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COVALENT BINDING OF BENZO[*a*]PYRENE TO RAT LIVER CYTOSOLIC PROTEINS AND ITS EFFECT ON THE BINDING TO MICROSOMAL PROTEINS

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Abstract—Benzo[*a*]pyrene will bind covalently to rat liver cytosolic proteins when incubated with microsomes and NADPH. The binding is most extensive when microsomes from 3-methylcholanthrene-treated rather than phenobarbital-treated or control rats are used. The binding to cytosolic proteins increases when incubations are performed with increasing concentrations of cytosol. At the same time the covalent binding of benzo[*a*]pyrene to microsomal proteins decreases. Two cytosolic polypeptides are the main targets for benzo[*a*]pyrene. These have the same mobility in polyacrylamide gels as the subunits of purified glutathione *S*-transferase B. These subunits also react covalently with benzo[*a*]pyrene when the transferase is incubated with microsomes and NADPH.

Several xenobiotics are converted to chemically reactive metabolites by the microsomal cytochrome P-450-linked monooxygenase system. These metabolites may bind covalently to tissue macromolecules [1–4] thereby eliciting their harmful effects. Covalent binding of xenobiotics to microsomal [3, 5], nuclear [4, 6, 7] and cytosolic [8–11] proteins has been reported. Recent evidence indicates that such binding occurs preferentially to some proteins [6, 12–15] rather than being a random event. Thus, ligandin is a preferred target in rat liver cytosol for polycyclic aromatic hydrocarbons administered *in vivo* [4, 8, 11]. Both 3-MC‡ and B[*a*]P will bind covalently to soluble proteins *in vitro* when incubated with postmitochondrial supernatant and reduced pyridine nucleotides [8, 10]. Therefore, metabolic activation of these compounds, presumably catalyzed by microsomal enzymes, seems to be required for binding.

In this report is shown that irreversible binding of B[*a*]P to cytosolic proteins is dependent on microsomes. It is also shown that the binding to cytosolic proteins results in a corresponding decrease in binding to microsomal proteins. This observation might be of importance in the assessment of the toxicity of polycyclic aromatic hydrocarbons and other compounds that undergo metabolic activation.

EXPERIMENTAL

Chemicals. [7,10-^{14}C]B[*a*]P (21.7 mCi/mmol) was obtained from the Radiochemical Centre, Amersham. Different forms of glutathione *S*-transferase (EC 2.5.1.18) purified according to [17] were a generous gift from Dr. B. Ketterer, London.

Animals and treatments. Male Sprague–Dawley rats (Anticimex, Stockholm) weighing 150–200 g, were allowed free access to food and water. The animals were treated with PB (0.1% in the drinking water for 5 days), 3-MC (one intraperitoneal injection of 80 mg/kg body weight in corn oil 24 hr before sacrifice), or were untreated (control animals).

Preparation of microsomes and cytosol. Microsomes were prepared as described earlier [12]. The cytosol was obtained as the 105,000 *g* supernatant, and was chromatographed through Sephadex G-25 (3 × 20 cm column) to remove low molecular weight material. The column was previously equilibrated and developed in 50 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, and 25 mM KCl. The material eluting with the void volume was collected. Both microsomes and cytosol were stored in aliquots at −20°.

Analytical procedures. Cytochrome P-450 was determined according to Omura and Sato [18]. The average cytochrome P-450 content was 0.60, 2.17 and 1.10 nmol/mg protein in microsomes from control, PB- and 3-MC-treated animals, respectively. Protein was determined by the Lowry procedure [19] using bovine serum albumin as standard.

Quantitative determination of covalently bound B[a]P. The binding of B[*a*]P to microsomal and cytosolic proteins was analysed as described in detail by Wallin *et al.* [20]. The incubations contained 10 mM Tris-HCl, pH 7.4, sample (0.20 mg microsomal and/or 0.72 mg cytosolic protein), 2 mM NADPH and B[*a*]P (added in 3 μl DMSO) in a volume of 200 μl. After incubation the proteins were precipitated on filterpaper discs and unbound B[*a*]P was extracted as described [20]. The incorporated radiolabel was measured in 0.4% Omnifluor (New-England Nuclear) in toluene. In experiments where microsomes and cytosol were separated after the incubation, the volume was scaled-up to 2.5 ml. These latter incubations were for 30 min and the samples were centrifuged at 105,000 *g* for 1 hr at 4°. The resulting microsomal pellet was suspended in

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‡ Abbreviations: B[*a*]P, benzo[*a*]pyrene; 3-MC, 3-methylcholanthrene; PB, phenobarbital.

1 ml of 50 mM Tris-HCl, pH 7.4, using a Potter homogenizer.

Analysis of protein binding patterns. Samples containing 70 μg of protein obtained from the incubations were treated for SDS-polyacrylamide gel electrophoresis and electrophoresced in slab gels as described earlier [21, 22]. After staining and destaining gels were treated with 2,5-diphenyloxazole and fluorographed [23] using Kodak X-Omat films.

RESULTS AND DISCUSSION

The covalent binding of B[*a*]P to rat liver cytosolic and microsomal components was examined first. In the presence of NADPH [^{14}C]B[*a*]P became bound irreversibly to microsomal proteins. This reaction was linear with time for around 20 min and reached a maximum after 30 min (Fig. 1). The rate of covalent binding (and also the maximum binding) was much higher into microsomes from 3-MC-treated rats than into microsomes from PB-treated or control rats (initial rates were 150, 50 and 15 pmol/min per mg protein, respectively). No significant binding occurred in the absence of NADPH indicating that a metabolic activation of B[*a*]P preceded binding.

The possibility that B[*a*]P would form adducts with cytosolic proteins upon incubation with cytosol and NADPH was then examined (Fig. 1). The specific

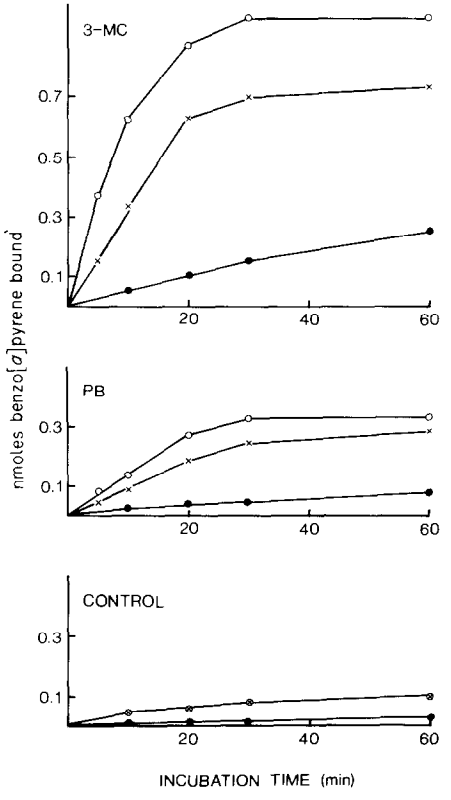

Fig. 1. Time courses of B[*a*]P binding to microsomal and cytosolic proteins. Microsomes (×), cytosol (●), or a microsome–cytosol mixture (○) were incubated and the binding analysed as described in Experimental. Liver microsomes and cytosol were in each case from control, 3-MC- or PB-treated rats.

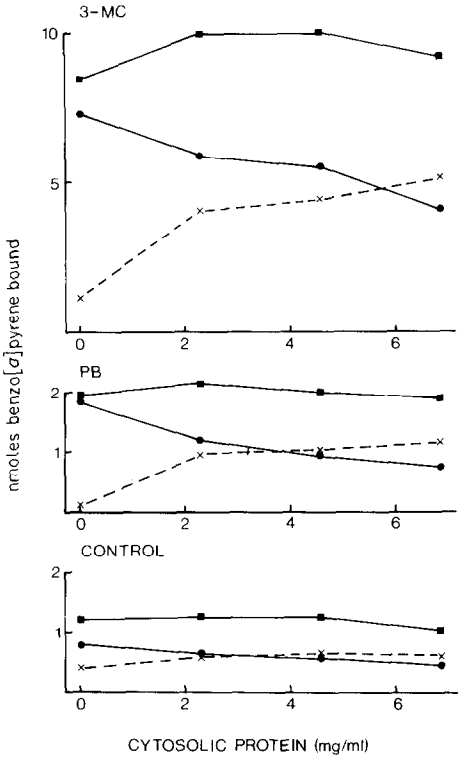

Fig. 2. The binding of B[*a*]P to soluble and microsomal proteins after incubation with increasing amounts of cytosol. Incubations were as described in Experimental. The soluble fraction (×) and microsomes (●) were separated by centrifugation after the incubation. The total binding (■) is also indicated.

irreversible binding observed with cytosol was approximately 20 times lower than with microsomes (note than 0.72 mg of cytosolic protein was used in the incubations compared to 0.20 mg of microsomal protein). α-Naphtoflavone, SKF 525A or CO inhibited this binding indicating that it was due to a slight microsomal contamination rather than to an intrinsic activity of the cytosol.

To examine whether microsomal enzymes would catalyse a binding of B[*a*]P into cytosolic components, these fractions were combined and incubated with NADPH (Fig. 1). The initial rate of B[*a*]P binding into the combined fractions isolated from 3-MC-treated rats was 50–100% higher than expected from the sum of the individual fractions. No such enhancement was seen in the combined fractions of PB-treated or control rats.

Since these experiments did not give clear evidence of a microsome-catalysed binding of B[*a*]P to cytosolic components, the matter was examined further by incubating combined fractions with B[*a*]P and NADPH, and separating the fractions by centrifugation before quantification of the binding (Fig. 2). It was then found that a substantial amount of B[*a*]P reacted covalently with the soluble fraction. Furthermore, if the amount of cytosol in the incubation mixture was increased relative to microsomes a progressively larger part of B[*a*]P became bound to the

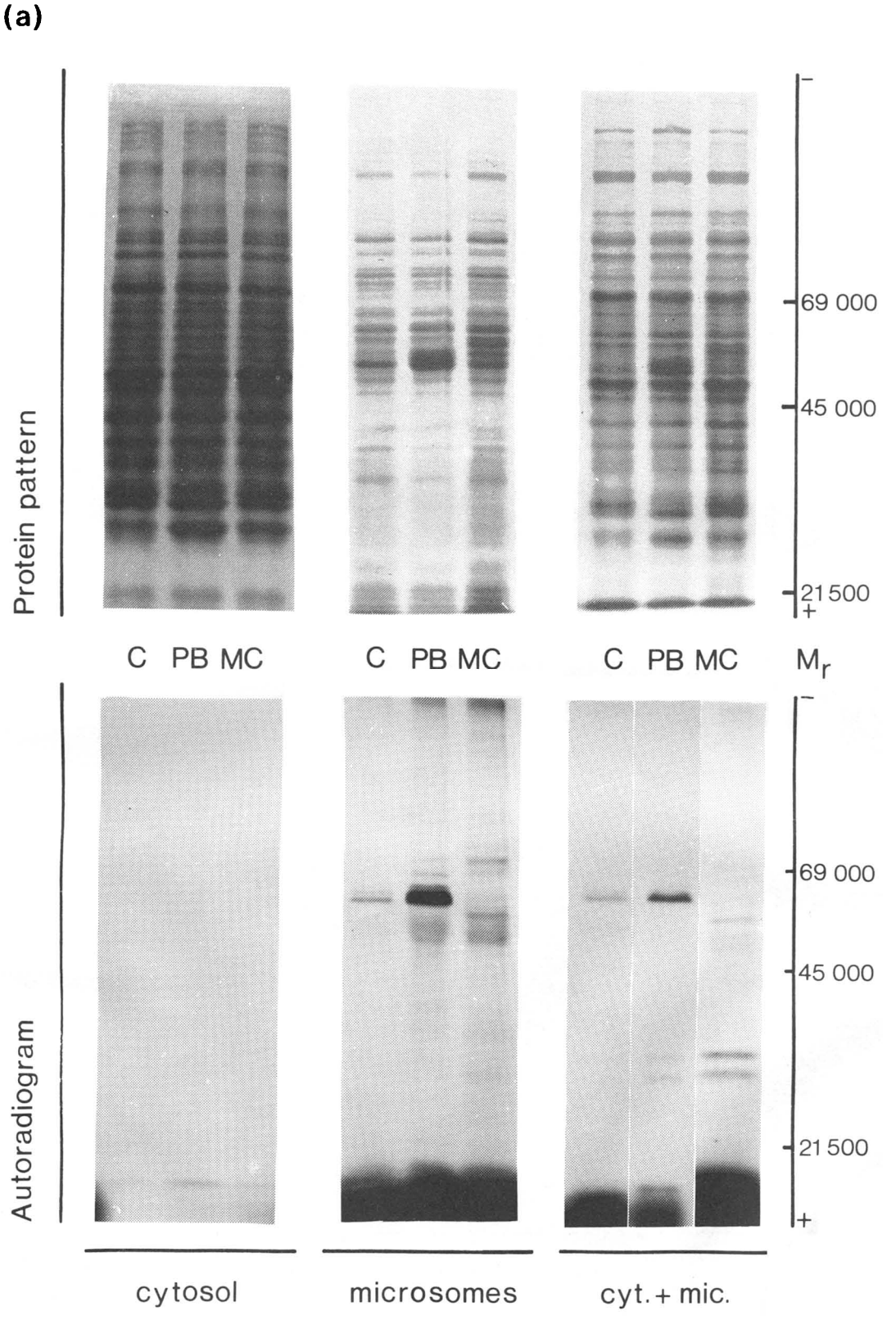

Fig. 3(a). Legend on p. 1504.

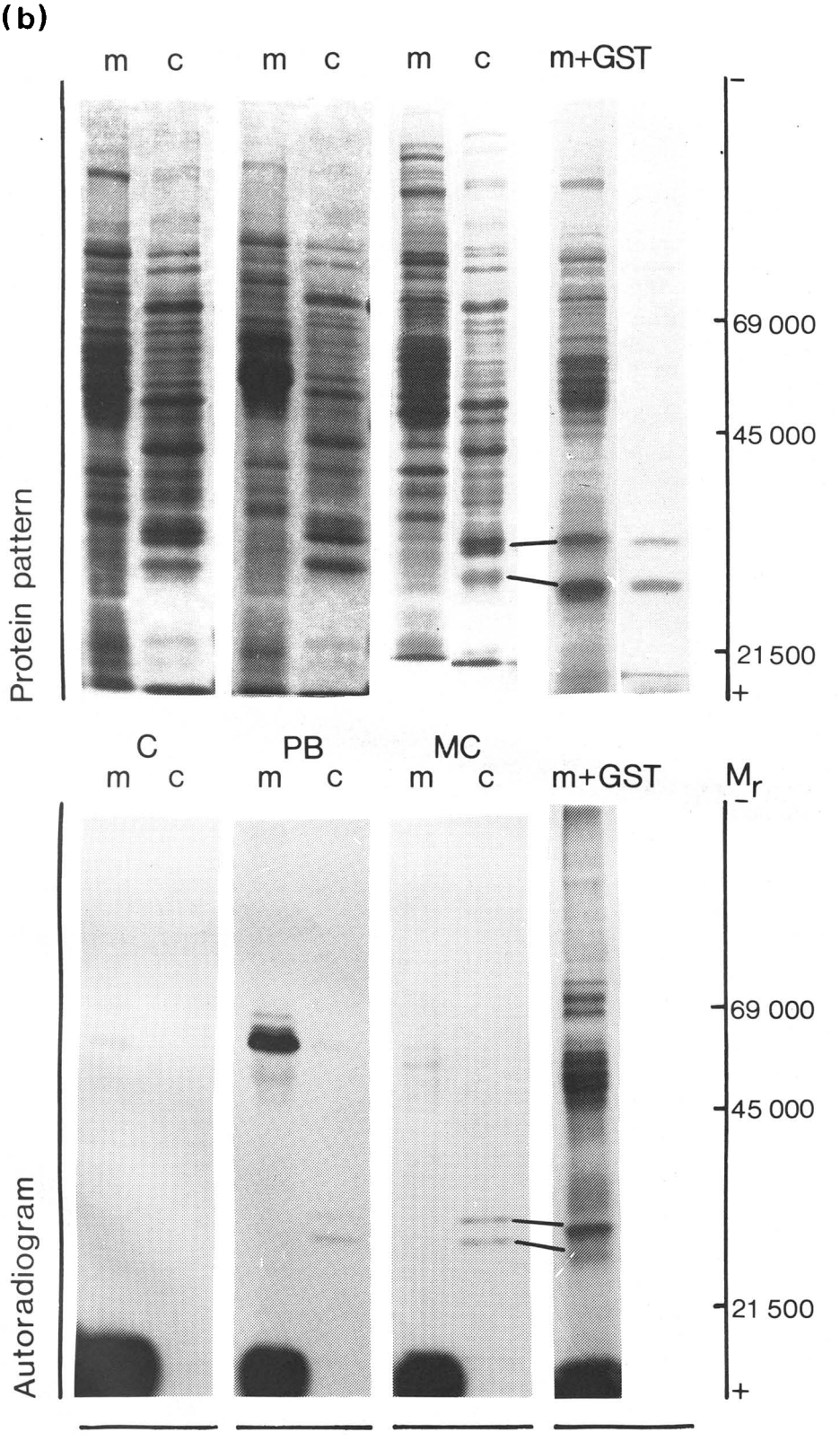

Fig. 3. B[*a*]P binding patterns. Samples prepared from control (C), PB- or 3-MC-treated rats were incubated with [^{14}C]B[*a*]P and NADPH and electrophoresed in SDS-polyacrylamide gels before fluorography. (a), protein patterns and fluorographs of cytosol, microsomes and cytosol–microsome mixtures. (b), protein patterns and fluorographs of microsomes (m) and cytosol (c) separated by centrifugation after incubation together. Microsomes from 3-MC-treated rats were also incubated with purified glutathione *S*-transferase B (m + GST). These latter patterns were obtained from electrophoretic runs with different separation in the low molecular weight region as indicated.

soluble fraction, while the binding to microsomes decreased correspondingly keeping the total binding constant. The binding to cytosolic components even exceeded that to microsomal components at high cytosol concentrations. A similar redistribution of bound B[a]P was observed regardless of whether the subcellular fractions were obtained from 3-MC- or PB-treated, or from control rats. The conclusion to be drawn is that microsomal enzymes catalyse a metabolic activation of B[a]P that leads to a covalent binding to soluble components. There also seems to be a competition between the microsomal and soluble fraction for the activated B[a]P.

The target specificity of the bound B[a]P was examined by electrophoresis in SDS-polyacrylamide gels and fluorography. As expected, no radioactive bands were detected in samples when cytosol alone had been incubated with radio-labelled B[a]P and NADPH (Fig. 3a). The patterns obtained with microsomes showed radiolabelled bands in the 50–70,000 M_r region. A dominating band (M_r = 60,000) was seen in PB-treated and control microsomes, while the binding was more equally distributed between several components in microsomes from 3-MC-treated animals. These patterns have been discussed in detail elsewhere [12, 13]. After incubation of cytosol and microsomes together two additional radiolabelled components (M_r 28,000 and 30,000) were evident when the subcellular fractions had been isolated from PB- or 3-MC-treated rats (Fig. 3b). Corresponding bands, although very faint, were seen in incubations from control animals. Further evidence that these additional bands belonged to the soluble fraction was obtained after centrifugation of the cytosol–microsome incubation mixture followed by electrophoresis and fluorography. Both these components remained in the supernatant, while the microsomal pellets yielded radiolabelled patterns similar to those obtained after incubation with microsomes alone. No other radiolabelled bands were detected in the supernatant.

The two soluble components that became modified through reaction with B[a]P could be identified as the subunits of glutathione *S*-transferase B (ligandin). When purified transferase was incubated with B[a]P, NADPH and liver microsomes, B[a]P was bound to two components with the same mobility in SDS-polyacrylamide gels as the soluble components of the above experiments and as the purified transferase (Fig. 3).

Our results thus confirm and extend the recent finding that B[a]P forms adducts with glutathione *S*-transferase B when incubated with postmitochondrial supernatant and NADPH [10]. In contrast to this work we found that both subunits of the transferase bound B[a]P. On the other hand, we did not observe the other binding components reported there. It is not clear whether these differences are due to different incubation conditions in the two cases.

The extent of B[a]P binding to cytosolic components can be calculated from the data of Fig. 2. Thus, approximately 0.7 nmoles bound per mg of cytosolic protein in the presence of liver microsomes from 3-MC-treated rats. Assuming that glutathione *S*-transferase B represents 2% of the total cytosolic protein, this binding is close to a one to one ratio between B[a]P and transferase subunit. This is a considerably lower value than the probably incorrect binding capacity reported in [10] (1 mole/mg of partly purified glutathione *S*-transferase B, i.e. close to one B[a]P incorporated per amino acid residue when corrected for transferase purity).

So far it is not known which reactive metabolite(s) of B[a]P will bind to the cytosolic proteins. Microsomal enzymes from both PB- and 3-MC-pretreated animals will catalyze the binding even though they yield different metabolites [24]. Thus both K- and bay-region epoxides as well as secondary dihydrodiol epoxides might be implicated in the covalent binding. We have shown earlier that metabolically activated B[a]P-7,8-dihydrodiol will bind more efficiently than other B[a]P metabolites to microsomal proteins [13].

The physiological consequences of the covalent binding of B[a]P to glutathione *S*-transferase B (or any other target protein in the cell) is still uncertain. A decline in the catalytic function of the enzyme on binding was observed in [10], but there is no information on how its ability to bind substances non-covalently is affected. One interesting observation, however, is the decrease in covalent binding of B[a]P to microsomal proteins found here in the presence of cytosol. The physiological significance of this competition between microsomal and cytosolic proteins for B[a]P metabolites is still a matter for speculation. It has been shown, however, that glutathione and glutathione *S*-transferase B will inhibit the incorporation of B[a]P and B[a]P metabolites into DNA of isolated hepatic nuclei [25].

In summary, B[a]P binds covalently to rat liver cytosolic proteins after metabolic activation in the presence of microsomes and NADPH. The two subunits of glutathione *S*-transferase B are the preferred targets. The binding of B[a]P into cytosolic proteins leads to a corresponding decrease in binding into microsomal proteins.

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