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THE EFFECT OF ALBUMIN ON THE METABOLISM OF ETHOXYRESORUFIN THROUGH *O*-DEETHYLATION AND SULPHATE-CONJUGATION USING ISOLATED RAT HEPATOCYTES*

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Abstract—Ethoxyresorufin was metabolised by suspensions of isolated rat hepatocytes through *O*-deethylation to resorufin, followed by sulphate-conjugation of the resorufin. The deethylation but not the conjugation was greatly induced by 3-methylcholanthrene pretreatment *in vivo*. Induction altered the apparent deethylation V_{max} but not the apparent K_m value. With control hepatocytes there was a 3-fold difference between the apparent V_{max} values for deethylation (0.07) and conjugation (0.22 nmole/min/10^6 hepatocytes) but none between the apparent K_m values (1.3 μM, deethylation and 1.0 μM, conjugation). After induction the deethylation V_{max} (10 nmole/min/10^6 hepatocytes) was almost 13-fold higher than the conjugation V_{max} (0.81 nmole/min/10^6 hepatocytes), but again there was no difference in the K_m values for the two reactions (2 μM). A significant proportion of the deethylation product, resorufin, passed out from the hepatocytes and then re-entered them in order to undergo conjugation. Extracellular bovine serum albumin inhibited the conjugation by binding resorufin that had left the hepatocytes. Albumin greatly increased the total resorufin formed from ethoxyresorufin, despite inhibiting very slightly the initial rate of deethylation.

The study of the hepatic metabolism of drugs, carcinogens, steroids and many other classes of xenobiotic or endogenous compounds has progresssed very far in the last twenty years, with the use of liver tissue sub-cellular fractions and purified enzymes. A major uncertainty with these techniques is the relevance of their results to the liver *in situ*. Suspensions of isolated hepatocytes are now being increasingly used in order to study the metabolic oxygenation and conjugation of drugs. It is considered that isolated cells provide an example of a physiological yet experimentally controllable environment [1–8].

One of the most important drug-metabolising enzymes, cytochrome P450, has been successfully measured with suspensions of isolated hepatocytes [9], or adrenal cells [10], together with measurement of the type 1 spectral change that characterises the binding of many substrates to cytochrome P450 [1, 9, 11]. Substrates whose oxygenative metabolism has been studied with isolateed hepatocytes include benzo(a)pyrene [2], *p*-nitroanisole [4], ethylmorphine [5], biphenyl [6] and naphthalene [7]. Conjugation reactions have been measured with isolated hepatocytes for *p*-nitrophenol [4], 4-hydroxybiphenyl [6] and 1-naphthol [7].

We describe here an investigation with isolated hepatocytes into the *O*-deethylation of ethoxyresorufin to resorufin and the subsequent sulphate-conjugation of resorufin. Ethoxyresorufin *O*-deethylation is catalysed by an abnormal form of cytochrome P450, cytochrome P448, which is induced by 3-methylcholanthrene and some other compounds [12–14]. We describe also the effect of extracellular BSA‡ on the intracellular deethylation reaction.

MATERIALS AND METHODS

Animals. Male Sprague–Dawley rats, 200 g, were used. They were allowed food (pellet diet "R3", supplied by Astra-Ewos, Sodertälje) and water *ad libitum*. Some rats were injected with 3-methylcholanthrene (20 mg/kg body wt, as a 5 mg/ml solution in corn oil; i.p., daily for 3 days), while others were injected with corn oil (0.5 ml, i.p., daily for 3 days).

Chemicals. Ethoxyresorufin (99 per cent pure by high pressure liquid chromatography) was synthesised as described elsewhere [12]. Resorufin was obtained from Matheson, Coleman and Bell (East Rutherford, NJ, U.S.A.).

Preparation of isolated hepatocytes. Hepatocytes were isolated by a modification of the method of Berry and Friend [15], as described by Högberg and Kristoferson [16], with a further variation essential for isolating hepatocytes from 3-methylcholanthrene pretreated rats: the initial perfusion with Hank's buffer containing 2% BSA and 0.5 mM EGTA was continued for the prolonged period of 7 min, followed by perfusion for another 6 min with Hank's buffer containing collagenase (1 mg/ml) and 4 mM $CaCl_2$. Very few hepatocytes were isolated from the pretreated rats unless the prolonged initial perfusion time was used. The average yield of hepatocytes

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‡ The abbreviation used is: BSA—bovine serum albumin.

was 25 per cent (30×10^6/g liver), of which more than 90 per cent excluded exogenous NADH [16] and Trypan Blue. These two tests indicated that the cells were of good condition.

Ethoxyresorufin and resorufin metabolism. For the determination of ethoxyresorufin *O*-deethylation, approximately 1×10^6 hepatocytes were suspended, in a 1 cm-square fluorimeter cell, in Krebs–Henseleit buffer (pH 7.4, saturated with an O_2 (95%)—CO_2 (5%) mixture) calculated to give a final volume of 2 ml after addition of substrate. The temperature of the cuvette contents was maintained at 37°. The buffer generally did not contain any albumin, because this binds resorufin and interferes with ethoxyresorufin deethylation (see Results). A baseline of fluorescence at 585 nm was recorded, using an Aminco–Bowman spectrofluorimeter with excitation light of 530 nm. After about 1 min, ethoxyresorufin (0.1–5.0 μM, as a 0.01 mM solution in Krebs–Henseleit buffer) was mixed into the cuvette and its deethylation to resorufin was measured by the increase in fluorescence at 585 nm. Routine measurements with a single substrate concentration used 0.5 ml of ethoxyresorufin (2.5 μM cuvette concentration). Normally, 1.5 mM salicylamide (a 10 μl aliquot of a 0.3 M solution in acetone) was included in the reaction mixture to inhibit sulphate and glucuronate conjugation [17, 18]. This concentration of salicylamide did not inhibit the deethylation reaction. The measurements were calibrated by adding 1 nmole of authentic resorufin (a 10 μl aliquot of a 0.1 mM solution in ethanol) to the cuvette after a suitable period of reaction. Exogenous NADPH did not affect ethoxyresorufin deethylation or resorufin conjugation with isolated hepatocytes. This result was as expected for hepatocytes with an undamaged cell membrane [6].

For the determination of resorufin conjugation (predominantly sulphate-conjugation), approximately 1×10^6 hepatocytes were suspended in albumin-free Krebs–Henseleit buffer, as above, but salicylamide and ethoxyresorufin were omitted. Resorufin (10–300 nM, as a 0.01 mM solution in Krebs–Henseleit buffer) was added and its conjugation was measured by the loss of fluorescence at 585 nm (λ excitation = 530 nm). The identities of the conjugates were determined by lysing the hepatocytes through addition to the incubation mixture of an equal volume of acetone, sedimenting them, evaporating the acetone from the supernatant, adjusting this to pH 5.5 with acetate buffer and incubating it with either β-glucuronidase (6.5×10^3 Fishman units, Type B-10 from the Sigma Chemical Company, St. Louis, MO) or aryl sulphatase (19 units, Type V from the Sigma Chemical Company, St. Louis, MO) for 3 hr at 37° in the dark. Saccharo-1,4-lactone (2.5 mM) was added with the aryl sulphatase in order to inhibit traces of β-glucuronidase activity. Resorufin liberated by hydrolysis was measured fluorimetrically. The small quantities of hydrolase proteins present (less than 0.1% w/v) did not affect the resorufin fluorescence. The use of 50% acetone to terminate the resorufin incubations ensured that the hepatocytes were totally disrupted and that all the non-metabolised resorufin and at least 90 per cent of the further metabolites of resorufin remained in the deproteinated supernatant.

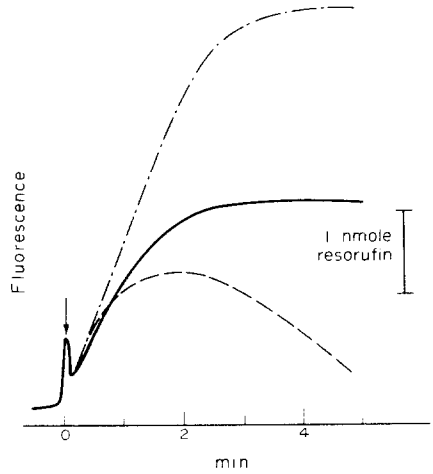

Fig. 1. Deethylation of ethoxyresorufin with isolated hepatocytes in the presence or absence of BSA or salicylamide 1×10^6 hepatocytes from a 3-methylcholanthrene pretreated rat were incubated with ethoxyresorufin (2.5 μM) in a fluorimeter cuvette, see Methods. The Fig. shows the change in sample fluorescence at 585 nm (λ excitation = 530 nm) after initiating the reaction by addition of the hepatocytes (marked by an arrow). The reaction was conducted with either ethoxyresorufin alone (– – – –), ethoxyresorufin plus 2% BSA (——), or ethoxyresorufin plus 1.5 mM salicylamide (– –).

Concentrations of resorufin greater than 500 nM were not tested for conjugation, since the relationship of fluorescence to concentration was not strictly linear above 400 nM resorufin.

RESULTS

Effects of BSA and salicylamide. When a suspension of hepatocytes isolated from a 3-methylcholanthrene pretreated rat was incubated with ethoxyresorufin in albumin-free buffer, there was first an increase and then a decrease in the deethylation product, resorufin, as measured by its characteristic fluorescence (Fig. 1). The presence of BSA (2%) in the buffer prevented the eventual loss of resorufin fluorescence, but inhibited slightly the rate of ethoxyresorufin deethylation. If instead of BSA the buffer contained salicylamide (1.5 mM), then much more resorufin was formed in a prolonged reaction. However, the initial rate was the same as with buffer alone and there was no subsequent decrease in resorufin fluorescence. An identical reaction rate profile was observed with salicylamide in the presence of 2% BSA.

The measured increase in fluorescence during the deethylation of ethoxyresorufin could be due to resorufin either within the hepatocytes or in the extracellular medium. It was fluorimetrically determined, after very slow speed centrifugation ($80 \times g$ for 1 min) of active hepatocytes in the presence of ethoxyresorufin, that the majority of free resorufin was distributed in the extracellular medium after 2 min of deethylation.

BSA-binding of resorufin. Since BSA affected the loss of resorufin much more so than its production from ethoxyresorufin, it was considered that any BSA-binding of ethoxyresorufin did not significantly influence the deethylation reaction. The lack of BSA-

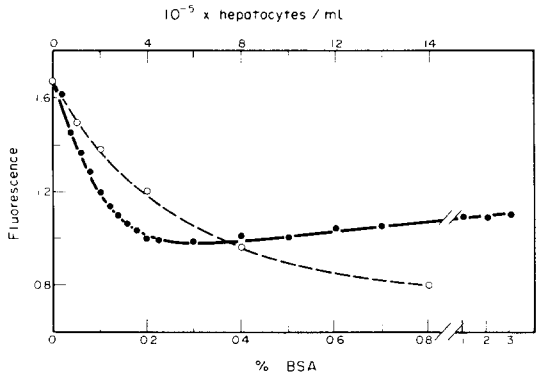

Fig. 2. Quenching of resorufin fluorescence by BSA or isolated hepatocytes

The fluorescence of resorufin (300 nM) in Krebs–Henseleit buffer containing a varying concentration of either BSA (——) or isolated hepatocytes from a 3-methylcholanthrene pretrated rat (----) was measured at 585 nm, with an excitation wavelength of 530 nm. The fluorescence values were measured relative to the fluorescence, at the same wavelengths, of a stable solution of rhodamine B (3.0 μg/ml in ethylene glycol; relative fluorescence = 20).

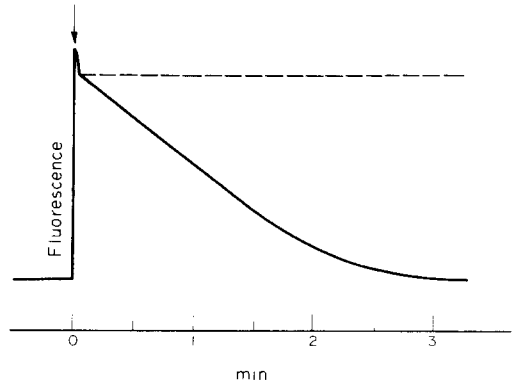

Fig. 3. Conjugation of resorufin with isolated hepatocytes in the presence or absence of salicylamide or BSA

1×10^6 hepatocytes from a 3-methylcholanthrene pretreated rat were incubated with resorufin (300 nM) in a fluorimeter cuvette, see Methods. The Fig. shows the change in sample fluorescence at 585 nm (λ excitation = 530 nm) after initiating the reaction by the addition of resorufin (marked by an arrow). The reaction was conducted with either resorufin alone (——) or resorufin plus either 1.5 mM salicylamide or 2% BSA (- - - -). Essentially similar results were obtained using isolated hepatocytes from a control rat.

binding of ethoxyresorufin was confirmed by a lack of any quenching-effect of BSA on ethoxyresorufin fluorescence (data not shown). In contrast the fluorescence of resorufin was markedly quenced by BSA (Fig. 2). This indicated that BSA-binding of resorufin occurred and might in some way account for the BSA-effect on ethoxyresorufin metabolism. A dissociation constant for the resorufin–BSA interaction was not obtained. Resorufin fluorescence was quenched also by the isolated hepatocytes themselves (Fig. 2). This was readily observed in BSA-free buffer, but was apparent in the presence of 2% BSA only at cell concentrations greater than 4.0×10^5 hepatocytes/ml. The percentage quenching:

$$100 - \left[\frac{\text{fluorescence with hepatocytes} \times 100}{\text{fluorescence without hepatocytes}}\right]$$

due to different hepatocyte concentrations in the presence of 2% BSA was: no cells—0%; 0.75×10^5 cells/ml—2%; 1.5×10^5 cells/ml—1%; 4.0×10^5 cells/ml—3%; 5.0×10^5 cells/ml—5%; 7.5×10^5 cells/ml—15%; 15×10^5 cells/ml—26%.

The 5 per cent quenching caused by the cell concentration (5×10^5/ml) normally used in the assay was corrected for by the calibration with standard resorufin.

Resorufin conjugation. Resorufin was metabolised by suspensions of isolated hepatocytes, from either control or 3-methylcholanthrene pretreated rats, to non-fluorescent products and this reaction was inhibited by salicylamide (Fig. 3). Since salicylamide inhibits glucuronate-[17] and sulphate-[18] conjugation reactions, it was presumed that resorufin was being converted to non-fluorescent conjugates. Hydrolysis with β-glucuronidase or aryl sulphatase confirmed that, for either control or 3-methylcholanthrene pretreated rats, 90 per cent of these were resorufin-sulphate conjugates and that no measurable glucuronides were formed (Table 1). The remaining 10 per cent of non-fluorescent resorufin metabolites, whose formation was also inhibited by salicylamide, were not identified. The presumed resorufin-sulphate conjugate was not isolated.

BSA in the cell-suspension buffer also inhibited the intracellular metabolism (presumably conjugation) of added resorufin to non-fluorescent products (Fig. 3). This was considered to be due to BSA binding of resorufin. A similar binding of resorufin

Table 1. Hydrolysis of non-fluorescent further metabolites of resorufin by β-glucuronidase or aryl sulphatase

Sample	Fluorescence
Hepatocytes and resorufin, before incubation	1.7 ± 0.2
Hepatocytes and resorufin, after incubation	0.1 ± 0.0
Post-incubation mixture, after β-glucuronidase	0.1 ± 0.0
Post-incubation mixture, after aryl sulphatase	1.4 ± 0.3

Resorufin (300 nM) was incubated for 20 min with hepatocytes isolated from a 3-methylcholanthrene pretreated rat, see Methods. Samples of zero-time incubation mixtures and of post-20 min incubation mixtures were mixed with an equal volume of acetone, their protein centrifuged down, the acetone evaporated by bubbling with nitrogen, and their fluorescence determined at 585 nm (excitation wavelength = 530 nm). Other samples of acetone-free, deproteinated post-incubation mixture were incubated with either β-glucuronidase or aryl sulphatase (+ 2.5 mM saccharo-1,4-lactone), see Methods, and their fluorescence determined (after readjusting their pH to 7.4). The fluorescence values are means ± S.D. for five samples. The relative fluorescence at the same wavelengths of rhodamine B (3.0 μg/ml in ethylene glycol) = 20. Virtually identical results were obtained in a similar experiment using isolated hepatocytes from a control rat.

Table 2. Apparent kinetics of ethoxyresorufin or resorufin metabolism with hepatocytes isolated from control or 3-methylcholanthrene pretreated rats

Reaction	K_m Control	V_{max} Control	K_m 3-MC	V_{max} 3-MC
Ethoxyresorufin deethylation	1.3 ± 0.2	0.07 ± 0.01	2.0 ± 0.2	10.21 ± 0.59
Resorufin conjugation	1.0 ± 0.2	0.22 ± 0.02	1.9 ± 0.2	0.81 ± 0.10

Values (K_m—μM and V_{max}—nmoles of resorufin formed (deethylation) or removed (conjugation)/min/10^6 hepatocytes) and means ± S.D. for four experiments. Rats were treated with either corn oil (control) or 3-methylcholanthrene (3-MC). Hepatocytes (for isolation see Methods) were incubated (see Methods) at a concentration of 0.5×10^6 cells per ml (total number of cells = 1×10^6) with ethoxyresorufin (0.1–5.0 μM) of resorufin (10–300 nM) in a 3 ml fluorimeter cuvette.

and resorufin conjugation. Apparent K_m and V_{max} values for ethoxyresorufin deethylation and for the presumed conjugation of authentic resorufin, with isolated hepatocytes of either control or 3-methylcholanthrene pretreated rats, are given in Table 2. The reactions were carried out in fluorimeter cuvettes, in air at 37°, using 1×10^6 hepatocytes/2 ml and either 0.1–5.0 μM ethoxyresorufin or 10–300 nM resorufin (in the absence of ethoxyresorufin). The initial reaction rates were measured during the first minute of reaction, before the cell suspension became appreciably anaerobic (as determined with an oxygen electrode). Ethoxyresorufin deethylation was measured in the presence of 1.5 mM salicylamide and the absence of BSA, while both were absent for determination of resorufin conjugation.

The deethylation and conjugation reactions with hepatocytes of control rats were characterised by the same apparent K_m value, which was not appreciably changed by 3-methylcholanthrene pretreatment *in vivo*. In contrast, the apparent V_{max} for the conjugation was 3-fold higher than the deethylation apparent V_{max}, with hepatocytes of control rats. 3-methylcholanthrene pretreatment *in vivo* induced the hepatocyte deethylation activity by a 40-fold greater extent than it induced the conjugation. Consequently, with hepatocytes of 3-methylcholanthrene pretreated rats the deethylation apparent V_{max} was 13-fold higher than the conjugation apparent V_{max}.

Reaction characteristics. In contrast to resorufin, ethoxyresorufin fluorescence was not affected by either hepatocytes, BSA, salicylamide or acetone at the concentrations used, nor did ethoxyresorufin up to 10 μM concentration affect the resorufin fluorescence.

With ethoxyresorufin deethylation measured using less than 10 μM ethoxyresorufin, substrate depletion and resorufin conjugation caused the eventual slowing-down of the rate of accumulation of resorufin. With higher ethoxyresorufin concentrations the apparent slowing-down was actually due to the accumulated resorufin concentration reaching levels where marked self-quenching of its fluorescence occurred. In these instances the deethylation reaction was calibrated by resorufin while the fluorescence was still increasing at a linear rate.

DISCUSSION

The opinion that measurement of monooxygenase and conjugating enzyme activities with isolated whole hepatocytes is the method of choice for extending to the physiological situation the knowledge gained from using isolated subcellular fractions, has been reviewed by Fry and Bridges [8]. For example, the relative rates of hydroxylation of naphthalene to 1-naphthol and naphthalene dihydrodiol are very different with liver microsomes compared to isolated hepatocytes [7]. There may be important differences also in conjugation reactions between isolated hepatocytes and liver microsomal preparations [7].

Ethoxyresorufin *O*-deethylase activity was over 100-fold higher with the isolated hepatocytes of 3-methylcholanthrene pretreated rats than with those of control rats. There was no difference between hepatocytes from control or pretreated rats in regard to resorufin conjugation, which might have influenced the apparent rate of deethylation. Thus, the very considerable induction of the deethylation, recorded previously with liver microsomal preparations [12], is a property of the intact hepatocyte and is not an artifact of subcellular fractionation. The sensitivity to α-naphthoflavone inhibition and refractivity to metyrapone inhibition, which are characteristic of microsomal ethoxyresorufin *O*-deethylase [14], and other cytochrome P448-associated enzymes [21–23], are also evident with isolated hepatocytes [19].

The many factors that could influence monooxygenase reactions with isolated hepatocytes makes it difficult to assess the significance of apparent enzyme kinetics. But the apparent kinetics are useful quantitative parameters for cellular reactions, and for ethoxyresorufin deethylation they were surprisingly similar to the values obtained with liver microsome preparations.

The apparent V_{max} for ethoxyresorufin *O*-deethylase with a mixture of hepatocytes isolated from a 3-methylcholanthrene pretreated rat, 10 nmoles/min/10^6 cells, was comparable with the apparent V_{max} for this enzyme measured with liver microsomes prepared from a 3-methylcholanthrene pretreated rat, 17.5 nmoles/min/mg protein [12], since this figure is equivalent to 4.5 nmoles/min/10^6 cells on the bases of 31 mg of microsomal protein [24], and 120×10^6 hepatocytes [25] per g of rat liver. The apparent K_m values with isolated

hepatocytes (1.3–2.0 μM) were approximately 10-fold higher than with liver microsomal preparations. This contrasts with the Phase 1 metabolism of alprenolol, which shows the same apparent K_m (10 μM) with either microsomes or hepatocytes [1], and of ethylmorphine, which shows a 5-fold lower apparent K_m with hepatocytes (50 μM) than with microsomal preparations [5]. It has been suggested that alprenolol enters the cell rapidly by a non-energy-requiring process [11], while ethylmorphine is actively concentrated within the hepatocyte [5]. The higher apparent K_m for ethoxyresorufin deethylation with hepatocytes compared to microsomes may be due to poor cellular absorbtion of the substrate, which is neither particularly water- nor lipid-soluble. Alternatively, it may reflect extensive binding of ethoxyresorufin to cellular protein.

It is important to know whether the oxygenative and conjugative phases of drug metabolism are linked in their function and control. The very large induction of ethoxyresorufin *O*-deethylase in hepatocytes by 3-methylcholanthrene *in vivo*, was not matched by a similar induction of resorufin conjugation. Furthermore, salicylamide inhibition of resorufin conjugation did not affect the initial rate of ethoxyresorufin deethylation with isolated hepatocytes. Therefore, it would appear that there was no linkage in either enzyme synthesis or function between the two phases of ethoxyresorufin metabolism. A similar independence of control of enzyme synthesis has been reported for the two phases of metabolism of *p*-nitroanisole [4] and naphthalene [7] with isolated hepatocytes.

Resorufin, at all concentrations up to 500 nM (the highest tested), was metabolised primarily to sulphate conjugates, with no apparent formation of glucuronides. This is similar to the observation of Moldéus *et al.* [4], that sulphate-conjugation of *p*-nitrophenol with isolated hepatocytes predominated over glucuronate-conjugation at low *p*-nitrophenol concentrations (< 25 μM), with glucuronides present as the major conjugates only at higher *p*-nitrophenol concentrations.

The effects of BSA on ethoxyresorufin and resorufin metabolism suggest that free resorufin, formed during the deethylation of ethoxyresorufin, passed rapidly out of the hepatocytes before being conjugated and could become bound to extracellular BSA. This binding then prevented the resorufin from re-entering the hepatocyte in order to undergo conjugation. A similar inhibitory effect of extracellular albumin-binding has been reported for bilirubin conjugation with cultured mouse hepatoma cells [26]. Thus, in the normal course of ethoxyresorufin metabolism with hepatocytes from 3-methylcholanthrene pretreated rats, a significant proportion of the sulphate conjugates was apparently formed with resorufin that had first left and then re-entered the cell. Our results, also indicate that an approximately equal proportion of the resorufin was conjugated immediately after its formation, before leaving the hepatocyte. Thus, more total unconjugated resorufin was formed in the presence of salicylamide, which could directly inhibit intracellular conjugation, than in the presence of BSA, which could inhibit conjugation only by extracellular trapping of resorufin that had passed out of the hepatocytes in the unconjugated form.

In conclusion, we have shown that isolated rat hepatocytes metabolised ethoxyresorufin through *O*-deethylation followed by sulphate-conjugation. The deethylation but not the conjugation was induced by 3-methylcholanthrene *in vivo*. A significant proportion of the deethylation product, resorufin, passed rapidly out from the hepatocytes and then re-entered them for conjugation. Extracellular albumin inhibited this conjugation by binding the resorufin that had left the hepatocytes. This phenomenon of extracellular albumin binding of unconjugated drug metabolites suggests a need for caution in designing and interpreting isolated cell experimental models for drug metabolism. BSA or other proteins are usually included in the suspension or culture media for such studies. Similar extracellular protein-binding might conceivably play a role in the accumulation of unconjugated metabolites from some xenobiotics *in vivo*. As the unconjugated metabolites are carried through the liver in the hepatic sinusoid bloodstream and the bile cannaliculi, re-entry into hepatocytes for the purposes of conjugation will be much more probable for free than for protein-bound metabolites of the type of resorufin.

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