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NADPH CYTOCHROME P-450 REDUCTASE IN RAT, MOUSE AND HUMAN BRAIN

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Abstract—NADPH cytochrome P-450 reductase (P-450 reductase), an essential component of the cytochrome P-450 mono-oxygenase system, has been estimated in rat and mouse brain, and seven human brains obtained at autopsy. The ratio of cytochrome P-450 to P-450 reductase is lower in the rat and mouse brains (2.5–4.0) as compared to the respective livers (10.0–11.0). The rat and mouse brain P-450 reductase were immunologically similar to the rat liver P-450 reductase as examined by immunochemical inhibition, Ouchterlony double diffusion and immunoblot. The antisera to rat liver P-450 reductase inhibited rat brain aminopyrine *N*-demethylase activity to the same extent as NADPH cytochrome *c* reductase, suggesting that the level of P-450 reductase controls the rate of this cytochrome P-450 mediated activity. The human brain NADPH cytochrome *c* reductase exhibited regional variation, maximal activity being observed in the brain stem region. Immunochemical inhibition and immunoblot studies revealed immunological cross-reactivity between rat liver reductase and human brain medulla, while none was observed in cortex or cerebellum. Immunocytochemical studies on human brain medulla using antisera to rat liver P-450 reductase indicated localization of the P-450 reductase in neuronal cell body.

NADPH cytochrome P-450 reductase (NADPH:ferricytochrome oxidoreductase, EC 1.6.2.4) is an essential component of the cytochrome P-450 mono-oxygenase system, supplying the reducing equivalents from NADPH to cytochrome P-450 (P-450‡). Cytochrome P-450 is involved in the metabolism of various xenobiotics (drugs, carcinogens) and endogenous compounds (steroids, fatty acids). The mono-oxygenase system consists of cytochrome P-450 (a heme containing protein) and NADPH cytochrome P-450 reductase. Although liver is the major organ involved in the P-450 mediated metabolism, it has also been detected in several extra-hepatic organs [1], including the brain [2]. Cytochrome P-450 has been detected in mouse [3] and rat brain [4] and its cellular localization in the CNS has been examined using immunocytochemical techniques [5, 6]. However, very little information is available regarding the nature of brain P-450 reductase. Immunocytochemical study of the rat brain P-450 reductase using antibody to the rat liver enzyme had revealed the presence of the enzyme in catecholaminergic neurons in substantia nigra, nucleus locus ceruleus and ventrolateral medullary region [7].

This paper reports for the first time, the presence of P-450 reductase in human brain. The immunological similarity of the rat and mouse brain P-450 reductase with the well characterized rat liver enzyme is also discussed.

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‡ Abbreviations: P-450 reductase; NADPH cytochrome P-450 reductase; P-450, cytochrome P-450; SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophotesis.

MATERIALS AND METHODS

Three-month-old male Wistar rats (200 g) and Swiss albino mice (30–35 g), from the colony of the animal house were used in this study. Animals had free access to pelleted diet (Hindustan Lever Ltd, Bombay, India) and water. Animals were anaesthesized with ether, perfused transcardially with normal saline (20 mL for rats and 5 mL for mice) prior to decapitation. The brain and liver were removed quickly and all subsequent operations were performed at 4°. The tissues were homogenized in 5 volumes of 0.1 M Tris buffer (pH 7.4) containing 20% glycerol, 1.15% potassium chloride, 1 mM EDTA, 0.1 mM dithiothreitol and 0.1 mM phenylmethyl sulphonyl fluoride, using a motor driven glass homogenizer and a Teflon pestle. The homogenate was centrifuged at 17,000 *g* for 30 min in a refrigerated centrifuge. The supernatant was collected and the pellet was rehomogenized in 5 volumes of the above buffer, and centrifuged at 17,000 *g* for 30 min. The supernatants were pooled and centrifuged at 100,000 *g* for 1 hr. The pellet was washed by resuspending in the homogenizing buffer and centrifuging for 1 hr at 100,000 *g*. The washed pellet was suspended in a small volume of Tris–glycerol and stored in liquid nitrogen and used within 1 week of perparation.

Seven human brains were collected at autopsy from cases of accidental death with no known neurological diseases. Liver was obtained from five of the above cases and kidney from two cases. The average interval between death and autopsy was

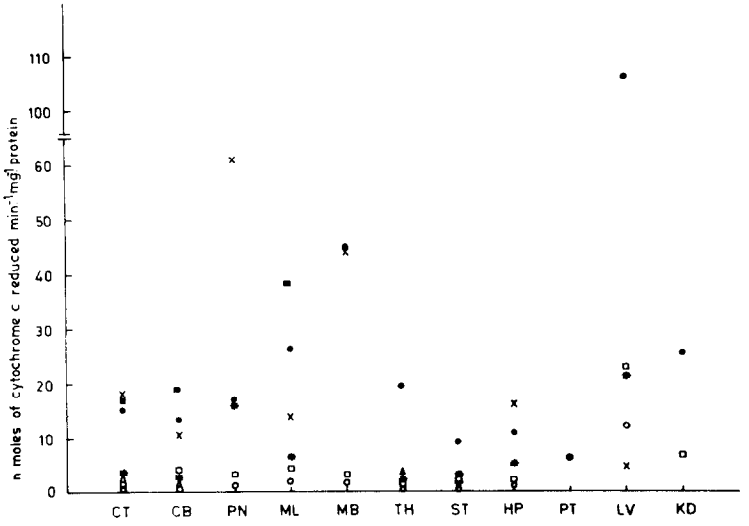


Fig. 1. Microsomal NADPH cytochrome *c* reductase activity in human liver, kidney and brain regions. The age of the deceased was as follows: case I, 70 years (△); case II, 73 years (×); case III, 55 years (○); case IV, 35 years (□); case V, 27 years (●); case VI, 70 years (*); and case VII, 18 years (■). Pituitaries were pooled from 10 autopsy cases. (⊗). The horizontal bars indicate the mean enzyme activity in the tissues. Cortex, CT; cerebellum, CB; pons, PN; medulla, ML; midbrain, MB, thalamus, TH; striatum, ST; hippocampus, HP; pituitaries, PT; liver, LV; and kidney, KD.

8.16 ± 1.6 hr; and the average age of the deceased was 49.7 ± 22.8 yr. Following the injury, the deceased were on ventilators for a period ranging from 12 to 144 hr. All patients received mannitol and glycerol, but no corticosteroids as antiedema measures. Case VI received phenobarbital (1 g) over 48 hr prior to death. None of the other cases received any anti-convulsant medication. Following autopsy, the brain was dissected into discrete regions using standard anatomical landmarks. The tissues were washed free of blood, stripped of blood vessels and meninges and stored at −70° prior to analysis. Microsomes were prepared from tissues as described above.

NADPH cytochrome P-450 reductase activity was assayed using cytochrome *c* as substrate [8]. Aminopyrine *N*-demethylase, morphine *N*-demethylase and ethoxycoumarin *O*-deethylase were assayed as described [9]. In experiments using antisera, microsomal incubations containing pre-immune and immune sera were kept at 37° for 30 min prior to addition of substrate.

NADPH cytochrome P-450 reductase was purified from rat liver as described [9]. The purity of the enzyme was established by SDS–PAGE. Antibodies to the reductase were raised in rabbits [10]. The antibodies were specific for the antigen as judged by the following criteria: Ouchterlony double diffusion of antigen with antisera showed a single precipitin line, inhibition of immunodiffusion and immunoblot staining with antisera preadsorbed with antigen. The lack of cross-reactivity between the antisera to reductase and purified cytochrome P-450 was also established.

SDS–PAGE was carried out on brain and liver microsomes [11], and the proteins were transferred to nitrocellulose membranes [12] and incubated with the antisera to rat liver reductase. The membranes were washed and immunostained to visualize the bands [13]. Ouchterlony double diffusion was performed on agar coated plates as described [14].

Table 1. Hepatic and cerebral cytochrome P-450 and NADPH cytochrome *c* reductase in rat and mouse

	P-450	Reductase	Ratio
Rat			
Liver	858.2 ± 16.0	77.4 ± 12.2	11.08
Brain	88.4 ± 7.3	22.3 ± 2.7	3.96
Mouse			
Liver	902.6 ± 38.0	84.6 ± 17.3	10.66
Brain	52.7 ± 7.5	21.1 ± 2.7	2.46

P-450 represents picomoles of cytochrome P-450/mg protein and P-450 reductase is measured as nanomoles of NADPH cytochrome *c* reduced/min/mg protein. Values are mean ± SD of three experiments with three different batches of microsomes.

Immunohistochemistry was carried out on paraffin sections of human medulla oblongata, frontal cortex and cerebellum fixed in 5% formalin, using rabbit antisera to rat liver reductase. The sections were incubated for 60 hr at 4° and the immune reactions were visualized by diaminobenzidine–hydrogen peroxide, following incubation with horse radish peroxidase linked swine antisera to rabbit IGg. Necessary controls were incorporated.

RESULTS

NADPH cytochrome *c* reductase levels were estimated in rat and mouse brain and liver. The specific activity of the enzyme in both rat and mouse brain was only 25% of the hepatic levels (Table 1). The

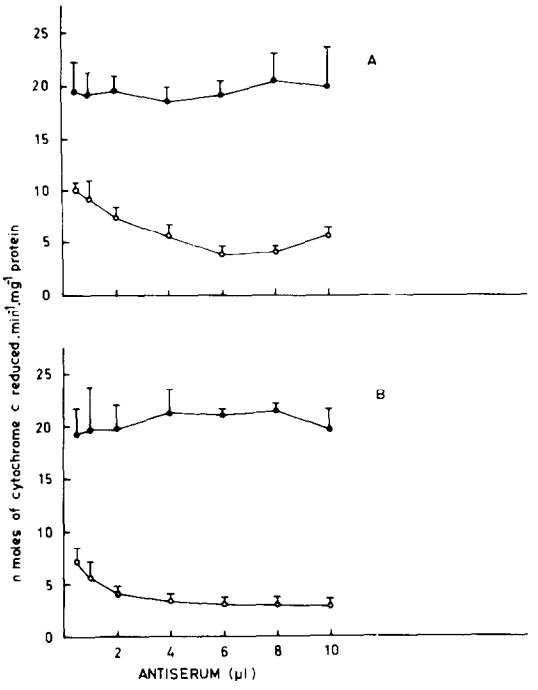


Fig. 2. Inhibition of (A) mouse brain and (B) rat brain NADPH cytochrome *c* reductase activity by antisera to rat liver reductase. Microsomes were incubated with (0–10 μL/mL incubation medium) preimmune sera (●—●) and immune sera (○—○) prior to addition of substrate and NADPH. Values are mean ± SD of three experiments.

above enzyme was also estimated in human brain regions, liver and kidney obtained at autopsy. The maximal activity was observed in midbrain, pons and medullary regions of the brain (Fig. 1). However, the enzyme activity in the brain regions was lower than that observed in the respective liver, although one exception was seen in the seven cases examined. The human kidney P-450 reductase activity was also lower than the hepatic levels in the two cases examined. In view of the elapsed time between death and autopsy, some autolytic changes could possibly have occurred in the human tissue. This data, hence, needs to be interpreted with caution.

Addition of the antisera prepared to rat liver P-450 reductase inhibited mouse and rat brain NADPH cytochrome *c* reductase in a dose-dependent manner (Fig. 2A and B). Maximal inhibition of rat brain NADPH cytochrome *c* reductase activity (84%) was observed following the addition of 4 μL of antisera, while twice the amount of antisera was required for similar inhibition of the enzyme activity in mouse brain microsomes. Addition of the above anti-sera to human brain microsomes prepared from cortex and cerebellum resulted in partial inhibition (40%) of NADPH cytochrome *c* reductase activity (Fig. 3A and B). However, the enzyme activity in microsomes prepared from human medulla was inhibited by a greater extent (75%) by the addition of the same amount of antisera (Fig. 3C).

The antisera to rat liver P-450 reductase also inhibited the P-450 associated mono-oxygenase activity in mouse and rat brain (Fig. 4A and B). Mouse brain aminopyrine *N*-demethylase and morphine *N*-demethylase were inhibited by 50% of their original activity after the addition of the antisera (Fig. 4A). In the rat brain, the inhibition of P-450 mono-oxygenases was varied; aminopyrine *N*-demethylase was inhibited by 70%, while morphine *N*-demethylase and ethoxycoumarin *O*-deethylase were inhibited to a lesser extent (50%) by the addition of the same quantity of antisera.

Ouchterlony double diffusion analysis of rat and mouse brain microsomes revealed a single precipitin line when incubated with the antisera to rat liver reductase (data not shown), indicating the immunological similarity between the liver and brain reductase. Immunoblots of mouse brain and liver microsomes revealed a single band that co-migrated, when immunostained with the above antisera (Fig. 5).

Human brain and liver microsomes were also subjected to similar immunoblot analysis using antisera to rat liver P-450 reductase. No bands were visualized in the microsomes from human liver or any of the brain regions except the medulla. The human medullary microsomes showed a single band when immunostained with the above antisera (Fig. 6). Similarly, immunohistochemical studies on human brain medulla revealed positive staining of the reticular neurons (Fig. 7A and B). The reactivity was seen in cell bodies and the dendrites. The sections of cortex and cerebellum did not stain significantly above the background level.

DISCUSSION

In this study NADPH cytochrome P-450 reductase has been shown to be present in mouse and rat brain and its immunological similarity to the well characterized rat liver P-450 reductase demonstrated. Although P-450 levels in the rat brain are one-tenth of that present in the liver (Table 1), the P-450 reductase levels in the brain are one-fourth of that present in the liver. This indicates that a lower P-450 to P-450 reductase ratio in the brain as compared to the liver, where a large excess of P-450 is known to exist as compared to P-450 reductase molecules [15].

Immunological similarity between hepatic P-450 reductase and those from extrahepatic organs in the same species of animals is known. The rat lung and liver P-450 do not seem to differ significantly [16]. Immunological similarities indicating similar peptide sequences presumably at the catalytic site are also noted between rat brain and liver P-450 reductase, in the present study. Earlier studies on hepatic P-450 reductases from different species have indicated a great degree of functional similarity among them, although immunological differences exist [17]. The cerebral P-450 reductase also seems to differ with respect to species. The immunoinhibition studies with rat and mouse brain NADPH cytochrome *c* reductase demonstrates this fact. The amount of antisera required for immunoinhibition of mouse brain enzyme was twice that required for rat brain enzyme.

Regional variation in NADPH cytochrome *c*

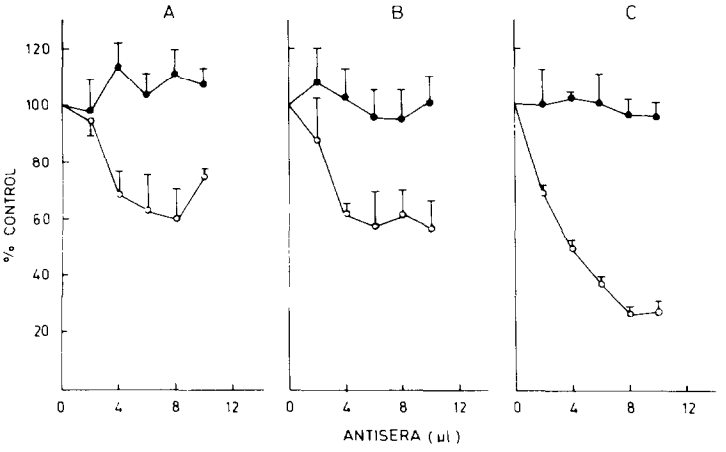


Fig. 3. Inhibition of human brain microsomal NADPH cytochrome *c* reductase by antisera to rat liver reductase. Microsomes were prepared from the following brain regions obtained from case VII: (A) cortex, (B) cerebellum and (C) medulla. Microsomes were incubated with (0–10 µL/mL incubation medium), preimmune (●—●) or immune (○—○) sera prior to the addition of substrate and NADPH. Values are mean ± SD of three experiments. Control values of P-450 NADPH cytochrome *c* reductase are nanomoles of cytochrome *c* reduced/mg protein.

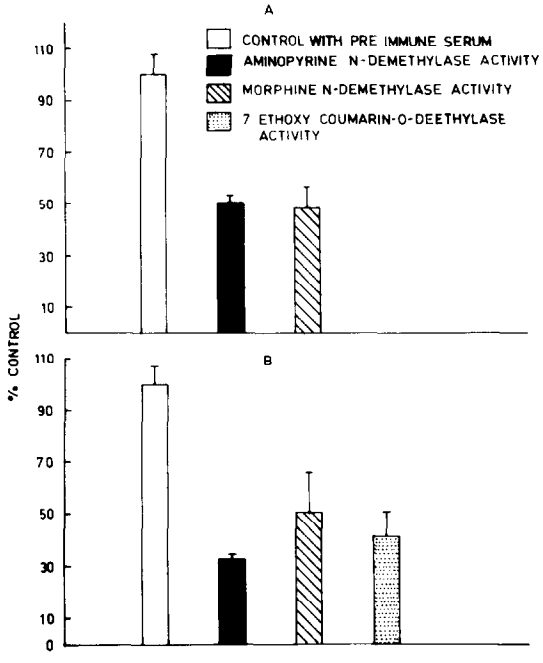


Fig. 4. Inhibition of P-450 associated mono-oxygenases by antisera to rat liver reductase in (A) mouse brain and (B) rat brain microsomes. Control incubations contained preimmune sera (10 µL) and all other incubations contained immune sera (10 µL), which were preincubated with the microsomes prior to the addition of substrate. Control activities are as follows. (A) aminopyrine *N*-demethylase and morphine *N*-demethylase 185.5 ± 10.5 and 37.8 ± 3.2 nanomoles of product formed/min/mg protein, respectively; (B) aminopyrine *N*-demethylase, morphine *N*-demethylase and ethoxycoumarin *O*-deethylase 181.2 ± 16.5, 125.4 ± 8.1 and 1.6 ± 0.2 nanomoles of product formed/min/mg protein, respectively. Values are mean ± SD of three experiments.

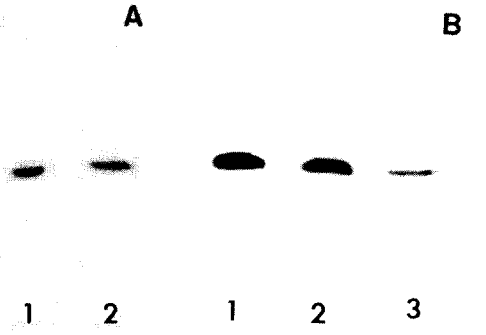
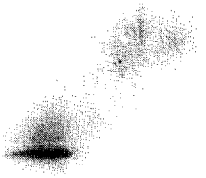


Fig. 5. Immunoblots of (A) mouse brain and (B) rat brain after immunostaining with antisera to rat liver reductase. (A) (1) mouse liver microsomes (32 µg) and (2) mouse brain microsomes (33 µg). (B) (1) purified rat liver reductase (1 µg), (2) rat liver microsomes (30 µg) and (3) rat brain microsomes (34 µg). The amount of protein loaded onto the cell is given in parenthesis.

reductase activity was observed in human brain, the highest activity being present in the brain stem region consisting of midbrain, pons and medulla. The P-450 linked mono-oxygenase activity has also been observed to be higher in the brain stem as compared to the other regions [18]. However, this result needs to be interpreted with caution in view of the possible autolytic changes that could have taken place during the period between death and autopsy.

The human liver reductase has been shown to contain some, but not all of the determinants of the rat liver enzyme and the differences between rat and human liver P-450 reductase have been explicitly demonstrated by experiments in which the NADPH cytochrome *c* reductase activity in purified preparations has been titrated with the antibody raised to the rat liver enzyme [17]. The lack of immunological



ST ML TH CT LV

Fig. 6. Immunoblot of microsomal protein from human brain regions and liver stained with antisera to rat liver reductase. Microsomes were prepared from tissues obtained at autopsy from Case VI. Striatum, ST (50 μg); medulla, ML (26 μg); thalamus, TH (42 μg); cortex, CT (49 μg); and liver, LV (43 μg). The amount of microsomal protein loaded onto the gel is given in parenthesis. Similar immunoblots were obtained from Case IV (see Fig. 4 legend).

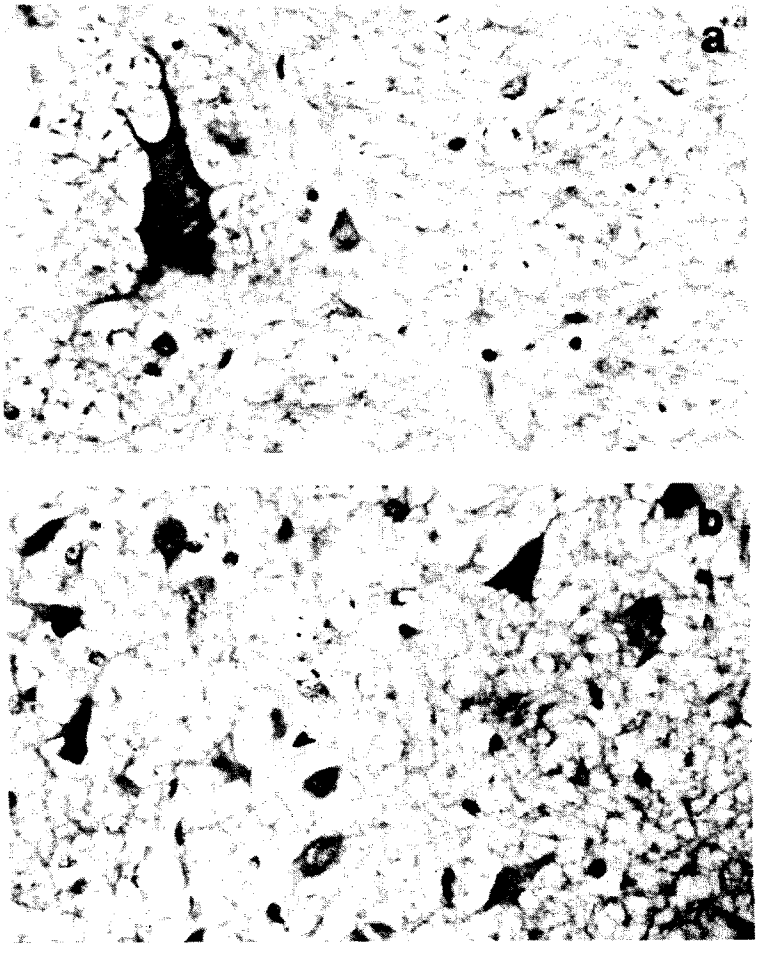


Fig. 7. Immunocytochemical localization of NADPH cytochrome P-450 reductase in (a) large reticular neuron (×360) and (b) neurons of cranial nerve nuclei (×320), in human brain medulla oblongata. The neurons of the cranial nerve are variably stained.

cross-reactivity between human and rat liver P-450 reductase has also been observed in the present study. The immunoblot analysis using rat liver P-450 reductase failed to reveal the presence of any immunoreactive bands in human liver microsomes. However, the microsomes prepared from human brain medulla oblongata revealed a protein band, when examined by immunoblot analysis using antisera to rat liver reductase. This band was absent in the other regions of the brain examined. Further, immunoinhibition studies using the same antisera, only partially inhibited (40%) NADPH cytochrome *c* reductase activity in the cortex and cerebellum, while almost completely inhibiting the activity in the medulla (75%). These results seem to indicate the presence of a form of P-450 reductase in human medulla that is immunologically similar to the rat liver P-450 reductase. Immunohistochemical localization of reductase in human brain revealed its presence in the reticular neurons and dendrites of the medulla oblongata. Earlier immunohistochemical studies on localization of P-450 in human brain have revealed a similar pattern of staining [19], indicating the co-localization of P-450 and P-450 reductase in the human brain medulla. The isolation and characterization of the human brain P-450 reductase and its regional variation may provide an insight into the nature and function of the enzyme.

REFERENCES

1. Gram TE, Okine LK and Gram RA, The metabolism of xenobiotics by certain extrahepatic organs and its relation to toxicity. In: *Annual Reviews of Pharmacology and Toxicology*, Vol. 26, pp. 259–291. Annual Reviews Inc., Palo Alto, CA, 1986.
2. Mesnil M, Testa B and Jenner P, Xenobiotic metabolism by brain mono-oxygenase and other cerebral enzymes. In: *Advances in Drug Research* (Ed. B. Testa), pp. 95–207. Academic Press, London, 1984.
3. Ravindranath V and Ananadtheerthavarada HK, High activity of cytochrome P-450 linked aminopyrine *N*-demethylase in mouse brain microsomes and associated sex-related difference. *Biochem J* **261**: 769–773, 1989.
4. Warner M, Kohler C, Hansson T and Gustafsson JA, Regional distribution of cytochrome P-450 in the rat brain: spectral quantitation and contribution of P-450 b,e and P-450 c,d. *J Neurochem* **50**: 1057–1065, 1988.
5. Kapitulnik J, Gelboin HV, Guengerich FP and Jacobowitz DM, Immunohistochemical localization of cytochrome P-450 in the rat brain. *Neuroscience* **20**: 829–823, 1987.
6. Kohler C, Eriksson LG, Hansson T, Warner M and Gustafsson JA, Immunohistochemical localization of P-450 in the rat brain. *Neurosci Lett* **84**: 109–114, 1988.
7. Haglund L, Kohler C, Haaparanta T, Goldstein M and Gustafsson JA, Presence of NADPH-cytochrome P-450 reductase in central catecholaminergic neurons. *Nature* **307**: 259–262, 1984.
8. Phillips AH and Langdon RG, Hepatic triphosphopyridine nucleotide–cytochrome *c* reductase: isolation, characterization and kinetic studies. *J Biol Chem* **237**: 2652–2660, 1962.
9. Guengerich FP, Microsomal enzymes involved in toxicology—analysis and separation. In: *Principles and Methods of Toxicology* (Ed. Hayes, AW), pp. 609–634. Raven Press, New York, 1982.
10. Kamataki T, Bekker DH and Neal RA, Studies on the metabolism of diethyl *p*-nitrophenyl phosphonothionate (parathione) and benzphetamine using an apparently homogenous preparation of rat liver cytochrome P-450: effect of a cytochrome P-450 antibody preparation. *Mol Pharmacol* **12**: 921–932, 1976.
11. Laemmli UK and Favre M, Maturation of the head of bacteriophage T_4, I. DNA packaging events. *J Mol Biol* **86**: 575–599, 1973.
12. Towbin H, Staehlin T and Gordon J, Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci USA* **76**: 4350–4354, 1979.
13. Guengerich FP, Wang P and Davidson NK, Estimation of isoenzymes of microsomal cytochrome P-450 in rats, rabbits and humans using immunostaining coupled with sodium dodecyl sulphate–polyacrylamide gel electrophoresis. *Biochemistry* **21**: 1698–1706, 1982.
14. Ouchterlony O, Diffusion-in-gel methods for immunological analysis II. In: *Progress in Allergy* (Eds. Kallas P and Waksman BH), Vol. VI, pp. 30–154, Karger, Basel, 1962.
15. McManus ME, Hall PM, Stupaus I, Brennan J, Burgess W, Robinson R and Birket DJ, Immunohistochemical localization and quantitation of NADPH-cytochrome P-450 reductase in human liver. *Mol Pharmacol* **32**: 189–194, 1988.
16. Philpot RM, Wolf CR, Slaughter SR, Bend JR, Robertson IGC, Zeiger E, Statham CN and Boyd MR, The role of cytochrome P-450-dependent mono-oxygenase system in pulmonary specific toxin effects of xenobiotics. In: *Microsomes, Drug Oxidations and Drug Toxicity* (Eds. Sato R and Kato R), pp. 487–494. Japan Scientific Societies Press, Tokyo and Wiley-Interscience, New York, 1982.
17. Guengerich FP, Wang P and Mason PS, Immunological comparison of rat, rabbit and human liver NADPH cytochrome P-450 reductases. *Biochemistry* **20**: 2379–2385, 1981.
18. Ravindranath V, Anandatheerthavarada HK and Shankar SK, Presence of drug metabolizing enzymes—cytochrome P-450 linked mono-oxygenases in human brain. In: *Proceedings of the Bidecennial Conference of the Indian Pharmacological Society*, pp. 65, 1987.
19. Ravindranath V, Anandatheerthavarada HK and Shankar SK, Xenobiotic metabolism in human brain—presence of cytochrome P-450 and associated monooxygenases. *Brain Research* **496**: 331–335, 1989.