Microsomal electron-transport reductase activities and fatty acid elongation in rat brain

Developmental changes, regional distribution and comparison with liver activity

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Gestational and postnatal changes of microsomal NADH:cytochrome *b*₅ reductase and NADPH:cytochrome *c* reductase activities were examined in rat brain. The specific activity of NADH:cytochrome *b*₅ reductase was high at 18–19 days of gestational age, decreased to a minimum at 4 to 6 days after birth and increased thereafter. An essentially similar developmental pattern was observed for the specific activity of NADPH:cytochrome *c* reductase. In contrast, the specific activities of these reductases in liver microsomes were low, did not display a peak during gestation and increased steadily to a maximum at 40–50 days after birth. The rate of incorporation of [2-¹⁴C]malonyl-CoA into palmitoyl-CoA in brain microsomes was found to be high in the foetus, sharply decreased to a minimum at the time of birth and increased thereafter. The activity of fatty acid elongation in liver microsomes was much less than that in brain during gestation and increased rapidly after birth to values at 50–60 days 20-fold greater than the foetal activity. NADH and NADPH were equally effective for brain microsomal fatty acid elongation. Regional distribution of cytochrome reductase activities and the activity of fatty acid elongation showed the lowest specific activity in cerebellum. These results suggest that brain microsomal electron transport may be correlated with the developmental alteration in fatty acid elongation.

Long-chain fatty acids are essential constituents of galactosphingolipids, which are highly enriched in myelin and accumulate during myelination. Microsomal fatty acid-elongating enzymes have high activities during the early postnatal period and reach a maximum at 20–30 days after birth in rat and mouse (Brophy & Vance, 1975; Bourre *et al.*, 1978; Murad & Kishimoto, 1978), whereas microsomal stearoyl-CoA and palmitoyl-CoA desaturase activities are reduced with age and development (Cook & Spence, 1973; Pullarkat & Reha, 1975; Carreau *et al.*, 1979). A microsomal electron-transport system in liver was shown to participate in the desaturation of fatty acids (Oshino *et al.*, 1971). Recently, involvement of microsomal electron transport in fatty acid elongation was also demonstrated in the liver of rats (Keyes & Cinti, 1980). Immunological studies and cytochrome *b*₅-depletion experiments in our laboratory have recently suggested that the NADH-dependent cytochrome *b*₅ reductase and cytochrome *b*₅ system is involved in transferring electrons to the elongation system in brain (Takeshita *et al.*, 1983).

We reported previously the postnatal development changes of microsomal reductases in rat brain (Takeshita *et al.*, 1982). In the present study we report the parallel decrease in microsomal cytochrome reductase activities and fatty acid elongation in rat brain during the gestational to neonatal stages. A comparison of brain microsomal cytochrome reductases and fatty acid elongation with those of liver preparations was also made.

Experimental

Materials

Chemicals. [2-¹⁴C]Malonyl-CoA (sp. radioactivity 45.5 Ci/mol) was purchased from New England Nuclear, Boston, MA, U.S.A. Cytochrome *c*, palmitoyl-CoA and malonyl-CoA were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. NADH and NADPH were the products of Boehringer-Mannheim, Mannheim, Germany. Trypsin was purchased from Miles Laboratories. EDTA was the product of Dojin Chemical Laboratories Inc.,

Kumamoto, Japan. Cytochrome b_5 was purified from pig liver microsomes by trypsin digestion by the method of Omura & Takesue (1970).

Animals. White Wistar rats of either sex were used throughout these experiments. Animals were bred in our laboratory. Gestational ages of foetal rats were determined by the impregnation date, and correlative body-length measurements were used as a further indication of relative maturity (Volpe & Kishimoto, 1972). Before being killed, suckling and newborn rats were kept with their mothers. Weaned and adult rats were regularly fed on laboratory diet obtained from Clea Japan Inc., Tokyo, Japan.

Methods

Preparation of microsomes. Rats were decapitated and the brains and livers were immediately removed. All subsequent operations were performed at 4°C. Brain tissues were homogenized in a glass tube, using a Teflon pestle, with 0.32 M-sucrose containing 0.1 M-potassium phosphate buffer, pH 7.0 (6 ml/g wet wt. of tissue) with six up-and-down strokes. A small portion of the whole homogenate was taken for assay of total activity of the cytochrome reductases. The homogenates were centrifuged at 17000 g_{av} for 30 min and the resultant supernatant was centrifuged at 105000 g_{av} for 60 min. The pellet was washed by suspending the residue in the above-defined sucrose/buffer, also containing 1 mM-EDTA, and centrifuged at 105000 g_{av} for 60 min and resuspended in 0.1 M-potassium phosphate buffer, pH 7.5. Liver microsomes were prepared by an essentially similar procedure to the brain preparation, except that the sucrose concentration used was 0.25 M.

Assay of the reductase activities. The reductase activities were assayed at 23°C. Ferricyanide reductase activity was assayed by the method of Mihara & Sato (1978). NADH:cytochrome b_5 reductase (EC 1.6.2.2) and NADPH:cytochrome c reductase (EC 1.6.2.4) activities were assayed by previously published procedures (Takeshita *et al.*, 1982).

Assay of fatty acid-elongating enzyme. The microsomal palmitoyl-CoA elongation was assayed by the method of Murad & Kishimoto (1978) with some modifications. The standard assay mixture contained 100 mM-potassium phosphate buffer, pH 7.2, 0.5 mM-NADH, 2 μ M-rotenone, 0.02 mM-palmitoyl-CoA, 0.05 mM-[2- 14 C]malonyl-CoA (sp. radioactivity 2 Ci/mol) and 0.4–0.6 mg of protein of brain and liver microsomes in a total volume of 0.5 ml. The reaction was started by adding microsomes, incubated at 37°C for 10 min, and was terminated with 0.5 ml of 15% KOH/methanol. The methylation, identification of fatty acid and radioactivity measurements were performed essentially by

the method of Keyes & Cinti (1980) using a Shimadzu GC-7AG gas-liquid chromatograph with flame-ionization detector, equipped with a Packard model 894 gas proportional counter. The column used for separation of the methylated fatty acids was stainless steel (3 mm internal diameter; 2 m long) packed with 10% Silar 10C (silicon) on 100/120 Gas-Chrom Q. The column temperature was 200°C and the flow rate of carrier helium was 50 cm³/min.

Protein determination. Protein was determined by the procedure of Lowry *et al.* (1951) with crystalline bovine serum albumin as standard.

Fig. 1. Rat brain microsomal cytochrome reductase activities at various stages of gestational and postnatal development

In each determination microsomes were prepared from one to six rats depending on the size of rat brain (six for rats younger than 4 days of age, five for rats of 4–10 days of age, two for rats of 16–25 days of age and one for rats older than 30 days). Tissues from all foetal rats of a litter were combined for one determination. Preparation of microsomes and assay conditions were as described in the Experimental section. Adult rats were 130–160 days of age. To show the general trends in the activity change, the lines were drawn smoothly among the probable values from the means of points at each age and the points around the respective mean values. (a) NADH:ferricyanide reductase; (b) NADH:cytochrome b_5 reductase; (c) NADPH:cytochrome c reductase.

Results

Microsomal reductase activities of rat brain and liver at various stages of development

Brain microsomal NADH:ferricyanide reductase, NADH:cytochrome b_5 reductase and NADPH:cytochrome c reductase activities were determined at various stages of gestational and postnatal development (Figs. 1a–1c). For the assay of NADH:cytochrome b_5 reductase activity, both ferricyanide and cytochrome b_5 reductase activity were measured. Although the elevation in the activity at prenatal ages was not distinct in the ferricyanide reductase, the cytochrome b_5 reductase showed significantly high activity during gestation and decreased to a minimum at 2–4 days after birth, reached a peak at 20–30 days after birth, slightly decreased to a plateau and then slightly increased again to a plateau in the adult brain (Figs. 1a and 1b). An essentially similar pattern was observed for

NADPH:cytochrome c reductase (Fig. 1c). An initial decrease in activity at the end of gestation and just after birth was not observed when these reductase activities were expressed as total activity per brain (Figs. 2a–2c). The specific activities of the liver microsomal reductases were low during gestation and increased steadily until reaching a maximum at approx. 40–50 days after birth; there was no distinct peak, as observed in the brain, throughout the foetal and postnatal stages (Figs. 3a–3c). In the case of liver ferricyanide reductase activity, a plateau values was observed between 10 and 20 days of ages, with a subsequent increase in activity up to the adult value (Fig. 3a). Similar plateau values were observed between 5 and 20, and 0 and 20 days of ages in liver cytochrome b_5 reductase and cytochrome c reductase activities respectively, though the points were scattered to some extent (Figs. 3b and 3c).

Fig. 2. Rat brain microsomal cytochrome reductase activities (total activity per brain) at various stages of gestational and postnatal development

Whole homogenate (100–150 μ g of protein) of the brain was subjected to the assay of cytochrome reductase activities by the methods described in the Experimental section. Adult rats were 150 days of age. The line was drawn by the method described in the legend to Fig. 1. (a) NADH:ferricyanide reductase; (b) NADH:cytochrome b_5 reductase; (c) NADPH:cytochrome c reductase.

Fig. 3. Rat liver microsomal cytochrome reductase activities at various stages of gestational and postnatal development

Preparation of liver microsomes and assay conditions were as described in the Experimental section. Adult rats were 130 to 160 days of age. The line was drawn by the method described in the legend to Fig. 1. (a) NADH:ferricyanide reductase; (b) NADH:cytochrome b_5 reductase; (c) NADPH:cytochrome c reductase.

Gestational and postnatal changes in brain and liver activities of fatty acid elongation

As the microsomal reductases showed a characteristic activity change during brain development, we investigated fatty acid elongation during the prenatal and postnatal stages. Palmitoyl-CoA was used as a substrate for fatty acid chain elongation, because the highest specific activity was obtained with palmitoyl-CoA as an acceptor for microsomal $[2-^{14}\text{C}]$ malonyl-CoA incorporation. The activity with longer fatty acids (such as stearoyl-CoA or behenyl-CoA) was less than one-fifth that with palmitoyl-CoA (M. Takeshita, M. Tamura & T. Yubisui, unpublished work). As has been reported by Brophy & Vance (1975) and Murad & Kishimoto (1978) the activities of fatty acid elongation in brain microsomes are increased with development of rat brain and reach a peak at 25 days of age (Fig. 4). Moreover, the specific activity was relatively high during gestation and decreased to a trough at birth.

Fig. 4. Age-related changes in the activity of $[2-^{14}\text{C}]$ malonyl-CoA incorporation into palmitoyl-CoA by microsomes from rat brain and liver

The numbers of organ tissues taken from foetal and adult rats that were used for each determination were the same as described in the legend to Fig. 1. Each point represents the mean of duplicate determinations. The reaction mixture contained 100 mM-potassium phosphate buffer, pH 7.2, 0.5 mM-NADH, $[2-^{14}\text{C}]$ malonyl-CoA (sp. radioactivity 2 Ci/mol) and microsomes (0.4–0.6 mg of protein) from brain or liver of rats in a total volume of 0.5 ml. The reaction was started by adding microsomes and incubated at 37°C for 10 min. Adult rats were 130–160 days of age. Symbols: ●, rat brain microsomes; ▲, rat liver microsomes.

Though specific activity was decreased during the final gestational stages towards birth, total activity per brain was approximately constant (results not shown), as noted above with cytochrome reductases. In contrast with the brain, specific activity of fatty acid elongation in liver was as low as 0.05 nmol/min per mg of microsomal protein in the prenatal periods, and the values increased by more than 20-fold at 40–50 days with a shoulder at 10–20 days after birth (Fig. 4). These results indicated that the patterns of the activity change in fatty acid elongation were essentially similar to the change in the activities of microsomal electron-transport reductases in both brain and liver.

Nicotinamide nucleotide cofactor requirements for palmitoyl-CoA elongation

In microsomal fatty acid-elongation system, a reduced nicotinamide nucleotide was required for maximum activity. Essentially no preference for either NADH or NADPH was observed (Table 1). In the presence of both nicotinamide nucleotides, the rate of malonyl-CoA incorporation was approximately the same level as when either nucleotide was used alone. In the absence of nicotinamide nucleotides the reaction rate decreased to less than 10% of the activity in the presence of either of these cofactors.

Regional distribution of cytochrome reductases and enzymes for fatty acid elongation

The cytochrome reductase activities and incorporation of $[2-^{14}\text{C}]$ malonyl-CoA into palmitoyl-CoA were measured in the microsomes prepared from different areas of rat brains at 50–60 days after birth (Table 2). In general, all specific activities were higher in the medulla oblongata and cerebral hemispheres and lowest in the cerebellum. An essentially similar pattern of distribution of cyto-

Table 1. Cofactor requirement for fatty acid elongation by rat brain microsomes

The assay system contained 0.5 mg of brain microsomal fractions from 25-day-old rats in the presence or in the absence of 0.5 mM-NADH and/or -NADPH. The other components of the system and the assay conditions were as described in the legend to Fig. 4. Results were expressed as means $\pm$ S.D. of five independent experiments.

Additions	Palmitoyl-CoA elongation (nmol/min per mg of protein)
NADPH	0.64 ± 0.22
NADH	0.50 ± 0.28
NADPH + NADH	0.61 ± 0.24
No nicotinamide nucleotide	0.04 ± 0.02

Table 2. *Cytochrome reductase activities and fatty acid elongation by microsomes from different regions of rat brain*
Two brains from 50–60-day-old rats were dissected into four distinct areas by the procedure reported previously (Takeshita *et al.*, 1982). The preparation of microsomes and assay conditions were as described in the Experimental section. Results are means $\pm$ s.d. of four independent determinations.

Region	NADH:ferricyanide reductase ($\mu\text{mol}/\text{min}$ per mg of protein)	NADH:cytochrome b_5 reductase (nmol/min per mg of protein)	NADPH:cytochrome c reductase (nmol/min per mg of protein)	Palmitoyl-CoA elongation (nmol/min per mg of protein)
Cerebral hemispheres	2.27 ± 0.27	17.4 ± 0.4	79.7 ± 2.9	0.50 ± 0.13
Mid-brain	2.04 ± 0.54	15.2 ± 3.8	60.9 ± 7.3	0.44 ± 0.12
Cerebellum	1.59 ± 0.26	11.3 ± 1.9	29.9 ± 9.6	0.22 ± 0.06
Medulla oblongata	3.42 ± 0.18	23.1 ± 4.0	47.9 ± 7.4	0.53 ± 0.08

chrome reductases was observed in 20-day-old rat brain (Takeshita *et al.*, 1982). More detailed inspection of the results revealed that NADH:cytochrome b_5 reductase activities were the highest in medulla oblongata, whereas NADPH:cytochrome c reductase activity was highest in the cerebral hemispheres. Incorporation of $[2-^{14}\text{C}]\text{malonyl-CoA}$ was high in the cerebral hemispheres and in the medulla oblongata.

Discussion

We previously reported postnatal developmental changes in NADH:cytochrome b_5 reductase and NADPH:cytochrome c reductase activities (Takeshita *et al.*, 1982). As the activity of fatty acid chain elongation showed essentially the same developmental alterations as those of the reductases (Brophy & Vance, 1975; Bourre *et al.*, 1978; Murad & Kishimoto, 1978), we have discussed the correlation of these reductases with fatty acid elongation in relation to myelination in the central nervous system (Takeshita *et al.*, 1982). In the present study we found that the specific activities of microsomal NADH:cytochrome b_5 reductase and NADPH:cytochrome c reductase were decreased in parallel with that of fatty acid elongation during late gestation and the neonatal period in the brain. These results suggest that microsomal cytochrome reductases might be functionally correlated with fatty acid elongation. The high foetal activity of reductases and fatty acid metabolism might be involved in the production of 'early' myelin (Agrawal *et al.*, 1970). Several reports have described a decrease in fatty acid desaturase activity during gestational and neonatal periods (Cook & Spence, 1973; Pullarkat & Reha, 1975; Carreau *et al.*, 1979). Fatty acid elongation in brain microsomes showed a peak activity in the foetal stages of rats, whereas that in liver microsomes showed no peak. The developmental changes in microsomal cytochrome reductases were similar to those of fatty acid elongation in both brain and liver. Although the specific activities of the

reductases and fatty acid elongation were decreased with development of the brain at the perinatal stages, the total activity per brain remained approximately the same. Pullarkat & Reha (1975) reported that the specific activity of rat brain fatty acid desaturase decreased after birth but total activity per brain was constant.

There is an absolute requirement for reduced nicotinamide nucleotides in microsomal fatty acid elongation. Aeberhard & Menkes (1968) observed no preference for either NADH or NADPH as cofactors for fatty acid elongation in brain, whereas Bourre *et al.* (1975, 1976) reported that their fatty acid chain-elongation system was specific for NADPH. In the present studies, NADPH and NADH were equally effective for palmitoyl-CoA elongation. If the microsomal electron-transport chain is needed for the transfer of reducing equivalents to fatty acid elongation, both NADH and NADPH must be involved in the reaction (Inoue & Shinagawa, 1965). The experiments by Bourre *et al.* (1975, 1976) involved elongation of behenyl-CoA, erucyl-CoA and the corresponding unsaturated fatty acyl-CoA species. It is of interest to study whether the electron-transport chain is involved in the elongation of very-long fatty acid chains. The activity in the presence of both NADH and NADPH was not exceeded by that in the presence of either nicotinamide nucleotide alone. The nicotinamide nucleotides might affect the common electron-transport component, presumably cytochrome b_5 , in the elongation system.

Although some minor variations were noted, the regional distribution pattern of microsomal reductases was roughly similar to that of fatty acid elongation. These activities were high in the cerebral hemispheres, medulla oblongata and mid-brain, and low in cerebellum. Pullarkat & Reha (1975) have also reported low stearoyl-CoA desaturase activity in cerebellum.

The activity of NADH:cytochrome b_5 reductase was high in the medulla oblongata, whereas that of NADPH:cytochrome c reductase was high in the

cerebral hemispheres. A similar pattern was also seen with 20 day-old rat brain (Takeshita *et al.*, 1982). Possmayer *et al.* (1979) suggested that NADPH:cytochrome *c* reductase may be present in the smooth-endoplasmic-reticulum system, which is responsible for fast axonal transport. It is not clear at present whether high NADPH:cytochrome *c* reductase activity in the cerebral hemispheres reflects any special role for this enzyme in the central nervous system.

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References

- Aeberhard, E. & Menkes, J. H. (1968) *J. Biol. Chem.* **243**, 3834–3840
- Agrawal, H. C., Banik, N. L., Bone, A. H., Davison, A. N., Mitchell, R. F. & Spohn, M. (1970) *Biochem. J.* **120**, 635–642
- Bourre, J. M., Daudu, O. L. & Bauman, N. A. (1975) *Biochem. Biophys. Res. Commun.* **63**, 1037–1034
- Bourre, J. M., Daudu, O. & Baumann, N. (1976) *Biochim. Biophys. Acta* **424**, 1–7
- Bourre, J. M., Pollet, S., Paturneau-Joas, M. & Baumann, N. (1978) in *Enzymes of Lipid Metabolism* (Gatt, S., Freysz, L. & Mandel, P., eds.), pp. 17–26, Plenum, New York
- Brophy, P. J. & Vance, D. E. (1975) *Biochem. J.* **152**, 495–501
- Carreau, J. P., Daudu, O., Mazliak, P. & Bourre, J. M. (1979) *J. Neurochem.* **32**, 659–660
- Cook, H. W. & Spence, M. W. (1973) *J. Biol. Chem.* **248**, 1793–1796
- Inoue, A. & Shinagawa, Y. (1965) *J. Neurochem.* **12**, 803–813
- Keyes, S. R. & Cinti, D. L. (1980) *J. Biol. Chem.* **255**, 11357–11364
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Mihara, K. & Sato, R. (1978) *Methods Enzymol.* **52**, 102–108
- Murad, S. & Kishimoto, Y. (1978) *Arch. Biochem. Biophys.* **185**, 300–306
- Omura, T. & Takesue, S. (1970) *J. Biochem. (Tokyo)* **69**, 249–257
- Oshino, N., Imai, Y. & Sato, R. (1971) *J. Biochem. (Tokyo)* **69**, 155–167
- Possmayer, F., Kleine, L., Duwe, G., Stewart-DeHaan, P. J., Wong, T., MacPherson, C. F. C. & Harding, P. G. R. (1979) *J. Neurochem.* **32**, 889–906
- Pullarkat, R. K. & Reha, H. (1975) *J. Neurochem.* **25**, 607–610
- Takeshita, M., Miki, M. & Yubisui, T. (1982) *J. Neurochem.* **39**, 1047–1049
- Takeshita, M., Tamura, M., Yoshida, S. & Yubisui, T. (1983) *Neurochem. Res.* in the press
- Volpe, J. J. & Kishimoto, Y. (1972) *J. Neurochem.* **19**, 737–753
- Yubisui, T. & Takeshita, M. (1980) *J. Biol. Chem.* **243**, 3834–3840