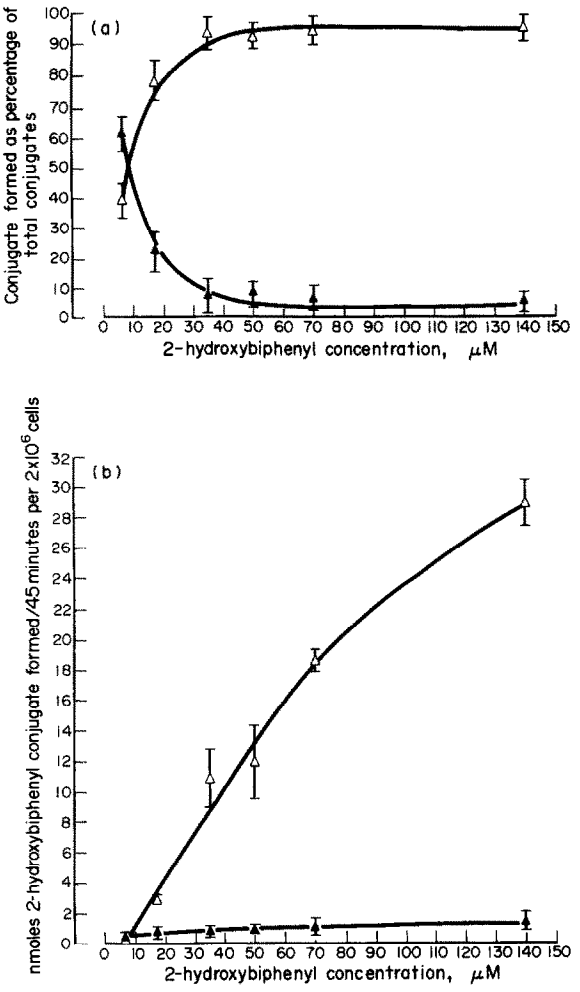

Fig. 7. Effect of 2-hydroxybiphenyl concentration on the formation of 2-hydroxybiphenyl sulphate and glucuronide in suspensions of rat hepatocytes from untreated animals. Cells (2×10^6/ml) were incubated with 7–140 μM 2-hydroxybiphenyl at 37° for 45 min. Conjugated 2-hydroxybiphenyl was estimated fluorimetrically as described in Methods. Each point is the mean of three separate experiments. (a) Relative proportion of conjugates formed; (b) amounts of conjugates produced. Bars are S.E.M. ▲—▲ 2-Hydroxybiphenyl sulphate; △—△ 2-hydroxybiphenyl glucuronide.

duced from newly formed 4-hydroxybiphenyl is a sulphate (Fig. 1) and this conjugate accounts for more than 75 per cent of the total 4-hydroxybiphenyl formed over a 45 min incubation period. The glucuronide conjugate produced represents only a minor (20 per cent) metabolite. (2) By use of high pressure liquid chromatography it has been possible to demonstrate that the minor metabolite previously ascribed to 2-hydroxybiphenyl comprises at least two metabolites, namely 2- and 3-hydroxybiphenyl, and that of these two metabolites the 3-hydroxybiphenyl is present in the greater amount (Table 2). Interestingly, the 2-hydroxybiphenyl was present solely as a glucuronide while the 3-hydroxy-isomer was present solely as a sulphate conjugate.

Biphenyl 4-hydroxylation is greatly induced in hepatocytes isolated from either phenobarbitone or 3-methylcholanthrene-pretreated rats (Table 1), phenobarbitone being the more potent inducer. These findings agree with earlier *in vitro* [1] and *in vivo* studies [17].

Phenobarbitone and 3-methylcholanthrene pretreatment of animals also affects the nature and extent of conjugates produced from newly formed 4-hydroxybiphenyl in the isolated hepatocytes. The increased rate of hydroxylation in the hepatocytes from induced rats leads to near-saturation of the sulphate conjugation pathway and thus any further free metabolite produced is conjugated with glucuronic acid (Table 1, Fig. 2). Because of this, the glucuronide becomes the major conjugate of 4-hydroxybiphenyl in situations of induced Phase I metabolism. Studies in which various levels of 4-hydroxybiphenyl were added to hepatocytes from control rats support these findings (Fig. 6), although total saturation of sulphate conjugation was not achieved.

The 'cross-over' of glucuronidation and sulphation of 4-hydroxybiphenyl in hepatocytes incubated with biphenyl (Fig. 4) and 4-hydroxybiphenyl (Fig. 6b) occurs at approximately the same point, i.e. when the rate for both conjugates reaches a value

of 2.5–3.0 nmoles produced/45 min/2 × 10^6 cells. This implies that the accessibility of 4-hydroxybiphenyl to both conjugating systems is the same whether the 4-hydroxybiphenyl is generated *in situ* or is added exogenously. With 2-hydroxybiphenyl the cross-over occurs at a rate of 0.5 nmoles produced/45 min/2 × 10^6 cells, illustrating that the position of the hydroxyl insertion on the biphenyl ring has a very significant influence on subsequent Phase II metabolism.

It is notable that despite the fact that 4-hydroxylation of biphenyl and glucuronidation are both located on the endoplasmic reticulum, it is sulphation, a cytoplasmic reaction, rather than glucuronidation that is the dominant conjugation mechanism for 4-hydroxybiphenyl at low biphenyl concentrations. This observation, together with the similarity of cross-over levels for 4-hydroxybiphenyl, suggests that there may be close association of the sulphation enzymes with the surface of the endoplasmic reticulum where the Phase I metabolites are generated.

Pretreatment with 3-methylcholanthrene *in vivo* has been shown to preferentially induce the 2-hydroxylation of biphenyl [17]. This can also be demonstrated using hepatocytes obtained from 3-methylcholanthrene rats (Fig. 3). However, by use of high pressure liquid chromatography we have shown that biphenyl 3-hydroxylation is also increased by this treatment (Table 2). The initial rate of total 2- and 3-hydroxylation is increased by 600 per cent in cells from 3-methylcholanthrene-induced animals (Fig. 3) with no increase detectable in biphenyl 2- and 3-hydroxylase activity in cells from phenobarbitone-induced animals (data not shown).

The large amounts of free 2-, 3- and 4-hydroxybiphenyls which escapes from the cells in the early phase of biphenyl metabolism indicates that there is no tight coupling of Phase I and Phase II reactions initially, rather the two phases may be largely independent of one another. Interactions between hydroxylation and glucuronidation systems, however, may occur later due to the activation of the glucuronidation reaction by the Phase I metabolites.

Lag in glucuronidation. We previously reported [1] a lag between the 4-hydroxylation of biphenyl and its subsequent conjugation, and postulated that either the cell isolation procedure in some way impaired conjugation, or that the presence of small amounts of 4-hydroxybiphenyl, arising as a Phase I metabolite, in some way 'activated' the conjugation. This conjugation lag phenomenon also exists in the formation of 2- and 3-hydroxybiphenyl and their subsequent conjugation and is especially seen in cells from 3-methylcholanthrene-induced animals.

Although the metabolic profile varied slightly between separate experiments, this lag was found to be only associated with the glucuronide conjugation of 4-hydroxybiphenyl, no such lag existing with sulphate conjugation (Fig. 1). Induction by phenobarbitone and 3-methylcholanthrene serves only to exaggerate this lag by increasing the rate of formation of free 4-hydroxybiphenyl during the first 15 min of incubation with biphenyl (Fig. 2).

The lag in glucuronidation occurs not only in isolated hepatocytes but also in the medium of liver slices incubated with biphenyl (unpublished work) and is therefore believed to be a true effect and not an artificial one arising from some peculiarity of the isolated hepatocyte system. It is of interest to note that activation of UDP-glucuronyl transferase in liver microsomal studies by detergents is well known and has been reported by several workers [18, 19]. The possibility that the galactose present as the sole sugar source in the L-15 medium by being metabolised to UDP-galactose, a known inhibitor of glucuronyl transferase [20], was responsible for the lag in 4-hydroxybiphenyl glucuronidation, was investigated by incubating hepatocytes in culture medium containing glucose instead of galactose. The extent and pattern of biphenyl metabolism (cf. Fig. 1) was the same irrespective of the sugar source used (unpublished work). Also at all time points assayed, the 4-hydroxybiphenyl glucuronide was only detected in the medium and thus a delay in excretion of the glucuronide from the cells could not account for this lag phenomenon.

Glucuronidation of 4-hydroxybiphenyl can be appreciably activated by preincubation of the cells with an alternative substrate (Table 3) although it must be emphasised that this activation did not eliminate the lag effect. Some activation of glucuronidation is even observed after preincubation in the absence of an alternative substrate and it is possible that this is due to replenishment, during the preincubation, of cofactor levels lowered during cell isolation. (It has been reported [21] that use of hyaluronidase in the cell isolation leads to a depletion of cellular glycogen stores and presumably UDPGA stores.) Sulphate conjugation was not stimulated by preincubation with the alternative substrate and in fact was depressed by preincubation in the absence of the alternative substrate. The reasons for this are not understood but may reflect re-channelling of the required cofactors to other cellular pathways. Thus, the evidence presented suggests that the lag in glucuronidation of Phase I metabolite generated *in situ* is due to activation of the glucuronidation pathway brought about either by the newly formed substrate or by increased levels of the necessary cofactors.

Acknowledgement—We wish to thank Miss Diane Benford for expert technical assistance with the high pressure liquid chromatographic studies.

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