J. Biochem. 125, 634-640 (1999)

7-Ethoxycoumarin Deethylation Activity in Perfused Isolated Rat Brain

Yoko Chikaoka[1] and Mamoru Tamura

Biophysics Division, Research Institute for Electronic Science, Hokkaido University, Sapporo 060-0812

Received August 3, 1998; accepted December 16, 1998

7-Ethoxycoumarin (7-EC) deethylation activity was measured in the perfused rat brain *in situ*. Infusion of 7-EC into a brain through an internal carotid artery resulted in the formation of 7-hydroxycoumarin (7-HC) and its conjugates in the effluent perfusate collected from the superior *vena cava*. The rate of formation of products was 200 nmol/h/g when 130 μM 7-EC was infused. This value was much higher (more than 100 times) than that determined from the brain microsomal activity (~1 nmol/h/g), indicating that the activity determined with microsomes was an underestimate. This value was comparable to the activity in the perfused liver (30-50%), suggesting that drug metabolizing enzymes can play important roles within the brain. Pretreatment of rats with P-450 inducers such as phenobarbital and β-naphthoflavone increased the deethylation activity in the perfused brain, as in the perfused liver. We conclude that the perfused brain is suitable for evaluating drug metabolizing activities under physiological conditions.

Key words: cerebral tissue, cytochrome P-450, drug-metabolizing activity, microsome, perfused brain.

Drug-metabolizing enzymes catalyze the incorporation of hydrophilic groups into xenobiotics and allow them to be excluded from the body (*1*). The reactions catalyzed by these enzymes are categorized into Phases I (functionalization) and II (conjugation), which have been studied mainly with liver preparations, because the majority of the enzymes exist in the liver. However, they are present in almost the whole body, including the brain, although the extrahepatic contents are reported to be very low. The brain has been demonstrated to contain almost all the drug metabolizing enzymes that exist in the liver (*2-8*). However, there is uncertainty about their activities. Extensive evidence thus far accumulated indicates that the levels of these enzymes in the brain are low and that their activities are much lower than those in the liver, while data obtained in one particular laboratory demonstrated higher activities (*9*). For example, the reported values for 7-ethoxycoumarin (7-EC) deethylation activity in rat brain microsomes ranged from 0.01 to 20% of that in the liver (*9-14*). As to the reason for the variance in the reported values, Bhagwat *et al.*, who reported the highest 7-EC deethylase activity in brain microsomes, claimed that microsomal specific activity might be underestimated due to a low recovery of brain microsomes, enzyme inactivation during preparation, and nonlinearity as to the reaction time and the amount of microsomal protein (*9*).

On the other hand, the significance of drug metabolizing enzymes in the brain is often overlooked, because of the existence of the blood-brain barrier (BBB), which restricts the entry of chemicals into the brain (*15*). In fact, perfused brains have been used for experiments focused on drug transport through the BBB (*16*). With perfused isolated organs one can directly determine the activity of the overall enzymatic reaction in a tissue. Here, the use of the perfused brain is expected to eliminate underestimation due to poor recovery or inactivation of enzymes during preparation (*17*). In the perfused brain the enzymes involved in reactions are present *in situ* in cells and tissues, where the localization of enzymes may affect metabolism in the brain. Thus, the assay conditions are nearly the same as *in vivo*, where cofactors for drug metabolism are highly coordinated and different types of cells assemble together in a limited structure.

The results obtained in this study showed that the drug-metabolizing activity of the perfused brain was much higher than that of microsomes and was comparable with that of the perfused liver.

MATERIALS AND METHODS

Preparation of Perfused Isolated Rat Brains—The perfused isolated rat brains were prepared according to the method previously reported by Inagaki and Tamura (*18*) with some modifications. A major improvement was the shortened preparation time, which was achieved by eliminating the complicated bypass cannulation method (*18*). To do this, the perfusate was infused through one of the two

[1] To whom any correspondence should be addressed at the present address: Department of Molecular Neurobiology, School of Human Science, Waseda University, 2-579-15 Mikajima, Tokorozawa, Saitama 359. Tel/Fax: +81-42-948-6883

Abbreviations: BA, 2-bromo-4′ aminonitrophene; BBB, blood-brain barrier; BSA, bovine serum albumin; CP, chloramphenicol; P-450, cytochrome P-450; 7-EC, 7 ethoxycoumarin; EEG, electroencephalogram; 7-HC, 7-hydroxycoumarin; NADPH, reduced β-nicotinamide adenine dinucleotide phosphate; α-NF, α-naphthoflavone; PB, phenobarbital.

carotid arteries. Since the flow rate was high enough to perfuse the whole brain through the circle of Willis, the cerebral tissue could be kept under aerobic conditions in the present modified procedure (see also "RESULTS").

The perfusion fluid was a perfluorocarbon FC-43 emulsion, which was composed of Krebs-Ringer bicarbonate buffer (pH 7.4) containing 20% FC-43, 2.55% Plurinic F-68, 3.0% hydroxyl starch, and 10 mM glucose. The emulsion was filtered through a 1.2 μm pore membrane (Millipore, USA) and then equilibrated with a gas mixture of 95% O_2+5% CO_2. P_{O_2} and P_{CO_2} of the FC-43 emulsion were adjusted to 490 and 28 mmHg, respectively. The flow rate was kept at 3.0 ml/min/g wet tissue, which meant the perfusion pressure was around 70 mmHg. The perfusion temperature was 30°C.

A Krebs-Ringer bicarbonate solution containing 3.0% dextran-70 and 10 mM glucose was substituted for the FC-43 emulsion in certain experiments. In these experiments, P_{O_2} and P_{CO_2} of the solution were maintained at 440 and 39 mmHg, respectively. The perfusion pressure was set at 160 mmHg to maintain a constant flow rate of the perfusate, and the temperature was 37°C.

At 37°C, the brains perfused with FC-43 emulsion ceased to exhibit EEG activity and we could not obtain reproducible data when the concentration of the infused 7-EC was changed successively, since the flow rate decreased suddenly with increases in the extremely high perfusion pressure, possibly due to edema. This phenomenon was not observed with the perfusion of the Krebs-Ringer solution at 37°C. 7-EC was added to the perfusate directly or by means of an infusion pump, in which case the concentration was controlled by the flow rate of the pump. The perfusate was sampled just before reaching the brain to determine the 7-EC concentration from the absorbance as described below.

Surgical Procedures—Male Wistar rats weighing 200–250 g were anaesthetized by intraperitoneal (i.p.) injection of sodium pentobarbital (50 mg/kg body weight) and then heparinized (100 units/kg body weight). After exposure of the common carotid arteries, both the external carotid arteries were ligated. Then one of these carotid arteries was cannulated with a polyethylene tube and the perfusion fluid was infused into the brain through the cannula by means of a peristaltic pump. The contralateral common carotid artery was then ligated. The effluent perfusate was collected *via* the superior *vena cava*. The heart was clamped to avoid circulation at the end of the procedure.

Liver Perfusion—The procedure for perfusion of the isolated rat liver was the same as that of Sugano *et al.* (*19*). The perfusate was 10 mM glucose-containing Krebs-Ringer bicarbonate buffer equilibrated with 95% O_2+5% CO_2, pH 7.4. The perfusion temperature was 37°C and the flow rate was above 3.0 ml/min/g wet tissue. The perfusate was pumped into the liver *via* a cannula placed in the portal vein. The effluent perfusate was collected *via* the *vena cava*. Bile was collected by cannulating the bile duct to check the viability of the liver as the bile-production rate.

Determination of Metabolites—The effluent collected from the brains perfused with the FC-43 emulsion was centrifuged to remove FC-43, and then the concentrations of the substrate and metabolites were measured. The non-specific binding of 7-EC and 7-HC to the FC-43 emulsion was examined by measuring the recovery of these chemicals after the removal of FC-43 by centrifugation. We found that FC-43 could partially bind 7-EC but not 7-HC. Separate experiments showed that the non-specific absorption of 7-EC to FC-43 depended on the period of incubation. Thus we infused 7-EC in the inflowing perfusate just before the brain. Centrifugation of the effluent was performed immediately after collecting each sample, and then the supernatants were stored. These procedures minimized the non-specific absorption of 7-EC to FC-43. The maximum absorption was ~30%, but by applying the above procedures, the absorption artifact became very small, and therefore we did not correct the obtained values for this non-specific absorption. For evaluation of the activity, we measured 7-HC and its conjugates in the effluent, which were not absorbed by the FC-43 emulsion. Therefore the observed activity was not affected by FC-43. The effluent from the brains perfused with the Krebs-Ringer solution was measured without centrifugation. The concentration of the substrate, 7-ethoxycoumarin (7-EC), was determined by measuring the absorbance difference at 330 and 259 nm. Since 7-EC in the FC-43 emulsion effluent was determined after centrifugation, it did not represent the total 7-EC added. The deethylated product of 7-EC, 7-hydroxycoumarin (7-HC), was found to be formed in the effluent in the free and conjugated forms of glucuronide and sulfate, respectively, as in the effluent from the liver (*20*). The concentration of 7-HC was determined fluorometrically (excitation, 390 nm; emission, 440 nm) after the addition of a 4-fold volume of 1.6 M glycine/NaOH buffer (pH 10.3) to the sample. The concentration of total metabolites including glucuronide and sulfate conjugates was determined by measuring the concentration of 7-HC formed on incubation with β-glucuronidase (420 units/ml) and sulfatase (80 units/ml) in 120 mM phosphate buffer (pH 7.4) for 1.5 h (*20*). Authentic 7-HC was used as the standard for fluorescence measurement.

Brain Microsomal Deethylation Activity—Brain microsomes were prepared for *in vitro* experiments by differential centrifugation (*21*). Essentially, a brain was perfused with ice-cold saline to remove blood, excised from the skull and then homogenized in 4 volumes of ice-cold 1.15% KCl. Each homogenate was centrifuged at 9,000 × *g* for 20 min and the resulting supernatant was centrifuged at 105,000 × *g* for 1 h. The microsomal pellet was resuspended in 10 mM phosphate buffer (pH 7.4). The protein concentration was determined by the method of Lowry *et al.* (*22*).

7-EC deethylation activity in the microsomes was measured according to the method previously reported (*23*). The reaction was carried out in 1.0 ml of 0.12 M phosphate buffer (pH 7.4), comprising 0.5 mM 7-EC, 5 mM $MgCl_2$, 1 mM NADPH, 10 mM glucose-6-phosphate, glucose-6-phosphate dehydrogenase (1 unit/ml), and 0.5–1.0 mg/ml of microsomes at 37°C. The reaction was started by adding NADPH and was stopped in 15 min with the same volume of 5% trichloroacetic acid. The protein precipitate was spun down and the pH of the mixture was brought to about 10 by adding 6 volumes of 1.6 M NaOH-glycine buffer, pH 10.3. 7-HC was measured fluorometrically as described above.

Enzyme Induction—For enzyme induction, rats were given phenobarbital (100 mg/kg body weight in 0.2 ml of propyleneglycol:ethanol:water=4:1.05:4.95) or β-naphthoflavone (30 mg/kg body weight in 1 ml of olive oil) by

i.p. injection for three days. Control animals were injected with the same volume of the vehicle.

Evans Blue Staining—To examine the breakdown of the blood-brain barrier (BBB), the penetration of Evans Blue-BSA (bovine serum albumin) was examined according to the method of Rapoport *et al.* (*24*). In the experiment, 0.3% BSA was added to a Krebs-Ringer solution containing 20 mg/ml Evans Blue. BSA binds to the dye and the bound form is excluded from the brain parenchyma when the BBB is intact.

Materials—7-Ethoxycoumarin was obtained from Nacalai, Tesque Japan. β-Glucuronidase was purchased from Wako Pure Chemicals, Japan. Sulfatase was obtained from Sigma. The FC-43 emulsion was purchased from Green Cross, Japan. All other chemicals used were of the highest purity commercially available. A stock solution of 7-ethoxycoumarin (1 M) was dissolved in *N,N'*-dimethylformamide and was added to the perfusate.

RESULTS

Viability of the Perfused Brain—Spontaneous electroencephalographic (EEG) profiles were recorded to monitor the viability of the perfused brain (data not shown) (*25*). Here, we employed an improved perfusion method, as described under "MATERIALS AND METHODS," where the brain was perfused *via* a single carotid artery. The EEG profiles exhibited the same amplitude in the right and left hemispheres, indicating that the whole brain was fully perfused through the ring of Willis, which links the four arteries to the brain. The EEG activity of the brains perfused with the FC-43 emulsion was retained for at least 90 min, but it rapidly disappeared when the perfusion was stopped. In contrast, the brains perfused with the Krebs-Ringer solution did not show any spontaneous EEG activity under the conditions employed here. The perfusion pressure in the brains perfused with the Krebs-Ringer solution remained abnormally high (see "MATERIALS AND METHODS). This is possibly due to the relatively low oxygen concentration of the Krebs-Ringer solution, but the actual reason is not clear yet. These unusual perfusion conditions might cause the breakdown of the BBB. However, the BBB was proved to be intact by the absence of staining with Evans Blue-BSA after 70 min of perfusion. Thus, all of the data below were obtained with brains perfused within 70 min. The various biochemical and physiological properties of the brain preparations perfused with the Krebs-Ringer solution were partly reported previously (*26*).

Elimination of Products from the Perfused Brain—We used 7-ethoxycoumarin (7-EC) as a substrate for the determination of drug-metabolizing activity. 7-HC in the effluent from a perfused brain was measured before and after hydrolysis with glucuronidase and sulfatase, and is presented as free and total 7-HC, respectively. This is the same method as that used for the perfused liver, in which 7-EC is deethylated to 7-hydroxycoumarin (7-HC) by several enzymes such as cytochrome P-450 (mainly 1A1 and 2B1), and 7-HC is conjugated to form glucuronides and sulfates (*20*). Figure 1A shows a typical example of the elimination of the product from a perfused rat brain when the concentration of 7-EC infused was changed in a stepwise manner. Here the data obtained for rats pretreated with phenobarbital are representatively shown. The elimination of 7-HC was more clearly seen than in non-treated rats when a low concentration of 7-EC was infused. Both total 7-HC (Fig. 1A) and the unreacted substrate (7-EC, Fig. 1B) in the effluent each increased in a stepwise manner and reached a constant level within 15 min after the 7-EC concentration was changed. It should be noted that there was almost no time delay between the change in the concentration of 7-HC formed and that of unreacted 7-EC, which was clearly demonstrated by the linear relationship between the formed 7-HC and unreacted 7-EC (Fig. 1C), even when the concentration of 7-EC infused was raised to 71 μM. The relative ratio of 7-HC to unreacted 7-EC (Fig. 1C) remained unchanged for the brains of untreated rats, where the same straight line was obtained (data not shown). Therefore, it is likely that there was almost no accumulation of the products in the brain, and the rate of elimination of 7-HC was thought to be almost equal to its production rate under these experimental conditions. From the concentration difference between total and free 7-HC, we could estimate the amounts of conjugated products. The fraction of the conjugated products in the total metabolites was found to be constant (Fig. 1D). This implied that in this

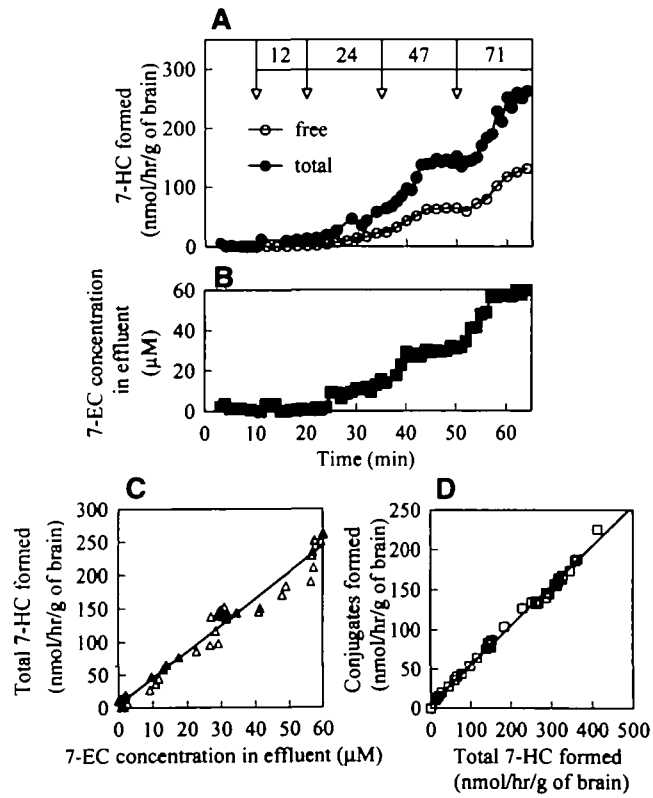

Fig. 1. **Formation of 7-HC and its conjugates in the perfused brain.** A rat was pretreated with phenobarbital as described under "MATERIALS AND METHODS." The brain was perfused with the FC-43 emulsion at 30°C through one of the internal carotid arteries. 7-EC was added to the influent at the concentrations indicated above the horizontal bar. The effluent was collected *via* the superior *vena cava*, and then the concentrations of the substrate and metabolites were analyzed. (A) Formation of total amounts of metabolites (Total, ●) and unconjugated metabolites (Free, ○). (B) Elimination of unreacted 7-EC. (C) Relationship between unreacted 7-EC elimination and 7-HC formation. (D) Relationship between formation of total 7-HC and that of conjugated metabolites.

7-EC production rate range, the conjugation reaction was not saturated, which therefore allowed the reaction to reach equilibrium.

7-EC Deethylation Activity of the Perfused Brain—Experiments similar to those in Fig. 1 were performed on the perfused brains of untreated rats, and the rate of 7-HC production was plotted against the concentration of infused 7-EC (Fig. 2). There was a relatively large scatter of the observed values, but the observed production rate increased with increased 7-EC infusion. With 50 μM 7-EC infusion, the rate was around 60 nmol/h/g, which was about half of that in a PB-pretreated rat (120 nmol/h/g) with 47 μM 7-EC infusion (Fig. 1). The maximum deethylation activity was 190 nmol/h/g when 150 μM 7-EC was infused.

For comparison of the activity to that of the perfused liver, we perfused the brain with the Krebs-Ringer solution and then measured the activities, which are also given in Fig. 2. The perfusion temperature was 37°C, since liver perfusion is usually performed at this temperature. We found that the effect of the perfusion temperature with the Krebs-Ringer solution was very small. For example, the deethylation rate at 30°C was 49 nmol/h/g, which was almost the same as the value at 37°C when 49 μM 7-EC was infused. Thus, the observed data with the Krebs-Ringer solution could be compared with those with the FC-43 emulsion, although the temperature differed. Although the scattering was almost twice as great with FC-43, the activities were almost the same in these two preparations (Fig. 2). The same results were obtained when the perfusate was switched to the other one during the perfusion (data not shown). From the data shown in Fig. 2, the maximum 7-EC deethylation activity in the perfused brain was estimated to be 200 nmol/h/g of brain with 130 μM 7-EC infusion.

7-EC Deethylation Activity of Brain Microsomes In Vitro—We determined the activity of microsomes to be 5.2 ± 0.9 pmol/min/mg in the presence of 0.5 mM 7-EC. This value is at the minimum end of the range of the large number of reported ones (*9-14*). The obtained value corresponded to about 1 nmol/h/g of brain, 3 mg of microsomes being obtained from 1 g of tissue in the present study. Compared to the above-mentioned activity of the perfused brain, the activity *in vitro* was much lower than that of the perfused brain *in situ*. Although the reasons for this difference are unclear, underestimation of the microsomal activity *in vitro* (*9*) could be one of them.

Comparison of the 7-EC Deethylation Activity of the Perfused Brain with That of the Perfused Liver—The deethylation activity of the perfused brain was compared with that of the perfused liver. As shown in Fig. 3, the activity of the perfused brain was found to range from 30 to 50% of that of the perfused liver with the concentrations of 7-EC examined up to 130 μM. This indicated that the brain

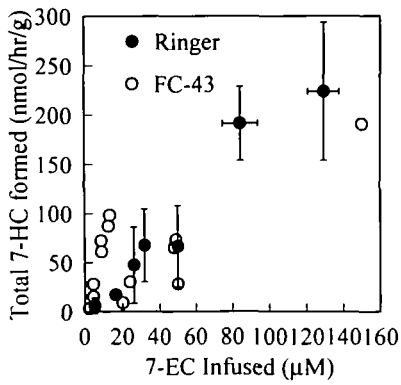

Fig. 2. **7-EC deethylation in the perfused brain.** The brains of non-treated rats were perfused with the FC-43 emulsion (○) at 30°C or the Krebs-Ringer solution at 37°C (●). The error bar indicates the standard error of three independent determinations for different animals.

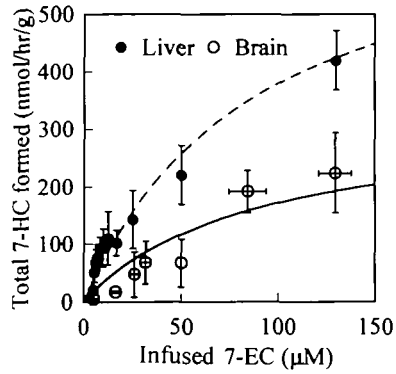

Fig. 3. **Comparison of 7-EC deethylation between the perfused brain and the perfused liver.** The brains and livers of non-treated rats were perfused with the Krebs-Ringer solution at 37°C. The error bar indicates the standard error of three independent determinations for different animals. The brain data are the same as in Fig. 2.

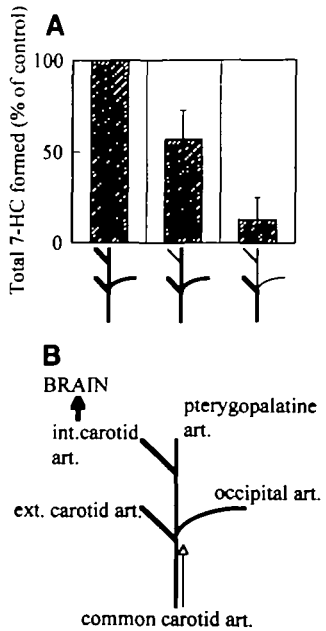

Fig. 4. **Effects of ligation of several arteries on 7-EC deethylation.** Non-treated rat brains were perfused with the Krebs-Ringer solution at 37°C. (A) Activity is expressed as a percentage of the control (first column). Bold lines indicate the arteries into which the perfusate was infused, while the fine lines show those with no influents. The 7-EC concentration in the influent was 50 μM. (B) Illustration of the arteries around the brain.

could possess drug-metabolizing activities comparable to those in the liver, which is the main site of drug metabolism in the body.

Contribution of Other Tissues to the Evaluation of Deethylation Activity—The contribution of other tissues, especially skin, to the formed 7-HC was examined. It is well known that the common carotid artery branches into four arteries, namely the internal carotid, occipital, pterygopalatine, and external carotid arteries, as shown in Fig. 4B. There may be anastomoses between these branched arteries. Of these arteries, only the internal carotid artery enters the brain. Therefore, the brain can be completely isolated when all other arteries and anastomoses are ligated. In the present experiments, only the external carotid arteries were usually ligated to reduce circulation to the skin. Therefore, the contribution of the skin to the enzymatic activity was estimated indirectly by ligation of the internal carotid artery. The perfusion was started from the no-ligation stage (control), where the whole head, including the brain and skin, was perfused, followed by ligation of one or more arteries. When the internal carotid artery was ligated to stop the perfusion into the brain, 7-HC formation decreased to 60% of the control value (Fig. 4A), which may suggest the existence of anastomoses between the internal carotid artery and the pterygoplatine artery. On the other hand, perfusion of the skin through the external carotid artery accounted for 13±12% of the control activity (Fig. 4A). Thus, more than 80% of the metabolites might originate from the cerebral tissue and its circulatory system.

Effect of Mannitol Infusion on the Evaluation of Deethylation Activity—Since 7-EC is a highly lipophilic compound, it is likely to pass through the BBB by simple diffusion (*15*), meaning that entry of 7-EC through the BBB is not rate-determining for the overall reaction. To examine this, we investigated the effect of opening of the BBB on the deethylation activity. To open the BBB, we infused a highly osmotic solution of saturated mannitol according to the method of Rapoport *et al.* (*24*). The opening of the BBB was confirmed by staining with Evans Blue-BSA (data not shown). As shown in Fig. 5, infusion of saturated mannitol did not change the rate of formation of 7-HC. The transient change in deethylation activity at the time of mannitol application might have been an artifact associated with switching of the perfusate. These results confirmed that the passive entry of 7-EC was not rate-determining.

Contribution of P-450 to the Deethlyation Activity—Phenobarbital (PB) and β-naphthoflavone (β-NF) were used to induce cytochrome P-450 in this experiment. Treatment of animals with these inducers for 3 days increased the rate of elimination of 7-HC from the perfused

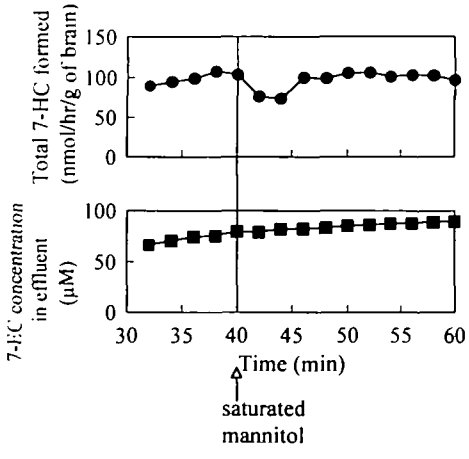

Fig. 5. **Effect of opening of the BBB with saturated mannitol on 7-EC deethylation.** A non-treated rat brain was perfused with the Krebs-Ringer solution at 37°C. Saturated mannitol was manually infused after 40 min perfusion for 30 s. Opening of the blood-brain barrier was confirmed by staining with Evans Blue-BSA complex. (A) 7-HC formation. (B) 7-EC concentration in effluent. The 7-EC concentration in the influent was 117 μM.

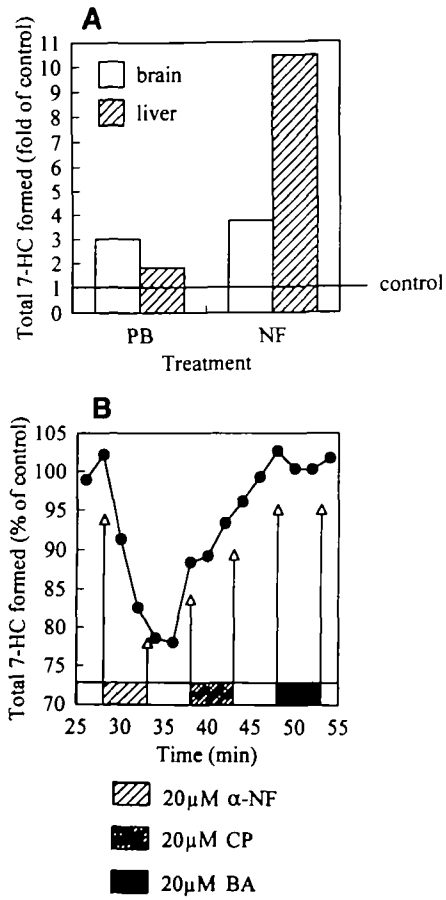

Fig. 6. **Effects of induction and inhibition of P-450 on 7-EC deethylation.** (A) Effects of *in vivo* enzyme induction and inhibition on 7-EC deethylation. Rats were pretreated with phenobarbital or β-naphthoflavone as described under "MATERIALS AND METHODS." Control animals were injected with the same vehicle as the experimental animals. Brains were perfused with the FC-43 emulsion at 30°C. Livers were perfused with the Krebs-Ringer solution at 37°C. The 7-EC concentration in the influent was 250 μM. 7-EC formation is indicated as the fold increase compared to the control level. (B) Effect of co-infusion of a P-450 inhibitor on 7-EC deethylation. A rat was pretreated with β-naphthoflavone as described under "MATERIALS AND METHODS." Its brain was perfused with the FC-43 emulsion at 30°C. 20 μM α-naphthoflavone (NF), 20 μM chloramphenicol (CP), or 20 μM 2-bromo-4'-aminonitrophene (BA) was infused into the influent by means of an infusion pump. The 7-EC concentration in the influent was 50 μM. 7-HC formation is indicated as a % of the control level. Control 7-HC formation was taken as that at 25 min of perfusion.

brain as well as from the perfused liver, which confirmed the enzyme induction. As shown in Fig. 6A, the effect of β-NF (1A1 inducer) was higher than that of PB (2B1 inducer), suggesting the major involvement of P-450 1A1 in this reaction and its higher inducibility.

The effects of P-450 inhibitors were also examined with the brain from a β-NF treated rat. Co-infusion of the P-450 1A1 inhibitor, α-naphthoflavone, decreased the activity, but that with chloramphenicol and bromoaminophene (2B1 inhibitors) did not (Fig. 6B). Therefore, the induced P-450 1A1 was shown to be acutely inhibited by its specific inhibitor in the brain.

DISCUSSION

We observed more than 100-times-higher 7-EC deethylation activity in the perfused brain compared to brain microsomal activity, assuming that this reaction only occurred in microsomes (the deethylation rate in the brain calculated from the activity in microsomes was ~1 nmol/h/g whereas the measured value was 200 nmol/h/g). The higher activity in the perfused brain might be partly explained by underestimation of the activity in microsomes obtained in this experiment, similar to in previous work (*13*). Moreover, brain mitochondria, which have a drug-metabolizing activity level similar to that of brain microsomes (*27*), could also be involved in metabolism in the whole brain. Although the degree of underestimation of the activity measured *in vitro* was not determined, the observed high activity supports the adequate viability of the perfused brain for determination of drug-metabolizing activity, even though EEG activity was lost with the Krebs-Ringer solution.

The high activity of the perfused brain, however, was unexpected because the substrate was suspected to be less accessible to enzymes in the whole organ than in isolated microsomes. In fact, perfused livers usually exhibit less activity than liver microsomes due to the concentration gradient of the substrate within the liver, depending on the physical properties of the substrate (*28*). The substrate gradient is thought to be formed only when the substrate is metabolized or eliminated before it is distributed throughout a tissue completely, according to the affinity of the drug to components in the tissue. Therefore, the high activity in the perfused brain suggested higher accessibility of the substrate to enzymes in the brain or slower elimination of xenobiotics from the brain.

There have been several reports supporting the higher accessibility to enzymes in the brain. Minn *et al.* (*5*) proposed the important role of the *metabolic* BBB, which refers to the localized drug-metabolizing enzymes in the BBB, such as in cerebral microvessels, the colloid plexus and circumventricular organs that can metabolize xenobiotics effectively before they enter the brain. The importance of localization of enzymes in the brain was also predicted by a computer simulation (*29*). Moreover, the effect of enzyme localization on the overall tissue metabolism was also reported for the perfused liver, where the activity was altered by changing the direction of perfusion flow from antegrade to retrograde (*30, 31*). Therefore, the localization of enzymes in the BBB may enable a substrate to gain access to enzymes effectively, and shows the importance of the architecture of the organ for effective drug metabolism.

Figure 6 demonstrates that the P-450 system mainly participates in the deethylation of 7-EC in the brain, whose activity is controlled by several biochemical factors (*32, 33*). For example, the NADPH supply is the rate-limiting step of this reaction, which is mainly the pentose phosphate pathway in the liver, but in the brain the NADP-NADH transhydrogenase reaction occurs in mitochondria. The comparable activities of 7-EC deethylation in the perfused brain and perfused liver can be explained by several characteristics of the P-450 reaction, although there is the large difference in the energy metabolism between the liver and brain. The effects of hypoxia, hypoglycemia and hyperglycemia on the drug-metabolizing activity are now being examined in our laboratory. An interesting finding is that hypoglycemia increased the activity of the brain but not that of the liver. The details will be reported elsewhere.

In conclusion, the use of the perfused brain can be recommended for the investigation of drug metabolism, because the perfused brain has some features that are the same as those *in vivo*. It is intact, has a preserved architecture, and contains all the systems required for drug-metabolism and disposition. Application of the perfused brain to determination of the activities of other drug metabolizing enzymes will provide valuable information that will help us understand drug-metabolizing activity *in vivo*. For this purpose, we can use brain preparations perfused with the Krebs-Ringer solution, which is a much more convenient procedure than that involving the FC-43 emulsion (*34*). We conclude that measurement of enzyme activity *in vitro* when the tissue structure is destroyed is insufficient for evaluating its biochemical significance in the whole organ and the whole body.

REFERENCES

1. Guengerich, F.P. (1990) Enzymatic oxidation of xenobiotic chemicals. *Biochem. Mol. Biol.* **25**, 97-153
2. Strobel, H.W., Geng, J., Kawashima, H., and Wang, H. (1997) Cytochrome P450-dependent biotransformation of drugs and other xenobiotic substrates in neural tissue. *Drug Metab. Rev.* **29**, 1079-1105
3. Ravindranath, V., Bhamre, S., Bhagwat, S.V., Anandatheerthavarada, H.K., Shankar S.K., and Tirumalai, P.S. (1995) Xenobiotic metabolism in brain. *Toxicol. Lett.* **82/83**, 633-638
4. Ravindranath, V. and Boyd, M.R. (1995) Xenobiotic metabolism in brain. *Drug Metab. Rev.* **27**, 419-448
5. Minn, A., Ghersi-Egea, J.F., Perrin, R., Leininger B., and Siest, G. (1991) Drug metabolizing enzymes in the brain and cerebral microvessels. *Brain Res. Rev.* **16**, 65-82
6. Lineweber, F.J. (1991) Drug disposition in the mammalian eye and brain: a comparison of mechanisms. *Drug Metab. Rev.* **23**, 133-246
7. Mesnil, M., Testa, B., and Jenner, P. (1984) Xenobiotic metabolism by brain mono-oxygenases and other cerebral enzymes in *Advances in Drug Research* Vol. 3, pp. 95-207, Academic Press, London
8. Mailman, R.B. (1980) Biochemical toxicology of the central nervous system in *Introduction to Biochemical Toxicology* (Hodgson, E. and Guthrie, F.E., eds.) pp. 224-244, Blackwell Science Publishers, Oxford
9. Bhagwat, S.V., Boyd, M.R., and Ravindranath, V. (1995) Rat brain cytochrome P450. Reassessment of monooxygenase activities and cytochrome P450 levels. *Drug Metab. Dispos.* **23**, 651-654
10. Anandatheerthavarada, H.K., Shankar, S.K., and Ravindranath, V. (1990) Rat brain cytochromes P-450: catalytic, immunochemical properties and inducibility of multiple forms. *Brain*

Res. **536**, 339-343
11. Warner, M., Köhler, C., Hansson, T., and Gustafsson, J.Å. (1988) 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
12. Näslund, B.M.A., Glaumann, H., Warner, M., Gustafsson, J.-Å., and Hanson, T. (1987) Cytochrome P-450 *b* and *c* in the rat brain and pituitary gland. *Mol. Phramacol.* **33**, 31-37
13. Srivastava, S.P., Seth, P.K., and Mukhtar, H. (1983) 7-Ethoxycoumarin O-deethylase activity in rat brain microsomes. *Biochem. Pharmacol.* **32**, 3657-3660
14. Guengerich, F.P. and Mason, P.S. (1978) Immunological comparison of hepatic and extrahepatic cytochromes P-450. *Mol. Pharmacol.* **15**, 154-164
15. Bonate, P.L. (1995) Animal models for studying transport across the blood-brain barrier. *J. Neurosci. Methods* **56**, 1-15
16. Rapoport, S.I., Ohno, K., and Pettigrew, K.D. (1979) Drug entry into the brain. *Brain Res.* **172**, 354-359
17. Benzi, G., Berté, F., Crema, A., and Frigo, G.M. (1967) Cerebral drug metabolism investigated by isolated perfused brain *in situ*. *J. Pharmac. Sci.* **56**, 1349-1351
18. Inagaki, M. and Tamura, M. (1993) Preparation and optical characteristics of hemoglobin-free isolated perfused rat head *in situ*. *J. Biochem.* **113**, 650-657
19. Sugano, T., Suda, K., Shimada, M., and Oshino, N. (1978) Biochemical and ultrastructural evaluation of isolated rat liver systems perfused with a hemoglobin-free medium. *J. Biochem.* **83**, 995-1007
20. Ji, S., Lemasters, J.J., and Thurman, R.G. (1981) A fluorometric method to measure sublobular rates of mixed-function oxidation in the hemoglobin-free perfused rat liver. *Mol. Pharmacol.* **19**, 513-516
21. Das, M., Seth, P.K., and Mukhtar, H. (1981) Characterization of microsomal aryl hydrocarbon hydroxylase of rat brain. *J. Pharmacol. Exp. Ther.* **216**, 156-161
22. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**, 265-275
23. Aitio, A. (1978) A simple and sensitive assay of 7-ethoxycoumarin deethylation. *Anal. Biochem.* **85**, 488-491
24. Rapoport, S.I., Fredericks, W.R., Ohno, K., and Pettigrew, K.D. (1980) Quantitiative aspects of reversible osmotic opening of the blood-brain barrier. *Am. J. Physiol.* **238**, R421-R431
25. Nomura, Y., Mastunaga, A., and Tamura, M. (1998) Optical characterization of heme a+a3 and copper of cytochrome oxidase in blood-free perfused rat brain. *J. Neurosci. Methods* **82**, 135-144
26. Tamura, M. and Nomura, Y. (1996) Organ perfusion in *Method for Biochemical Experiment* (Shimonisi, Y., Nagai, K., Hase, S., and Honda, T., eds.) pp. 41-57, Kagakudojin, Kyoto
27. Walther, B., Ghersi-Egea, J.F., Jayyosi, Z., Minn, A., and Siest, G. (1987) Ethoxyresorufin O-deethylase activity in rat brain subcellular fractions. *Neurosci. Lett.* **76**, 58-62
28. Roberts, M.S. and Rowland, M.J. (1986) Correlation between in-vitro microsomal enzyme activity and whole organ hepatic elimination kinetics: analysis with a dispersion model. *J. Pharm. Pharmacol.* **38**, 177-181
29. Britto, M.R. and Wedlund, P.J. (1992) Cytochrome P-450 in the brain: Potential evolutionary and therapeutic relevance of localization of drug-metabolizing enzymes. *Drug. Metab. Dispos.* **20**, 446-450
30. Häussinger, D. (1983) Hepatocyte heterogeneity in glutamate and ammonia metabolism and the role of an intercellular glutamine cycle during ureogenesis in perfused rat liver. *Eur. J. Biochem.* **133**, 269-275
31. Conway, J.G., Kauffman, F.C., Tsukuda, T., and Thurman, R.G. (1987) Glucuronidation of 7-hydroxycoumarin in periportal and pericentral regions of the lobule in livers from untreated and 3-methylcholanthrene-treated rats. *Mol. Pharmacol.* **33**, 111-119
32. Turman, R.G. and Kauffman, F.C. (1980) Factors regulating drug metabolism in intact hepatocytes. *Pharmacol. Rev.* **31**, 229-251
33. Iyanagi, T., Suzuki, T., and Kobayashi, S. (1981) Oxidation-reduction states of pyridine nucleotide and cytochrome P-450 during mixed-function oxidation in perfused rat liver. *J. Biol. Chem.* **256**, 12933-12939
34. Kawaji, A., Isobe, M., Tochino, Y., Takabatake, E., Chikaoka, Y., Nomura, Y., and Tamura, M. (1998) Flavin containing monooxygenase mediated metabolism of benzydamine in perfused brain and liver. *Biochem. Biophys. Acta* **24663**, 1-6