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The 8S Benzo(a)pyrene-Binding Protein Is an Aldehyde Dehydrogenase Regulated by the Ah Receptor

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8S Benzo(a)pyrene-binding proteins from liver cytosol of mouse and rabbit have been partially purified by gel permeation chromatography and affinity chromatography on 1-aminopyrene-Sepharose columns. These proteins, which bind polycyclic aromatic hydrocarbons and daunorubicine, have been identified, by microsequencing, as aldehyde dehydrogenases composed of polypeptides of 54 kDa. Using Ah receptor-deficient (AHR-/-) transgenic mice it has been shown that the amount as well as the binding capabilities of 8S protein was strongly altered in these mice, suggesting that its expression was partially under the control of the Ah receptor. The function of these proteins is currently unknown. © 1998 Academic Press

Benzo(a)pyrene (BP) and other polycyclic aromatic hydrocarbons (PAHs), which are potential carcinogens in animals and possibly in humans, bind to several cellular components as lipoproteins, Ah receptor and 4S protein (1). More recently, another protein called 8S BP-binding protein, was detected and characterized in the cytosol of several animal species and human (2), but no biological function has been defined for this binding protein. In order to further advance in this field the purification of the protein was required. In this report we describe a two-step purification procedure which led us to identify the 8S BP-binding protein from mouse and rabbit as aldehyde dehydrogenases (AHD-2).

MATERIALS AND METHODS

Animals. C57BL/6 (B6) mice were from Iffa-Credo (Les Oncins, France). Genetically engineered AHR-deficient (AHR-/-) mice were from our laboratory. Male New Zealand rabbits were from INRA (Toulouse, France). Tricolor guinea-pigs were obtained from Cob-Labo-Lap (Yffiniac, France).

Materials. [^{3}H]BP, radiochemical purity 98.2%, was purchased from Amersham Corp. (Buchs, England). Nonradioactive BP and other PAHs, daunorubicine (DNR), aldehyde dehydrogenase (AHD) from bakers yeast and acetaldehyde were from Sigma (Saint Louis, MO). Sepharose 4B and Sephacryl S-300 HR were from Pharmacia. Immobilon-P^{SQ} transfer membranes were from Millipore Corp. (Bedford, MA).

Synthesis of 1-aminopyrene-Sepharose 4B. 1-Aminopyrene-Sepharose 4B (1-AP-4B) was prepared by coupling techniques previously described by Lesca (3). Briefly, the cyanogen bromide-activated Sepharose was sequentially coupled with octamethylene diamine and succinic anhydride then with 1-aminopyrene, at pH 4.8, for 20 h at 20°C in presence of *N*-ethyl-*N′*-(3-diaminopropyl)-carbodiimide (25 mg/ml of gel suspension. The 1-aminopyrene content of the preparation, determined as described (4), was 5 μmoles ligand/ml packed gel.

Purification of liver 8S BP-binding proteins. The binding proteins from mice and rabbit were purified from the liver cytosol by a two step procedure. All steps were carried out at 4°C. During each step in the purification, the appropriate fractions were loaded by gravity flow, eluting proteins were monitored either by the radioactivity determined by liquid scintillation counting or by the absorbance at 280 nm. The appropriate fractions were pooled, concentrated by centrifugation at 1,000 × g in Centriflo membrane cones CF25 (Amicon) and assayed for specific binding activity using sucrose gradient analysis.

Briefly, cytosols were prepared by centrifugation of liver homogenates at 9,000 × g for 20 min and the supernatants from these were centrifuged at 100,000 × g for 1h to yield supernatant cytosol fractions. The protein concentrations (20-25 mg/ml) were determined by the method of Lowry et al. (5). The cytosols, incubated with 20 nM [^{3}H]BP, for 1h at 4°C, then treated with dextran charcoal (10 mg charcoal/ml), were loaded onto a Sephacryl S-300 HR gel permeation column (2.5 × 90 cm) equilibrated with HEDGM buffer (25 nM Hepes, 1.5 mM EDTA, 1 mM dithiothreitol, 20 mM sodium molybdate and 10% glycerol) pH 7.6. The proteins were eluted with the same buffer. From the 128 fractions (3 ml/fraction) collected, the 8S protein-enriched fractions (90-100) were pooled and concentrated. The samples were incubated with 1-AP-4B gel, for 2h at 4°C, in HEDGM buffer containing 0.1M NaCl. Typically, 10 ml rabbit 8S protein from Sephacryl chromatography (90 mg protein; 42,570 pmoles BP binding activity) were incubated with 80 ml 1-AP-4B suspension (400 μmoles 1-aminopyrene). The suspension was poured into a 1.6 × 23 cm column (bed volume 50 ml) and the unbound proteins were two-fold recycled to the column. Nonspecific proteins were collected and the column washed with the same buffer. Elution of proteins specifically bound to 1-AP-4B, was performed with HEDGM buffer containing 1M sodium thiocyanate. Eluted protein fractions were concentrated and dialyzed against HEDGM buffer. The final volume (4.2 ml) contained 6,420 pmoles 8S BP-binding protein (yield 15%).

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Electrophoresis. 7.5% SDS-polyacrylamide gel electrophoresis was performed as described by Laemmli (6) using molecular weight protein standards from Sigma.

Benzo(a)pyrene binding assay. The [^{3}H]BP specific binding to various 8S protein fractions and the competition experiments were carried out as described previously (2) with 20 nM or 1 μM [^{3}H]BP respectively.

In vivo [^{3}H]BP treatment of mice. Three male B6 mice (20 g) received, by i.p. route, a single treatment of 50 mg/kg [^{3}H]BP (143 μCi/mouse) dissolved in 0.2 ml of sunflower oil. Mice were killed

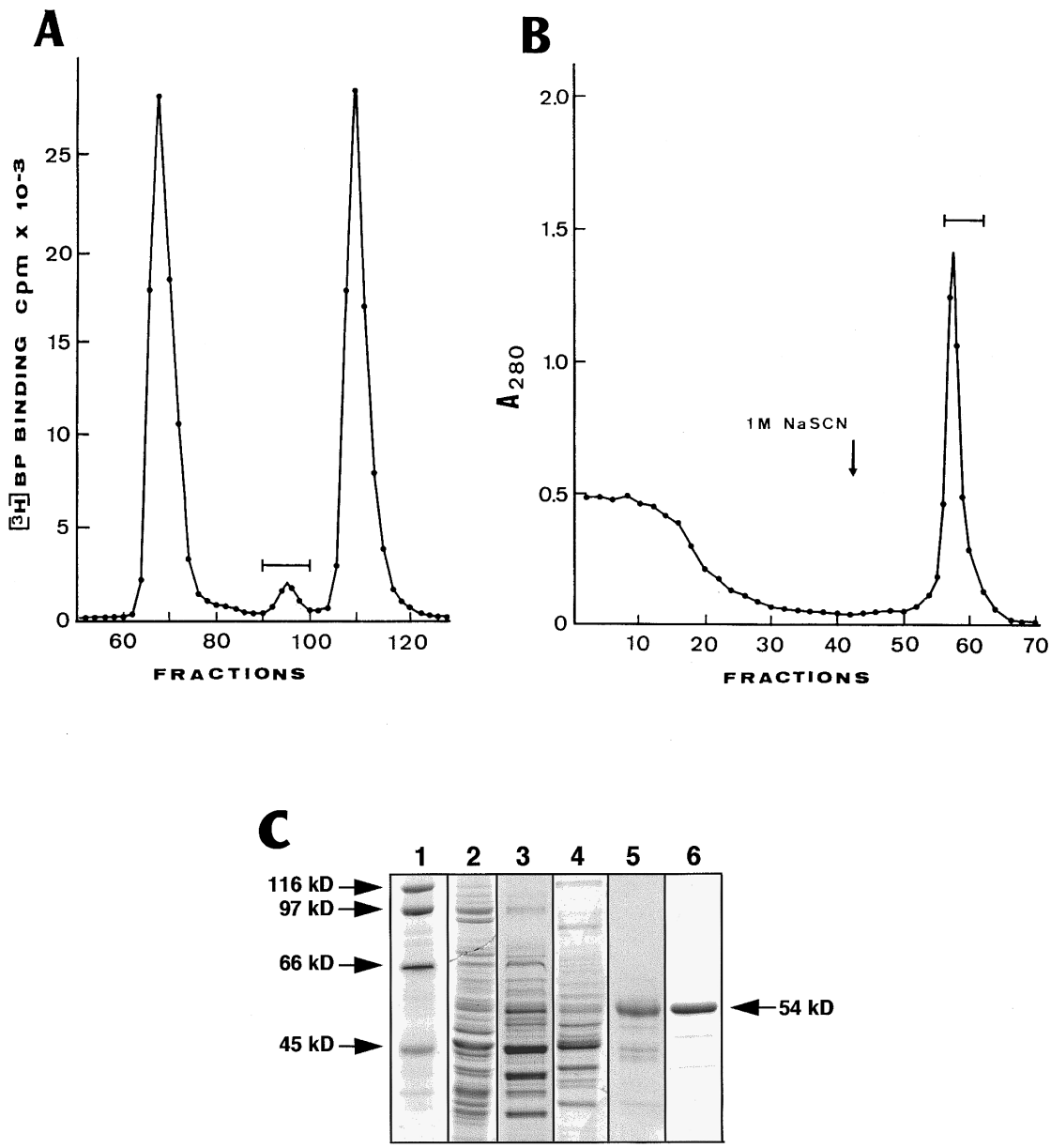

FIG. 1. (A) Gel permeation chromatography of rabbit liver cytosol. Rabbit liver cytosol (8 ml, 20 mg protein/ml) incubated with 20 nM [^{3}H]BP, then treated with dextran-coated charcoal, was loaded onto a Sephacryl S-300 HR column equilibrated with HEDGM buffer, pH 7.6. The column was eluted with the same buffer and the radioactivity of fractions was determined by liquid scintillation counting. Fractions 90-100 were pooled and concentrated. (B) Affinity chromatography to 1-aminopyrene-Sepharose 4B. The pooled fractions from Sephacryl chromatography (S-300) were incubated with 1-AP-4B gel in HEDGM buffer containing 0.1 M NaCl and then was poured into a column. The unbound proteins were two-fold recycled to the column. Nonspecific proteins were collected and, after washing of the column, the specifically bound proteins were eluted with HEDGM buffer containing 1 M sodium thiocyanate. The eluted proteins (fractions 56-62) were concentrated and dialyzed against HEDGM buffer. (C) SDS-PAGE analysis. Various samples, from cytosol to 1-AP-4B fractions were examined. Lanes: (2) whole cytosol B6 mouse (20 μg); (3) S-300 B6 mouse (10 μg); (4) S-300 AHR-/- mouse (10 μg); (5) 1-AP-4B B6 mouse (20 μg); (6) 1-AP-4B rabbit (10 μg). Electrophoresis was through a 7.5% polyacrylamide gel stained with Coomassie blue. Mobility of molecular weight standards (kDa) is indicated lane 1.

4h after injection and the cytosol was prepared as described above.

Sequencing of peptides from 54 kDa bands. 8S BP-binding proteins from B6 mouse (20 μg) or from rabbit (50 μg) were electrophoresed on denaturing 7.5% polyacrylamide gel. Gel was placed on a 13 x 10 cm Immobilon polyvinylidene difluoride membrane presoaked, for 3 sec. in methanol, then immersed in water and finally equilibrated in buffer transfer. Proteins were electroblotted from gel onto Immobilon sheet at a constant current of 175 mA for 1h at room temperature. The sheet was stained with Coomassie blue for 5 min. and destained with a mixture of 50% methanol, 2% acetic acid and 48% water. The 54 kDa bands were excised and prepared for trypsinisation according to Fernandez et al.(7). The resulting digests were separated by reversed phase HPLC on a C8 column eluted by an acetonitrile gradient in 0.01% trifluoroacetic acid; after a second purification on a C18 column, selected peptides were sequenced on a Procise (Perkin-Elmer, Foster City, CA, USA) sequencer.

AHD assay. The enzyme assay, with acetaldehyde as substrate, was performed at 37°C as described by Manthey et al. (8) except that 50 mM Tris-HCl pH 8 was used instead of pyrophosphate buffer. The appearance of NADH was monitored at 340 nm.

RESULTS AND DISCUSSION

Purification of the 8S BP-binding proteins. As shown Fig. 1, the two-step procedure used leads to a partial purification of 8S proteins from mouse and rabbit. For the two animal species, a major protein, of apparent molecular weight 54 kDa, is detected but slightly contaminated by non identical discrete proteins of lower molecular weights (Fig. 1C, lanes 5 and 6). The first purification step (Fig. 1A) allowed to separate, by gel permeation chromatography, the 8S protein from the other BP binding components, namely the lipoproteins (fractions 62-76), the Ah receptor (around the fraction 83) and the 4S BP-binding protein (fractions 105-120). From the liver cytosol, the 8S proteins (fractions 90-100) were 4-fold enriched with a yield of 84%. The second step, affinity chromatography to 1-AP-4B (Fig. 1B), had been previously used by Collins et al. to partially purify the 4S BP-binding protein (9). The choice of 1-AP, coupled to Sepharose 4B, to purify 8S proteins was based on its relative affinity for these proteins. In previous competition assays conducted with various compounds, including PAHs, 1-AP appeared as the most effective (2). Preliminary tests were performed to determine the optimal concentration of 1-AP bound to Sepharose 4B, which allowed the removing of the nonspecific BP-binding proteins and to retain to the gel the 8S protein, subsequently released by 1M sodium thiocyanate solution. This concentration was 5 μmoles 1-AP/ml packed gel.

Samples from gel permeation chromatography and from 1-AP-4B affinity chromatography, incubated with [^{3}H]BP, were analyzed on 5-20% sucrose density gradients. Fig. 2 demonstrates that they contain a BP binding protein sedimenting in the 8S region of gradients. It can be noted, Fig. 2, that the BP-binding capability of 8S protein is strongly altered in AHR-/- mice, suggesting a relationship between the 8S protein and the Ah receptor. In fact, the SDS-PAGE analysis of 8S proteins from B6 and AHR-/- mice (Fig. 1C) reveals that the amount of 8S protein is lower in AHR-/- mice (lane 4) than in B6 mice (lane 3). This result demonstrates that the expression of the 8S protein gene is partially under the control of the Ah receptor. From the data of Fig. 2 it may be inferred that the final yield of the purification of mouse 8S protein was 12.7%.

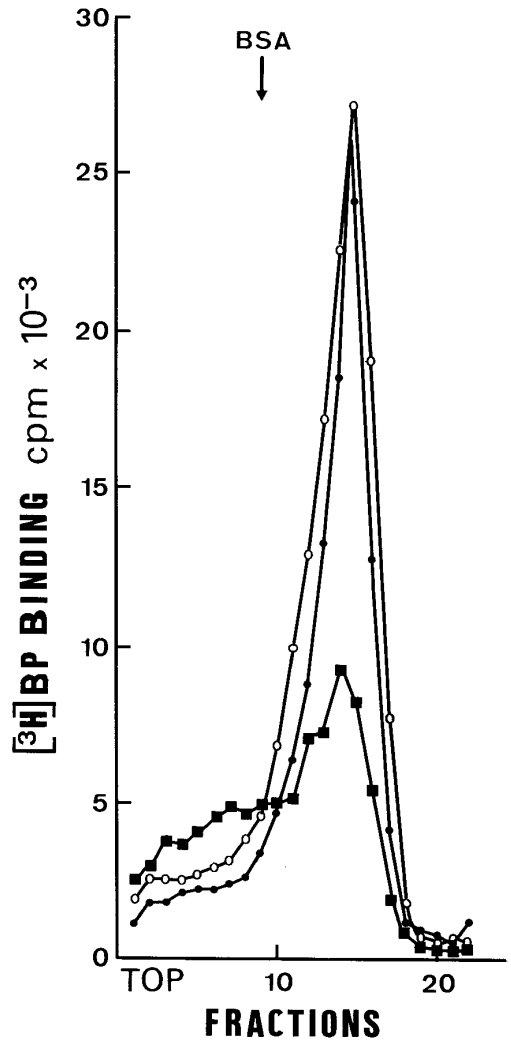

FIG. 2. Detection and characterization of 8S BP-binding proteins from B6 mouse and AHR-/- mouse livers. Fractions 90-100 from gel permeation chromatography of liver cytosols from B6 mouse (○) and AHR-/- mouse ( ) and fractions 56-60 from 1-AP-4B affinity chromatography of B6 mouse (l) were pooled and concentrated. The samples were incubated, for 1h at 4°C, with 20 nM [^{3}H]BP. Then 300 μl was placed onto 5-20% sucrose density gradients. The gradients were centrifuged, for 2h at 372,000 x g in a vertical rotor. Fractions were collected and the radioactivity was measured by liquid scintillation counting.

Identification of the 8S BP-binding protein. Samples of purified 8S BP binding proteins from B6 mouse and rabbit liver were electrophoresed on a 7.5% SDS-

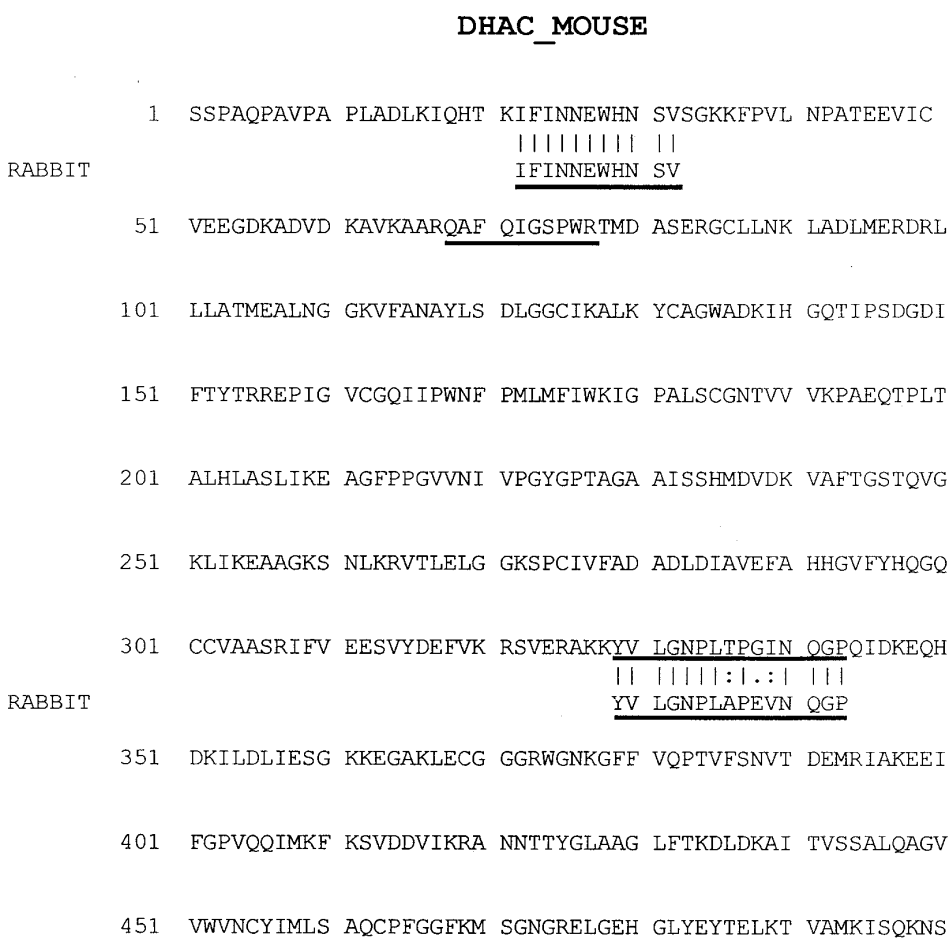

FIG. 3. Partial amino acid sequencing of mouse and rabbit liver AHD. The 54 kDa 8S BP-binding proteins from purified samples were transferred to Immobilon membrane and then digested with trypsin as indicated in the text. The tryptic digests were separated by reverse phase HPLC, and peptides were sequenced as described in the text. The sequences were matched against those in the Swiss-Prot data base. The sequences of two informative peptides so obtained from both mouse and rabbit 8S proteins are underlined and identities or homologies are scored.

polyacrylamide gel and transferred to Immobilon membrane. The 54 kDa proteins were digested with trypsin and sequenced. The peptide sequences are indicated (underlined) in Fig. 3. Comparison of the micro-sequenced peptides from 8S proteins with the Swiss-Prot data base using the Fasta method revealed their unambiguous identity with aldehyde dehydrogenases. To further ascertain this identity, the AHD activity of several samples of mouse and rabbit 8S proteins was measured with acetaldehyde as substrate. The data shown in Table 1 indicate that the two 8S proteins are able to reduce NAD to NADH in the presence of a saturating concentration of acetaldehyde. Conversely, the aldehyde dehydrogenase from bakers yeast (Sigma) was able to bind [^{3}H]BP as demonstrated by the sedimentation of the labeled protein in the 8S region of 5-20% sucrose density gradient (Fig. 4).

Functional relationships between the 8S BP binding protein and the Ah receptor, especially the decrease of the 8S protein in AHR-/- mice (Fig. 1C and Fig. 2), become understandable by the fact of its identification as an aldehyde dehydrogenase since it is known, for a

TABLE 1

AHD Activity of Various Samples from Mouse and Rabbit Purified 8S BP-Binding Proteins

8S protein	Protein (mg/ml)	AHD activity (nmol NADH/min/mg)
B6 mouse	0.6	180
	0.8	147
	0.5	208
Rabbit	1.2	125

Note. 50-μl samples of 8S protein were incubated, for 10 min at 37°C, with 50 mM acetaldehyde as substrate and 4 mM NAD. The appearance of NADH was monitored at 340 nm and the blank values were subtracted.

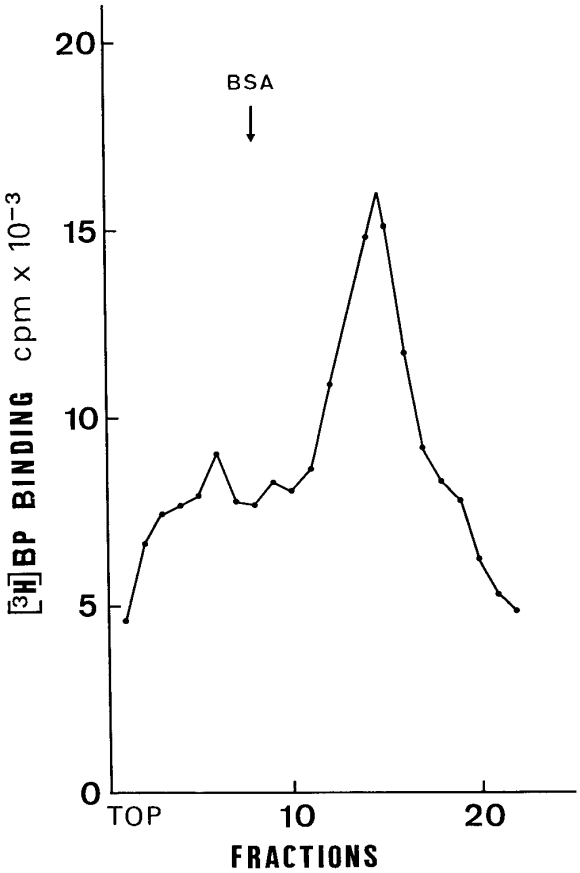

FIG. 4. Characterization of the bakers yeast AHD as a 8S BP-binding protein. 300 μg of bakers yeast AHD (Sigma) was solubilized in 300 μl HEDGM buffer, pH 7.6, and then incubated, for 1h at 4°C, with 1 μM [^{3}H]BP. The sample was placed onto a 5-20% sucrose density gradient, and then the gradient was centrifuged, for 2h at 372,000 × g in a vertical rotor. Fractions were collected and the radioactivity was measured by liquid scintillation counting.

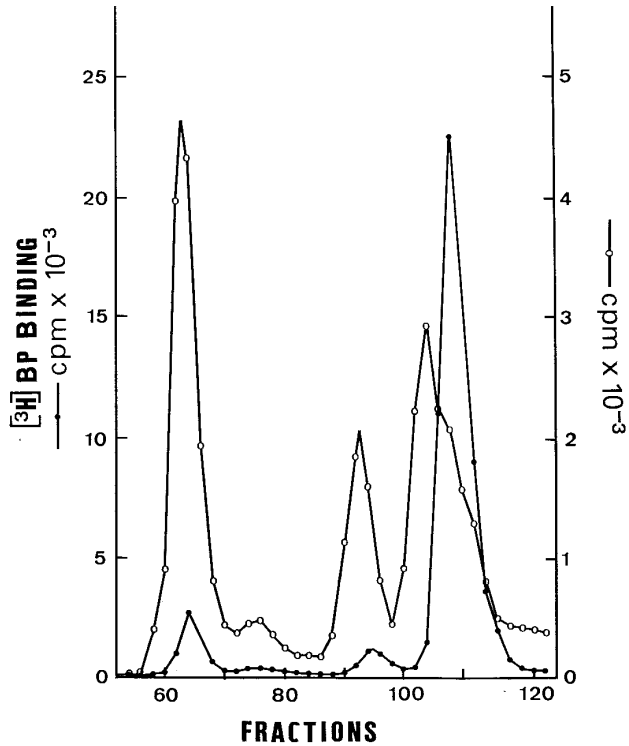

FIG. 5. Comparative profiles of gel permeation chromatography of B6 mouse liver cytosols, either incubated *in vitro* with [^{3}H]BP or obtained after *in vivo* administration of [^{3}H]BP. B6 mice were either untreated or treated, during 4h, with 50 mg/kg [^{3}H]BP, and then the liver cytosols were prepared as described under Materials and Methods. A 9-ml sample from untreated mice was incubated, for 1 h at 4°C, with 20 nM [^{3}H]BP and, following dextran-charcoal treatment, the sample was loaded onto a Sephacryl S-300 HR column (2.5 × 90 cm) equilibrated with HEDGM buffer, pH 7.6. The proteins were eluted with the same buffer and the radioactivity of fractions was counted (l). Following dextran-charcoal treatment, a 9 ml-sample of cytosol from [^{3}H]BP-treated mice was chromatographed and analyzed under the same conditions (○).

TABLE 2

Effect of Various Chemicals as Competitors with [^{3}H]BP for Guinea-Pig 8S BP-Binding Protein

Competitors	[3H]BP-binding (%)
Benzo(a)pyrene (BP)	10
1-Aminopyrene (1-AP)	20
Pyrene (P)	26
7, 12-Dimethylbenz(a)anthracene (DMBA)	29
Daunorubicine (DNR)	34
2-Acethylaminofluorene (2-AAF)	35
3-Methylcholanthrene (3-MC)	42
β-Naphthoflavone (BNF)	53
2,3,7,8-Tetrachlordibenzofuran (TCDF)	80
Phenobarbital (PB)	100

Note. 8S protein from guinea-pig liver (0.177 mg protein/ml), prepared by gel permeation chromatography, was incubated, for 1h 30 min at 4°C, with 1 μM [^{3}H]BP in the absence or presence of 100 μM competitors. Specific binding was determined by sucrose gradient sedimentation. The values represent the percentages of [^{3}H]BP binding in the presence of a 100-fold excess of competitor.

long time, that this enzyme is regulated by the Ah receptor (10) and is inducible by 2,3,7,8,-tetrachlorodibenzo-p-dioxin or benzo(a)pyrene. In that point of view, the residual amount of the 54 kDa protein observed in AHR-/- mice after electrophoresis of the 8S protein in SDS polyacrylamide gel (Fig. 1C, lane 4), represents the constitutive expression of the AHD gene.

Binding properties of 8S proteins. As previously reported, the 8S proteins were isolated from the liver cytosol of several animal species, through their binding with the carcinogen benzo(a)pyrene (2). Curiously, a similar protein was isolated, from the rat liver soluble fraction, thanks to its binding to the anthracycline antibiotic daunorubicine used as a cancer chemotherapeutic agent. (11). The daunorubicine binding protein (DNR-BP54) was further identified as an aldehyde dehydrogenase (12) exhibiting the same sedimentation properties, in sucrose gradient, than the 8S BP-binding protein (11,2). We think that

the 8S BP-binding protein and the DNR-BP54 are one and the same protein because DNR is able to displace [^{3}H]BP bound to 8S protein and because the two proteins are endowed with the same AHD enzyme activity. The guinea-pig 8S protein was used to perform competition experiments between 1 μM [^{3}H]BP as a radioligand and various chemicals including PAHs and daunorubicine. The data presented in Table 2 show that DNR is a good ligand of 8S protein but exhibiting a lower competitive effect than BP, 1-AP, P and DMBA. In contrast, the best ligands of the Ah receptor (TCDF, 3-MC and BNF) exhibit a poorer competitive efficiency than DNR.

Currently, the role of the 8S BP-binding protein in the cell is unknown. The possibility of a function in PAH transport would be in good accordance with the binding characteristics (high capacity and low affinity) (2) of 8S proteins determined *in vitro*. But, the presence in the cytosol of other BP-binding components as lipoproteins, Ah receptor and 4S protein, which impede a significant binding of [^{3}H]BP to 8S protein, makes difficult to consider such a transport role. Nevertheless, when radioactive BP is administered to mice, a strong increase in the binding to 8S protein occurs, as shown in Fig. 5, allowing the easy detection of 8S protein (fractions 88-98) in the cytosol from treated mice. In contrast, the [^{3}H]BP binding to the 4S protein (fractions 100-116) seems to be unchanged. It can be noted, Fig. 5, that after *in vivo* administration of 50 mg/kg [^{3}H]BP both 8S protein and 4S protein, bound to [^{3}H]BP, appear as slightly heavier complexes than after *in vitro* incubation with 20 nM [^{3}H]BP. Actually, studies are in progress to elucidate this physiological event and to know the fate of the 8S protein-[^{3}H]BP complex in the cell. These investigations could help us to better understand the role of the 8S protein.

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