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Cytochrome P-450 b and c in the Rat Brain and Pituitary Gland

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SUMMARY

A quantitative assessment of the levels of cytochromes P-450 b and P-450 c in the brains and pituitary glands of untreated and β-naphthoflavone (BNF)-pretreated rats was made with polyclonal antibodies raised against hepatic P-450 b and c and the sensitive fluorometric assay of P-450 catalytic activity, namely, the *O*-deethylation of ethoxycoumarin (ETC). In the microsomal fraction of brains of untreated rats, the rate of formation of 7-hydroxycoumarin from ETC ranged between 0.1 and 20 pmol/min/mg of microsomal protein, which is approximately 0.01–2% of the level of hepatic microsomes of phenobarbital-induced rats. This brain activity was completely inhibited by anti P-450 b antibodies but was unaffected by anti P-450 c antibodies. As with hepatic P-450 b, metyrapone and chloramphenicol (100 μM) were good inhibitors of catalytic activity, whereas α-naphthoflavone (1 μM) was a poor inhibitor. No ETC *O*-deethylase activity was detectable in microsomes prepared from the pituitary glands of untreated rats. Upon pretreatment of rats with BNF, there was induction of ETC *O*-deethylase activity in the pituitary gland to a level of 3.3 ± 1.5 pmol/min/mg of microsomal protein, but there was no significant increase in the level of activity in brain microsomes. Despite this, there was evidence of induction of P-450 c in both the brain and pituitary of BNF-pretreated rats since anti P-450 c antibodies inhibited brain activity by 55% and pituitary activity by 84%. The regional distribution of P-450 b and c in the hypothalamic-preoptic area and olfactory bulbs was examined. The level of ETC *O*-deethylase activity in the hypothalamic-preoptic area was not different from that in the whole brain, but in the olfactory bulbs activity was higher than that in whole brain, with a range of 0.1–52 pmol/min/mg of microsomal protein. The catalytic activity in the whole brain and in the olfactory bulbs was inhibited by anti $P\text{-}450_b$ but not by anti $P\text{-}450_c$ antibodies. Neither estradiol, testosterone, dehydrotestosterone, nor 5α-androstane,3β,17β-diol (100 μM) competitively inhibited ETC *O*-deethylase activity, indicating that P-450 b is not responsible for the steroid hydroxylations previously reported in the brain. BNS pretreatment of rats did not cause a consistent increase in ETC *O*-deethylase upon BNF induction. However, there was an induction of P-450 c in the olfactory bulbs since catalytic activity was inhibited with anti $P\text{-}450_c$ antibodies. Western immunoblots with microsomes from whole brain, hypothalamic-preoptic area, or olfactory bulbs of control or BNF-pretreated rats gave no signals with either P-450 b or P-450 c antibodies. This is perhaps not surprising, since the level of these enzymes is less than 0.1% of that in liver microsomes, which means that the P-450 proteins constitute approximately 20 ng/mg of microsomal protein and this is below the level of detectability of the Western immunoblots.

The hepatic microsomal P-450 (P-450) monooxygenase system plays an important role in the metabolism of a wide variety of xenobiotics as well as endogenous compounds. Multiple forms of P-450 have been purified from livers of untreated rats and of rats treated with different inducers (1, 2). P-450s immunologically similar to those in the liver are also found in extrahepatic tissues (3–8).

The presence of P-450-catalyzed steroid metabolism in mammalian brain is well established (9, 10). The HPOA and the amygdala are the main sites for aromatization of androgens (11), whereas the hypothalamus and the PG exhibit a high capacity for 2/4-hydroxylation of estrogens (12, 13). Involvement of multiple P-450s in the latter reaction and differential distribution of these enzymes in rat brain have been suggested (13).

Microsomal fractions from whole rat brain also metabolize a variety of xenobiotic substrates, known to be substrates for liver P-450(s), such as benzo(*a*)pyrene, (3), parathion (14), aminopyrine (15), and ETC (16, 17). The specific activity of constitutive brain monooxygenases, expressed per mg of microsomal protein, is very low, approximately 0.01–0.2% of the corresponding activities in liver microsomes. Slight induction

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ABBREVIATIONS: P-450, cytochrome P-450 enzyme; EDTA, ethylenediaminetetraacetate; HEPES, 4-2-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; BNF, β-naphthoflavone(5,6-benzoflavone); ANF, α-naphthoflavone(7,8-benzoflavone); 5α-A-3α,17β-diol, 5-androstane-3α,17β-diol; 5α-A-3β,17β-diol, 5α-androstane-3β,17β-diol; DHT, 5α-dihydrotestosterone; ATD, 1,4,6-androstatriene-3,17-dione; ETC, 7-ethoxycoumarin; $P\text{-}450_c$, the major P-450 inducible in the rat liver by β-naphthoflavone; $P\text{-}450_b$, the major P-450 inducible in the rat liver by pituitary gland; OB, olfactory bulb; HPOA, hypothalamic-preoptic area; PG, pituitary gland.

of microsomal monooxygenase activities by BNF and methylcholanthrene in rat brain has been reported (3, 16–18).

The aim of the present study was to use antibodies against two of the major inducible hepatic forms of P-450, namely P-450$_b$ and P-450$_c$, to characterize the P-450(s) in rat brain. The *O*-deethylation of ETC was chosen as a measure of catalytic activity because it is a catalytic activity characteristic of both P-450s b and c and because of its sensitivity.

Materials and Methods

Chemicals. α-Naphthoflavone(5,6-benzoflavone), β-naphthoflavone(7,8-benzoflavone), ETC, and 7-hydroxycoumarin were obtained from Aldrich-Chemie (Steinheim, German Democratic Republic). DL-Isocitrate, isocitrate dehydrogenase, NADP, NADPH, chloramphenicol, metyrapone (2-methyl-1,2-di-pyridol-1-propanone), testosterone, estradiol-17β,5α-androstane-3α,17β-diol, 5α-androstane-3β,17β-diol, and 5α-dihydrotestosterone were obtained from Sigma Chemical Co. (St. Louis, MO). 1,4,6-Androstatriene-3,17-dione was purchased from Steraloids Inc. (Wilton, NH). All other chemicals used were of analytical grade.

Treatment of animals. Adult Sprague-Dawley rats were obtained from ALAB Labtjänst AB (Stockholm, Sweden). The animals were housed for 5 days before use in plastic cages with hardwood bedding in a room with controlled temperature, humidity, and light (12-hr light cycle). Food and water were available *ad libitum*. Male rats (2–18 animals) weighing 280–320 g were treated with two single daily intraperitoneal injections of 40 mg/kg BNF as a 1% (w/v) solution in corn oil. The control rats received an identical volume of the vehicle. The animals were sacrificed by decapitation 24 hr after treatment.

Homogenization procedures and isolation of subcellular fractions. The rats were killed by decapitation and the brains were excised. Pooled brains of 2–13 rats were washed with 0.15 M KCl and cleared from visible blood vessels. The tissue was cut and homogenized in 4 volumes of 0.1 M Tris-acetate buffer (pH 7.4) containing 0.1 M KCl, 1 mM EDTA, and 20 μM butylated hydroxytoluene (19) by using an Ultraturrax for 20 sec at 550 W and, thereafter, a Potter-Elvehjem Teflon-glass homogenizer with four strokes at 1400 rpm. After addition of phenylmethylsulfonyl fluoride to a final concentration of 0.1 mM, the 20% (w/v) homogenate was sonicated at 15 μm for 30 sec with an MSE 150-W ultrasonic disintegrator (MSE Scientific Instruments, West Sussex, England). This tissue homogenate (15–20 mg of protein/ml) was used as the enzyme source in some experiments.

The following procedure was used for studies on distribution of marker enzymes in subcellular fractions of rat brain. The tissue homogenate was centrifuged at 1,240 × *g* for 10 min to give pellet I (P I), which was resuspended by hand in half the original volume of the homogenization buffer and recentrifuged. The resulting supernatants were pooled and centrifuged at 10,000 × *g* for 20 min to yield the pellet (P II) called mitochondrial pellet and Sup II. The P II pellet was resuspended by hand in half the original volume of the homogenization buffer and recentrifuged to give washed pellet II (P IIw) and Sup IIw. This supernatant was pooled with Sup II and centrifuged at 100,000 × *g* for 60 min to obtain the final microsomal pellet (P III) and Sup III.

The standard procedure for preparation of microsomes from whole brain and different regions of the brain did not include the centrifugation at 1240 × *g*. Microsomes from different regions were prepared as described for whole brain with tissues pooled from 2–18 animals. The final microsomal pellet was resuspended in 10 mM Tris-acetate buffer (pH 7.4) containing 1 mM EDTA and 20% glycerol, to give a protein concentration of 5–20 mg/ml. All fractions not immediately analyzed were quick-frozen as 0.5- to 1-ml aliquots in liquid N_2 before storage at −70°. The protein was determined (20) following TCA precipitation, using bovine serum albumin as the standard.

Enzymatic assays. The ETC *O*-deethylase activity was measured essentially according to a previously described method (21). The incubations were performed with 1–3 mg of protein of the subcellular fractions from whole brain or with 0.1–1.5 mg of protein from homogenates or microsomal preparations from the discrete regions. The incubation medium contained 0.5 μmol of NADP, 4 μmol of DL-isocitrate, 15 μmol of $MgCl_2$, 0.1 μmol of EDTA, 0.3 μmol of ETC, and 0.6 unit of isocitrate dehydrogenase in a final volume of 1 ml of 0.05 M HEPES buffer (pH 7.5). After 3 min of preincbuation at 37°, the reaction was started by the addition of DL-isocitrate and allowed to proceed for 60 min. The incubation was terminated by the addition of 0.1 ml of 2 M HCl; after extraction with chloroform and 30 mM sodium borate, the 7-hydroxycoumarin formed was determined with a Shimadzu RF-510 spectrofluorophotometer. The wavelengths for excitation and emission were at 368 and 456 nm, respectively. When the substrate dependence of the microsomes was determined, ETC concentrations ranging between 3 and 300 μM were used. Incubations with microsomes without NADP and DL-isocitrate, alternatively excluding ETC, were performed and served as blanks. In some experiments various steroids and P-450 inhibitors were added in 5 μl of acetone (ANF) or in 10 μl of methanol to the incubation mixture. Chloroamphenicol was preincubated for 5 min before the assay as described earlier (22). The enzyme activity toward ETC, which was linear with respect to time (for at least 60 min) and protein (up to at least 3 mg) in both microsomes and tissue homogenates, did not change as a result of freezing and retained the same activity for at least 1 month at −70°.

Antibody inhibition of the brain ETC *O*-deethylase activity was performed with microsomes from control and BNF-pretreated rats. Preincubation was carried out for 20 min at room temperature with 2 mg and 0.1–0.4 mg of microsomal protein from whole brain and OB, respectively, and at increasing amounts of antibodies ranging from 0 to 2 mg. The polyclonal antibodies were raised in rabbits against rat liver P-450$_c$ and P-450$_b$. Details of the antibodies used in this investigation are described elsewhere (23). The control incubations contained microsomes and the corresponding concentrations of preimmune IgG. After addition of substrate, buffer, and cofactors, the incubation was allowed to proceed for 60 min.

NADPH-cytochrome *c* reductase activity was determined at 30° in 0.3 M potassium phosphate buffer (pH 7.7) and measured at 550 nm as described earlier (19). KCN was included to avoid interference from contaminating mitochondrial cytochrome *c* oxidase. This enzyme activity was assayed according to the method described earlier (24). Cytochrome *c* was reduced by addition of ascorbate and removal of excess of Sephadex G-25 gel filtration.

Western immunoblotting. Western immunoblotting was used to detect P-450$_c$ and P-450$_b$ in microsomes obtained from whole brain. HPOA, and OB microsomes of control or BNF-pretreated rats (23).

Light and electron microscopic analysis. The brain homogenate was fixed in Histofix (a buffered fixative containing formaldehyde), embedded, sectioned, and stained according to routine procedures, and then examined by light microscopy. The mitochondrial and microsomal fractions were fixed in 3% (w/v) glutaraldehyde, containing 0.1 M sucrose and 0.1 M phosphate buffer (pH 7.2), and processed for electron microscopy according to the methods described earlier (25).

Results

Subcellular fractionation of whole rat brain. Light microscopic analysis of fractions following each of the different homogenization steps showed that Ultraturrax treatment for 20 sec, followed by homogenization in a Potter-Elvehjem glass homogenizer at 1400 rpm with four up and down strokes, and then by ultrasonication at 15 μm for 30 sec was necessary for an efficient disruption of the cells. In particular, ultrasonication seemed to be the crucial step in obtaining microsomes in relatively high yield since, by this approach, the recovery of microsomal protein increased from 1.8 ± 0.27 to 5.2 ± 0.44 mg/g wet weight (five experiments) of whole brain tissue. A parallel increment of recovery of both the ETC *O*-deethylase and the

NADPH-cytochrome *c* reductase in the microsomal fraction was observed following this treatment. Electron microscopic analysis revealed that P III (microsomal fraction) was enriched in microsomal vesicles mostly free of ribosomes that apparently had been detached by the homogenization and/or ultrasonication treatment (Fig. 1a). The pattern of distribution of the ETC *O*-deethylase and NADPH-cytochrome *c* reductase activity was similar in subcellular fractions obtained from control and BNF-pretreated rats. The ETC *O*-deethylase (0.77 pmol/min/mg) and NADPH-cytochrome *c* reductase (24 nmol/min/mg) activities were 3- to 4-fold enriched in the P III fraction compared to the homogenate. The total recovery of ETC *O*-deethylase and NADPH-cytochrome *c* reductase activities from homogenate in the microsomal fraction was 36% and 26%, respectively (Table 1). Contamination of the microsomal fraction with mitochondrial cytochrome *c* oxidase activity was around 10%. Ultrasonication of homogenate did not change the specific activity of ETC *O*-deethylase or that of NADPH-cytochrome *c* reductase in the microsomal fraction, nor was there evidence for disruption of mitochondrial membranes (Fig. 1b). The contamination of the mitchondrial fraction with microsomal vesicles was morphometrically estimated to be approximately 20%. This is consistent with the specific activity of ETC *O*-deethylase in the P II fraction, which was about 20% (0.16 pmol/mg/min) of that in the P III fraction (Table 1).

The recovery of microsomal protein from OB, HPOA, and PG was 6.5 ± 1.0, 5.4 ± 1.4, and 11.4 ± 1.5 mg/g wet weight of tissue (five experiments), respectively. The subcellular distribution pattern and the microsomal recovery of ETC *O*-deethylase (34 ± 7%) and NADPH-cytochrome *c* reductase (27 ± 1%) activities from OB homogenate were comparable to those for the whole brain.

Control rat brain. The ETC *O*-deethylase activity could be measured in microsomes from whole brain (1.6 ± 5.0 pmol/min/mg), OB (17.2 ± 19.8 pmol/min/mg), and HPOA (0.31 ± 0.15 pmol/min/mg) but not in microsomes from the PG (<0.1 pmol/min/mg) (Fig. 2). The constitutive activity in the OB and whole brain exhibited a wide variation ranging from 0.1 to 52 pmol/min/mg in OB and from 0.1 to 20 pmol/min/mg in whole brain. This variability was observed in microsomal preparations from pooled tissues (5–15 rats) as well as homogenates prepared from individual animals and in both male and female rats. Increasing the pool size when preparing microsomes did not decrease the variability in the ETC *O*-deethylase activity of different preparations. The production of 7-hydroxycoumarin as the product in preparations with both high and low activity was confirmed by thin layer chromatographic analyses.

P-450$_b$ antibodies completely inhibited the *O*-deethylation in microsomes from OB (Fig. 3) and whole brain (Table 2), respectively, whereas P-450$_c$ antibodies had no significant effect on this activity in OB and whole brain microsomes from untreated animals (Fig. 4).

Inhibitors of P-450$_b$, metyrapone (26), and chloramphenicol (27) at 100 μM decreased the ETC *O*-deethylase activity in OB microsomes by 74% and 72%, respectively. A similar effect of metyrapone was also seen in HPOA microsomes. ANF, which at a concentration of 1 μM preferentially inhibits P-450$_c$, was not, at this concentration, a good inhibitor of constitutive ETC *O*-deethylase activity in OB and HPOA microsomes (Table 3).

Testosterone, DHT, 5α-A-3α,17β-diol, and 5α-A-3β,17β-diol, and the aromatase inhibitor, ATD (28), when added to the incubations at a concentration of 100 μM, did not inhibit the constitutive ETC *O*-deethylase activity in microsomes from OB or HPOA (Table 4).

Testosterone significantly stimulated the ETC *O*-deethylase activity 1.7- and 4.5-fold over the control value in OB and HPOA microsomes, whereas the same concentration of this androgen decreased the activity in liver microsomes from 0.89 to 0.66 nmol/min/mg.

BNF-pretreated brain. Upon BNF treatment, the catalytic activity ranged from 0.7 to 52 pmol/mg/min in OB and from 0.1 to 20 pmol/mg/min in whole brain. The mean values of the activity did not differ between the control and BNF-pretreated groups. Despite this, there was evidence of P-450$_c$ induction since P-450$_c$ antibodies inhibited the *O*-deethylase activity in OB and whole brain microsomes obtained from BNF-pretreated rats by 55% and 60%, respectively, whereas the catalytic activity in untreated animals was reduced only slightly (Fig. 4). ANF, at a concentration of 1 μM, resulted in a 33, 32, and 59% inhibition of the activity in microsomes from whole brain, OB, and HPOA, respectively, from BNF-pretreated rats, whereas the level of inhibition by ANF was only 10–14% in the corresponding control preparations (Table 3).

No constitutive ETC *O*-deethylase activity could be measured in PG microsomes, but, upon BNF pretreatment, the catalytic activity was clearly measurable, with a mean value of 3.3 ± 1.5 pmol/min/mg. The activity was completely inhibited by P-450$_c$ antibodies (data not shown) and 84% by ANF (1 μM) (Table 3).

Western immunoblots with partly purified P-450 from whole brain, HPOA, or OB obtained from control or BNF-pretreated rats gave no signals with either P-450$_b$ or P-450$_c$ antibodies (data not shown).

Discussion

The involvement of P-450 in the brain in both steroid (9, 10) and xenobiotic metabolism (3, 14–16, 18) has been demonstrated. The low P-450 catalytic activities in microsomal fractions prepared from whole brain tissues may in part be due to the heterogeneity of cells in the brain and dilution of cells that contain the catalytic activity with those that do not. This is the case for aromatization of androgens and 2/4-hydroxylation of estrogens (11–13). The current estimation of P-450 content by spectral measurements in microsomal fractions from whole brain gives values of P-450 levels that are 100-fold lower than those in liver (29–31), whereas the catalytic activities expressed per mg of microsomal protein are 1000-fold lower than the corresponding hepatic activities. In view of these results, questions arise as to which forms of P-450 are present in brain and about the regional distribution of P-450 in the brain with regard to region as well as cell type.

In the present study, the contribution to brain P-450 of two well characterized forms of hepatic P-450(s), P-450$_b$ and P-450$_c$, was evaluated by using polyclonal antibodies against P-450$_b$, which recognizes also P-450$_e$ and P-450$_c$, which recognizes both P-450$_c$ and P-450$_d$. Recognition of brain P-450 by these antibodies does not necessarily mean identity but could also be cross-reactivity with similar forms of P-450.

ETC *O*-deethylase activity in the brains of untreated rats, expressed as pmol of 7-hydroxy-ethoxycoumarin formed /min/mg of microsomal protein, was highly variable, particularly in microsomes from OB and whole brain, with a range from 0.1

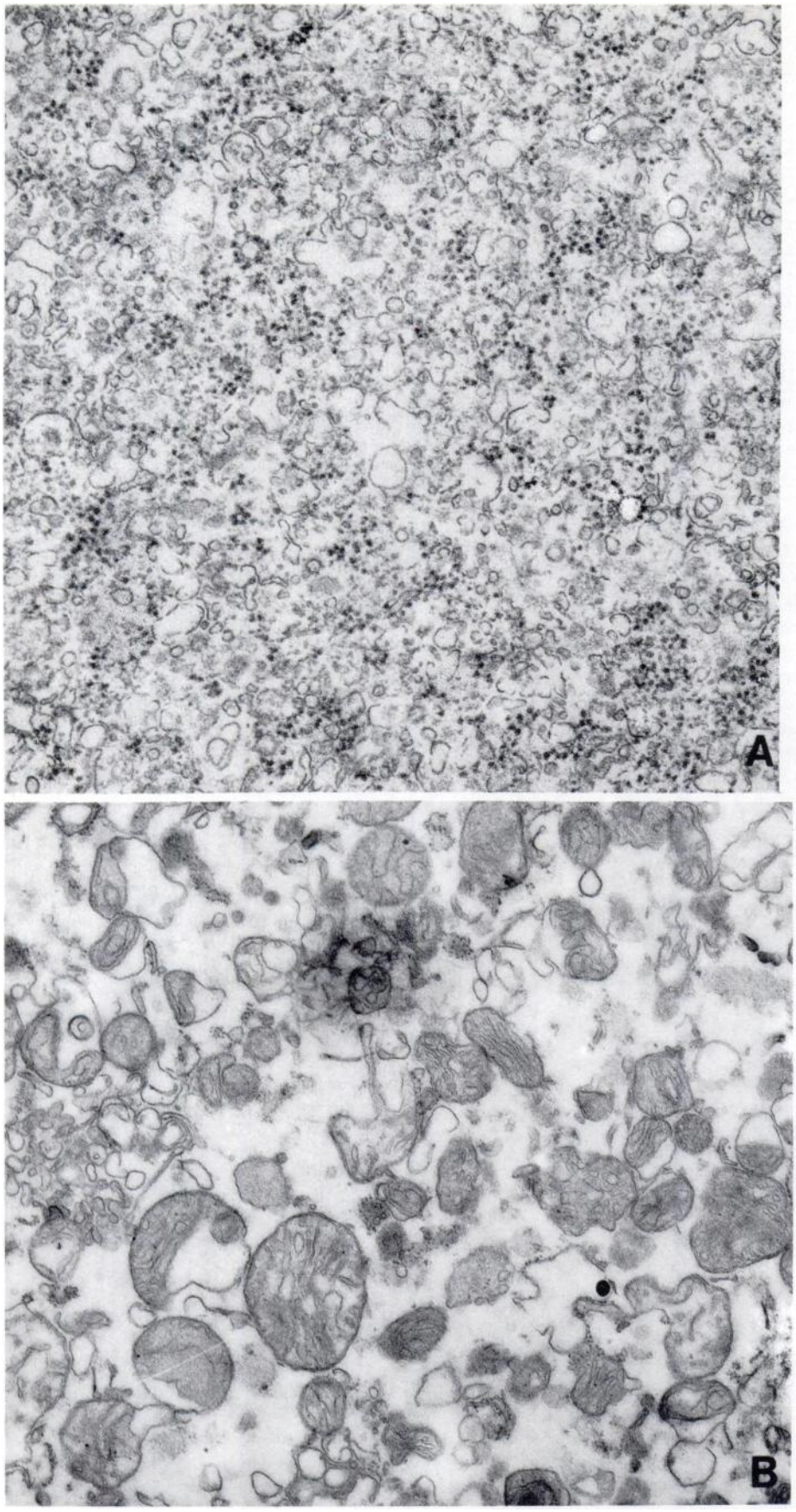

Fig. 1. A. Electron micrograph of the microsomal fraction. The fraction consists of vesicular structures and free (detached) ribosomes. These might have been stripped off by the homogenization and/or ultrasonication procedure. Magnification × 44,000. B. Electron-microscopic appearance of the mitochondrial fraction. Note comparatively well preserved mitochondria and few nonidentifiable structures. Magnification × 21,000.

TABLE 1

Distribution of marker enzymes in subcellular fractions of the brains of BNF-pretreated rats

Fraction	Ethoxycoumarin O-deethylase		NADPH-cytochrome c reductase		Cytochrome c oxidase	
	pmol/mg/min	% in fraction	nmol/mg/min	% in fraction	nmol/mg/min	% in fraction
Homogenate	0.27	100	5.1	100	180	100
1,240-g Pellet (P I)	0.19	27.9	1.7	30.2	200	65.7
10,000-g Pellet (P II)	0.16	6.8	8.8	5.7	1020	31.4
10,000-g Pellet, washed (P II_w)	0.14	4.2	9.2	4.2	1470	31.8
100,000-g Supernatant	<0.05	ND[a]	6.0	20.6	1	0.1
100,000-g Pellet	0.77	35.7	24.1	25.7	99	2.8
Recovery		67.8		80.7		100

[a] ND, not determined.

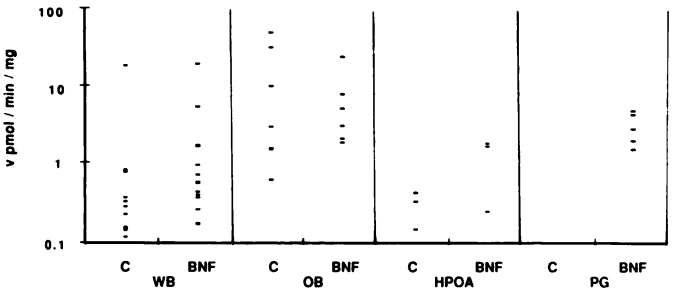

Fig. 2. Regional distribution of ETC *O*-deethylase activity in brain microsomes obtained from untreated and BNF-pretreated animals. The rats were given two single daily intraperitoneal injections of 40 mg/kg BNF in corn oil. Control animals received the vehicle only. After 24 hr, microsomes from pooled tissues of 2–13 whole brains (*WB*), 5–16 OBs, 5–16 HPOAs, and 8–16 PGs, respectively, were prepared. The ETC *O*-deethylase activity was measured by incubation in duplicate with 3 mg of microsomal protein from whole brain or with 0.1–1.5 mg from discrete brain regions and for 60 min. Control values were (pmol/min/mg): whole brains, 1.6 ± 5.9; HPOAs, 0.3 ± 0.2; OBs, 17.2 ± 20.0; PGs, < 0.1. Values for BNF-pretreated rats were: whole brains, 2.4 ± 5.2; HPOAs, 1.3 ± 0.9; OBs, 6.7 ± 7.8; PGs, 3.3 ± 1.5. Values are expressed as mean ± standard deviation.

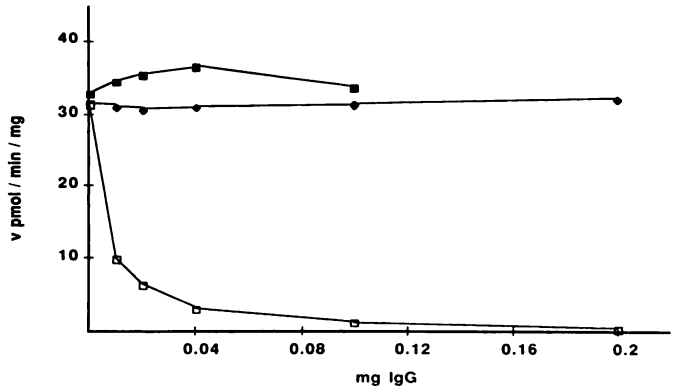

Fig. 3. Effects of polyclonal antibodies against liver cytochrome $P\text{-}450_b$ or $P\text{-}450_c$ on the ETC *O*-deethylase activity in microsomes obtained from OBs of control rats. The antibodies and microsomes were incubated as described in the legend to Fig. 4. The amount of microsomal protein was 0.1 mg/ml incubation and the control activity was 32 pmol/min/mg. □, microsomes from untreated animals + preimmune IgG; ♦, microsomes from untreated animals + anti-$P\text{-}450_b$; ■, microsomes from untreated animals and anti-$P\text{-}450_c$.

to 52 pmol/min/mg and from 0.1 to 20 pmol/min/mg, respectively. The activity within the lower range, 0.1 pmol/min/mg, is 10,000-fold lower than the liver value (31). This constitutive catalytic activity was entirely mediated by a form or forms of P-450 immunologically similar to $P\text{-}450_b$, since there was a

TABLE 2

Inhibition of the ETC *O*-deethylase activity by polyclonal antibodies against liver cytochrome $P\text{-}450_b$ in whole brain microsomes from untreated rats

The antibodies were preincubated with microsomes and buffer (10 mM Tris-acetate buffer, pH 7.4, containing 20% glycerol and 1 mM EDTA) in a total volume of 125–200 μl for 20 min at room temperature prior to the assay. The amount of microsomal protein was 1.5 mg/ml incubation.

Antibody	Dose	Enzyme activity	% of Control activity
	mg	*pmol/min/mg*	
Control		0.51	100
+ Preimmune IgG	0.15	0.41	84
	0.8	0.39	76
	3.0	0.43	85
+ Anti $P\text{-}450_b$	0.15	ND[a]	0
	0.8	ND	0
	1.5	ND	0
	3.0	ND	0

[a] ND, none detectable (<0.1 pmol/min/mg).

complete inhibition by $P\text{-}450_b$ antibodies in microsomes from OB and whole brain. $P\text{-}450_c$ was not constitutive since $P\text{-}450_c$ antibodies did not affect ETC *O*-deethylase activity in brain microsomes of untreated animals. Induction of $P\text{-}450_c$ in whole brain and OB microsomes by BNF was evidenced by inhibition of ETC *O*-deethylase activity by $P\text{-}450_c$ antibodies (50–55%) and the increased sensitivity to inhibition by 1 μM ANF in microsomes from BNF-pretreated rat than in control microsomes. Although the fold induction of $P\text{-}450_c$ may be infinitely large because there is very low activity in non-induced animals, the total activity in brain microsomes is small compared to that found in hepatic microsomes of BNF-pretreated rats. There are two possible explanations for this low overall content of $P\text{-}450_c$. The first is that there is a very low level of induction all over the brain, and the second is that very few cells are induced, and these cells have very high levels of $P\text{-}450_c$. This issue can only be resolved by immunohistochemical techniques.

It has recently been shown immunohistochemically that $P\text{-}450_c$ immunoreactivity is present even in the brains of untreated rats (32). It is possible that the immunoreactivity is not due to $P\text{-}450_c$ itself but to a cross-reactivity with a similar from of P-450. This issue could be resolved by Western immunoblotting of brain microsomes. However, no $P\text{-}450_b$ or $P\text{-}450_c$ was detected on Western immunoblots with brain microsomes of untreated or BNF-pretreated rats. This again may be a problem of the low concentration of these enzymes in the brain and the heterogeneity of cells within this tissue. With immunohistochemistry, individual forms of P-450 can be detected in single cells. This degree of sensitivity is not possible with Western

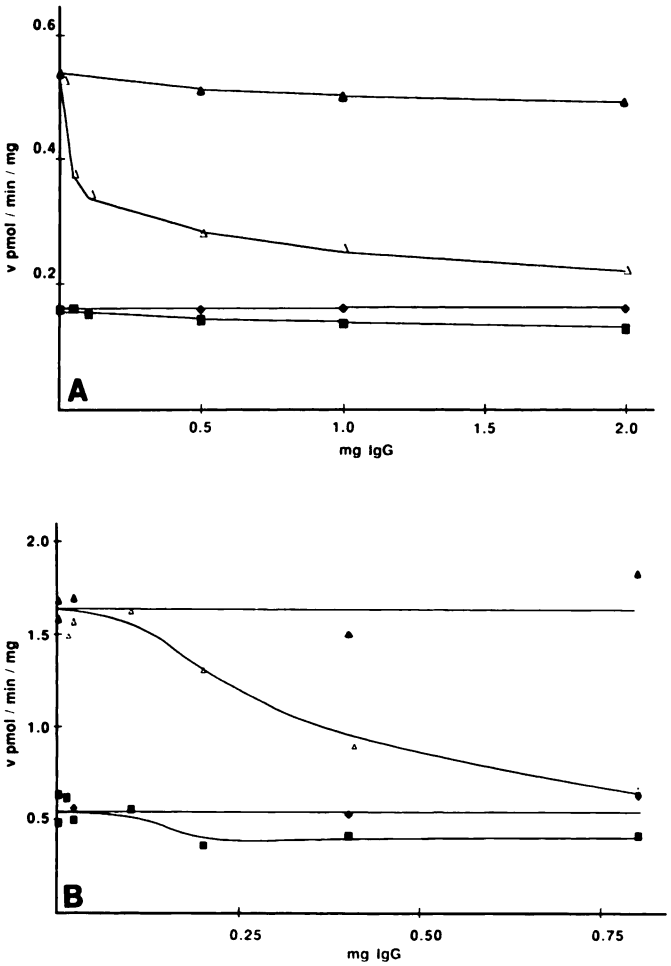

Fig. 4. Effects of polyclonal antibodies against liver cytochrome P-450$_c$ on the ETC *O*-deethylase activity in microsomes obtained from whole rat brain (A) and OBs (B) of untreated or BNF-pretreated animals. The antibodies were preincubated with microsomes and buffer (10 mM Tris-acetate, pH 7.4, containing 20% glycerol and 1 mM EDTA) in a total volume of 125–200 μl for 20 min at room temperature prior to the assay. The amount of microsomal protein from whole brain was 2 mg/incubation of 1 ml and the control activities were 0.15 pmol/min/mg and 0.53 pmol/min/mg in microsomes from untreated and BNF-pretreated rats, respectively (A). The amount of microsomal protein from OBs was 0.4 mg/incubation of 1 ml and the activities were 0.56 pmol/min/mg and 1.64 pmol/min/mg in microsomes from untreated and BNF-pretreated rats, respectively (B). ■, microsomes from untreated animals + preimmune IgG; ♦, microsomes from untreated animals + anti-P-450$_c$; △, microsomes from BNF-treated animals + preimmune IgG; ▲, microsomes from BNF-pretreated animals + anti-P-450$_c$.

immunoblots because of the dilution of any one form of P-450, which may be present only in a limited number of cells, with other more abundant forms of the enzyme. The concentration of P-450$_b$ in hepatic microsomes of phenobarbital-induced rats is approximately 1.0 nmol/mg of protein (33). The concentration of this enzyme in brain microsomes where catalytic activity is 0.01–0.2% of that in the liver is, at maximum, 1 pmol/mg of microsomal protein. Similar levels of P-450$_c$ are also present in the brains of BNF-pretreated rats. It is therefore not surprising that no signals with either anti-P-450$_b$ or anti-P-450$_c$ antibodies could be detected upon Western immunoblots of brain microsomes. Positive identification of P-450$_b$ and P-450$_c$ will be done upon purification of these enzymes from brain microsomes.

In PG, which has high levels of estrogen 2/4-hydroxylase (10) and 3β-diol hydroxylase (34), ETC *O*-deethylase activity was not detected. Upon BNF treatment, this activity was measurable, 3 pmol/min/mg, was completely inhibited by anti P-450$_c$ antibodies, and was inhibited by 85% by ANF.

The P-450(s) mediating aromatization of androgens, 2/4-hydroxylations of estrogens, and 6,7-hydroxylations of 3β,5α-androstanediol probably are not involved in *O*-deethylation of ETC since addition of various steroid substrates (testosterone, estradiol, 3β,5α-androstanediol, DHT) or an aromatase inhibitor (ATD) did not inhibit the catalytic activity in either OB or HPOA microsomes.

In conclusion, we have shown that, in the rat brain, P-450$_b$ or a form of P-450 which is immunologically similar is constitutive, and P-450$_c$ is induced by pretreatment of rats with BNF. Since P-450$_b$ and P-450$_c$ participate in activation of a variety of xenobiotics to cytotoxic and/or carcinogenic metabolites (35), the presence of these forms of P-450(s) in brain raises questions about their role in central nervous system toxicity.

TABLE 3

Effect *in vitro* of ANF (1 μM) on the ETC *O*-deethylase activity in brain microsomes from control and BNF-pretreated rats

Results are expressed as per cent ± standard deviation of the control incubation without inhibitor added. Values in parentheses indicate number of experiments. Values in brackets represent activity expressed as pmol/min/mg ± standard deviation in the control incubation.

	Control	BNF-treated
OB	86 ± 12 (10)[a] [20 ± 15]	68 ± 19 (8)[a] [6.4 ± 7.9]
POA	89 (1) [0.6]	41 ± 30 (4)[b] [0.6 ± 0.7]
PG	ND[c]	16 ± 17 (3)[b] [2.7 ± 0.5]
Whole brain	88 ± 11 (3) 0.5 ± 0.3	67 ± 25 (5)[b] 0.4 ± 0.2

[a] $p < 0.01$ when compared to control incubation without inhibitor added.
[b] $p < 0.05$ when compared to control incubation without inhibitor added.
[c] ND, not detectable (<0.1 pmol/min/mg).

TABLE 4

Effects *in vitro* of inhibitors and steroids on the ETC *O*-deethylase activity in brain microsomes from control rats

Microsomes were prepared from pooled tissue of whole brain, olfactory bulbs, hypothalamic-preoptic area, and pituitary gland from 5–18 control or BNF-treated (twice at 40 mg/kg) rats. The microsomal protein (0.1–3 mg) was incubated with the inhibitor or steroid added in 5 μl of acetone or 10 μl of methanol. Chloramphenicol was preincubated with microsomes for 5 min in a complete system without ethoxycoumarin before assay. The control incubations contained the corresponding solvent. The data are expressed as percentage of control and the number of experiments is given in parentheses. Results are expressed as per cent ± standard deviation of the control incubation without test substance added. Statistical significance was assessed with the paired *t* test.

Test substance	Concentration	% of Control activity	
		OBs	HPOA
	μM		
Metyrapone	100	26 (1)	33 (1)
Chloroamphenicol	100	28 ± 20 (5)[a]	NA[b]
Testosterone	100	167 ± 69 (7)[c]	451 ± 31 (2)[c]
DHT	100	137 ± 64 (3)	246 (1)
ATD	100	164 ± 101 (2)	115 (1)
Estradiol-17β	100	106 ± 1 (2)	127 (1)
5α-A-3α,17β-diol	100	92 ± 7 (2)	123 (1)
5α-A-3β,17β-diol	100	92 ± 3 (2)	112 (1)
Control activity (pmol/min/mg ± SD)		12 ± 12 (15)	0.6 ± 0.1 (2)

[a] $p < 0.01$ when compared to control incubation without test substance added.
[b] NA, not analyzed.
[c] $p < 0.05$ when compared to control incubation without test substance added.

The localization of these enzymes into specific cells may play a part in *in situ* production of toxic compounds and may explain the selective action of certain neurotoxic agents.

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