A novel 4 S [³H]β-naphthoflavone-binding protein in liver cytosol of female Sprague–Dawley rats treated with aryl hydrocarbon receptor agonists

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β-Naphthoflavone (β-NF) is a widely used inducer of phase-I and phase-II enzymes controlled by aryl hydrocarbon receptor (AhR). Studies of competitive binding with ³H-labelled 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), 3-methylcholanthrene (3-MC) and benzo[*a*]pyrene (B[a]P) have shown that β-NF is a high-affinity ligand for AhR and also for polycyclic aromatic hydrocarbon (PAH)-binding protein, both soluble proteins of rat liver in 8 S and 4 S fractions, respectively, of sucrose gradients. This study examined binding of [³H]β-NF to liver cytosolic proteins of female Sprague–Dawley rats. Treatment of rats with β-NF, 3-MC, TCDD or α-naphthoflavone (α-NF) increased the specific [³H]β-NF binding to liver cytosol up to 125-fold that of vehicle (corn oil)-treated rats (< 100 fmol/mg of protein). Sucrose gradients revealed a large 4 S and a small 8 S peak of radioactivity from [³H]β-NF binding to cytosols of β-NF-, 3-MC-, TCDD- or α-NF-treated rats. Whereas co-incubation with the unlabelled β-NF eliminated both peaks, co-incubation with 2,3,7,8-tetrachlorodibenzofuran (TCDF) eliminated only the 8 S peak. The sucrose density gradient from [³H]TCDD binding to cytosol of β-NF- or TCDD-treated rats yielded a small 4 S and a larger 8 S peak; only the latter was abolished by co-incubation with TCDF. Thus, the patterns of sedimentation, distribution and elimination of radioactivity from the 8 S fraction of the liver cytosols from β-NF-, 3-MC-, TCDD- or α-NF-treated rats were characteristic for the AhR, whereas those from the 4 S fraction appeared specific for [³H]β-NF binding. The data

indicate that potent AhR agonists, TCDD, 3-MC and β-NF, and to a lesser extent α-NF, a weak AhR agonist, induce a 4 S [³H]β-NF-binding protein in liver cytosol of female rats. α-NF, β-NF and 3-MC were effective competitors (80–85 % inhibition) of the [³H]β-NF-specific binding to the β-NF-, 3-MC- or TCDD-induced 4 S protein, whereas several PAHs including B[a]P and benzo[*e*]pyrene were only weak competitors. The increased [³H]β-NF binding was not associated with glycine N-methyltransferase activity. Hence, the 4 S [³H]β-NF-binding protein described herein differs from the constitutive 4 S PAH-binding protein of rat liver cytosols in the inducibility by β-NF and 3-MC, ligand-binding characteristics, and lack of glycine N-methyltransferase activity. Gel filtration on Sephacryl of liver cytosols from β-NF-treated rats indicated a molecular mass of ≈ 42 kDa for [³H]β-NF-bound protein and suggested that it was derived from a large mass component that before the radioligand binding was eluted with the void volume of the gel and sedimented in a 7 S fraction of the sucrose gradient. The [³H]β-NF binding activity was not eluted with glutathione S-transferase Ya, aldehyde-3-dehydrogenase or DT-diaphorase [NAD(P)H:quinone oxidoreductase] activities, which are AhR-controlled and β-NF-inducible. Further studies are needed to determine the identity and function of this novel protein which may be involved either directly or indirectly (as a carrier protein) in xenobiotic metabolism *in vivo*.

Key words: 4 S binding protein, rat liver, soluble proteins.

INTRODUCTION

In mammalian and non-mammalian species, hydrophobic xenobiotics bind non-covalently to proteins and lipoproteins, which may facilitate their storage, transport and intracellular distribution and result in gene transcription and activation [1–4]. Two soluble proteins, aryl hydrocarbon receptor (AhR) and polycyclic aromatic hydrocarbon (PAH)-binding protein, which may be distinguished by their sedimentation in the 8 S and 4 S fractions of sucrose density gradients, respectively, have been studied extensively in rodent livers [2,4–11]. The AhR binds several different structural classes of aromatic compounds: halogenated aromatics, e.g. 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD); PAHs, e.g. 3-methylcholanthrene (3-MC) and benzo[*a*]pyrene (B[a]P); and heteropolynuclear aromatic hydro-

carbons, e.g. β-naphthoflavone (β-NF; 5,6-benzoflavone). The AhR is a ligand-dependent transcription factor, which via a complex signal-transduction pathway involving interactions with other proteins induces expression of certain phase-I (the CYP1 family of cytochrome P450s) and phase-II {e.g. glutathione S-transferase (GST) Ya, aldehyde-3-dehydrogenase and DT-diaphorase [NAD(P)H:quinone oxidoreductase]} enzymes in xenobiotic metabolism [2–4]. In addition to binding to the AhR, the PAHs, but not TCDD, bind to a 4 S protein [6,9,11,12]. However, certain highly specific ligands for a 4 S protein with little or no binding for the AhR, e.g. benzo[*e*]pyrene (B[e]P), induce *CYP1A1* gene expression, and thus a function similar to but independent from AhR for the 4 S PAH-binding protein was suggested [13]. In the rat liver, the latter has been identified as glycine N-methyltransferase (GNMT) [14], which elicits different

Abbreviations used: AhR, aryl hydrocarbon receptor; PAH, polycyclic aromatic hydrocarbon; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; TCDF, 2,3,7,8-tetrachlorodibenzofuran; β-NF, β-naphthoflavone; α-NF, α-naphthoflavone; 3-MC, 3-methylcholanthrene; B[a]P, benzo[*a*]pyrene; B[e]P, benzo[*e*]pyrene; BA, benz[*a*]anthracene; DMBA, 7,12-dimethylbenz[*a*]anthracene; B[*ghi*]Per, benzo[*ghi*]perylene; 2-AAF, 2-acetylaminofluorene; DHEA, dehydroepiandrosterone; GST, glutathione S-transferase; GNMT, glycine N-methyltransferase; EROD, ethoxyresorufin-O-de-ethylase; SD, Sprague–Dawley; i.p., intraperitoneal; b.wt., body weight.

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functions depending on the subunit configurations, e.g. the B[a]P-binding unit of GNMT is a homodimer [15]. The studies in cell cultures support GNMT as a protein which binds PAHs to mediate the induction of CYP1A1 via an AhR-independent mechanism [16,17]. However, the evidence from other laboratories supports neither the identity of the 4 S PAH-binding protein as GNMT nor its transcriptional function in the gene expression [18,19]. The dispute suggests that both the identity and role of this protein are yet to be elucidated.

The binding characteristics of a 4 S protein were determined with the use of ^3H -labelled 3-MC and B[a]P as ligands [5,6,20,21]. The affinities of other compounds for this protein were determined from their competitive effects on the binding of the above ligands. Among the competitors tested, β -NF was shown to significantly block specific binding of [^3H]3-MC [5,20], and that of [^3H]B[a]P [21], thus revealing its high-to-moderate affinity for a 4 S protein in liver cytosols of male Sprague–Dawley (SD) rats. β -NF has been also shown to bind to AhR of rat liver cytosol with an affinity less than that of TCDD and greater or similar to that of B[a]P [10,22]. Binding of β -NF to liver cytosolic proteins of female SD rats was of particular interest to us since, in our previous study, treatment of these rats with β -NF decreased ($\approx 70\%$) the levels of oestrogen receptor in liver cytosols to an even greater extent than did ovariectomy ($\approx 30\%$ decrease) [23]. Although β -NF is not a ligand for oestrogen receptor, its 'anti-oestrogen-like' effect resembled that of TCDD and hence might be mediated via AhR [4].

The purpose of the present study was to determine binding of β -NF to liver cytosolic proteins of female SD rats with the use of [^3H] β -NF as the ligand to gain insight into its interaction(s) with relevant receptors. Since specific binding of [^3H] β -NF to a 4 S protein was found herein to be significantly induced by treatment of rats with β -NF, the effects of treatment with other potent AhR agonists, TCDD or 3-MC [4], and α -naphthoflavone (α -NF; 7,8-benzoflavone), an antagonist of TCDD-induced activation of AhR in rat liver cytosol [24], on the [^3H] β -NF binding activity were also examined. In addition, ligands with known affinities for the 4 S PAH-binding protein were evaluated for competition with [^3H] β -NF binding to a 4 S protein of β -NF-, 3-MC- and TCDD-treated rats.

MATERIALS AND METHODS

Materials

The following were purchased from the sources indicated: β -NF, α -NF, charcoal (activated), Sephacryl S-200 HR, Blue Dextran, alcohol dehydrogenase (yeast), BSA, cytochrome *c* (horse heart), catalase (bovine liver) were from Sigma (St. Louis, MO, U.S.A.); Dextran T-70 was from Pharmacia Biotech (Piscataway, NJ, U.S.A.); 3-MC was from Eastman Kodak (Rochester, NY, U.S.A.); 2,3,7,8-tetrachlorodibenzofuran (TCDF) was from Ultra Scientific (North Kingstown, RI, U.S.A.); *S*-[methyl- ^3H]adenosyl-L-methionine (14.6 Ci/mmol) was from DuPont NEN Research Products (Boston, MA, U.S.A.); and [$3',5'$ - ^3H] β -NF (47.7 Ci/mmol) was from Chemsyn Science Lab. (Lenexa, KS, U.S.A.). HPLC of [^3H] β -NF with the mobile phase of methanol/ H_2O (85:15) showed 98.3% radioactivity associated with the peak of β -NF ($t_{\text{R}} = 8.18$ min). [^3H]TCDD (30 Ci/mmol) was a generous gift from Dr Steven H. Safe, Texas A&M University, College Station, TX, U.S.A. All other chemicals were of the highest quality from commercial sources.

Hazardous chemicals, e.g. TCDD, PAHs and 2-acetylaminofluorene (2-AAF), were handled and disposed of according to instructions in their respective *Material Safety Data Sheets*.

Animals and treatment

Rats were housed in the facilities accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International, and cared for in accordance with [25]. Female SD rats (Specific Pathogen Free from Harlan Sprague Dawley, Indianapolis, IN, U.S.A.) were maintained on Teklad Certified Rodent Diet (Harlan Teklad, Madison, WI, U.S.A.) and water *ad libitum*. Rats were treated with β -NF at 40 mg/kg body weight (b.wt.) or the vehicle (corn oil) at 2.5 ml/kg b.wt. by daily intraperitoneal (i.p.) injections on days 41–49 of age as described previously [23]. Separate rats were injected intraperitoneally once with β -NF, 3-MC or α -NF at 40 mg/kg b.wt. or the vehicle (corn oil) at 5 ml/kg b.wt. on day 50 ± 2 of age. Four male SD rats were also injected intraperitoneally once with β -NF or vehicle at the above dose levels. All rats were killed (by decapitation under asphyxia with CO_2) 24 h after the injection. Treatment of female rats (≈ 55 days old) with one i.p. injection of TCDD at 25 $\mu\text{g/kg}$ b.wt. or the vehicle (corn oil) at 4 ml/kg b.wt. 72 h before killing was adapted from the published protocols [26,27] and carried out in the laboratory of Dr Karl K. Rozman, University of Kansas Medical Center, Kansas City, KS, U.S.A.

Preparation of liver microsomal and cytosolic fractions

The livers were perfused, removed and frozen as described previously [23]. Hepes buffer, pH 7.6, consisting of 25 mM Hepes, 1.5 mM EDTA, 20 mM sodium molybdate, 1 mM dithiothreitol, 0.5 mM PMSF and 10% glycerol was used unless stated otherwise. The homogenization of livers and fractionation were described previously [23]. Protein concentration was determined by the method of Bradford [28].

Assay of specific binding of [^3H] β -NF and [^3H]TCDD to liver cytosol and sucrose-gradient analysis

Liver cytosols (protein amounts are stated in the Figure or Table legends) from β -NF-, 3-MC-, α -NF-, TCDD- or corn oil-treated rats were incubated for 1 h at 4 °C with 10 nM [^3H] β -NF (unless stated otherwise) or 5 nM [^3H]TCDD in the presence and absence of a 100- or 200-fold excess of the unlabelled β -NF or TCDF added in ethanol (3.3% final concentration), in a total volume of 150 μl of the Hepes buffer. The unbound ^3H -labelled compounds were removed by addition of 50 μl of a charcoal suspension (3% charcoal in 0.3% Dextran T-70), incubation at 4 °C for 10 min and sedimentation at 1500 *g* for 10 min. Aliquots (100 μl) of the supernatants were layered on to a linear 10–30% sucrose density gradient (in the Hepes buffer without glycerol) in 4.9 ml OptiSeal Beckman vertical rotor tubes. The gradients were centrifuged at 4 °C for 2 h (unless stated otherwise) at 372408 *g* in a Beckman VTi 65.2 rotor, using slow acceleration and deceleration modes. After piercing the bottom of each tube, fractions (≈ 5 drops) were added to 5 ml of an Ultima Gold scintillation cocktail (Packard Instrument Co., Meriden, CT, U.S.A.) and the radioactivity was determined with a 1900 CA TriCarb Liquid Scintillation Analyzer (Packard Instrument Co., Downers Grove, IL, U.S.A.). BSA (4.4 S) and catalase (11.3 S) were layered on to separate sucrose gradients as external sedimentation standards.

Assay of competition for [^3H] β -NF binding to liver cytosols from β -NF-, 3-MC- or TCDD-treated rats

Incubation mixtures consisted of aliquots of cytosols containing 75 μg of protein, 500 nM TCDF (added to block [^3H] β -NF

binding to an 8 S protein), 10 nM [3 H] β -NF and a 100-fold excess of the unlabelled competitor dissolved in ethanol (final concentration of 3.3%) in a total volume of 150 μ l of the Hepes buffer (without PMSF). After incubation for 4 h at 4 °C, the unbound [3 H] β -NF was removed by addition of 300 μ l of a suspension of 0.5% charcoal in 0.05% Dextran T-70, incubation at 4 °C for 10 min and centrifugation at 1500 *g* for 15 min. The radioactivity of supernatants was determined in 200 μ l aliquots as described above.

Determination of binding kinetics of [3 H] β -NF to liver cytosol of β -NF-treated rats

[3 H] β -NF (20 nM) with and without a 200-fold excess of the unlabelled β -NF was incubated at 4 °C with liver cytosol at 0.2, 0.5 and 1.0 mg of protein/ml. At eight time intervals between 0.5 and 24 h, duplicate aliquots of 150 μ l were withdrawn from each incubation mixture and combined with 300 μ l of a charcoal suspension to remove the unbound [3 H] β -NF. The charcoal was sedimented and the radioactivity in 200 μ l aliquots of the supernatants was determined as described above for calculation of [3 H] β -NF-specific binding. The apparent half-life of association of [3 H] β -NF with the cytosolic protein(s) was determined by non-linear regression analysis using GraphPad Prism version 2.0 (GraphPad Software, San Diego, CA, U.S.A.).

Dissociation of [3 H] β -NF from its cytosolic binding protein(s) was determined by employing the principles of infinite dilution and rebinding prevention [9]. In the first approach, 20 nM [3 H] β -NF with and without a 200-fold excess of the unlabelled β -NF and cytosol (1.0 mg of protein/ml) were incubated at 4 °C for 15 h. The unbound β -NF was then removed by addition of 2 vol. of a charcoal suspension and sedimentation. Duplicate aliquots of 150 μ l were withdrawn from each supernatant at seven time intervals between 0 and 24 h, treated with a charcoal suspension to remove the dissociated β -NF, centrifuged and radioactivity in the supernatants was determined. In the second approach, a charcoal sediment was added to the incubation mixture after 15 h at 4 °C. After removal of the unbound [3 H] β -NF, unlabelled β -NF (4 μ M) was added to the supernatant and duplicate aliquots of 150 μ l were withdrawn at the same time intervals as above. The dissociated [3 H] β -NF was determined by addition of 300 μ l aliquots of a charcoal suspension, centrifugation and radioactivity measurements of the supernatants.

Gel-filtration chromatography

A Sephacryl S-200 HR column (100 cm $\times$ 1.6 cm inner diameter) and the molecular-mass markers Blue Dextran (2000 kDa), alcohol dehydrogenase (150 kDa), BSA (66 kDa) and cytochrome *c* (12.4 kDa) were used. Liver cytosolic proteins from β -NF-treated rats were separated on the column at 4 °C before (i) and after (ii) incubation with [3 H] β -NF. In (i), an aliquot of cytosol (150 mg of protein in 4.5 ml) was applied to the column and eluted with 25 mM Tris buffer, pH 7.6, containing 1.5 mM EDTA, 5 mM sodium molybdate, 25 mM NaCl, 1 mM dithiothreitol and 0.5 mM PMSF. Fractions ($\approx$ 1.7 ml) were collected, frozen in liquid nitrogen and stored at -80 °C for assays of [3 H] β -NF-specific binding and enzyme activities. Aliquots (150 μ l) of individual fractions were incubated with 20 nM [3 H] β -NF for 20 h at 4 °C with and without a 200-fold excess of the unlabelled β -NF, and after removal of the unbound [3 H] β -NF through charcoal adsorption and sedimentation, their radioactivities were determined as described above. In (ii), aliquots of cytosols (4.8 mg of protein in 1 ml) were incubated with [3 H] β -NF with and without the unlabelled β -NF as described above.

After treatment with charcoal and sedimentation, the supernatants were applied to the column, and eluted with the above buffer. The fractions were collected and their radioactivities determined as described above.

Assays of enzyme activities

Liver microsomal ethoxyresorufin-O-de-ethylase (EROD) [29] and UDP-glucuronosyltransferase [30] activities, cytosolic GNMT [31], DT-diaphorase [32], GST [33] and aldehyde-3-dehydrogenase [34] activities were determined by the published procedures.

Statistical analysis

Values are expressed as means $\pm$ S.E.M. Significance of difference was assessed by Student's *t* test (see Table 1 below) or one-way ANOVA followed by Tukey's post test (see Table 2) [35]. $P \leq 0.05$ was considered statistically significant. The effects of competitors on [3 H] β -NF binding (see Table 3) were examined using a multivariate ANOVA [35]. Percentage binding was compared among the treatment groups for each of the 17 competitors used. Pairwise comparisons of means were conducted using *t* tests. In addition, *t* tests were used to determine whether percentage binding for each competitor differed from 100%, i.e. the control value obtained in the absence of competitor. All tests were adjusted using the Bonferroni method [35], with the overall level of significance set to $\alpha = 0.05$.

RESULTS

Effects of treatment of rats with β -NF on specific binding of [3 H] β -NF to liver cytosol

Hepatic cytosols used herein were devoid of EROD and UDP-glucuronosyltransferase activities and thus considered free of microsomal contamination. After treatment of rats with β -NF (nine i.p. injections at 40 mg/kg b.wt.), the level of [3 H] β -NF binding to liver cytosol was 1467 ± 260 fmol/mg of protein, whereas that of control (corn oil-treated) rats was 51 ± 35 fmol/mg of protein, thus yielding a 30-fold increase. The [3 H] β -NF binding was non-covalent since it was released by denaturation of the labelled protein(s) with methanol, acetone or trichloroacetic acid. The sucrose density gradients from [3 H] β -NF binding to cytosol of β -NF-treated rats contained a large 4 S and a small 8 S peak of radioactivity, indicating binding to two protein fractions (Figure 1, panels A1 and A2). Whereas co-incubation with the unlabelled β -NF eliminated both peaks, co-incubation with TCDF eliminated only the 8 S peak. The sucrose density gradient from [3 H] β -NF binding to cytosol of corn oil-treated rats showed only a trace amount of radioactivity in the 4 S region (Table 1). These results indicate that a constitutive 4 S binding protein is essentially absent but that it is induced 1000-fold by treatment of rats with β -NF. The sucrose density gradient from [3 H]TCDD binding to cytosol of β -NF-treated rats yielded a small 4 S peak and a larger 8 S peak (Figure 1, panel B). The 8 S, but not the 4 S, peak was abolished by co-incubation with TCDF, which was analogous to the effect on [3 H] β -NF binding (Figure 1, panels A1 and A2). Thus the patterns of sedimentation, distribution and elimination of radioactivity from the 8 S fraction were characteristic for the AhR [6,11], whereas those from the 4 S fraction appeared to be specific for β -NF binding. Specific binding of [3 H]TCDD to an 8 S protein was 3-fold greater from cytosols of β -NF- than corn oil-treated rats (Table 1). Whereas the β -NF-treatment-dependent increases in [3 H]TCDD binding to an 8 S protein and microsomal

Figure 1 Binding of [^3H] β -NF and [^3H]TCDD to liver cytosol of β -NF-treated rats

Preparation of liver cytosol from β -NF-treated rats (nine i.p. injections at 40 mg/kg b.wt.) and the binding assay are described in the Materials and methods section. Aliquots of cytosol containing 0.84 mg of protein were incubated with 10 nM [^3H] β -NF (○), 10 nM [^3H] β -NF + 2 μM β -NF (●) or 10 nM [^3H] β -NF + 1 μM TCDF (□) (A1 and A2; A2 depicts an enlarged area of A1), and with 5 nM [^3H]TCDD (△) or 5 nM [^3H]TCDD + 1 μM TCDF (▲) (B). After removal of the unbound compounds with charcoal and sedimentation, aliquots (100 μl) were applied to sucrose gradients and analysed as described in the Materials and methods section. Arrows denote the positions of the sedimentation standards BSA (4.4 S) and catalase (11.3 S) analysed on separate sucrose gradients. The results are typical of at least three separate experiments.

EROD activity are consistent with properties of AhR agonists, the prominent induction of a 4 S [^3H] β -NF-binding protein is to our knowledge a novel property of β -NF.

Comparison of the effects of treatment of rats with β -NF, TCDD, 3-MC or α -NF on specific binding of [^3H] β -NF to liver cytosol

The effects of treatment of rats with the AhR agonists other than β -NF, i.e. TCDD or 3-MC [4], or with α -NF, an antagonist of TCDD-induced activation of AhR [24], on the specific binding

Table 1 Effect of treatment of rats with β -NF on specific binding of [^3H] β -NF and [^3H]TCDD to liver cytosolic proteins and on cytosolic GNMT and microsomal EROD activities

Treatment of rats [nine i.p. injections of β -NF at 40 mg/kg b.wt. or corn oil (vehicle) at 2.5 ml/kg b.wt.], fractionation of liver, assays of specific binding and enzyme activities are described in the Materials and methods section. Aliquots containing 0.84 mg of cytosolic protein were incubated at 4 °C with 20 nM [^3H] β -NF or 5 nM [^3H]TCDD for 20 or 1 h, respectively, with and without a 200-fold excess of the unlabelled β -NF or TCDF. Specific binding is that of [^3H] β -NF to a 4 S and of [^3H]TCDD to an 8 S protein after sucrose-gradient sedimentation for 3 and 2 h, respectively. The values are the means $\pm$ S.E.M. from duplicate assays with individual livers of four rats. * $P < 0.05$, significantly different from the corresponding values for corn oil-treated rats.

Treatment	Specific binding (fmol/mg of cytosolic protein)		Activity (pmol/min/mg of protein)	
	[^3H] β -NF	[^3H]TCDD	GNMT	EROD
Corn oil	1.07 $\pm$ 0.74	15.30 $\pm$ 1.69	440 $\pm$ 155	88 $\pm$ 7
β -NF	1033 $\pm$ 29*	43.90 $\pm$ 3.32*	161 $\pm$ 5*	1694 $\pm$ 235*

Table 2 Effect of treatment of rats with 3-MC, β -NF, α -NF or TCDD on [^3H] β -NF binding to liver cytosol

Treatment of rats by one i.p. injection of the compounds or vehicle and [^3H] β -NF binding assay are described in the Materials and methods section. Aliquots of liver cytosol containing 30 μg of protein were incubated with 20 nM [^3H] β -NF at 4 °C for 20 h with and without a 200-fold excess of the unlabelled β -NF in a total volume of 150 μl of the buffer, and the unbound [^3H] β -NF was removed with charcoal. The values are the means $\pm$ S.E.M. from duplicate assays with livers of 3 or 4 rats (the number of rats in each treatment group is indicated in parentheses). *Significantly different ($P < 0.05$) from the values for corn oil-treated rats. †Significantly different ($P < 0.05$) from the values for α -NF-treated rats.

Treatment (n)	Dose [mg (μmol)/kg b.wt.]	Specific [^3H] β -NF binding (fmol/mg of protein)
3-MC (4)	40 (149)	11193 $\pm$ 2277*†
β -NF (4)	40 (147)	8024 $\pm$ 306*†
α -NF (4)	40 (147)	1928 $\pm$ 349*
Corn oil (4)	—	89 $\pm$ 38
TCDD (3)	0.025 (0.08)	11301 $\pm$ 482*
Corn oil (3)	—	94 $\pm$ 42

of [^3H] β -NF to liver cytosol and the 4 S fraction was determined by sucrose-gradient analysis. Preliminary experiments established that one i.p. injection of β -NF at 40 mg/kg b.wt. compared with corn oil (vehicle) led to the increased [^3H] β -NF binding to liver cytosol, with the maximal increase 24 h after treatment. Hence, the effect of 3-MC or α -NF treatment on the specific binding of [^3H] β -NF to liver cytosol was examined 24 h after one i.p. injection of a dose equimolar to that of β -NF. Treatment of rats with β -NF, 3-MC or α -NF increased the [^3H] β -NF binding to liver cytosol 90-, 125- or 22-fold, respectively (Table 2). Since treatment of male SD rats with β -NF (one i.p. injection at 40 mg/kg b.wt.) increased the [^3H] β -NF binding to liver cytosol $\approx$ 23-fold, i.e. from 310 to 7180 fmol/mg of protein (based on the averages from two rats in the vehicle- and β -NF-treated groups), the effect of β -NF was not sex-specific. The sucrose-density-gradient analyses of [^3H] β -NF binding in liver cytosols from female rats treated with β -NF, 3-MC or α -NF (one i.p. injection; Figure 2) showed a similar pattern of distribution of radioactivity to that from rats treated with β -NF (nine i.p. injections; Figure 1, panels A1 and A2). Thus large and small amounts of radioactivity were associated with a 4 S and an 8 S

Figure 2 Binding of [3 H] β -NF to liver cytosol of β -NF-, 3-MC-, α -NF- or corn oil-treated rats

Treatment of rats (one i.p. injection of β -NF, 3-MC or α -NF at 40 mg/kg b.wt. or corn oil at 5 ml/kg b.wt.), preparation of liver cytosols, and the binding assay are described in the Materials and methods section. Aliquots of cytosol containing 0.4 mg of protein were incubated: from β -NF-treated rats with 10 nM [3 H] β -NF (○); from 3-MC-treated rats with 10 nM [3 H] β -NF (□), 10 nM [3 H] β -NF + 2 μ M β -NF (●) or 10 nM [3 H] β -NF + 1 μ M TCDF (■); from corn oil-treated rats with 10 nM [3 H] β -NF (×) (A); and from α -NF-treated rats with 10 nM [3 H] β -NF (△), 10 nM [3 H] β -NF + 2 μ M β -NF (▲) or 10 nM [3 H] β -NF + 1 μ M TCDF (◇) (B). After removal of the unbound compounds with charcoal and sedimentation, aliquots (100 μ l) were applied to sucrose gradients and analysed as described in the Materials and methods section. Arrows denote positions of the sedimentation standards BSA (4.4 S) and catalase (11.3 S) analysed on separate sucrose gradients. The results are typical of two separate experiments.

fraction, respectively. Both peaks of radioactivity were eliminated by co-incubation with the unlabelled β -NF and the 8 S but not the 4 S peak by co-incubation with TCDF (shown in Figure 2, panels A and B, with cytosols from 3-MC- and α -NF-treated rats, respectively). In TCDD-treated rats (72 h after one i.p. injection), the [3 H] β -NF binding to liver cytosol was 120-fold greater than in the corn oil-treated controls (Table 2). Sucrose-gradient analyses of [3 H] β -NF and [3 H]TCDD binding to liver cytosol of TCDD-treated rats showed that the patterns of distribution of the radioactivity and its suppression in the presence of the unlabelled β -NF or TCDF (Figure 3) were similar to those with liver cytosols from β -NF-, 3-MC- or α -NF-treated rats (Figure 2). Co-incubation with TCDF also eliminated the background binding (Figures 2 and 3). The data indicate that the AhR agonists, TCDD, 3-MC and β -NF, induce a 4 S [3 H] β -NF-binding protein in rat liver cytosol. The lower level of induction of [3 H] β -NF binding activity and protein in the liver cytosol of α -NF-treated rats ($\approx$ 20–25% the level of 3-MC- or β -NF-treated rats) suggests that α -NF displays a weak AhR agonist activity *in vivo*, which may be similar to that reported for rat liver cytosol *in vitro* [36].

Figure 3 Binding of [3 H] β -NF and [3 H]TCDD to liver cytosol of TCDD- or corn oil-treated rats

Treatment of rats (one i.p. injection of TCDD at 25 μ g/kg b.wt. or corn oil at 4 ml/kg b.wt.), preparation of liver cytosols and the binding assay are described in the Materials and methods section. Aliquots of cytosol containing 0.86 mg of protein were incubated: from TCDD-treated rats with 10 nM [3 H] β -NF (○), 10 nM [3 H] β -NF + 2 μ M β -NF (●) or 10 nM [3 H] β -NF + 1 μ M TCDF (□) (A); 5 nM [3 H]TCDD (△) or 5 nM [3 H]TCDD + 1 μ M TCDF (▲) (B); and from corn oil-treated rats with 10 nM [3 H] β -NF (×) (A). After removal of the unbound compounds with charcoal and sedimentation, aliquots (100 μ l) were applied to sucrose gradients and analysed as described in the Materials and methods section. Arrows denote positions of the sedimentation standards BSA (4.4 S) and catalase (11.3 S) analysed on separate sucrose gradients. The results are typical of two separate experiments.

Competitors of [3 H] β -NF binding to liver cytosols from β -NF-, 3-MC- or TCDD-treated rats

The patterns of elimination of [3 H] β -NF binding to liver cytosols after co-incubation with the unlabelled β -NF and TCDF indicated that β -NF competed with [3 H] β -NF for binding to both the 4 S and 8 S proteins whereas TCDF competed for the 8 S protein (Figures 1–3). Therefore, competition by other compounds with [3 H] β -NF for binding to the 4 S protein was examined in the presence of TCDF to eliminate the binding to the 8 S protein (and also the background binding). Competitors of [3 H] β -NF binding were determined with liver cytosols from β -NF-, 3-MC- or TCDD-treated rats (Table 3). No statistically significant differences were found among the three treatment groups for any of the 17 compounds examined [$F(2,8) = 0.42$, $P = 0.6681$]. Binding of [3 H] β -NF was significantly inhibited by α -NF, β -NF, 3-MC, B[a]P, benz[a]anthracene (BA), 7,12-dimethylbenz[a]anthracene (DMBA), B[e]P, benzo[ghi]perylene (B[ghi]Per) and 2-AAF (listed in the order of the magnitude of their effects). The high level of inhibition ($\approx$ 80–85%) determined in the presence of α -NF, β -NF or 3-MC indicated that these compounds were particularly effective in replacing [3 H] β -NF from its binding

Table 3 Competition for [^3H] β -NF binding to liver cytosols from β -NF-, 3-MC- or TCDD-treated rats

Treatment of rats (one i.p. injection of β -NF or 3-MC at 40 mg/kg b.wt. or TCDD at 25 $\mu\text{g/kg}$ b.wt.), assay of [^3H] β -NF binding and statistical analysis are described in the Materials and methods section. All incubations with 10 nM [^3H] β -NF and a 100-fold excess of the unlabelled competitor, including the controls to which no competitor was added, contained TCDF to block [^3H] β -NF binding to an 8 S protein. The values are the means $\pm$ S.E.M. from duplicate assays with separate cytosols from four (β -NF- or 3-MC-treated) and three (TCDD-treated) rats. *Significantly different ($P < 0.05$) from the control value of 100%.

Competitor	Binding of [^3H] β -NF (% of control)		
	β -NF	3-MC	TCDD
α -NF	18.08 $\pm$ 1.31*	16.80 $\pm$ 1.31*	15.20 $\pm$ 1.52*
β -NF	18.95 $\pm$ 0.95*	17.45 $\pm$ 0.95*	16.17 $\pm$ 1.10*
3-MC	22.88 $\pm$ 1.16*	20.80 $\pm$ 1.16*	18.63 $\pm$ 1.34*
B[a]P	65.43 $\pm$ 1.75*	65.40 $\pm$ 1.75*	62.47 $\pm$ 2.02*
BA	71.88 $\pm$ 1.76*	72.83 $\pm$ 1.76*	71.60 $\pm$ 2.04*
DMBA	82.08 $\pm$ 1.55*	83.70 $\pm$ 1.55*	82.03 $\pm$ 1.79*
B[a]P	83.65 $\pm$ 1.68*	85.65 $\pm$ 1.68*	86.77 $\pm$ 1.94*
B[ghi]Per	92.58 $\pm$ 0.98*	95.20 $\pm$ 0.98*	93.97 $\pm$ 1.13*
2-AAF	92.78 $\pm$ 1.01*	94.65 $\pm$ 1.01*	93.20 $\pm$ 1.17*
Metyrapone	98.18 $\pm$ 0.54*	99.95 $\pm$ 0.54	99.90 $\pm$ 0.63
Indole-3-carbinol	100.00 $\pm$ 1.17	103.35 $\pm$ 1.17*	101.80 $\pm$ 1.35
Bilirubin	101.25 $\pm$ 0.91	103.38 $\pm$ 0.91*	102.80 $\pm$ 1.05*
17 β -Oestradiol	100.93 $\pm$ 0.96	102.18 $\pm$ 0.96	102.10 $\pm$ 1.11
Oestrone	101.43 $\pm$ 1.44	101.95 $\pm$ 1.44	102.00 $\pm$ 1.66
DHEA	102.48 $\pm$ 1.33	103.88 $\pm$ 1.33	105.27 $\pm$ 1.54*
Progesterone	100.15 $\pm$ 0.67	101.60 $\pm$ 0.67	102.67 $\pm$ 0.78*
Dexamethasone	100.35 $\pm$ 1.24	102.83 $\pm$ 1.24	102.83 $\pm$ 1.44

Figure 4 Kinetics of [^3H] β -NF binding to liver cytosol of β -NF-treated rats

Treatment of rats (one i.p. injection of β -NF at 40 mg/kg b.wt.) and assays of [^3H] β -NF-specific binding are described in the Materials and methods section. Association of [^3H] β -NF with cytosol at the protein concentrations of 0.2, 0.5 and 1.0 mg/ml (A); and dissociation from cytosol (1 mg of protein/ml) of [^3H] β -NF that was bound during a 15 h incubation (B). The points are mean values from two separate liver cytosols, each assayed in duplicate.

site(s) on the 4 S cytosolic protein. [^3H] β -NF binding was inhibited by ≈ 28 –38 %, < 20 % and < 10 % by co-incubations with B[a]P or BA, DMBA or B[a]P, and B[ghi]Per or 2-AAF, respectively. Thus, the latter PAHs appeared to have much lower binding affinities than α -NF, β -NF or 3-MC for the [^3H] β -NF-binding protein inducible by treatment of rats with β -NF, 3-MC or TCDD. The other compounds tested had no significant effects on [^3H] β -NF binding, except for a low level of inhibition (≈ 2 %) by metyrapone in the cytosol of β -NF-treated rats, or stimulation (≈ 3 –5 %) by indole-3-carbinol and bilirubin in the cytosol of 3-MC-treated rats and by bilirubin, dehydroepiandrosterone (DHEA) and progesterone in the cytosol of TCDD-treated rats. An apparent stimulation of [^3H] β -NF binding may be caused by blocking non-specific binding sites for β -NF by these compounds.

Characterization of [^3H] β -NF-binding protein

Sucrose-density-gradient analysis suggested that [^3H] β -NF binding to a 4 S fraction was saturable since addition of a 200-fold excess of the unlabelled β -NF completely blocked the binding (Figure 1, panels A1 and A2). Since equilibrium saturation analysis of [^3H] β -NF binding to liver cytosols of β -NF-treated rats yielded non-linear and poorly reproducible Scatchard plots (results not shown), the interaction of [^3H] β -NF was examined by analyses of association of the free and dissociation of the protein-bound radioligand, both as a function of time (Figure 4). The half-lives of association [$\ln(2)/\text{observed rate constant}$] were 1.1, 2.1 or 5.6 h for 1.0, 0.5 or 0.2 mg of protein/ml, respectively, suggesting an apparent dependence of the rate constant for association on protein concentration. Determination of dissociation, either by infinite dilution or rebinding prevention [9] showed that only a fraction (22–40 %) of total specifically bound [^3H] β -NF was dissociable; a half-life for dissociation of the radiolabel was ≈ 4 h. Addition of a 100-fold excess of TCDF to block [^3H] β -NF binding to the 8 S fraction had negligible effect on the dissociation curve, suggesting that the observed partial dissociation of [^3H] β -NF is from its binding to the 4 S fraction. No degradation of the [^3H] β -NF-binding protein occurred during the entire time (15 + 24 h) of the dissociation experiment since the [^3H] β -NF-binding capacity of the cytosol stored at 4 $^{\circ}\text{C}$ for 39 h remained unchanged. The association–dissociation kinetics data indicate that [^3H] β -NF binding to liver cytosol of β -NF-treated rats does not obey the law of mass action, as assumed for classical receptor–ligand binding.

Liver cytosol from β -NF-treated rats subjected to chromatography on Sephacryl S-200 HR before incubation with [^3H] β -NF yielded the [^3H] β -NF-binding component in the void volume (Figure 5A, dashed line), suggesting its association with a large protein complex or aggregation. By contrast, incubation of cytosol with [^3H] β -NF and removal of the unbound [^3H] β -NF before the chromatography yielded a major radioactive peak with an apparent molecular mass of ≈ 42 kDa and a minor radioactive peak eluted with the void volume (Figure 5A, solid line). These results suggest that the 42 kDa component is derived from a large β -NF-binding protein aggregate in the cytosol. Consistent with the above results, liver cytosol subjected to sucrose-density-gradient centrifugation before incubation with [^3H] β -NF showed specific binding of the radioligand to a denser component (≈ 7 S) than the 4 S component of the cytosol preincubated with [^3H] β -NF (Figure 5B). The molecular mass of a 7 S component is estimated at ≈ 120 kDa [37].

Since AhR agonists including β -NF induce several cytosolic enzyme activities, e.g. those of GST Ya, aldehyde-3-dehydrogenase and DT-diaphorase [4], their elution profiles on Sephacryl

Figure 5 Elution profiles of [^3H] β -NF-binding protein subjected to gel-filtration chromatography on Sephacryl S-200 HR (A) and sucrose-density-gradient centrifugation (B) before and after incubation of rat liver cytosol with [^3H] β -NF

Liver cytosols from β -NF-treated rats with nine and one i.p. injections at 40 mg/kg b.wt. were used in (A) and (B), respectively. Gel-filtration chromatography (A) before (i) and after (ii) incubation of the cytosol with [^3H] β -NF is described in the Materials and methods section. The elution of [^3H] β -NF-binding protein from (i) is indicated by a dashed line, and that from (ii) by dotted or solid lines, corresponding to incubation in the presence or absence of the unlabelled β -NF, respectively. Arrows indicate the positions of the void volume (V_0) and molecular-mass markers: alcohol dehydrogenase (ADH), 150 kDa; BSA, 67 kDa; and cytochrome *c* (cyt *c*), 12.4 kDa. Sucrose-gradient centrifugation (B) is described in the Materials and methods section, except that the Hepes buffer with glycerol and dithiothreitol was used and the gradient centrifugation was for 3 h. The sedimentation of [^3H] β -NF-binding protein was determined before (○) and after (●) incubation of the cytosol with [^3H] β -NF as follows. Cytosol (100 μl) containing 2 mg of protein was applied to the sucrose gradient, and each collected fraction was divided into two portions that were incubated with 20 nM [^3H] β -NF at 4 °C for 4 h with or without a 200-fold excess of the unlabelled β -NF to determine specific binding. Also, aliquots of cytosol containing 75 μg of protein were incubated with [^3H] β -NF as above in a total volume of 150 μl of Hepes buffer, the unbound radioligand was removed with charcoal, and 100 μl aliquots were applied to sucrose gradients. Arrows denote positions of the sedimentation standards BSA (4.4 S) and catalase (11.3 S) analysed on separate gradients. The results are typical of at least three separate experiments.

S-200 were compared with that of [^3H] β -NF-binding activity (Figure 6). However, the latter did not co-elute with any of the above enzyme activities. Furthermore, treatment of rats with β -NF significantly decreased (by 63 %) the GNMt activity of liver cytosol (Table 1). Although this activity was ascribed to a 4 S

Figure 6 Elution profiles on Sephacryl S-200 HR of [^3H] β -NF-specific binding and enzyme activities from liver cytosol of β -NF-treated rats

Preparation of liver cytosol from β -NF-treated rats (nine i.p. injections at 40 mg/kg b.wt), gel-filtration chromatography according to procedure (i), assays of [^3H] β -NF-specific binding (○), GST (●), aldehyde-3-dehydrogenase (□) and DT-diaphorase (△) activities, and protein concentration (dashed line) are described in the Materials and methods section. Points are mean values from two separate determinations.

PAH-binding protein [14], a later report did not confirm this finding [18].

DISCUSSION

β -NF was shown to be a high-affinity ligand for a constitutive 4 S PAH-binding protein because it significantly blocked the specific binding of [^3H]3-MC [5,20] and that of [^3H]B[a]P [21] in liver cytosols of untreated male SD rats. Hence, these rats (either of unknown source, or CD rats from Charles River, Margate, Kent, U.K.) as well as male Wistar [38,39] and SD rats from Sasco (Omaha, NE, U.S.A.) [11,12,40] are considered to be '4-S-positive'. By contrast, binding of [^3H]B[a]P to a 4 S protein was undetectable in male Harlan SD rats from Houston, TX, U.S.A. (see [12]), which were thus considered to be '4-S-negative' [12]. In the 4-S-positive male SD rats, the specific [^3H]B[a]P-binding activity was increased by pretreatment with phenobarbital or isosafrole, but not with 3-MC [40]. However, in phenobarbital-treated SD rats (Janvier, Le Genest, France), β -NF exerted only a small competitive effect on the specific binding of [^3H]3-MC to a 4 S protein [10]. In our study, there was little or no binding of [^3H] β -NF to a 4 S protein in the liver cytosols of the control (corn oil-treated) female Harlan SD rats from Indianapolis, IN, U.S.A. (Table 1) suggesting that they were 4-S-negative. Treatment of these rats with the AhR agonists TCDD, 3-MC or β -NF [4], and to a lesser extent with α -NF, a weak AhR agonist [36], induced the [^3H] β -NF-binding activity (Table 2). This is commensurate with a 4 S [^3H] β -NF-binding protein (Figures 1–3), for which α -NF, β -NF and 3-MC were high-affinity ligands blocking the [^3H] β -NF binding by $\geq 80\%$ (Table 3). A potent inhibition of the [^3H] β -NF binding by α -NF was in contrast with its moderate-to-weak affinity for the constitutive 4 S PAH-binding protein [5,20]. On the other hand, the PAHs, e.g. B[a]P, B[a]P, B[ghi]Per, DMBA or BA, reported to be the high-affinity ligands for the constitutive 4 S PAH-binding protein of liver cytosols from male SD [5,20] or Long-Evans [7] rats, were only weak ligands for the induced 4 S protein described herein, inhibiting the [^3H] β -NF binding by $\approx 35\text{--}5\%$ (Table 3). Furthermore, metyrapone,

shown to compete with [^3H]B[a]P for binding to the constitutive 4 S PAH-binding protein of rat liver cytosol [21], was only a very weak competitor ($\approx 2\%$ inhibition) of the [^3H] β -NF binding to the β -NF-inducible 4 S protein. The differences between these two 4 S proteins discussed above indicate that the [^3H] β -NF-binding protein induced herein by AhR agonists is a novel 4 S protein. The common characteristics of the constitutive 4 S PAH-binding protein and the inducible 4 S [^3H] β -NF-binding protein include the high affinities of both for 3-MC and virtual lack of affinities for steroid hormones (Table 3) [5,20,21]. The latter suggest that these 4 S proteins do not function as steroid hormone receptors.

A 4 S PAH-binding protein was localized in the mouse hepatocytes throughout the liver and was relatively evenly distributed in the cytoplasm of the cells, which correlated with high levels of [^3H]B[a]P binding in the liver cytosol [41]. Since treatment of mice with β -NF did not affect the localization, intensity of staining and [^3H]B[a]P-binding constants, these data support the conclusion of our study that the β -NF-inducible 4 S [^3H] β -NF-binding protein is different from the constitutive 4 S PAH-binding protein. The latter does not appear to be induced by PAHs since, in the livers of 3-MC-treated rats, the specific [^3H]B[a]P binding to a 4 S fraction was not increased [40] and the cytoplasmic 4 S [^3H]3-MC-binding protein was transferred to nuclei without an apparent increase in its overall content [39]. The molecular mass of the 4 S [^3H] β -NF-binding protein from β -NF-treated rats was ≈ 42 kDa (Figure 5A), and thus was similar to that of the 4 S PAH-binding protein determined with [^3H]3-MC (≈ 39 kDa) [38] or [^3H]B[a]P (≈ 40 kDa) [18] as ligands. Since the [^3H] β -NF-binding protein was eluted with the void volume of Sephacryl S-200 gel filtrate before the radioligand binding, but was eluted as a 42 kDa component after binding of [^3H] β -NF, it appears that the large complex or aggregate disassembles upon binding to yield the 42 kDa protein (Figure 5A). Liver cytosol subjected to sucrose-gradient analysis yielded the [^3H] β -NF binding component sedimenting at ≈ 7 S before and at ≈ 4 S after the radioligand binding (Figure 5B). The data indicate that the 7 S protein observed herein is different from light-density protein(s) detected in the top (non-sedimenting) fractions of the sucrose gradients [42]. These protein(s) were found to be capable of binding TCDD, B[a]P and 3-MC.

Sucrose-density-gradient analysis suggested that binding of [^3H] β -NF to a 4 S fraction was saturable since addition of a 200-fold excess of β -NF completely blocked binding of the labelled compound. However, the equilibrium saturation analysis using liver cytosols from β -NF-treated rats yielded non-linear and poorly reproducible Scatchard plots. Non-linear Scatchard plots for binding of TCDD to AhR of rat liver cytosol were also reported and attributed to a high level of non-specific binding [43]. However, minimizing the non-specific [^3H] β -NF binding did not improve our plots. The half-life for association of the [^3H] β -NF increased from 1.1 to 5.6 h with decreasing protein concentration from 1.0 to 0.2 mg/ml (Figure 4A). Furthermore, only a fraction (22–40%) of the total specifically bound [^3H] β -NF was dissociable, with a half-life of ≈ 4 h for dissociation of the radiolabel determined at 1.0 mg of protein/ml (Figure 4B). Hence, the [^3H] β -NF binding characteristics were inconsistent with classical association–dissociation kinetics and may reflect the influence on the rate of an enzymic reaction(s) and/or conformational changes of the protein. However, it is unlikely that the ligand itself is metabolized in the absence of microsomes. The association of the TCDD–AhR complex in rat liver cytosol was similarly irreversible and hence the dissociation kinetics could not be determined [43]. When a stable preparation of AhR from mouse liver cytosol and ^{125}I -labelled dioxin ligand were

used, the apparent equilibrium dissociation constant was shown to be directly related to the receptor concentration, and ligand binding to the AhR was interpreted as a two-step process characterized by rapid ligand dissociation from the initial complex and a much slower ligand dissociation rate from a transformed complex [44]. Clearly, elucidation of the nature of [^3H] β -NF binding requires further studies with a purified 4 S [^3H] β -NF-binding protein from liver cytosols of β -NF-treated rats.

The 4 S PAH-binding protein in the livers of untreated or B[a]P-treated male SD rats has been identified as GNMT [14,15]. Apparently, this protein exhibits diversity in function since different subunit configurations may be involved in its enzymic role as a methyltransferase and as a PAH-binding protein. In our study, induction of [^3H] β -NF binding activity by treatment of rats with β -NF significantly suppressed rather than increased the GNMT activity of liver cytosol (Table 1), strongly suggesting that a 4 S [^3H] β -NF-binding protein is different from the 4 S PAH-binding protein exhibiting GNMT activity. On the other hand, the latter has been also reported to be devoid of GNMT activity [18,19]. The induction of the 4 S [^3H] β -NF-binding protein by treatment of rats with the AhR agonists suggested that this binding activity may be associated with an enzyme known to be under AhR control, such as GST Ya, aldehyde-3-dehydrogenase or DT-diaphorase [4]. However, gel-filtration chromatography of the above enzymes showed their different migration patterns from that of [^3H] β -NF-binding protein (Figure 6). In addition, bilirubin or DHEA, high- and moderate-affinity ligands, respectively, for GST Ya [45], did not compete with [^3H] β -NF for binding to a 4 S protein (Table 3). This finding is consistent with the lack of affinity of β -NF for GST Ya [45].

In view of the widespread use of β -NF as a non-carcinogenic inducer of phase-I and phase-II enzymes in the rodent tissues for studies of xenobiotic metabolism and carcinogen detoxification [46] and as a blocking agent of mammary gland carcinogenesis in female rats [47], further studies on the identity and function of the novel 4 S [^3H] β -NF-binding protein inducible by treatment of rats with β -NF or other AhR agonists are required. Conceivably, this protein may serve as a carrier protein for β -NF to microsomes since the [^3H] β -NF-binding activity was also found in this fraction (723 fmol/mg of protein versus that of 840 fmol/mg of cytosolic protein) after treatment of rats with β -NF (nine i.p. injections). This idea is also supported by the evidence that a B[a]P-binding protein of rat liver cytosol was shown to transport B[a]P and its oxidized products to and from the microsomes [48]. Hence, the potential function of the induced β -NF-binding protein described herein as a carrier protein in metabolism of carcinogens *in vivo* remains to be investigated.

We thank Professor Karl K. Rozman for helpful discussions and comments, and his assistant Margitta Lebofsky for treatment of rats with TCDD and shipment of the frozen livers. We thank Professor Steven H. Safe for the ^3H -labelled TCDD. We thank Robin L. Bliss (University of Minnesota Comprehensive Cancer Center, Minneapolis, MN, U.S.A.) for statistical analyses. We also thank Mark A. Reichert for technical assistance. This study was supported by Biomedical Research Funds, the U.S. Department of Veterans Affairs, and a grant from the National Cancer Institute (CA-28000), the U.S. Public Health Service.

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Received 16 September 1999/3 February 2000; accepted 3 March 2000