Biochemical Pharmacology, Vol. 27, pp. 693–702. Pergamon Press, 1978. Printed in Great Britain.

STUDIES ON THE METABOLISM AND EXCRETION OF BENZO(a)PYRENE IN ISOLATED ADULT RAT HEPATOCYTES

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(*Received* 19 *August* 1977; *accepted* 6 *September* 1977)

Abstract—[^{3}H]Benzo(a)pyrene is metabolised by isolated rat hepatocytes to both ethyl acetate-soluble metabolites, which co-chromatograph with 4,5-dihydro-4,5-dihydroxybenzo(a)pyrene, 7,8-dihydro-7,8-dihydroxybenzo(a)pyrene, 9,10-dihydro-9,10-dihydroxybenzo(a)pyrene and 3-hydroxybenzo(a)pyrene and its sulphate ester, benzo(a)pyren-3-yl-hydrogen sulphate, and to water-soluble metabolites. Hydrolysis of the water-soluble metabolites with β-glucuronidase release ethyl acetate-soluble metabolites which co-chromatograph with 7,8-dihydro-7,8-dihydroxybenzo(a)pyrene, 4,5-dihydro-4,5-dihydroxybenzo(a)-pyrene, 9,10-dihydroxybenzo(a)pyrene (9,10-catechol) and 3-hydroxybenzo(a)pyrene. During the incubation significant differences in the distribution of metabolites between the cells and the extracellular medium are observed. Initially the cells produce predominantly ethyl acetate-soluble metabolites, which are only partly released into the extracellular medium, but at later times in the incubation a greater percentage of the metabolites are further metabolised to water-soluble conjugates which are very readily released from the cells. Individual ethyl acetate-soluble metabolites show significant distributional differences. Monohydroxybenzo(a)pyrenes accumulate intracellularly and only low amounts are released into the medium. Sulphate esters of monohydroxybenzo(a)pyrenes such as benzo(a)pyren-3-yl-hydrogen sulphate also accumulate intracellularly, although to a lesser extent than the monohydroxybenzo(a)pyrenes. 4,5-Dihydro-4,5-dihydroxybenzo(a)pyrene and 7,8-dihydro-7,8-dihydroxybenzo(a)pyrene are distributed more evenly between cells and medium whereas 9,10-dihydro-9,10-dihydroxybenzo(a)pyrene is found mainly in the medium. Significant amounts of radioactivity are bound irreversibly to cellular macromolecules.

Polycyclic aromatic hydrocarbons are distributed widely in our environment and have been shown to cause carcinogenic, mutagenic and cytotoxic effects in various species and tissues [1, 2]. As a necessary pre-requisite to these effects, metabolic activation of the parent compound by inducible NADPH-dependent mono-oxygenases is required [3–5].

An important member of this class of compounds is benzo(a)pyrene and its metabolic fate has been the subject of extensive study. It is metabolised to a complex mixture of products including organic-soluble metabolites such as epoxides, dihydrodiols, diol-epoxides, catechols, quinones, phenols and their sulphate esters and to water-soluble conjugates [5–9]. Initially, highly reactive epoxide intermediates are formed which are capable of (i) binding to nucleophilic tissue components or (ii) undergoing re-arrangement to phenols or (iii) undergoing further metabolism to dihydrodiols in a reaction catalysed by epoxide hydratase or (iv) forming glutathione conjugates in a reaction catalysed by glutathione *S*-transferase [5, 6]. Many of the primary metabolites may also be further metabolised by the NADPH-dependent mono-oxygenases thus extending the life of these products within the cell.

Much of the extremely complex metabolism of benzo(a)pyrene has now been elucidated by the use of isolated microsomal preparations [5, 10, 11] or cultured cell-lines [12]. However, the ultimate carcinogenicity and toxicity of this and other carcinogens is dependent in part on their overall metabolic fate in the body in both susceptible and non-susceptible tissues. Thus the balance between the formation of primary metabolites and their further metabolism by either oxidative or conjugation mechanisms is crucial in determining possible levels of reactive metabolites, which may be formed in different tissues. It is therefore necessary for the study of compounds which require metabolic activation to use a drug metabolising system resembling as closly as possible the *in vivo* situation. The advantages of freshly isolated adult mammalian hepatocytes for such studies has been recently reviewed [13–15]. These studies have clearly demonstrated that the metabolism of xenobiotics by isolated viable hepatocytes in suspension much more closely resembles the *in vivo* situation than does metabolism of xenobiotics in isolated subcellular organelles [13], largely because, in the hepatocyte suspensions, the biochemical and structural organization at the cellular level is intact. Consequently, the isolated hepatocyte system appears to be a useful model for study of the *in vivo* metabolism of xenobiotics. Numerous investigators have studied metabolism of the lung and skin carcinogen benzo(a)pyrene in liver microsomes and have discussed their findings in relation to benzo(a)pyrene-induced lung cancer. However, many of the factors that may be important in determining the role of hepatic metabolism in benzo(a)pyrene-induced cancer can only be adequately studied at the intact cell level. Such factors include cell membrane permeability for benzo(a)pyrene and/or metabolites produced by hepatic and extrahepatic organs, the overall metabolite pattern and inter-relationship between Phase I and Phase II meta-

bolism, the degree of binding of xenobiotic/metabolites to cellular organelles and the subsequent rate of release of free and conjugated metabolites from the cell into the extracellular environment.

In the present study, isolated viable rat hepatocytes have been used to study the Phase I and Phase II metabolism of benzo(a)pyrene with particular attention to the fate of the metabolites. The cells were obtained from uninduced animals to ensure no distortion (by enzyme induction) of the basic metabolic profile as it was felt that the uninduced state would be more likely to appertain to the human situation in which benzo(a)pyrene exposure is largely by inhalation. One aspect that we have particularly studied is the distribution of metabolites between the cells and the extracellular medium since it is possible that selective release from, or retention by, the cells of key metabolites may be important in determining the biological consequences of benzo(a)pyrene administration. A previous report [16] has drawn attention to some cell distributional differences between dihydrodiol and phenolic metabolites. However, largely because the thin-layer chromatography system employed in that study was unable to satisfactorily resolve the dihydrodiols and other metabolites migrating near the origin, such as sulphate esters of monohydroxybenzo(a) pyrenes and tetrahydrotetrahydroxy compounds, their findings and conclusions warranted further investigation.

Although benzo(a)pyrene is not a proven hepatocarcinogen it is known to form highly reactive intermediates and to be extensively metabolised in liver. The present work was undertaken using isolated adult hepatocytes from untreated rats to elucidate further the fate and distribution of benzo(a)pyrene and its major metabolites in this tissue.

MATERIALS AND METHODS

[^{3}H]Benzo(a)pyrene (sp. act. 25 Ci/m-mole) was obtained from the Radiochemical Centre, Amersham, and after further purification, according to the method of De Pierre *et al.* [17] was added to unlabelled benzo(a)pyrene (Koch-Light Laboratories Ltd., Bucks.) to the appropriate concentration. The unlabelled reference compounds 4,5-dihydro-4,5-dihydroxybenzo(a)pyrene (4,5-dihydrodiol), 7,8-dihydro-7,8-dihydroxybenzo(a)pyrene (7,8-dihydrodiol), 9,10-dihydro-9,10-dihydroxybenzo(a)pyrene (9,10-dihydrodiol) and 3-hydroxybenzo(a)pyrene were prepared essentially by the method of Sims [18] and their identity confirmed by comparing their u.v. and fluorescence excitation and emission spectra with those reported in the literature.

Collagenase (type CLS 1) was obtained from Worthington, Cambrian Chemicals, Croydon. Hyaluronidase (type II), β-glucuronidase (type H-I, containing some sulphatase activity) and arylsulphatase (type H-I) were obtained from Sigma Chemicals Ltd., London. Pure β-glucuronidase (ketodase) was obtained from Warner-Chilcott Labs., NJ, U.S.A. Leibovitz L-15 medium, containing 2 mM glutamine and 10% foetal calf serum, was obtained from Flow Labs., Scotland. All other reagents were of highest obtainable purity.

Thin layer chromatogram (t.l.c.) aluminium sheets precoated with silica gel 60 (0.20 mm thickness) were obtained from Merck Darmstadt, Germany. Fluorimetric and radioactivity measurements were performed using a Perkin–Elmer MPF-3 fluorescence spectrophotometer and a Packard Tri-Carb scintillation counter, model 3320 respectively. The radiochromatograms were scanned with a Varian–Berthold scanner.

Animals. Male Wistar albino rats (60–100 g), bred at the University of Surrey were maintained on wood shavings and allowed food (Spillers No. 1 Laboratory Diet, Spillers Ltd., Croydon) and water *ad libitum.*

Cell isolation. Rats were killed by cervical dislocation and the liver lobes quickly excised. Hepatocytes were isolated by the method of Fry *et al.* [19]. Slices of liver were washed in 0.5 mM ethyleneglycol-bis-(β-aminoethylether) *N,N'*-tetra-acetic acid (EGTA) in phosphate buffered saline (lacking calcium and magnesium salts) and digested with collagenase/hyaluronidase enzymes (0.05%/0.10%, w/v) for 60 min at 37°. Isolated hepatocytes were suspended in Leibovitz *L*-15 medium containing 10% foetal calf serum, 10% tryptose phosphate broth, and penicillin (100 i.u./ml) and streptomycin (100 μg/ml). Their viability, as assessed by the Trypan Blue dye exclusion test [20] was greater than 90 per cent. Cell counts were made using an improved Neubauer counting chamber (Gelman Hawksley, Sussex).

Metabolism of [^{3}H]benzo(a)pyrene by isolated hepatocytes. Unlabelled and [^{3}H]benzo(a)pyrene in *N,N*-dimethyl formamide (DMF) were added to culture medium. Incubations were carried out in 10 ml conical flasks at 37° in a shaking water-bath (approx. 90 oscillations/min). The final incubation mixture, volume 1 ml, contained 2×10^6 viable hepatocytes, 80 μM benzo(a)pyrene (approx. 9 μCi), and 0.2% v/v DMF. Control incubations were also carried out at 0° with hepatocytes and at 37° when the hepatocytes were omitted. After timed intervals, the reaction was stopped by placing the flasks in ice. The cells were sedimented by centrifugation (50 *g* for 45 sec) at 0° and the extracellular medium was removed. The cells were washed with 2 × 2 ml portions of culture medium. The washings and extracellular medium from each incubate were pooled and aliquots taken for determination of radioactivity. The scintillation cocktail contained 0.02% w/v 1,4-bis-2-(4-methyl-5-phenyl-oxazoyl)-benzene, 0.5% w/v 2,5-diphenyloxazole, 33% v/v 'Synperonic NXP' (I.C.I. Ltd., Teeside) in sulphur-free toluene.

The cell pellet was resuspended in 5 ml water and thoroughly mixed on a rotary mixer to lyse the cells. Lysis was assessed by the Trypan Blue exclusion method [20]. Aliquots were taken for determination of radioactivity. Each aliquot was digested in Soluene-350 (Packard Instrument Co.) and counted in the scintillation cocktail omitting 'Synperonic NXP'.

The medium and cells were both extracted with ethyl acetate (2 × 1 vol.) and the extracts dried with anhydrous sodium sulphate. Aliquots were taken for radioactivity estimation. The extracts were examined by t.l.c. in benzene–ethanol (9:1, v/v) as described previously [21]. The metabolites were identified by co-chromatography with unlabelled markers and by their fluorescence excitation and emission spectra, fol-

lowing elution of the relevant area of the chromatogram into ethanol (spectroscopic grade).

Mean recovery of total radioactivity used in three experiments was 84 per cent (range from all samples was 75–91 per cent). In experiments where 3-hydroxybenzo(a)pyrene or one of the dihydrodiols was incubated separately with isolated cells, recoveries of greater than 96 per cent were obtained.

Enzymic hydrolyses for investigation of water-soluble metabolites. The water-soluble metabolites, remaining in the aqueous medium after the organic extraction, were subjected to deconjugating enzymes prior to extraction and analysis. For estimation of the total conjugation, 55 Fishman units of crude β-glucuronidase in 0.2 ml of 0.2 M acetate buffer, pH 5.0, were added to each ml of incubate and the samples incubated overnight in a shaking water-bath at 37°. Levels of glucuronic acid conjugation were determined following an overnight incubation with 0.5 ml ketodase solution (2500 Fishman units/ml incubate). Sulphate conjugation was determined following overnight incubation with arylsulphatase (350 Fishman units/ml incubation) in 0.2 M acetate buffer, pH 5.0. Since this preparation of arylsulphatase contained some β-glucuronidase activity, 20 mM saccharo-1,4-β-lactone was added to inhibit the β-glucuronidase activity. Controls were run simultaneously to which no deconjugation enzymes were added. Deconjugated products were extracted with ethyl acetate and the extracts analysed as described above.

Benzo(a)pyrene 3-monooxygenase assay, (aryl hydrocarbon hydroxylase). Benzo(a)pyrene 3-monooxygenase activity was determined essentially by the method of Nebert and Gelboin [22]. Benzo(a)pyrene (80 μM) was incubated with 2×10^6 viable cells at 37° in 1 ml volumes for 60 min. The reaction was terminated after timed intervals by placing the flasks on ice and the cells and extracellular medium separated as described above. The amount of 3-hydroxybenzo(a)pyrene in both cells and medium was then determined [22]. The water-soluble metabolites, after having been subjected to the deconjugation procedure described above, were extracted with ethyl acetate and the organic-soluble monohydroxybenzo(a)pyrenes determined [22].

Estimation of radioactivity covalently bound to cellular material. Cellular macromolecules were precipitated by the addition of an equal volume of ice-cold 1*N*-perchloric acid. The pellet was washed twice with 0.5 *N*-perchloric acid. Non-covalently bound radioactivity was removed according to the method of Siekevitz [23]. The precipitates were digested in 1 *N*-sodium hydroxide and radioactivity determined.

RESULTS

Cell viability. After incubation of the isolated rat liver cells with 80 μM benzo(a)pyrene, the viability remained greater than 90 per cent, as assessed by the Trypan Blue exclusion method.

Uptake and release of benzo(a)pyrene by cells. Benzo(a)pyrene was very rapidly taken up by the cells, a maximal cellular level of 22 nmoles per 10^6 cells being reached after 5 min incubation. The total amount of radioactivity within the cells remained constant for about 20 min and then slowly declined

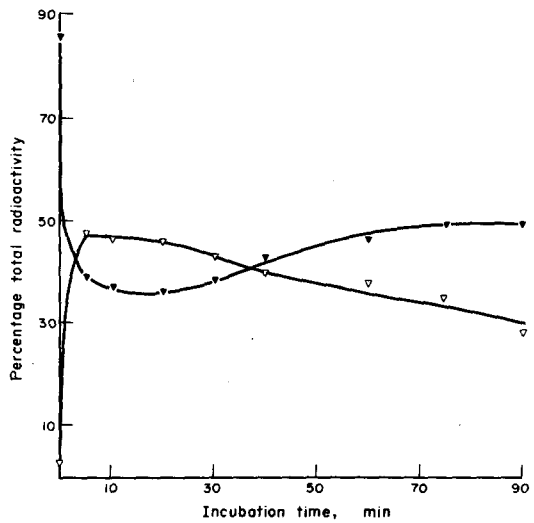

Fig. 1. Time course of the distribution of radioactivity associated with hepatocytes (▽——▽) and the extracellular medium (▼——▼) on incubation of rat hepatocytes with [^{3}H]benzo(a)pyrene. Viable rat hepatocytes (2×10^6 cells per ml) were incubated with benzo(a)pyrene (80 μM) at 37°. At various times the incubation was stopped, the cells and extracellular medium separated and the total radioactivity in both determined.

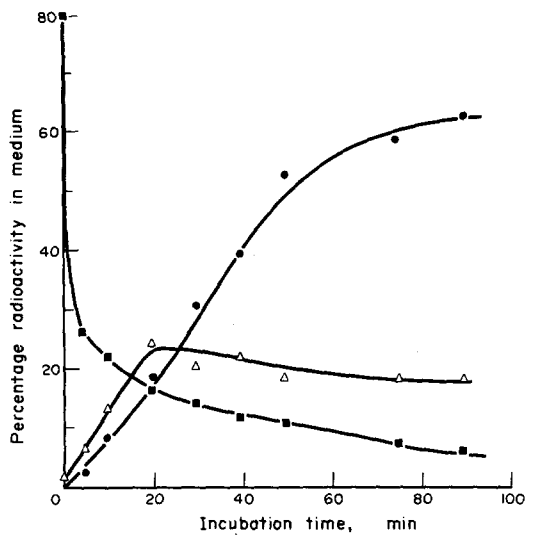

Fig. 2. Time course of the percentage of the radioactivity in the extracellular medium associated with ethyl acetate-soluble metabolites (△——△), water-soluble (radioactivity) metabolites (●——●) and unchanged benzo(a)pyrene (■——■). Viable rat hepatocytes (2×10^6 cells per ml) were incubated with [^{3}H]benzo(a)pyrene (80 μM) at 37°. At various times the incubation was stopped and the cells and extracellular medium separated. The extracellular medium together with washings of the cells were extracted with ethyl acetate (2 × 1 vol.) and the radioactivity not extractable with ethyl acetate was designated as water-soluble radioactivity. The ethyl acetate-soluble radioactivity products were separated by t.l.c. in benzene and ethanol (9:1, v/v). The unchanged [^{3}H]benzo(a)pyrene was quantified following cutting of the chromatogram and liquid scintillation counting and this value subtracted from the total ethyl acetate-soluble radioactivity in order to obtain a value for the ethyl acetate-soluble metabolites.

as metabolites were released from the cells, Fig. 1. The amount of radioactivity within the medium reflected the changes observed in the cells, i.e. an initial rapid fall due to uptake of benzo(a)pyrene into the cells followed later by an increase due to release of metabolites from the cells (Fig. 1).

In order to obtain an estimate of the half-life ($t_{\frac{1}{2}}$) of benzo(a)pyrene in the isolated hepatocytes, its disappearance from the medium was determined from the amounts of benzo(a)pyrene left unmetabolised as determined by thin-layer chromatography of the appropriate ethyl acetate extract and eluting the band corresponding to benzo(a)pyrene. An estimated value for the half-life of 53 min was obtained using 80 μM benzo(a)pyrene with 2×10^6 liver cells.

The metabolites released into the medium during the first 25 min of incubation were largely extractable with ethyl acetate (64 per cent of metabolites at 25 min), with measurable amounts present within the first few minutes incubation. With time, however, the metabolites released had undergone further metabolism and/or conjugation to form water-soluble products (Fig. 2). After 90 min incubation, 78 per cent of the metabolites in the extracellular medium were water-soluble.

Analysis of ethyl-acetate soluble metabolites. Benzo(a)pyrene was metabolised to ethyl acetate-soluble metabolites which co-chromatographed with 3-hydroxybenzo(a)pyrene, 4,5-dihydrodiol, 7,8-dihydrodiol and 9,10-dihydrodiol (Fig. 3). Marked differences in the distribution of individual ethyl-acetate soluble metabolites were found between the cell contents and the surrounding medium (Fig. 3). Significantly higher levels of the free phenols were found in the cells compared to those in the extracellular medium. Maximal levels of the free phenols within the extracellular medium never exceeded 100 *p*moles/10^6 cells during the incubation period, whereas the intracellular level of phenol was approximately 500 *p*moles/10^6 cells after 60 min incubation (Fig. 4).

Significant amounts of another metabolite(s) migrating beyond 3-hydroxybenzo(a)pyrene were also observed within the cell (Fig. 3). This material co-chromatographed with benzo(a)pyrene quinones. On the basis of its chromatographic behaviour and red colour a major portion of these quinones was tenta-

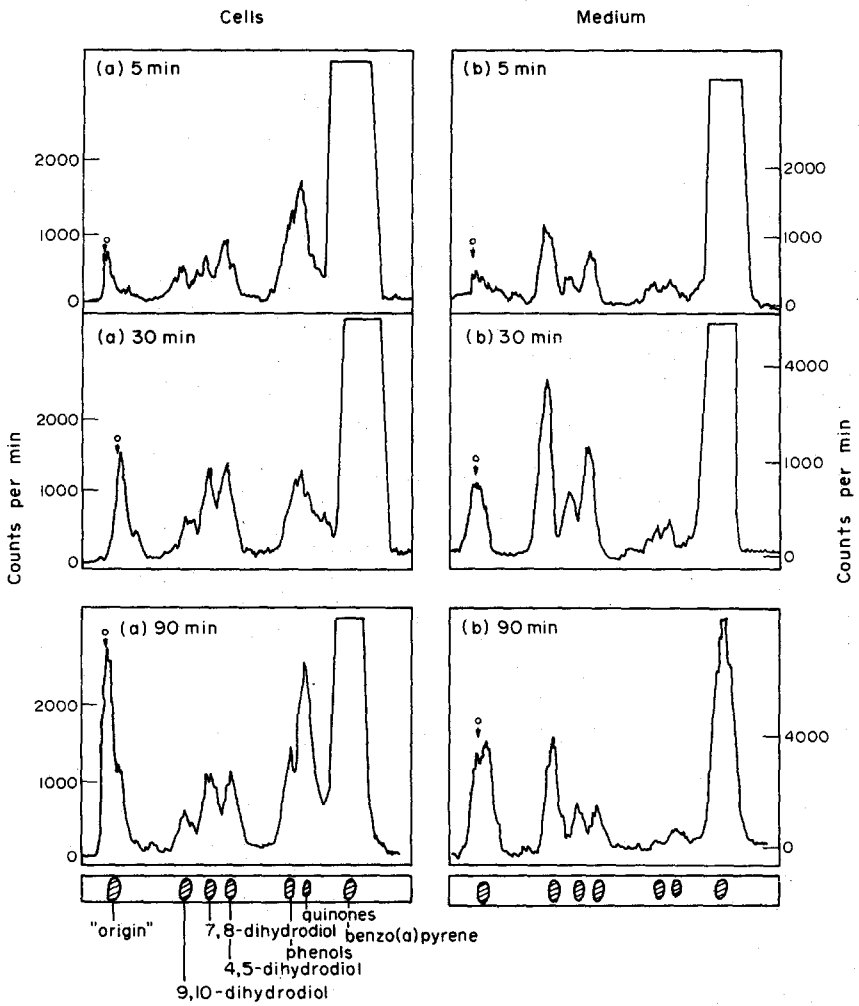

Fig. 3. Tracings of radioscans of thin-layer radiochromatograms of ethyl acetate soluble-metabolites in (a) the hepatocytes and (b) the extracellular medium. Viable rat hepatocytes (2×10^6 cells per ml) were incubated for various times with [^{3}H]benzo(a)pyrene (80 μM). The cells and extracellular medium were separated, extracted with ethyl acetate and the ethyl acetate-soluble radioactive products were separated as described in the legend to Fig. 2.

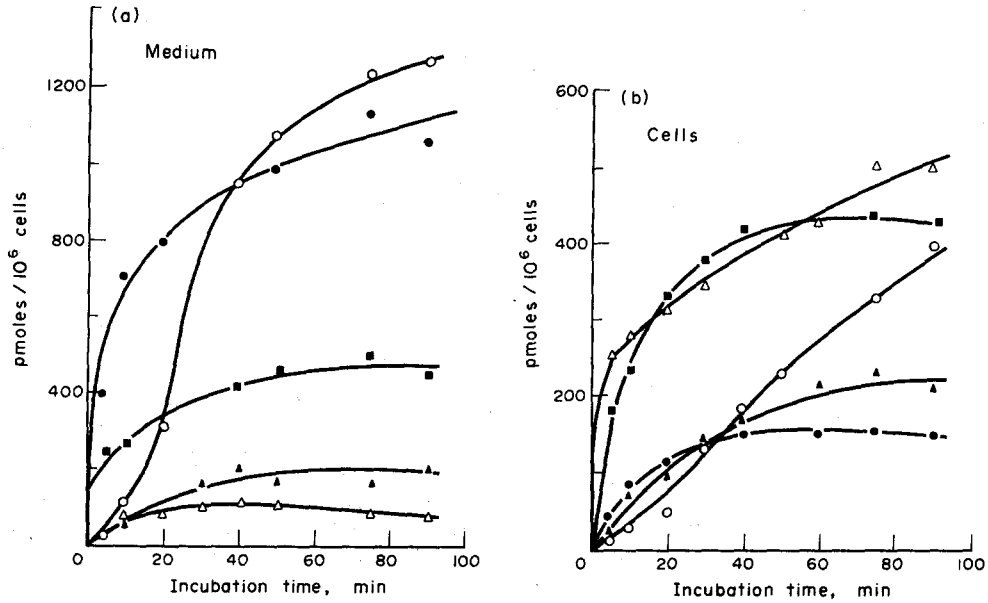

Fig. 4. Distribution of ethyl acetate-soluble metabolites of benzo(a)pyrene between (a) the extracellular medium and (b) hepatocytes. Following incubation of [^{3}H]-benzo(a)pyrene (80 μM) with hepatocytes the ethyl acetate soluble-metabolites were separated and quantified as described in the legend to Fig. 2. 3-Hydroxybenzo(a)pyrene △——△; 4,5-dihydrodiol ■——■; 7,8-dihydrodiol ▲——▲; 9,10-dihydrodiol ●——●; metabolite(s) at or near origin ○——○.

tively identified as benzo(a)pyrene 3,6-quinone. It is difficult to discern whether these quinones are formed metabolically or arise as an artifact formed by air oxidation during the isolation and separation of metabolites.

Using the benzene–ethanol (9:1) t.l.c. system, the three principal dihydrodiols formed could be satisfactorily resolved. The major dihydrodiol found in the extracellular medium was 9,10-dihydrodiol whereas 4,5-dihydrodiol was the major one in the cells (Fig. 4). The extent to which free dihydrodiol egressed from the cells into the extracellular medium differed markedly with respect to each dihydrodiol (Fig. 3). Throughout the incubation most of the 9,10-dihydrodiol was present in the medium although significant amounts were still measurable within the cells. At 90 min, 88 per cent of the radioactivity associated with 9,10-dihydrodiol was found in the extracellular medium and the remaining 12 per cent was associated with the hepatocytes (Table 1).

Table 1. Percentage radioactivity within hepatocytes during incubation

Metabolite	5 Min	50 Min	90 Min
3-Hydroxybenzo(a)pyrene	94	77	86
4,5-Dihydrodiol	44	46	45
7,8-Dihydrodiol	60	46	50
9,10-Dihydrodiol	17	12	12
"Material at Origin"	47	30	26

Benzo(a)pyrene (80 μM) was incubated with isolated rat hepatocytes. The cells and extracellular medium were separated, and extracted with ethyl acetate. The ethyl acetate-soluble radioactivity was separated by t.l.c. in benzene–ethanol (9:1, v/v) and the metabolites quantified by cutting the t.l.c. plates into segments and determining the radioactivity. The percentages represent the relative proportion of each individual metabolite present in the cell, the remainder of each metabolite being in the extracellular medium. The "Material at Origin" represents the most polar organic-soluble metabolites which remain at the origin when chromatographed in benzene–ethanol (9:1, v/v). These metabolites include sulphate conjugates of monohydroxybenzo(a)pyrenes, 7,8,9,10-tetrahydro-7,8,9,10-tetrahydroxybenzo(a)pyrene as well as other as yet uncharacterised metabolites.

Both 7,8-dihydrodiol and 4,5-dihydrodiol were distributed more evenly between the cells and medium; thus the percentage of these metabolites found within the cells were much greater than for 9,10-dihydrodiol (Table 1). Slightly more 7,8-dihydrodiol than 4,5-dihydrodiol was retained within the cells (Table 1).

The material associated with the radioactive peak at the origin of the chromatograms was examined by fluorescence spectroscopy. This material had similar fluorescence excitation and emission spectra to the sulphate conjugate of 3-hydroxybenzo(a)pyrene (benzo(a)pyren-3-yl-hydrogen sulphate) [9]. However other unidentified products such as polyhydroxylated phenols, tetrahydrotetrols and other sulphate conjugates may also have been present, but were not resolved. The radioactivity, associated with this mixture of metabolites within the cells remained high during the incubation (30 per cent at 50 min, Table 1).

Analysis of water-soluble metabolites. Water-soluble conjugates were detected in the extracellular medium after 5 min incubation. After 90 min incubation, these constituted the major metabolites (78 per cent of total metabolites formed). Throughout the incubation little or no water-soluble metabolites were detected within the cells suggesting that on formation they passed rapidly into the extracellular medium. Following enzyme hydrolysis of the water-soluble metabolites, ethyl acetate-soluble metabolites which co-chromatographed with 3-hydroxybenzo(a)pyrene and 4,5-di-

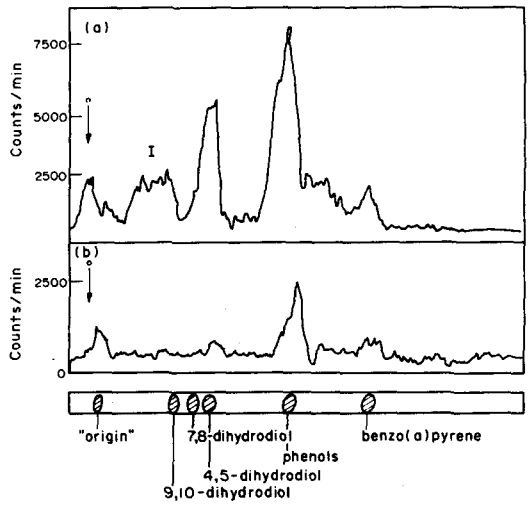

Fig. 5. Enzymic hydrolysis of water-soluble metabolites from benzo(a)pyrene. Water-soluble metabolites from the extracellular medium, after 90 min incubation of viable rat hepatocytes (2×10^6 cells/ml) with [^{3}H]benzo(a)pyrene (80 μM) were hydrolysed with either (a) β-glucuronidase or (b) buffer alone. The ethyl acetate-soluble radioactivity was separated by t.l.c. in a mixture of benzene and ethanol (9:1, v/v) and a tracing of the radioscan of the thin-layer chromatogram is shown above.

hydrodiol were obtained (Fig. 5). Little or no radioactivity was observed which co-chromatographed with 9,10- or 7,8-dihydrodiols. However, significant amounts of radioactivity were observed in region I (Fig. 5a), chromatographing just prior to 9,10-dihydrodiol in benzene–ethanol (9:1, v/v). Some phenols were partially released even in the controls (Fig. 5b) suggesting that some of the phenol conjugates are relatively labile. Only small amounts of the sulphate conjugate of 3-hydroxybenzo(a)pyrene were present in the water-soluble metabolites as it had been very largely removed by the initial ethyl acetate extraction.

Relative rates of 3-hydroxybenzo(a)pyrene conjugation. In order to ascertain the relative importance of glucuronide and sulphate conjugation in the further metabolism of 3-hydroxybenzo(a)pyrene formed on incubation of the hepatocytes with benzo(a)pyrene, cells and extracellular medium were hydrolysed with either ketodase or arylsulphatase and the levels of monohydroxybenzo(a)pyrenes determined by the fluorescence method [12]. 3-Hydroxybenzo(a)pyrene was extensively metabolised to both sulphate and glucuronide conjugates, approximately two-thirds of the conjugates being glucuronides (Fig. 6), at 60 min. The maximal rates of formation of monohydroxybenzo(a)-pyrenes and their glucuronide and sulphate conjugates were 230, 99 and 64 pmoles product/min/10^6 cells respectively.

Glutathione conjugates. After the free metabolites and those conjugated with sulphate or glucuronic acid had been removed from the medium, some radioactivity still remained (6 per cent at 60 min). Following acetone precipitation of the protein material the aqueous supernatants were resolved in a t.l.c. system containing butanol–propanol–2M NH_3 (2:1:1). At

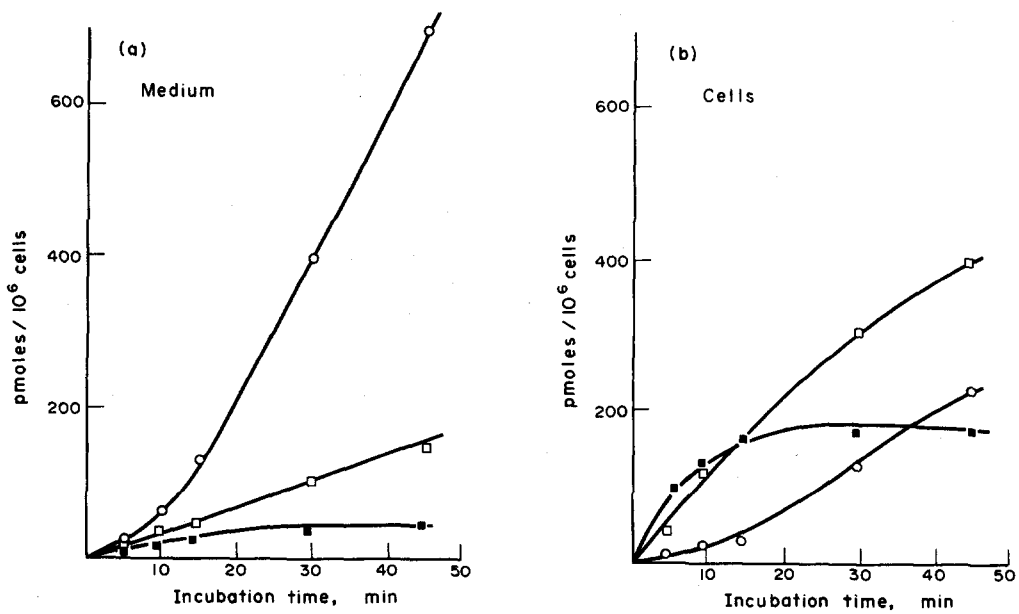

Fig. 6. Distribution of 3-hydroxybenzo(a)pyrene (■——■), its glucuronide conjugate (○——○) and its sulphate ester, (□——□) between (a) extracellular medium and (b) hepatocytes. Hepatocytes (2×10^6 cells per ml) were incubated with benzo(a)pyrene (80 μM), the cells and extracellular medium separated and the amounts of 3-hydroxybenzo(a)pyrene determined by the fluorescence method of Nebert and Gelboin [22]. The amounts of glucuronide and sulphate conjugates were determined from the 3-hydroxybenzo(a)pyrene present following enzymic hydrolysis with either β-glucuronidase (with saccharo-1,4-β-lactone) or aryl sulphatase respectively.

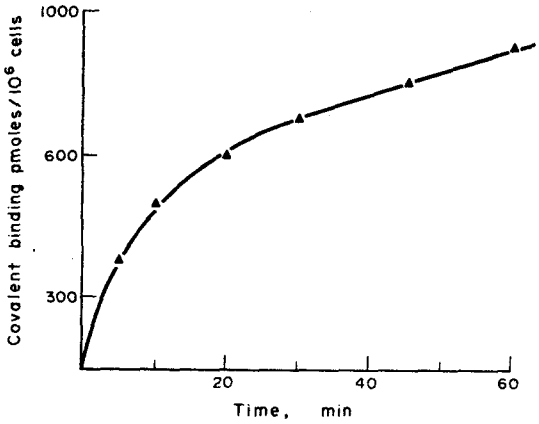

Fig. 7. Covalent binding of [^{3}H]benzo(a)pyrene related radioactivity to viable rat hepatocytes. Hepatocytes (2×10^6 cells per ml) were incubated with [^{3}H]benzo(a)pyrene (80 μM) for various times. The cellular macromolecules were precipitated with perchloric acid and the non-covalently bound radioactivity removed by exhaustive solvent extraction. The radioactivity remaining associated with the precipitate is referred to as covalently bound.

least two metabolites were detectable by radio scanning. These were possibly glutathione conjugates but have not been further characterised.

Covalently bound metabolites. After 60 min incubation, approximately 3 per cent of the added radioactivity was bound to cellular material and could not be removed by repeated washing with organic solvents. The proportion of radioactivity bound increased with time (Fig. 7) indicating that certain metabolites of benzo(a)pyrene irreversibly bind to hepatocyte macromolecules.

DISCUSSION

Incubation of rat hepatocytes with [^{3}H]benzo(a)pyrene demonstrated a very rapid uptake of radioactivity into the cells which plateaued after 5 min incubation (Fig. 1). This coincided with a disappearance of radioactivity from the medium and the appearance in the cells and medium of a range of ethyl acetate-soluble and water-soluble products. The initial increase of radioactivity in the cells was due to uptake of benzo(a)pyrene but at the plateau an equilibrium between the uptake of substrate and efflux of metabolites was achieved.

The major metabolite detected within the cells was 3-hydroxybenzo(a)pyrene (Fig. 4). This metabolite was barely detectable in the medium throughout the course of the incubation. The *in vivo* significance of this intracellular accumulation of 3-hydroxybenzo(a)pyrene is at present uncertain although it is interesting to note that the cytotoxicity produced by benzo(a)pyrene to cells in culture, is believed to be due to the production of 3-hydroxybenzo(a)pyrene [24].

It is not clear from our experiments whether the formation of quinones arises metabolically or as an artifact during isolation. If some of these quinones arise metabolically it is expected that such metabolites would accumulate within cells, awaiting further reductive or oxidative metabolism, since quinones such as benzo(a)pyrene-1,6- and 3,6-quinones do not form glucuronic acid conjugates when incubated with a liver microsomal system fortified with UDP-glucuronic acid as they are not substrates for UDP-glucuronosyl transferase [25]. The accumulation of metabolically formed quinones within the cell may be partially responsible for the cytotoxicity attributed to 3-hydroxybenzo(a)pyrene.

The 3-hydroxybenzo(a)pyrene produced by the hepatocytes was further metabolised to both glucuronide and sulphate conjugates. Benzo(a)pyren-3-yl hydrogen sulphate was identified as a major ethyl acetate-extractable metabolite formed from benzo(a)pyrene by isolated rat hepatocytes. This is in agreement with our previous observations when this metabolite was also formed by short-term organ cultures of rat, hamster and human lung [9]. Of particular interest were the experiments using the fluorescence assay (Fig. 6) which showed that benzo(a)pyren-3-yl hydrogen sulphate was concentrated in cells to a far greater extent than would be expected based on its lipophilicity alone. This was supported by the results in the experiments using radioactivity, when proportionally greater amounts of radioactivity associated with metabolites migrating near the origin were found within the cells (Fig. 3) than would be expected due to their lipid solubility. However, it must also be remembered that the metabolites migrating near the origin, in the solvent system used in the present experiments, will include known metabolites such as sulphate esters of monohydroxybenzo(a)pyrenes, and 7,8,9,10-tetrahydro-7,8,9,10-tetrahydroxybenzo(a)pyrene as well as other as yet unidentified metabolites. Benzo(a)pyren-3-yl hydrogen sulphate formed within the cells may itself be biologically active or it may be further metabolised by the microsomal mixed function oxidase system to active or inactive metabolites or be split by sulphohydrolases present in tissues, thus acting as a reservoir for 3-hydroxybenzo(a)pyrene. The 3-hydroxybenzo(a)pyrene thus formed may then be activated to a form which binds to DNA [26].

The 3-hydroxybenzo(a)pyrene formed from benzo(a)pyrene within the hepatocytes, was also metabolised to a water-soluble metabolite. This was identified as the glucuronide conjugate of 3-hydroxybenzo(a)pyrene since (i) 3-hydroxybenzo(a)pyrene was released from the water-soluble metabolites in the medium following enzymic hydrolysis with β-glucuronidase and identified from its fluorescence characteristics and (ii) benzo(a)pyren-3-yl hydrogen sulphate was removed by prior ethyl acetate extraction.

Recent studies using high pressure liquid chromatography to separate benzo(a)pyrene metabolites have shown that rat liver microsomes can form four monohydroxybenzo(a)pyrenes. 3-Hydroxybenzo(a)pyrene has been identified as the major metabolite with smaller amounts of 1,7- and 9-hydroxybenzo(a)pyrene also observed [27]. These monohydroxybenzo(a)pyrenes will also form sulphate esters [9, 28] and glucuronide conjugates [25]. As the t.l.c. system used in the present study will not resolve these different monohydroxybenzo(a)pyrenes it is possible that small amounts of glucuronide and sulphate conjugates of other monohydroxybenzo(a)pyrenes besides 3-hydroxybenzo(a)pyrene were also present.

Significant levels of the glucuronide conjugate of 3-hydroxybenzo(a)pyrene were not found in the extracellular medium until after 5 min incubation. As the

Table 2. Concentration of metabolites in cells and extracellular medium

Time min	3-Hydroxybenzo(a)-pyrene nmole ml^{-1}		Material at origin nmole ml^{-1}		9,10-Dihydrodiol nmole ml^{-1}		4,5-Dihydrodiol nmole ml^{-1}		7,8-Dihydrodiol nmole ml^{-1}	
	M	C	M	C	M	C	M	C	M	C
10	0.12	66.2	0.22	6.5	1.41	21.2	0.53	55.6	0.12	17.7
30	0.18	82.0	1.00	30.4	2.48	30.3	1.17	90.7	0.33	28.4
90	0.16	118.3	2.50	96.3	2.09	35.3	0.91	88.3	0.41	48.9

Benzo(a)pyrene was incubated with isolated rat hepatocytes. The metabolites in the cells (C) and extracellular medium (M) were separated and estimated as described in the legend to Table 1. The amounts of metabolites are expressed per ml of extracellular medium or intracellular volume. In the latter calculation 2×10^6 hepatocytes occupied a packed volume of 8 μl (based on the assumption that each hepatocyte was a sphere of mean diameter 22 μ), in an incubation containing 1 ml extracellular medium.

glucuronide conjugate was not detectable in the cells at any stage during the incubation, this lag in glucuronide appearance in the medium must be due to a delay in its formation rather than to a delay in release from the cells. We have previously observed a similar lag in glucuronidation of other newly-formed Phase I metabolites [29], and interpreted this as indicating activation of the required UDP-glucuronosyl-transferase by the newly-formed Phase I metabolite together with a delay in resynthesis and/or rechanneling of UDP-glucuronic acid required for the conjugation process.

Marked differences were also observed in the distribution of dihydrodiols between the cells and the extracellular medium (Fig. 3). The retention of 4,5- and 7,8-dihydrodiols within the cells was significantly greater than for 9,10-dihydrodiol particularly if the results for the cells were calculated on a unit volume basis (Table 2). Similar findings have been observed with ferret and hamster hepatocytes (unpublished work). It is unlikely that the observed differences in dihydrodiol retention can be explained simply in terms of the relative partition coefficients of the respective dihydrodiols, which if calculated using the Hansch method are essentially the same [30]. It is possible that the differences in dihydrodiol retention within the cells may be related to their affinities for intracellular binding proteins or lipid membranes. These possibilities are currently being investigated.

Enzymic hydrolysis with β-glucuronidase, of the water-soluble metabolites of the medium after incubation of the hepatocytes with benzo(a)pyrene, yielded ethyl acetate-soluble metabolites which co-chromatographed with 3-hydroxybenzo(a)pyrene and 4,5-dihydrodiol (Fig. 5a) but little or no radioactivity co-chromatographed with 9,10- and 7,8-dihydrodiol although some metabolite(s) with associated radioactivity chromatographed very close to and just prior to 9,10-dihydrodiol (region I, Fig. 5a). These observations, in agreement with the relative rates of glucuronide conjugation of 3-hydroxybenzo(a)pyrene and 4,5-, 7,8- and 9,10-dihydrodiols using microsomes from rat liver fortified with UDP-glucuronic acid [25], demonstrate that the glucuronide conjugates of 3-hydroxybenzo(a)pyrene and 4,5-dihydrodiol are the major water-soluble metabolites of benzo(a)pyrene formed by isolated hepatocytes. The results in the present study do not exclude the possibility of the formation of a sulphate conjugate of 4,5-dihydrodiol. The results obtained with isolated hepatocytes in the present study are also in fairly good agreement with our previous results of the metabolic fate of benzo(a)-pyrene and its metabolites using short-term organ cultures of hamster lung. Then benzo(a)pyrene was metabolised to water-soluble metabolites which on enzyme hydrolysis yielded ethyl acetate-soluble metabolites which co-chromatographed with 3-hydroxybenzo(a)pyrene, 4,5- and 7,8-dihydrodiols, but little or no 9,10-dihydrodiol was observed [31]. 4,5-Dihydrodiol was metabolised to a glucuronide conjugate with little or no sulphate conjugate formed [32] and 7,8-dihydrodiol was metabolised to an ethyl acetate-soluble metabolite identified as 7,8,9,10-tetrahydro-7,8,9,10-tetrahydroxybenzo(a)pyrene and to a water-soluble conjugate, most likely a glucuronide conjugate. No glucuronide conjugate of 9,10-dihydrodiol was observed [31]. The only slight inconsistencies in the results obtained with short-term organ cultures of lung and the present study appeared to be that in the hepatocytes little or no 7,8-dihydrodiol was conjugated and possibly small amounts of a conjugate of 9,10-dihydrodiol were also observed. In order to try and resolve these discrepancies we have carried out some preliminary incubations of isolated rat hepatocytes with 7,8- and 9,10-dihydrodiols. In these experiments 7,8-dihydrodiol (8 μM) was metabolised both to an ethyl acetate-soluble metabolite which co-chromatographed with and had similar excitation and emission spectra to 7,8,9,10-tetrahydro 7,8,9,10-tetrahydroxybenzo(a)pyrene and to water-soluble metabolites, tentatively identified as the glucuronide and sulphate conjugates of 7,8-dihydrodiol. 9,10-Dihydrodiol (8 μM) was metabolised by isolated hepatocytes to a water-soluble metabolite which has been identified as the glucuronide conjugate of the catechol, 9,10-dihydroxybenzo(a)pyrene, whereas little or no 9,10-dihydroxybenzo(a)pyrene and 7,8,9,10-tetrahydro-7,8,9,10-tetrahydroxybenzo(a)pyrene were observed as ethyl-acetate-soluble metabolites. The most likely metabolic fate of 9,10-dihydrodiol in the intact hepatocyte would therefore appear to be conversion to its catechol followed by rapid conjugation. This pathway would then explain part of the results of enzymic hydrolysis of the water-soluble metabolites from the hepatocytes and benzo(a)pyrene (Fig. 5a), i.e. (i) the presence of little or no 9,10-dihydrodiol and (ii) the presence of a metabolite(s) in the region migrating just prior to 9,10-dihydrodiol (Region I, Fig. 5a) as this is the area where 9,10-dihydroxybenzo(a)pyrene migrates in this solvent system. The reason for the

presence of little or no 7,8-dihydrodiol, following enzymic hydrolysis of the water-soluble metabolites of the medium from hepatocytes incubated with benzo(a)pyrene, is not clear. It is possible that most of the 7,8-dihydrodiol formed metabolically from benzo(a)pyrene is metabolised to the tetrahydrotetrol or by some yet unidentified pathway. The formation of significant amounts of 7,8,9,10-tetrahydro-7,8,9,10-tetrahydroxybenzo(a)pyrene from 7,8-dihydrodiol, whilst from 9,10-dihydrodiol, 9,10-dihydroxybenzo-(a)pyrene was mainly formed, is consistent with the observations of Booth and Sims [8] using isolated liver microsomes to study the metabolic fate of these dihydrodiols. The formation of 7,8,9,10-tetrahydrotetrol from 7,8-dihydrodiol also suggests that some of the ethyl acetate-soluble metabolites from benzo-(a)pyrene, which migrate at or near the origin in the solvent system used in this study (Fig. 3) may contain this tetrahydrotetrol as well as benzo(a)pyren-3-yl hydrogen sulphate together with other as yet unidentified metabolites.

It is of interest to note formation of 7,8,9,10-tetrahydrotetrol from 7,8-dihydrodiol, thus indicating that hepatocytes are capable of producing the diol-epoxide 7,8-dihydro-7,8-dihydroxybenzo(a)pyrene 9,10-oxide, the postulated ultimate carcinogen of benzo(a)pyrene (7,33). The possibility therefore exists that 7,8-dihydrodiol retained within hepatocytes may act as a "store" of slowly released metabolite, capable of being metabolised to the ultimate carcinogen.

Since the 9,10-dihydrodiol was the major ethyl acetate-soluble product formed by the cells, it is necessary to consider the possible significance of the metabolism of 9,10-dihydrodiol to its diol-epoxide, 9,10-dihydro-9,10-dihydroxybenzo(a)pyrene 7,8-oxide. In the light of our findings with isolated hepatocytes we consider this pathway to be of relatively minor significance because; (a) newly-formed 9,10-dihydrodiol is readily eliminated from the hepatocytes and (b) the dihydrodiol remaining in the cells is metabolised to the catechol which is then conjugated and eliminated. In similar experiments with short-term organ culture of hamster lung, 9,10-dihydrodiol was metabolised to a small extent, presumably via diol-epoxide, to 7,8,9,10-tetrahydro-7,8,9,10-tetrahydroxybenzo(a)pyrene. Little or no 9,10-dihydroxybenzo(a)pyrene was found as an ethyl-acetate soluble metabolite but small amounts of unidentified water-soluble metabolites were obtained [31]. A major portion of these minor unidentified water soluble metabolites have subsequently been identified as the glucuronide conjugate of 9,10-dihydroxybenzo(a)pyrene (Moore and Cohen, unpublished observations). As the formation of the catechol 9,10-dihydroxybenzo(a)pyrene from 9,10-dihydrodiol appears to be much slower in lung tissue than in hepatocytes, it is possible that more diol-epoxide may also be formed from 9,10-dihydrodiol in lung tissue than in liver.

Whilst much of the above discussion has emphasised the relative retention within the hepatocyte of 3-hydroxybenzo(a)pyrene, quinones and 7,8- and 4,5-dihydrodiols, it may also be of significance that such metabolites also escape into the medium (Table 2). Release of small amounts of metabolites from the hepatocyte *in vivo* may be of biological significance. 7,8-Dihydrodiol, a proximate carcinogen, released from the liver, a relatively insensitive organ to hydrocarbon carcinogenesis, may be taken up by other more susceptible tissues such as lung, where the dihydrodiol may be converted to the ultimate carcinogen. In these experiments using hepatocytes it is not possible to discern whether metabolites found in the medium could reach the systemic circulation or be excreted in the bile *in vivo*. In *in vivo* experiments with rats injected with benzo(a)pyrene, only very small amounts of ethyl acetate-soluble metabolites were excreted in the bile. No 7,8,- 4,5- and 9,10-dihydrodiols or 3-hydroxybenzo(a)pyrene and only small amounts of benzo(a)pyren-3-yl hydrogen sulphate were excreted in the bile (Haws and Cohen, unpublished observations). Thus it is possible that the liver may be a source of potentially toxic metabolites, as metabolites released by the hepatocytes could reach other tissues by the systemic circulation.

In this study we have demonstrated the covalent binding of benzo(a)pyrene metabolites with cellular constituents (Fig. 7). The nature of these metabolites and the consequence of this binding has not been investigated in this study but such covalent binding and the factors affecting it may be of importance in the process of tumour initiation.

In conclusion, this study has demonstrated that benzo(a)pyrene is extensively metabolised by viable hepatocytes isolated from untreated rats to give a wide range of Phase I and Phase II metabolites. Certain of these metabolites (notably 3-hydroxybenzo(a)pyrene and its sulphate ester, benzo(a)pyren-3-yl hydrogen sulphate, and the 4,5- and 7,8-dihydrodiols) are selectively retained by the hepatocytes possibly by binding to intracellular binding proteins or lipid membranes. Whilst the *in vivo* significance of this selective metabolite retention by the hepatocytes is uncertain, it is possible that it may lead to the maintenance of high intracellular levels of substrates capable of being metabolised to the ultimate carcinogen. Finally, as isolated rat hepatocyte suspensions have been clearly shown in the present study to be of great value in studying the metabolism of benzo(a)pyrene, it is to be hoped that use of similar cell systems derived from different tissues and species and the influence of various drug metabolism inducers and inhibitors on them will lead to a greater understanding of the tissue and species differences in benzo(a)pyrene carcinogenicity.

Acknowledgements—Miss C. A. Jones is in receipt of a Science Research Council Grant. The work was also partly sponsored by grants from the Cancer Research Campaign and Medical Research Council.

REFERENCES

1. *Natn Acad. Sci.* Biological Effects of Atmospheric Pollutants, Particulare Polycyclic Organic Matter, Washington, D.C. (1972).
2. C. Heidelberger, *Ann. Rev. Biochem.* **44**, 79 (1975).
3. E. Boyland, *Fedn. Eur. Biochem. Soc. Symp.* **16**, 183 (1969).
4. H. V. Gelboin, N. Kinoshita and F. J. Wiebel, *Fedn Proc.* **31**, 1298 (1969).
5. P. Sims and P. L. Grover, *Adv. Cancer Res.* **20**, 165 (1974).
6. D. M. Jerina and J. W. Daly, *Science N.Y.* **185**, 573 (1974).

7. P. Sims, P. L. Grover, A. Swaisland, K. Pal and A. Hewer, *Nature, Lond.* **225,** 326 (1974).
8. J. Booth and P. Sims, *Biochem. Pharmac.* **25,** 979 (1976).
9. G. M. Cohen, S. M. Haws, B. P. Moore and J. W. Bridges, *Biochem. Pharmac.* **25,** 2561 (1976).
10. G. Holder, H. Yagi, P. Dansette, D. M. Jerina and W. Levin, *Proc. natn. Acad. Sci., U.S.A.* **71,** 4356 (1974).
11. J. K. Selkirk, R. G. Croy, P. P. Roller and H. V. Gelboin, *Cancer Res.* **34,** 3474 (1974).
12. D. W. Nebert and H. V. Gelboin, *J. biol. Chem.* **243,** 6250 (1968).
13. J. R. Fry and J. W. Bridges, in *Progress in Drug Metabolism* (Eds J. W. Bridges and L. F. Chasseaud). Vol. 2, p. 71. Wiley & Sons, London (1977).
14. J. R. Fry and J. W. Bridges, *Biochem. Pharmac.*, **26,** 969 (1977).
15. J. L. Holtzman, V. Rothman and S. Margolis, *Biochem. Pharmac.* **21,** 581 (1972).
16. H. Vadi, P. Moldéus, J. Capdevila and S. Orrenius, *Cancer Res.*, **35,** 2083 (1975).
17. J. W. DePierre, M. S. Moron, K. A. M. Johannesen and L. Ernster, *Analyt. Biochem.* **63,** 470 (1975).
18. P. Sims, *Biochem. Pharmac.* **19,** 795 (1970).
19. J. R. Fry, C. A. Jones, P. Wiebkin, P. Bellemann and J. W. Bridges, *Analyt. Biochem.* **71,** 341 (1976).
20. J. Paul, *Cell and Tissue Culture* 4th edition, (Eds E. and S. Livingstone), p. 91. Edinburgh (1970).
21. G. M. Cohen and B. P. Moore, *Biochem. Pharmac.* **25,** 1623 (1976).
22. D. W. Nebert and H. V. Gelboin, *J. biol. Chem.* **243,** 6242 (1968).
23. P. Siekevitz, *J. biol. Chem.* **195,** 549 (1952).
24. H. V. Gelboin, E. Huberman and L. Sachs, *Proc. natn. Acad. Sci., U.S.A.* **64,** 1188 (1969).
25. N. Nemoto and H. V. Gelboin, *Biochem. Pharmac.* **25,** 1221 (1976).
26. J. Capdevila, B. Jernström, H. Vadi and S. Orrenius, *Biochem. biophys. Res. Commun.* **65,** 894 (1975).
27. J. K. Selkirk, R. G. Croy and H. V. Gelboin, *Cancer Res.* **36,** 922 (1976).
28. G. M. Cohen, B. P. Moore and J. W. Bridges, *Biochem. Pharmac.* **26,** 551 (1977).
29. P. Wiebkin, J. R. Fry, C. A. Jones, R. Lowing and J. W. Bridges, *Xenobiotica* **6,** 725 (1976).
30. A. Leo, C. Hansch and D. Elkins, *Chem. Rev.* **71,** 525 (1971).
31. G. M. Cohen and B. P. Moore, *Biochem. Pharmac.* **26,** 1481 (1977).
32. B. P. Moore, G. M. Cohen, J. W. Bridges and D. V. Parke, *Biochem. Soc. Trans.*, (in press).
33. R. F. Newbold and P. Brookes, *Nature, Lond.* **261,** 52 (1976).