MOLECULAR PHARMACOLOGY, 18, 529–535

Irreversible Binding of Isolated Benzo[a]pyrene Metabolites to Specific Rat Liver Microsomal Proteins

CECILIA SCHELIN, ANDERS TUNEK,* BENGT JERNSTRÖM† AND BENGT JERGIL

*Biochemistry 1, Chemical Centre, University of Lund, S-220 07 Lund, Sweden, *Institute of Environmental Health, University of Lund, S-22362 Lund, Sweden, and †Department of Forensic Medicine, Karolinska Institutet, S-104 01 Stockholm, Sweden*

Received April 8, 1980; Accepted June 17, 1980

SUMMARY

SCHELIN, C., A. TUNEK, B. JERNSTRÖM AND B. JERGIL. Irreversible binding of isolated benzo[a]pyrene metabolites to specific rat liver microsomal proteins. *Mol. Pharmacol.* 18: 529–535 (1980).

[^{3}H]Benzo[a]pyrene and seven isolated [^{3}H]benzo[a]pyrene metabolites were incubated with liver microsomes from control, phenobarbital-, and 3-methylcholanthrene-treated rats, and irreversible binding to microsomal proteins was studied. For all compounds tested, including benzo[a]pyrene-4,5-oxide, the binding was greatly enhanced in the presence of a NADPH-generating system. All metabolites except 3-hydroxybenzo[a]-pyrene bound more extensively to microsomal proteins from 3-methylcholanthrene-treated rats than to proteins from phenobarbital-treated or control rats. Benzo[a]pyrene-7,8-dihydrodiol bound much more efficiently than the other metabolites. The protein binding patterns of the metabolites were examined by SDS–polyacrylamide gel electrophoresis and fluorography. The most extensive binding occurred to a few proteins in the MW region of 45,000–70,000, and there were differences in the patterns between different metabolites. The composite pattern of the metabolites corresponded to the binding pattern obtained with benzo[a]pyrene. A major target protein in microsomes from 3-methylcholanthrene-treated rats had the same mobility as purified cytochrome *P*-448. Most binding to microsomes from phenobarbital-treated animals occurred to a 60,000 MW component. Binding also occurred to a protein which comigrated with cytochrome *P*-450. The specificity and extent of metabolite binding to proteins may be of importance for the development of cytotoxicity. Factors affecting the binding patterns are discussed.

INTRODUCTION

Polycyclic aromatic hydrocarbons (PCH)[1] are primarily metabolized by the cytochrome *P*-450-linked monooxygenase system located mainly in the endoplasmic reticulum. The result of the metabolism is more polar products such as phenols, dihydrodiols, or further oxygenated compounds which become water soluble and readily excretable after conjugation. During this metabolism reactive intermediates are formed which may bind covalently to cellular macromolecules (1, 2).

BP is the most thoroughly studied PCH, and it is metabolized to several products in rat liver (3). The metabolite pattern obtained depends on the state of activation of the inducible cytochrome *P*-450-linked monooxygenase system. Phenobarbital, for instance, induces certain species of cytochrome *P*-450 and, thereby, a preferential metabolism in the 4,5 region of the BP molecule (4). MC, on the other hand, induces a different form of the cytochrome (cytochrome *P*-448) which increases the metabolic rate of BP and shifts the metabolism to the 7,8 and 9,10 region of the molecule (4, 5). BP-7,8-dihydrodiol is considered to be a proximate carcinogen of BP (6) and is converted to the ultimate carcinogen by further oxygenation in the 9,10 region to isomeric BP-7,8-dihydrodiol-9,10-oxides (7, 8). These may bind covalently to DNA (9).

There are several reports on the binding of PCH and PCH metabolites to DNA. Much less is known of their binding to cellular proteins, and in particular to microsomal proteins, i.e., at the site of metabolic activation. However, several toxic and carcinogenic compounds including BP have been shown to bind covalently to micro-

This work was supported by John and Augusta Persson's Foundation, the Swedish Cancer Society, and a personal grant to C. S. from the foundation "Bengt Lundquists minne."

[1] Abbreviations used: BP, benzo[a]pyrene; 3-OH-BP, 3-hydroxybenzo[a]pyrene; 9-OH-BP, 9-hydroxybenzo[a]pyrene; BP-4,5-dihydrodiol, 4,5-dihydro-4,5-dihydroxybenzo[a]pyrene; BP-7,8-dihydrodiol, 7,8-dihydro-7,8-dihydroxybenzo[a]pyrene; BP-9,10-dihydrodiol, 9,10-dihydro-9,10-dihydroxybenzo[a]pyrene; BP-4,5-oxide, benzo[a]pyrene-4,5-oxide; BP-3,6-quinone, benzo[a]pyrene-3,6-quinone; MC, 3-methylcholanthrene; PCH, polycyclic aromatic hydrocarbons.

0026-895X/80/060529-07$02.00/0

somal proteins after metabolic activation (10). We have found that BP, MC, and the toxic aromatic compounds phenol, benzene, and chlorobenzene bind irreversibly and specifically to a few microsomal proteins after metabolic activation (11). Although the major DNA-binding species of BP are further activated primary metabolites, there is no information available on the binding of such metabolites to microsomal or other proteins. We have examined the incorporation of BP and individual BP metabolites to microsomal proteins.

MATERIALS AND METHODS

Chemicals. [G-^{3}H]BP (19 Ci/mmol) was purchased from the Radiochemical Centre, Amersham. [^{3}H]BP-4,5-oxide was a generous gift from Dr. Franz Oesch, Mainz. It was also obtained from the National Cancer Institute Carcinogenesis Research Program, Bethesda, Md. Isotopically labeled BP metabolites (3-OH-BP, 9-OH-BP, BP-4,5-dihydrodiol, BP-7,8-dihydrodiol, BP-9,10-dihydrodiol, and BP-3,6-quinone) were obtained by incubating [^{3}H]BP with liver microsomes from MC-treated rats. The metabolites were isolated as described by Jernström *et al.* (12). They were pure according to high-pressure liquid chromatography and absorption spectra when compared to authentic BP metabolites. All other chemicals used were of analytical grade.

Animals and treatments. Male Sprague–Dawley rats (Anticimex, Stockholm), weighing 150–200 g, were allowed food and water *ad libitum*. Phenobarbital (80 mg/kg) was injected intraperitoneally on 3 consecutive days and the rats were killed on the fourth day. MC (80 mg/kg in corn oil) was injected once intraperitoneally 24–35 h before sacrifice. Control animals were untreated, but we have ascertained in other experiments that the injection of saline or corn oil does not affect the results.

Preparation of microsomes. The rats were stunned by a blow on the head and bled to death by severing the carotid artery. Livers were excised immediately, chilled, and homogenized in 2.5 ml of buffer (50 mM Tris–HCl, pH 7.4, 10 mM $MgCl_2$, 25 mM KCl, 0.35 M sucrose) per g of tissue using a Potter–Elvehjem homogenizer with a Teflon pestle. Microsomes were obtained by sedimenting the postmitochondrial (10,000*g* for 10 min) supernatant at 105,000*g* for 90 min. The pellet was washed once by suspension in the buffer and sedimentation as above. The final pellet was suspended in buffer without sucrose at a protein concentration of 8–12 mg/ml, and aliquots were stored under N_2 at −70°C.

Analytical procedures. Protein concentrations were determined by the procedure of Lowry *et al.* (13) using bovine serum albumin as the standard. Cytochrome *P*-450 was determined according to Omura and Sato (14). The average cytochrome *P*-450 concentrations were 0.7 nmol/mg protein in control microsomes, 2.4 nmol/mg protein after induction with phenobarbital, and 1.2 nmol/mg protein after induction with MC.

Quantitative determination of irreversible metabolite binding to proteins. The binding of BP or isolated BP metabolites to microsomal proteins was performed in the presence of a NADPH-generating system as previously described in detail (11). The quantitative binding was determined after extractions as described in (15). BP or BP metabolites were added to the incubation mixture dissolved in 10 μl of methanol, except for BP-3,6-quinone and BP-4,5-oxide, which were added in 10 μl of dimethyl sulfoxide. Amounts and specific activities of the substrates and the incubation times used in different experiments are indicated in each case.

Analysis of protein binding patterns by electrophoresis. Samples containing 100 μg of membrane protein obtained from the incubations were treated for SDS–polyacrylamide gel electrophoresis and electrophoresed in slab gels as described earlier (11, 16, 17). After staining and destaining, gels were treated with 2,5-diphenyloxazole (18) before fluorography. Kodak X-Omat films were exposed to the gels at −70°C for 1–6 weeks.

RESULTS

Metabolic activation is required for irreversible metabolite binding. Appreciable irreversible binding of BP or its metabolites to microsomal proteins occurred only after metabolic activation. Incorporation of metabolites to proteins in liver microsomes from phenobarbital- or MC-treated rats in the absence of NADPH was below 10% of the binding obtained in the presence of a NADPH-generating system. In microsomes from control rats, where the incorporation was comparatively low for most of the metabolites tested, the corresponding figure was approximately 20%. BP-4,5-oxide, which in itself is supposed to be a reactive intermediate, did not bind more than the other metabolites in the absence of the NADPH-generating system.

Time course of metabolite binding. The irreversible binding of BP and seven BP metabolites to liver microsomes from control, phenobarbital-, and MC-treated rats after metabolic activation was studied as a function of time at a substrate concentration of 0.8 μM (Fig. 1). The extent of binding was highest to microsomes from MC-treated rats for all substrates tested except for 3-OH-BP, which initially bound more efficiently to microsomes from phenobarbital-treated rats. BP-7,8-dihydrodiol bound most extensively of all substrates examined to microsomes from MC-treated animals. This reaction appeared to be comparatively rapid, since the binding was complete within the first 10 min of incubation. In contrast, the binding of the other substrates progressed with a significant rate for at least 30 min. The time-dependent binding was also examined at a substrate concentration of 8 μM (not shown). The incorporation curves were very similar to those obtained at 0.8 μM, except that the incorporation was more extensive (cf. Fig. 2).

Effect of metabolite concentration on binding. The irreversible binding of BP metabolites to microsomal proteins after further activation was examined at various metabolite concentrations (Fig. 2). For most metabolites the extent of binding to microsomes from both treated and untreated animals increased roughly linearly with increasing substrate concentrations.

At all substrate concentrations examined the binding to microsomes from MC-treated rats was quantitatively higher than the binding to microsomes from control or phenobarbital-treated rats. An exception was 3-OH-BP,

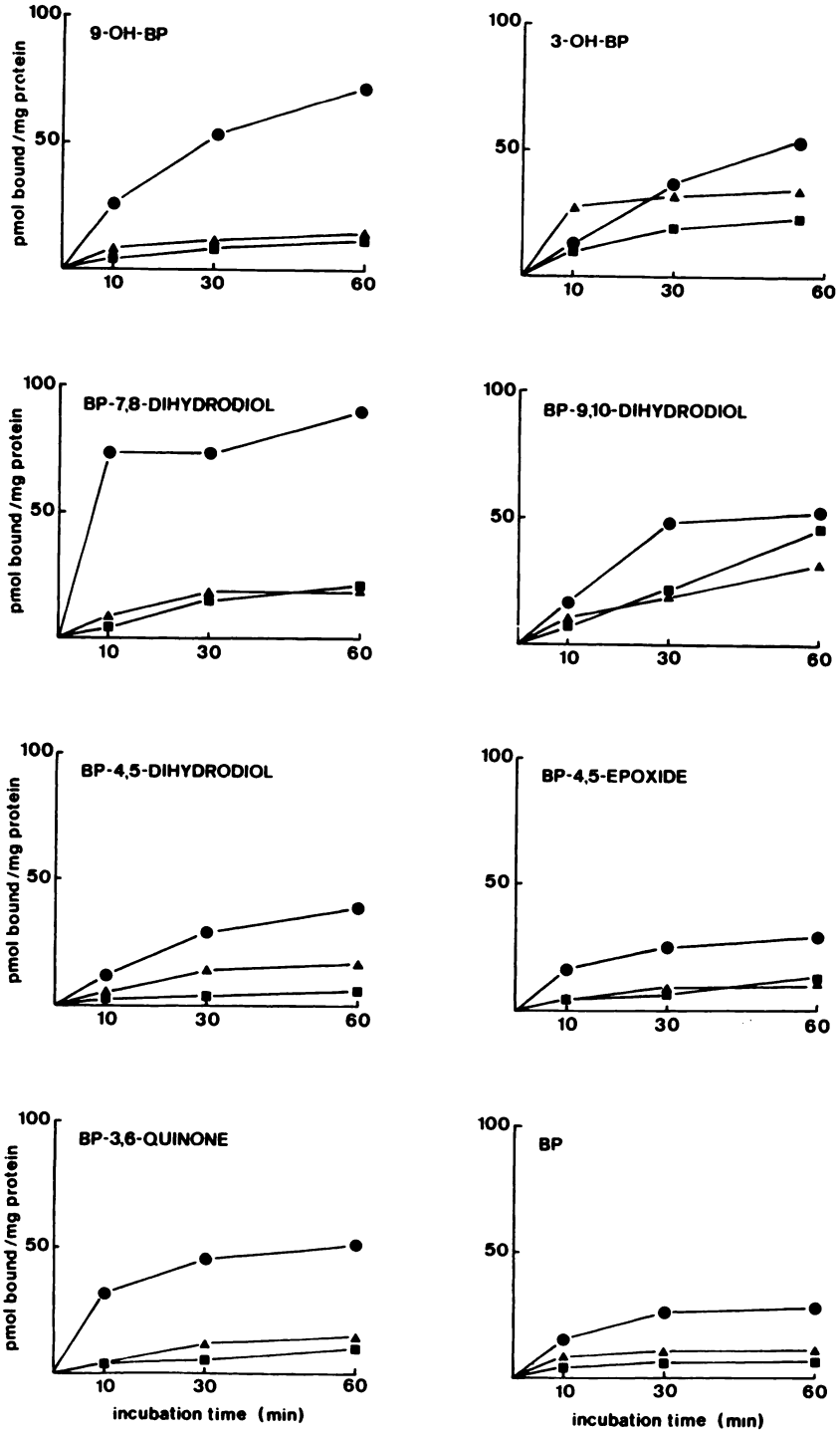

FIG. 1. *Quantitative determination of the irreversible binding of BP and BP metabolites to liver microsomes from control (■), phenobarbital (▲)-, and MC (●)-pretreated rats*

Incubations were performed as described in the experimental section using a substrate concentration of 0.8 μM and the following specific activities: BP, 19 Ci/mmol; 3-OH-BP, 0.84 Ci/mmol; 9-OH-BP, 0.66 Ci/mmol; BP-7,8-dihydrodiol, 0.53 Ci/mmol; BP-9,10-dihydrodiol, 0.47 Ci/mmol; BP-4,5-dihydrodiol, 0.54 Ci/mmol; BP-4,5-oxide, 0.44 Ci/mmol; and BP-3,6-quinone, 1.32 Ci/mmol.

which bound preferentially to microsomes from phenobarbital-treated animals. Of the compounds tested, BP-7,8-dihydrodiol was activated to products that bound more extensively to microsomes from MC-treated rats (1.1 nmol incorporated/mg membrane protein) than the products from the other compounds (less than 0.4 nmol). The incorporation into microsomes from phenobarbital-treated or control animals was 0.1 nmol/mg protein or less. In no case was a saturation in metabolite binding observed. Concentrations exceeding 8 μM were not tested due to a limited supply of metabolites. However, we have tested the incorporation of BP up to a concentration of 50 μM without obtaining a saturation in the binding.

Binding patterns to microsomal proteins. The protein

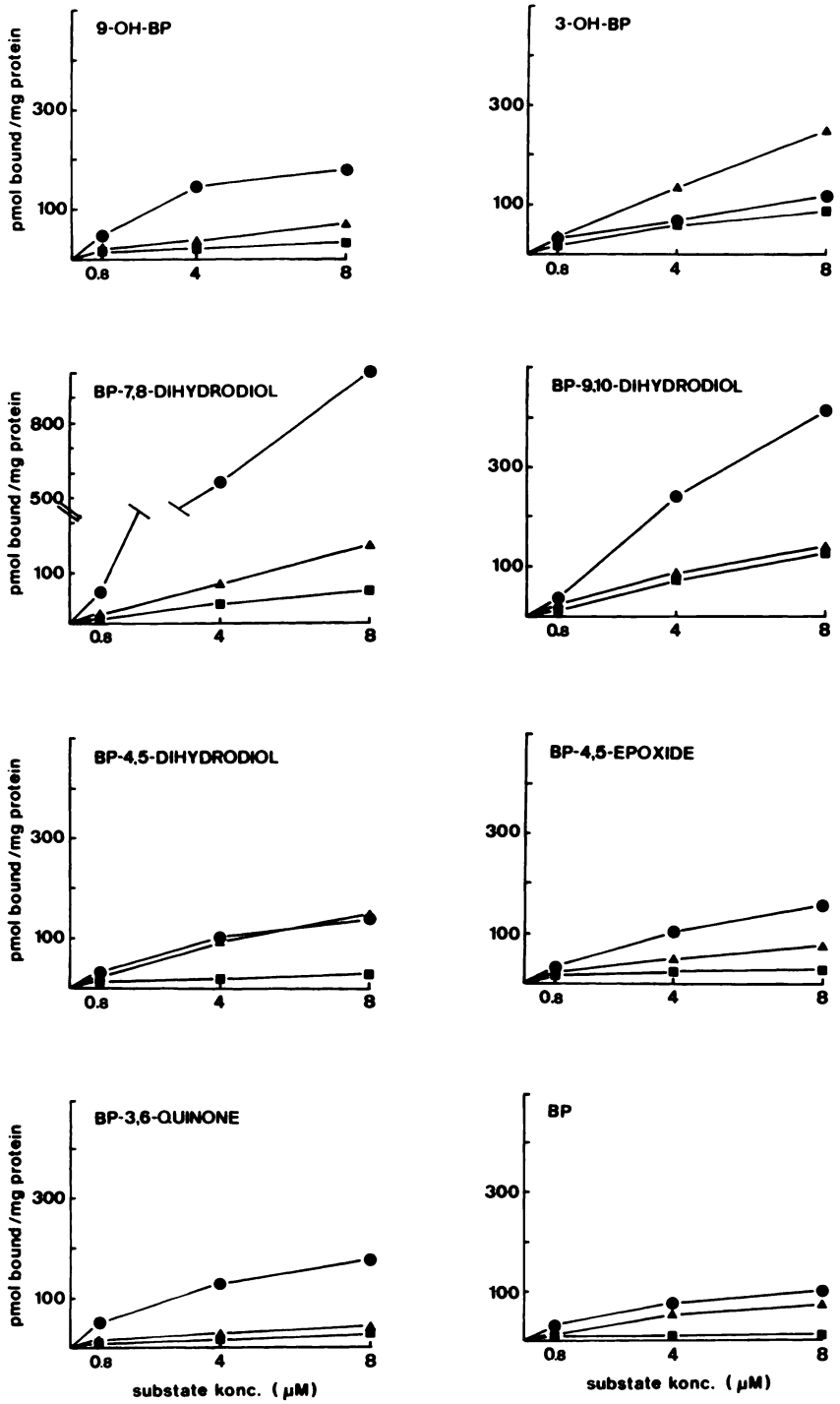

FIG. 2. *Effect of substrate concentration on the irreversible binding of BP and BP metabolites to liver microsomes from control* (■), *phenobarbital* (▲)-, *and MC* (●)-*pretreated rats*

Incubations were performed as described in the experimental section using three substrate concentrations. The incubation time was 30 min. The specific activities at 0.8 μM were the same as in Fig. 1. At 4 μM they were reduced to 1/10, except for BP-4,5-oxide and BP-3,6-quinone, which had the same specific activities as at 0.8 μM. The specific activities at 8 μM were (given in the same order as in Fig. 1.) 0.95, 0.042, 0.033, 0.027, 0.024, 0.027, 0.44, and 1.32 Ci/mmol, respectively.

binding patterns of BP or individual BP metabolites were examined by SDS–polyacrylamide gel electrophoresis and fluorography after incubation of microsomes with BP or metabolites and a NADPH-generating system (Fig. 3). In essence, appreciable binding occurred to a few proteins mainly in the MW region of 45,000–70,000. There were differences, however, in the binding patterns of different metabolites, but the composite patterns essentially add up to the pattern of BP itself.

To facilitate a comparison, schematic binding patterns in the most interesting region are shown in Fig. 4. The major binding components have been assigned according

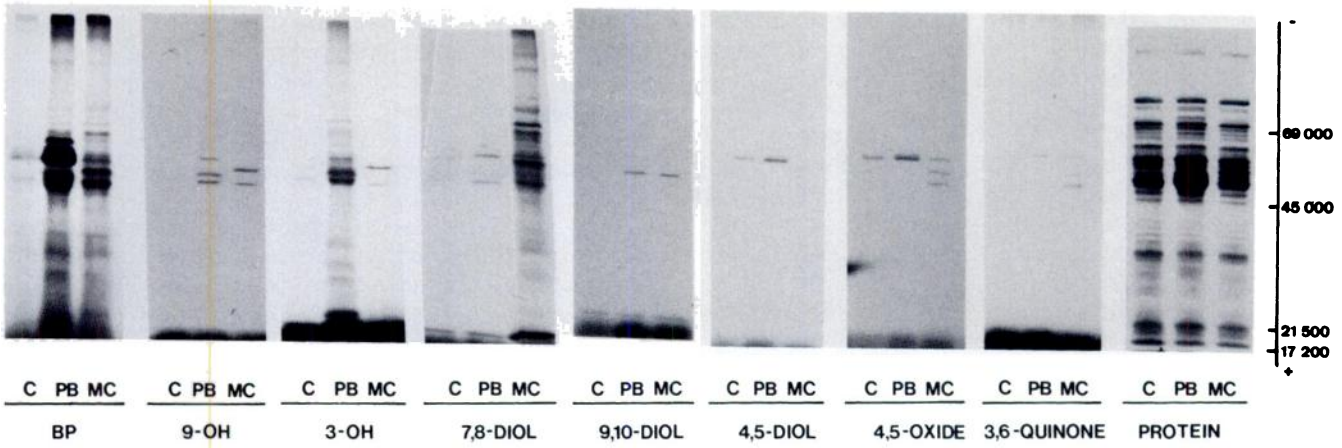

FIG. 3. *Binding patterns of BP and BP metabolites to microsomal proteins from livers of control (C), phenobarbital (PB)-, and MC-pretreated rats*

Incubations were performed for 30 min at a substrate concentration of 13 μM. The specific activities used were the same as in Fig. 1. Electrophoresis and fluorography were performed as described in the experimental section.

to their molecular weights. The most striking differences in binding specificity between microsomes from phenobarbital- and MC-treated rats occurred in two components, one with an apparent MW of 52,000 present in microsomes from phenobarbital-treated rats and one with a MW of 56,000 present in microsomes from MC-treated animals. These components had the same mobility in the gel as two prominent protein bands seen after phenobarbital or MC treatment, respectively. We have found in other experiments that these bands have the same mobilities as purified cytochromes *P*-450 and *P*-448. Another difference observed was in the binding to a 60,000 MW component, which was more prominent in microsomes from phenobarbital-treated rats than from MC-treated rats. The binding to control microsomes was comparatively weak for all substrates.

A comparison of the protein binding patterns of BP and BP metabolites to microsomes from phenobarbital-treated rats reveals that different metabolites yielded different patterns. The principal target for BP-4,5-dihydrodiol, BP-4,5-oxide, and BP was the 60,000 MW component. BP-7,8-dihydrodiol, on the other hand, bound equally well to this component and to the 50,000 MW component, while 3-OH-BP and 9-OH-BP bound more to the 50,000 and 52,000 MW components and less to that of 60,000 MW. There was also a strong radioactive band at MW 20,000 in both the 3-OH-BP and the BP patterns (Fig. 3).

The protein binding pattern of BP-7,8-dihydrodiol to microsomes from MC-treated animals differed markedly from those of the other metabolites. There was little radioactivity associated with the otherwise conspicuous 56,000 MW component, and the pattern was dominated by the 50,000 MW component together with 58,000 and 72,000 MW bands that were specific for this metabolite. In contrast, BP, 3-OH-BP, and 9-OH-BP bound preferentially to the 56,000 MW component, but also to the 52,000 MW component. The intensity of the binding patterns obtained with the other metabolites was generally very weak (Fig. 3).

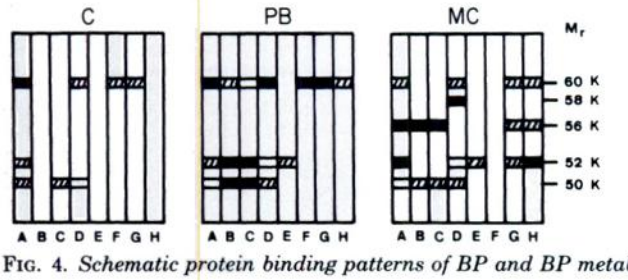

FIG. 4. *Schematic protein binding patterns of BP and BP metabolites*

The electrophoretic patterns of Fig. 3 in the MW region 45,000–70,000 have been redrawn. A, BP; B, 9-0H-BP; C, 3-OH-BP; D, BP-7,8-dihydrodiol; E, BP-9,10-dihydrodiol; F, BP-4,5-dihydrodiol; G, BP-4,5-oxide; H, BP-3,6-quinone.

DISCUSSION

The results presented in this paper show both quantitative and qualitative differences in the irreversible binding to microsomal proteins of further activated BP metabolites. In general, the extent of binding was higher to microsomes from phenobarbital-treated and, particularly, from MC-treated animals than to microsomes from untreated animals. The requirement of a NADPH-generating system shows that metabolic activation is a prerequisite for significant binding to occur.

The metabolism of BP is enhanced in the 7,8 and 9,10 regions of the molecule when the reaction is catalyzed by microsomes from MC-treated rats, resulting in an increase in the formation of BP-7,8-dihydrodiol and 9-OH-BP (4). BP-7,8-dihydrodiol is further activated to the corresponding 9,10-oxide (7, 8, 12), whereas the active form of 9-OH-BP is probably the corresponding 4,5-oxide (12), both of which are major DNA binding species (12, 19, 20). We found here that BP-7,8-dihydrodiol bound more extensively than other metabolites to proteins in microsomes from MC-treated rats. The most likely ultimate binding species in this case was BP-7,8-dihydrodiol-9,10-oxide, in analogy with the activation preceding binding to DNA (9). It should be pointed out, however, that a final identification of binding metabolites has to await their isolation from microsomal proteins. The only BP

metabolite isolated after hydrolysis of microsomal proteins so far is BP-4,5-dihydrodiol, indicating that the binding agent in this case was BP-4,5-oxide (21) or a derivative of this oxide.

It is expected that BP-4,5-oxide should be one of the activated metabolites which bind irreversibly to microsomal proteins. However, the binding of BP-4,5-oxide was greatly enhanced in the presence of a NADPH-generating system, suggesting that an additional activation is required before binding. This indicates that the metabolic activation of BP starting with oxide formation in the 4,5 position might be more complicated than is presently recognized, and that several metabolic steps are required before binding to proteins.

The electrophoretic studies showed that the binding patterns to microsomal proteins differed both for the different metabolites studied and after various treatments of the animals. The binding pattern of BP to liver microsomes from phenobarbital-treated rats was dominated by a 60,000 MW band. Some of the BP metabolites examined, particularly BP-4,5-diol, BP-4,5-oxide, and BP-7,8-diol, also bound extensively to this component. However, there was a very low binding of BP and BP metabolites to the 60,000 MW component in microsomes from control or MC-treated animals. A possible explanation for the difference is that the component is induced by phenobarbital treatment. This appears less likely, however, since there is no visible enhancement in protein staining at 60,000 MW. We have also found in preliminary experiments that the binding of BP to 60,000 MW material in microsomes from control rats is enhanced after the addition of cytochrome *P*-450 purified from phenobarbital-treated rats.

An important question then is which factor(s) determines the specificity in the binding of BP metabolites to their target proteins. One possibility is that reactive metabolites will bind close to their site of activation in the membrane. This suggestion is borne out by the binding of metabolites to the 56,000 MW component in microsomes from MC-treated rats. This component comigrates with purified cytochrome *P*-448 in the gel, indicating that the binding protein is cytochrome *P*-448, an enzyme which participates in the metabolic activation of BP and its metabolites. Similar evidence applies to the 52,000 MW component in microsomes from phenobarbital-treated animals which comigrates with purified cytochrome *P*-450. We have also found in preliminary experiments that BP will bind irreversibly to a protein which comigrates with cytochrome *P*-450 on incubation with a reconstituted activating system consisting of NADPH–cytochrome *P*-450 reductase, cytochrome *P*-450, and NADPH. The identities of the metabolite binding components of 50,000 and 60,000 MW are not known presently, but we are examining whether the former might be epoxide hydratase.

The most likely metabolic route leading to covalent binding of metabolites to microsomal proteins is through the formation of reactive oxides by the mixed-function oxidase system. Provided that protein binding occurs close to the site of activation in the membrane, similar binding patterns would be expected for different BP metabolites after activation to oxides. The patterns differed markedly, however, and other additional explanations for the binding specificity have to be found. One possibility is that the kind of metabolite formed is crucial; important factors may be the positions of the reactive oxide or other substitutions in the molecule or the kind of primary metabolite that is activated (quinone, dihydrodiol, or phenol).

It is also possible that metabolic conversions other than oxide formation will produce reactive metabolites capable of binding covalently to proteins. For instance, dihydrodiols may be dehydrogenated to catechols with a subsequent formation of semiquinones or quinones. However, this pathway is catalyzed by dihydrodiol dehydrogenase, which is a soluble enzyme (22) and therefore most likely absent from our incubations.

It has been suggested that there is a connection between the binding of toxic compounds to proteins and their toxicity (10, 23). In order to evaluate this possibility intracellular target proteins for the toxic compounds should be isolated, and the effect of binding examined. So far, a few cytosolic proteins have been identified which bind polycyclic hydrocarbons irreversibly (24), but nothing is known of the identity of target proteins in other subcellular fractions. The present work shows that only a few microsomal proteins will bind metabolites of BP and that the protein binding patterns differ for the metabolites. Of prime importance now is to isolate microsomal target proteins and to examine in which manner the binding will affect their function.

REFERENCES

1. Heidelberger, C. Chemical carcinogenesis. *Annu. Rev. Biochem.* **44:** 79–121 (1975).
2. Weisburger, E. K. Mechanisms of chemical carcinogenesis. *Annu. Rev. Pharmacol. Toxicol.* **18:** 395–415 (1978).
3. Sims, P. The metabolism of benzo(a)pyrene by rat-liver homogenates. *Biochem. Pharmacol.* **16:** 613–618 (1967).
4. Fahl, W. E., G. R. Jefcoate and C. B. Kasper. Characteristics of benzo(a)pyrene metabolism and cytochrome P-450 heterogeneity in rat liver nuclear envelope and comparison to microsomal membrane. *J. Biol. Chem.* **253:** 3106–3113 (1978).
5. Kinoshita, N., B. Shears and H. V. Gelboin. K-region and non K-region metabolism of benzo(a)pyrene by rat liver microsomes. *Cancer Res.* **33:** 1937–1944 (1973).
6. Jerina, D. M., R. Lehr, M. Shaefer-Ridder, H. Yagi, J. M. Karle, D. R. Thakker, A. W. Wood, A. Y. H. Lu, D. Ryan, S. West, W. Levin and A. H. Conney. Bay region epoxides of dihydrodials: A concept which explains the mutagenic and carcinogenic activity of benzo(a)pyrene and benzo(a)anthracene, in *Origin of Human Cancer* (H. H. Hiatt, J. D. Watson and J. A. Winstein, eds.). Cold Spring Harbor Laboratory, New York, Vol. 4, 639–658 (1977).
7. Thakker, D. R., H. Yagi, A. Y. H. Lu, W. Levin, A. H. Conney and D. M. Jerina. Metabolism of benzo(a)pyrene: Conversion of (±)-trans-7,8-dihydroxy-7,8-dihydrobenzo(a)pyrene to highly mutagenic 7,8-diol-9,10-epoxides. *Proc. Nat. Acad. Sci. USA* **73:** 3381–3385 (1976).
8. Huberman, E., L. Sachs, S. K. Yang and H. Gelboin. Identification of mutagenic metabolites of benzo(a)pyrene in mammalian cells. *Proc. Nat. Acad. Sci. USA* **73:** 607–611 (1976).
9. Sims, P., P. L. Grover, A. Swaisland, K. Pal and A. Hewer. Metabolic activation of benzo(a)pyrene proceeds by a diolepoxide. *Nature* **252:** 326–328 (1974).
10. Uehleke, H. The model system of microsomal drug activation and covalent binding to endoplasmic proteins. *Proc. Eur. Soc. Study Drug Toxic.* **15:** 119–129 (1973).
11. Tunek, A., C. Schelin and B. Jergil. Microsomal target proteins of metabolically activated aromatic hydrocarbons. *Chem. Biol. Interact.* **27:** 133–144, (1979).
12. Jernström, B., H. Vadi and S. Orrenius. Formation of DNA-binding products from isolated benzo(a)pyrene metabolites in rat liver nuclei. *Chem. Biol. Interact.* **20:** 311–321 (1978).
13. Lowry, O. H., N. J. Rosebrough, A. L. Farr and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275 (1951).

14. Omura, T., and R. Sato. The carbon monoxide-binding pigment of liver microsomes. II. Solubilization, purification and properties. *J. Biol. Chem.* **239**: 2379–2385 (1964).
15. Tunek, A., K. L. Platt, P. Bentley and F. Oesch. Microsomal metabolism of benzene to species irreversibly binding to microsomal protein and effects of modifications of this metabolism. *Mol. Pharmacol.* **14**: 920–929 (1978).
16. Henriksson, T., and B. Jergil. Protein kinase activity and endogenous phosphorylation in subfractions of rat liver mitochondria. *Biochim. Biophys. Acta* **588**: 380–391 (1979).
17. Neville, D. M., Jr. Molecular weight determination of protein-dodecyl sulfate complexes by gel electrophoresis in a discontinuous buffer system. *J. Biol. Chem.* **246**: 6328–6334 (1971).
18. Bonner, W. M., and R. A. Laskey. A film detection method for tritium-labelled proteins and nucleic acids in polyacrylamide gels. *Eur. J. Biochem.* **46**: 83–88 (1974).
19. King, H. W. S., M. H. Thompson and P. Brookes. The role of 9-hydroxybenzo(a)pyrene in the microsome mediated binding of benzo(a)pyrene to DNA. *Int. J. Cancer.* **18**: 339–344 (1976).
20. Jernström, B., S. Orrenius, O. Undeman., A. Gräslund and A. Ehrenberg. Fluorescence study of the DNA-binding metabolites of benzo(a)pyrene formed in hepatocytes isolated from 3-methylcholanthrene-treated rats. *Cancer Res.* **38**: 2600–2607 (1978).
21. Raha, C. R., C. H. Gallagher, P. Shubik and S. Peratt. Covalent binding to protein of the K-region oxide of benzo(a)pyrene formed by microsome incubation. *J. Nat. Cancer Inst.* **57**: 33–38 (1976).
22. Jerina, D. M., H. Ziffer and J. W. Daly. The role of the arene oxide-oxepin system in the metabolism of aromatic substrates. IV. Stereochemical considerations of dihydrodiol formation and dehydrogenation. *J. Amer. Chem. Soc.* **92**: 1056–1061 (1970).
23. Brodie, B. B., W. D. Reid, A. K. Cho, G. Sipes, G. Krishna and J. R. Gillette. Possible mechanism of liver necrosis caused by aromatic organic compounds. *Proc. Nat. Acad. Sci. USA* **68**: 160–164 (1971).
24. Smith, G. J., V. S. Ohl and G. Litwack. Ligandin, the glutathione-S-transferase, and chemically induced hepatocarcinogenesis: A review. *Cancer Res.* **37**: 8–14 (1977).

Send reprint requests to: Cecilia Schelin, Biochemistry 1, Chemical Centre, P.O. Box 740, S-220 07 Lund, Sweden.