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THE WATER-SOLUBLE AND PROTEIN-BOUND METABOLITES OF BENZO(a)PYRENE FORMED BY RAT LIVER

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Abstract—An optimal incubation system to study benzo(a)pyrene metabolism to its polar derivatives is described. The system using the 20,000 *g* supernatant enzymes of rat liver converts up to 80 per cent of micromolar concentrations of [^{14}C]benzo(a)pyrene to water-soluble products of which about 10 per cent become covalently bound to protein, are trichloroacetic acid-precipitable, not extractable into organic solvents and are stable to polyacrylamide gel electrophoretic and gel filtration separation techniques.

Four (I–IV) discrete[^{14}C]benzo(a)pyrene–protein complexes have been purified by ion exchange Sephadex gel filtration from post-incubation cytosols. Protein I has the highest binding specificity, a molecular weight estimated at 44,500, migrates as a dimer in sodium dodecyl sulphate polyacrylamide gel, and has glutathione *S*-transferase activity. This binding protein is believed to be at least similar to, if not identical with, ligandin. Proteins II–IV are also active as glutathione *S*-transferases. This enzymic function of the binding proteins is markedly reduced by the covalent attachment of the metabolised benzo(a)pyrene, so that the de-activating conjugation with GSH of reactive metabolites is inhibited. The carcinogen, under these conditions hampers its own detoxification reaction.

Certain metabolic inhibitors reduce specific binding of the carcinogen to protein I and enhance the binding to the other proteins. The less specific binding to proteins II–IV may be important to the carcinogenic process.

Studies of benzo(a)pyrene (B(a)P) metabolism in cultured cell systems, isolated hepatocytes or *in vivo* have revealed that, in contrast to metabolism in microsomal systems *in vitro*, a large proportion of the carcinogen is converted to water-soluble polar and potentially excretable products such as conjugates with glutatione, glucuronic acid or sulphate [1, 2, 3]. In addition, adducts of B(a)P have been described with at least two cytosolic binding proteins, ligandin and the "h" protein [4–7].

RNA or protein might be intracellular targets of polycyclic hydrocarbon carcinogens, but major interest recently has centred on the covalent binding of carcinogen metabolites to DNA as the critical event in tumorigenesis. Nevertheless the reported relatively specific covalent binding of polycyclic hydrocarbons to a small number of soluble proteins suggests that such binding may play a role in carcinogenesis.

In order to elucidate such a possible role, we here report studies on the water-soluble products of B(a)P formed *in vitro* by rat liver preparations, their binding to soluble proteins, and modification of this metabolism by inducers and inhibitors which have been shown to alter B(a)P-induced carcinogenicity, mutagenicity or binding to DNA.

MATERIALS AND METHODS

Tissue preparation. Livers from male Wistar rats (200–300 g), routinely induced by intraperitoneal (i.p.) injection of unlabelled B(a)P (45 mg/kg body weight dissolved in safflower oil) 16 hr prior to slaughter, were homogenised in cold 0.25 M sucrose, using a glass Dounce homogeniser (Blaessig Glass, Rochester, N.Y.) to provide a 20% (w/v) suspension. The pellets obtained by centrifugation at 700 *g* for 10 min, 9000 *g* for 10 min and 20,000 *g* for 20 min (Sorvall RC-2B refrigerated centrifuge), were successively discarded, and the final 20,000 *g* supernatant (SN) fraction used as the enzyme source. All procedures were carried out at 0–2°. Protein was quantitated by the method of Lowry *et al.* [8].

Incubation system. The reaction medium, in a final volume of 1 ml, contained 0.5 mM NADPH, 2 mM $MgCl_2$, 50 mM Tris–HCl buffer pH 7.4, 1.5 μM [7,10-^{14}C]-B(a)P sp. act. 25.3 mC/mmole (Radiochemical Centre, Amersham) and the 20,000 *g* SN fraction equivalent to 50 mg wet liver. When required, inhibitors were included, 3,3,3-trichloropropylene oxide (TCPO, Aldrich) and diethyl maleate (DEM, Aldrich) dissolved in ethanol, styrene oxide (StO, Aldrich) in aqueous-ethanol, and *N*-ethylmaleimide (NEM), bromosulphophthalein (BSP), β-oestradiol, testosterone, retinol, bilirubin, 2,4-dinitrophenol (2,4-DNP) and dithiothreitol (DTT) from Sigma Chemical Co., MO, USA, in aqueous solutions. Incubation was for 30 min at 37° in a shaking water bath in the dark, and was generally terminated by the addition of 3 ml cold acetone/ethyl acetate, 1:2, followed by immediate vortex mixing for 30 sec and brief centrifugation at 2° to aid in

phase separation. The ^{14}C content of the aqueous layer, measured in Instagel (Packard Instrument Co., IL., USA) in a Packard Liquid Scintillation spectrometer, provided a measure of the water soluble metabolites. Protein-bound metabolites were measured by precipitation by trichloroacetic acid (TCA) onto filter paper disks, followed by an organic solvent washing procedure [9].

Purification of binding proteins. Large volume (8 ml) incubations with [^{14}C]-B(a)P were terminated by ultracentrifugation (Beckman Spinco L-2) at 100,000 *g* for 60 min, and the cytosol fractionated on a Sephadex G50 column at 2° eluted with 0.01 M Tris–HCl buffer, pH 7.5. The ^{14}C-containing protein band was collected, concentrated by ultrafiltration (Amicon XM50 membrane) and then fractionated on a DEAE-Sephadex A50 column at 2°. The breakthrough proteins (peak I) were eluted with 0.01 M Tris–HCl buffer, pH 7.5, and a linear gradient of 0–0.5 M sodium chloride then eluted up to 3 further protein bands (peaks II–IV). All four protein peaks were found to be radiolabelled. They were concentrated separately by ultrafiltration and stored at −10°. The same binding proteins were also purified from incubations in the absence of the carcinogen.

Characterisation of binding proteins. Molecular weight was determined by Sephadex G200 gel filtration [10–12] calibrated by standard proteins.

Polyacrylamide disc gel electrophoresis of the native binding proteins was performed as described by Davis [13], and of the sodium dodecyl sulphate (SDS)-denatured proteins according to Shapiro *et al.* and Kuroki and Heidelberger [14, 15].

Proteolytic digestion of the [^{14}C]-B(a)P-proteins was carried out with an equal volume of 1% (w/v) suspension of pronase (Sigma) at 37° for one hour in the dark. The TCA-precipitable ^{14}C, and ethyl acetate soluble ^{14}C were monitored before and after digestion. Absorption spectra of the ethyl acetate extracts after digestion were measured between 200 and 400 nm with a double beam Beckman recording spectrophotometer.

Glutathione (GSH) *S*-transferase activity was assayed with substrates 1,2-epoxy-3-(*p*-nitrophenoxy)propane (ENPP, Eastman) according to Clifton *et al.* [16] and 1-chloro-2,4-dinitrobenzene (DNCB, Univar) according to Habig *et al.* [17].

RESULTS

Metabolism to water-soluble products

The rate of *in vitro* production of water-soluble metabolites of [^{14}C]-B(a)P was linear for 30 min for a range of [^{14}C]-B(a)P concentrations (Fig. 1). The rate was also shown to be inversely dependent on the concentration of [^{14}C]-B(a)P (Table 1). At best, at 1.5 μM [^{14}C]-B(a)P, the substrate concentration routinely used, the system converted 42.5 per cent of the total [^{14}C]-B(a)P to water-soluble products and 3.14 per cent to protein-bound ^{14}C after 30 min incubation at 37°. Under control conditions, approximately 8–10 per cent of the water-soluble metabolites always became bound to protein. The enzymes could be inactivated by heating at 100° for 10 min, but a low level of spontaneous reaction remained (Table 1).

Metabolism was inducible by phenobarbital (PB), and, more strongly, by pretreatment with unlabelled B(a)P (Table 2).

Inhibition of metabolism to water-soluble products

Inhibitors of the microsomal enzymes involved markedly reduced the water-soluble metabolites (Table 3). Inhibition of aryl hydrocarbon hydroxylase by 7,8-benzoflavone (7,8-BF) and of epoxide hydrase by TCPO or StO, as well as the presence of sulphydryl-binding reagents NEM and DEM and of the ligandin substrate BSP, not only reduced the aqueous products, but increased their proportional binding to protein about two-fold. Conversely

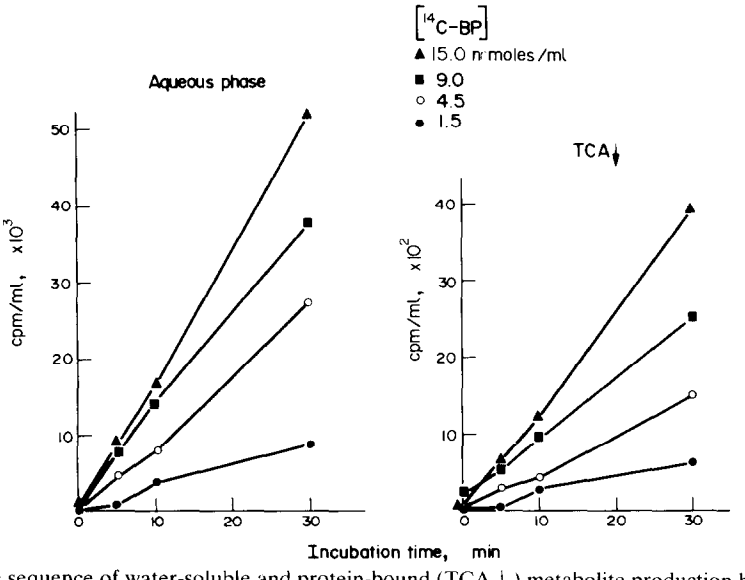

Fig. 1. Time sequence of water-soluble and protein-bound (TCA ↓) metabolite production by the liver from [^{14}C]-B(a)P at varying concentrations.

Table 1. [^{14}C]-B(a)P concentration-dependence of water-soluble metabolite production and protein-binding, measured as a percentage of total ^{14}C initially added, including a boiled enzyme preparation

[^{14}C]-B(a)P concn	% Total ^{14}C Added			
	Cystolic ^{14}C		Bound ^{14}C	
	t_0	t_{30}	t_0	t_{30}
15.0 μM		25.5		1.96
9.0		31.3		2.12
4.5		37.5		2.53
1.5	0.41	42.5	0.03	3.14
1.5 μM boiled enzyme	0.39	0.53	0.09	0.09

Table 2. Effect of induction on liver metabolism

	[^{14}C]-B(a)P concn (pmoles/ml)			
	Aqueous phase		TCA ↓	
	t_0	t_{30}	t_0	t_{30}
Normal Rat	3.5	305.0	3.5	24.4
PB-induced	3.6	752.8	4.8	32.0
Normal Rat	3.6	123.0	2.3	8.5
B(a)P-induced	4.9	1158.8	2.9	90.4

[^{14}C]-B(a)P = 1.5 nmoles/ml.

whereas bilirubin and retinol, and to a smaller degree the steroid hormones and 2,4-DNP were also inhibitory to the production of water-solubles, they had no influence on the binding of the carcinogen to protein.

Inhibition by sulphydryl reagents could be reversed by thiols. When DEM was administered i.p. 2 hr prior to preparation of the liver enzymes, in order to deplete the liver of GSH, the subsequent depression of *in vitro* metabolism was prevented either by *in vitro* addition of GSH, or by intraperitoneal cysteine 15 min prior to slaughter (Table 4).

Purification of [^{14}C]-*B(a)P-protein complexes*

The protein and ^{14}C profiles eluting from DEAE-Sephadex showed four separate radioactive cytosolic proteins (Fig. 2). Peak I, the breakthrough protein, had the highest specific activity of ^{14}C-binding. The gradient-eluted peaks II and III and the less well-defined peak IV were radiolabelled to a lesser degree. Under the influence of different metabolic inhibitors, the ^{14}C-binding to peak I was reduced and ^{14}C-binding to the other less-specific proteins was increased (Table 5 and Fig. 2). From the specific

Table 3. Effect of inhibitors *in vitro* on [^{14}C]-B(a)P metabolism by B(a)P-induced rat liver enzymes to water-soluble products and their binding to cytosolic protein

	Cytosolic [^{14}C]-B(a)P concn (μmoles/ml)			
	Total	% Control	TCA ↓	% Protein bound
Control	1.57	100.0	0.19	12.4
Flavone				
7,8-BF 0.1 mM [37]*	0.67	42.7	0.09	47.4
Epoxides				
TCPO 3 mM [26, 30]	0.62	37.4	0.14	23.4
StO 3 mM	0.71	45.0	0.15	21.0
SH-Reagents				
NEM 1 mM [28, 38]	0.77	50.4	0.15	20.0
DEM 3 mM	1.27	82.5	0.26	20.3
Thiols				
DTT 3 mM [39, 40]	1.50	100.0	0.22	14.9
Ligandin substrates				
BSP 1 mM	0.92	61.5	0.27	29.0
Bilirubin 3 mM	0.86	55.1	0.10	11.3
Steroid hormones				
Oestradiol 3 mM	1.19	80.4	0.15	12.9
Testosterone 3 mM	0.81	54.8	0.13	15.6
Miscellaneous				
2,4-DNP 3 mM [41]	1.05	70.1	0.15	14.5
Retinol 3 mM [42, 43]	0.30	20.5	0.15	17.6

* References in square brackets.

Table 4. Effect of *in vivo* DEM inhibition on subsequent [^{14}C]-B(a)P metabolism *in vitro*

Animal	*In vitro* addition	Cytosolic [^{14}C]-B(a)P concn (pmoles/ml) Total	% Control	TCA ↓	% Protein bound
Control	—	801.7	100	74.5	9.3
	GSH 1 mM	800.2	99.8	71.2	8.9
	Cysteine 1 mM	797.4	99.5	61.6	7.7
DEM i.p.	—	739.5	92.2	107.8	14.6
	GSH 1 mM	770.6	96.1	74.0	9.6
	Cysteine 1 mM	714.4	89.1	104.8	14.7
DEM i.p. + Cysteine i.p.	—	763.8	95.3	95.8	12.5

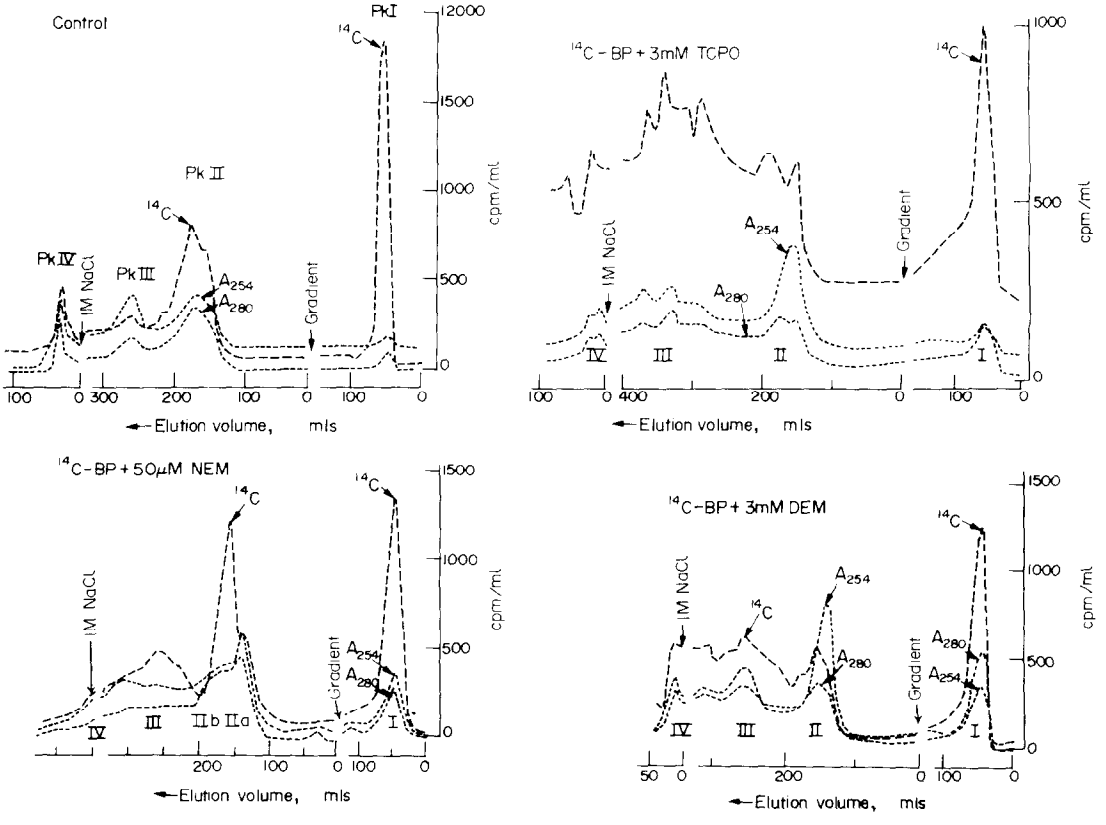

Fig. 2. Ion exchange gradient elution of [^{14}C]-B(a)P-incubated cytosols, showing effect of three metabolic inhibitors.

Table 5. Specificity of binding of [^{14}C]-B(a)P to binding proteins purified from *in vitro* incubated cytosols

Incubation	Bound [^{14}C]-B(a)P (cpm/μg protein) Peak I	Peak II	Peak III	Peak IV
Control	44.8	6.5	2.8	2.6
+ 3 mM TCPO	6.8	5.0	5.6	—
+ 3 mM DEM	6.3	6.4	9.7	6.9
+ 50 μM NEM	12.3	7.8	5.0	—

activity of the [^{14}C]-B(a)P used, it is calculated that the binding capacity of peak I was 0.999 μmoles/mg protein.

Determination of molecular weight

Of the purified [^{14}C]-B(a)P-proteins, only peak I in its native state migrated electrophoretically in polyacrylamide gel towards the anode (mobility 0.319 ± 0.025). Peak II migrated slowly towards the cathode if the polarity was reversed but peak III was immovable.

The SDS-denatured peak I resolved into several components; the major ^{14}C band, which was a dimer, provided a measure of its subunit molecular weight (25,600). Elution from Sephadex G200 indicated molecular weights of 44,500 for peak I, 29,300 for peak II and 31,300 for peak III.

GSH S-transferase activity

From the activities measured on the available binding proteins purified with and without bound carcinogen, and from untreated as well as B(a)P-induced rats, it emerged that *S*-transferase activity was detectable in all four proteins, peak I having the highest specific activity for *S*-aryl (DNCB) transferase and peak III for *S*-epoxide (ENPP) transferase (Table 6).

The *S*-aryl transferase activity was B(a)P-inducible and was found with high specificity in peak I, increasing about 10-fold by the purification of the protein from whole cytosol. Furthermore, the activity was markedly reduced by the covalent attachment of [^{14}C]-B(a)P mediated by the *in vitro* incubation. The *S*-epoxide activity was not inducible by B(a)P pre-treatment, but was similarly markedly reduced by the binding of [^{14}C]-B(a)P.

"Pronase" digestion of [^{14}C]-B(a)P proteins

The action of pronase removed 88–95 per cent of the protein from the carcinogen, which was no longer TCA-precipitable, and 35–36 per cent of the originally protein-bound ^{14}C was now extractable into ethyl acetate (Table 7).

The ultraviolet absorption spectra (200–400 nm) of the ethyl acetate extract of each digested B(a)P-protein shows three regions of absorption, around 200, 240 and 280 nm (Fig. 3). Peaks III and IV have a major absorption peak around 240 nm, which is detectable but not marked in peaks I and II, suggesting that there may be two different B(a)P metabolites involved in protein-binding (Fig. 3). The absence of any absorbance corresponding to the 267 nm peak of chrysene [18] indicated that the covalent bonds to protein were not *via* the 4C–5C K-region. Absorbance at 240 nm however may correspond to the major 238 nm peak in the spectrum of pyrene suggesting that the polycyclic structure had been opened between C atoms 7–10. Recent research suggests that a 7,8-diol-9,10-epoxide of B(a)P is the ultimate form which binds to DNA [19–21]. This region may also be responsible for the binding to the cytosolic proteins.

DISCUSSION

The *in vitro* incubation system described utilises both activating and de-activating enzymes, and provides optimal metabolism of B(a)P to polar deriva-

Table 6. GSH *S*-transferase activities of post-incubation whole cytosols and purified [^{14}C]-B(a)P-binding proteins (peaks I to IV)

	Cytosol	Peak I	Peak II	Peak III	Peak IV
	Δ OD/hour/mg protein				
GSH *S*-ENPP Transferase					
Non-induced, incubated-B(a)P		1.398			
Induced, incubated-B(a)P	0.584	1.032			
Induced, incubated + [^{14}C]-B(a)P	0.598	0.263	0.439	0.688	0.410
	Δ OD/minute/mg protein				
GSH *S*-DNCB Transferase					
Non-induced, incubated-B(a)P	3.03	22.95			
Induced, incubated-B(a)P	3.97	40.00			
Induced, incubated + [^{14}C]-B(a)P	5.57	3.15	0.69	0.13	0.05

Table 7. TCA-Precipitable and ethyl acetate-soluble ^{14}C remaining from [^{14}C]-B(a)P-proteins purified from control incubations following treatment with pronase

[^{14}C]-B(a)P-protein peak	% Total ^{14}C concn	
	TCA-precipitable ^{14}C	Ethyl acetate-soluble ^{14}C
I	5.0	47.5
IIa	5.5	35.4
IIb	9.9	47.3
III	8.7	56.0
IV	12.4	50.9

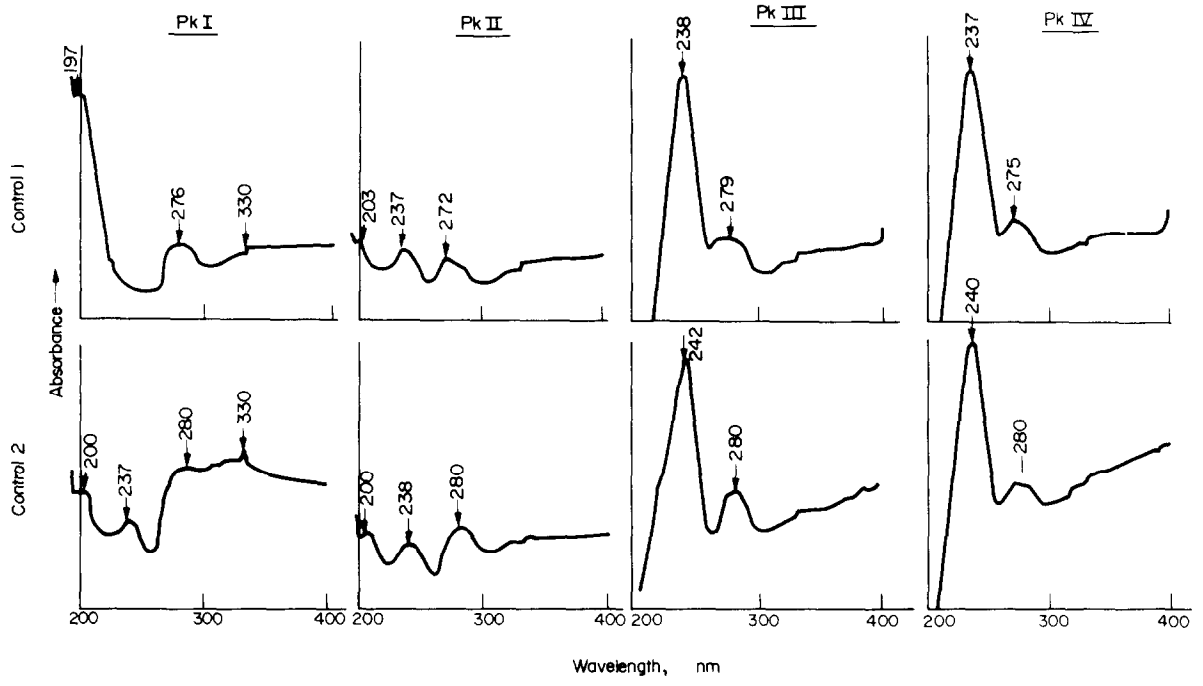

Fig. 3. U.v. absorption spectra of ethyl acetate extracts of duplicate pronase-digested B(a)P–protein complexes, (Peaks I–IV) purified from two separate incubations with [^{14}C]-B(a)P.

tives, simulating the *in vivo* situation. Induced rat liver enzymes converted up to 80 per cent of micro-molar concentrations of B(a)P to water-soluble products no longer extractable into ethyl acetate. Metabolism proceeded more efficiently as the B(a)P concentration was reduced, and could be markedly induced by pretreatment of animals with phenobarbital or B(a)P. Such conditions of repeated exposure to small amounts of carcinogens in the environment probably prevail *in vivo*.

About 10 per cent of the aqueous metabolites became bound to cytosolic proteins and could be precipitated from solution by TCA. When partially purified from post-incubation cytosols, the four [^{14}C]-B(a)P-protein complexes eluted as single peaks from Sephadex gels, and migrated electrophoretically in polyacrylamide gels. Following "pronase" digestion of the protein moiety, the ^{14}C portion became ethyl acetate-soluble, and showed absorption spectral characteristics of ring fission, possibly at two regions of the polycyclic structure. The carcinogen–protein bond was therefore deemed to be a covalent linkage in agreement with other workers [18, 22–24].

In response to inhibitors, water-soluble metabolites were reduced, but epoxide hydrase inhibitors (TCPO, StO) and SH-reagents (DEM, NEM), two classes of compounds which have repeatedly been shown to enhance either the binding of polycyclic hydrocarbon metabolites to DNA [25–28], their mutagenicity [29] or their initiation of skin tumours [26, 30], were shown to cause an increased proportional binding of aqueous B(a)P forms to protein. Their inhibitory effect on the metabolism resulted in reduced B(a)P binding to the major binding protein (peak I) but increased binding to the less-specific binders (peaks II–IV). Peak I is believed to be similar if not identical with ligandin, because of its molecular weight, migration as a dimer in SDS-acrylamide gels, activity as a GSH *S*-transferase but not as the major *S*-epoxide transferase, and inhibited capacity to bind B(a)P in the presence of its well-known ligand, BSP.

All four binding proteins are active GSH *S*-transferases. The enzymic function of binding protein I is reduced by the covalent attachment of B(a)P; thus the carcinogen effectively inhibits one major route of its own detoxification, viz. by its enzymatically catalysed conjugation with GSH. Non-covalent ligands such as BSP, bilirubin, oestradiol sulphate and indocyanine green, are also known to reduce ligandin's enzyme activity [33–35].

A recent report [36] described the noncovalent *in vitro* binding, in the absence of microsomal enzymes, of 3-methylcholanthrene to a rat liver cytosolic protein of similar molecular weight (44,600) to peak I. The noncovalent binding capacity was approximately one thousandth of the covalent binding we report; however, the carcinogen was transferable to nuclei without prior enzymatic alteration. Studies to test whether covalently bound B(a)P can also be intracellularly translocated are currently under way.

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