# Possible Essentiality of Docosahexaenoic Acid in Japanese Monkey Neonates: Occurrence in Colostrum and Low Biosynthetic Capacity in Neonate Brains

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**The importance of mother's milk as a source of docosahexaenoic acid (DHA) in Japanese monkey neonates was investigated. The DHA content in monkey colostrum total lipids was 2.2%, similar to or slightly higher than in humans. A comparison of the biosynthetic capacity of brain microsomes from monkeys of different age (up to 10 years) showed that chain elongation/desaturation of linolenic acid and eicosapentaenoic acid in neonates was significantly less pronounced than in adults. In particular, the formation of DHA, which is the product of Δ4 desaturase, was negligible. These results suggest that milk is an important source of DHA in Japanese monkey neonates.**
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Long-chain polyunsaturated fatty acids (PUFA), which are major fatty acid components at the 2-position of glycerophospholipids in membranes, are thought to be essential for maintaining fluidity and physiological activity. Docosahexaenoic acid (DHA, 22:6n-3) is particularly abundant in phosphatidylethanolamine of brain grey matter (1) and retina (2). In these tissues, most of the n-3 PUFA is DHA, not eicosapentaenoic acid or linolenic acid. DHA is known to be selectively incorporated into the developing brain (3,4). Holman *et al.* (5) reported a neurological disturbance in a 6-year-old girl who was maintained on parenteral nutrition that lacked n-3 PUFA. Neuringer *et al.* (6,7) found that dietary n-3 fatty acid deprivation caused subnormal visual acuity in rhesus monkeys. Feeding of n-3 PUFA-deficient oils to rats was shown to decrease DHA level in brain phospholipids (8–10) and be associated with inferior learning capacity (8,9), whereas supplementation with DHA partially restored learning ability (11). These findings suggest that DHA might be essential in maintaining nervous system function.

In animals, long-chain PUFA such as 20:4n-6, 22:4n-6 and 22:6n-3 are synthesized from dietary $C_{18}$ precursors, 18:2n-6 and 18:3n-3, which are synthesized *de novo* in plants. Animal studies have suggested that the capacity for chain elongation and desaturation is species and age dependent. Indirect evidence indicated that newborn human infants cannot produce sufficient quantities of long-chain PUFA from $C_{18}$ precursors and, hence, depend on maternal supply (12,13). This would explain why DHA is present in human milk, although its content is variable and partially dependent on dietary fat intake (14). However, even the milk from vegetarians contains a considerable amount of DHA (15). The milk from various animals (16), including rats (17–21), rabbits (22), guinea pigs (22) and pigs (23), contains only trace or negligible amounts of DHA, whereas fish-eating marine animals have high DHA levels (16). Human milk provides DHA for neonates, whose DHA biosynthetic activity may be low at the time the human brain develops (12,13).

In the present study, Japanese monkeys were used as experimental models to investigate the effects of age on the biosynthetic capacity of long-chain PUFA in brain. First, the fatty acid composition of colostrum lipids was analyzed for comparison with that of humans. Then, the ability of brain microsomes to produce long-chain PUFA from their precursors was determined *in vitro* for animals of different age.

To test whether there is any relationship between the local PUFA content and the local synthetic activity of PUFA in cerebral cortex, three major regions of cerebral cortex were compared in respect to both long-chain PUFA content and synthetic capacity to produce long-chain PUFA.

## MATERIALS AND METHODS

*Materials.* [1-$^{14}$C]Linolenic acid (2.07 GBq/mmole) and [1-$^{14}$C]eicosapentaenoic acid (2.17 GBq/mmole) were purchased from Amersham Japan (Tokyo, Japan). Radioactive purities were checked by high performance liquid chromatography (HPLC) and liquid scintillation counting as described below. Non-radioactive linolenic and eicosapentaenoic acids were obtained from Sigma Chemical Co. (St. Louis, MO). Octadecatetraenoic (n-3), eicosatetraenoic (n-3) and docosapentaenoic (n-3) acids used as standards were provided by Nippon Suisan Kaisha (Tokyo, Japan). Arachidonic and octadecatetraenoic acids (n-3) were provided by Idemitsu Petrochemical Co. (Tokyo, Japan). Coenzymes were purchased from Oriental Yeast Co. (Tokyo, Japan).

*Animals.* Cerebral cortices of Japanese monkeys [*Macaca fuscata fuscata;* two newborns, (a male and a female), a male 2 years of age, a male 6.5 years of age, and a male 10 years of age] were obtained from the Primate Research Institute, Kyoto University, Inuyama, Japan. Animals were fasted for 24 hr prior to killing. Tissues were frozen and kept at −80°C until use. The frontal lobe from a 6.5-year-old animal and the occipital lobes from a 2-, a 6.5- and a 10-year-old animal were used for the preparation of microsomes. The specific origin of the samples from the two neonatal brains was unknown.

The colostrum was obtained from a 10-year-old female animal four days after delivery. All animals had been fed a commercial monkey diet, Oriental APF (Oriental Yeast Co.).

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Abbreviations: DHA, docosahexaenoic acid; GLC, gas-liquid chromatography; GSH, glutathione (reduced form); HPLC, high performance liquid chromatography; PUFA, polyunsaturated fatty acids.

*Isolation of microsomes.* The cerebral cortex was homogenized using a Potter-Elvehem homogenizer in 5 vol of a solution containing 0.25 M sucrose, 0.15 M KCl, 5 mM $MgCl_2$, 1.5 mM glutathione (reduced form) (GSH) and 50 mM $K_2HPO_6$ at pH 7.0. The homogenate was centrifuged at 800 × *g* for 10 min to remove cell debris, and then the supernatant was centrifuged at 10,000 × *g* for 20 min. The supernatant at 10,000 × *g* was further centrifuged at 100,000 × *g* for 1 hr to obtain the microsomal fraction. The microsomal pellet was suspended in a buffer containing 0.15 M KCl, 5 mM $MgCl_2$, 0.15 mM GSH and 50 mM $K_2HPO_4$ at pH 7.0. The entire isolation procedure was carried out at 0–4°C. The protein content of the microsomal solution was measured using the method of Lowry *et al.* (24).

*Assay of PUFA chain elongation-desaturation.* The assay conditions were basically those of the procedure described by Purvis *et al.* (25). Typically, reaction mixtures contained 100 mM fatty acids, 150 mM $MgCl_2$, 1.5 mM GSH, 5 mM ATP, 1.25 mM NADH, 1.25 mM NADPH, 0.3 mM CoA, 0.3 mM malonyl CoA and 50 mM $K_2HPO_6$ at pH 7.0 in a final volume of 0.5 mL. The fatty acids were a mixture of labeled and unlabeled 18:3 or 20:5 and were used in experiments after conversion to ammonium salt and suspension in 1% aqueous solution of Triton WR1339 containing fatty acid-free bovine serum albumin (Sigma Chemical Co., St. Louis, MO) at a molar ratio of 4:1. The radioactivities in reaction mixtures were in the range of 5.0 to 8.3 × $10^3$ Bq. Incubation was initiated by adding 0.5 to 1.0 mg of microsomal protein to the reaction mixture and carried out at 37°C for 20 min. Assays were terminated by addition of 0.5 mL of 20% KOH containing 1 mg BHT. The mixtures were saponified at 80°C for 30 min, acidified with 0.5 mL of 5 M HCl, and the fatty acids were extracted with chloroform/methanol (2:1, v/v). After evaporation to dryness, the mixture was methylated with 13% (w/v) $BF_3$/methanol at 80°C for 5 min (26). Reaction mixtures without microsomes were used for control experiments. Recovery of radioactivity in the control experiment was 72.6%.

*Separation of fatty acids and liquid scintillation counting.* Fatty acid methyl esters were separated by HPLC essentially as described by Hirata *et al.* (27) using a Hitachi chromatograph model 635A (Hitachi, Tokyo) fitted with two Lichrosorb RP-18 columns (5 μm particle size, 4 × 250 mm connected with 7 μm particle size, 4 × 250 mm; Kanto Chemical Co., Tokyo) in series. The methyl esters were eluted stepwise with aqueous methanol (14%, 10% and 6%) at a flow rate of 0.6 mL/min. Fractions were detected with a UV monitor set at 210 nm and identified by comparison of their retention times with those of standards.

The radioactivities of the methyl esters were determined by counting fractions collected at 1-min intervals in scintillation vials containing 6 mL of NT scintillation fluid (28) using an Aloka liquid scintillation system, LSC-903 (Aloka, Tokyo).

*Lipid analysis.* Colostrum lipids were extracted by the Bligh-Dyer method (29). The lipid classes were separated by silicic acid and Florisil column chromatography (30). The lipids of the diet and tissues were extracted by the Folch method (31). Tissue lipids were fractionated into lipid classes on a Sep-Pak Cartridge Silica (Waters Associates, Milford, MA) (32). Lipids were transmethylated with sodium methylate. Microsomal lipids were saponified, extracted with chloroform/methanol (2:1, v/v), and then methylated with 13% $BF_3$/methanol as described above. Fatty acid methyl esters were analyzed by gas-liquid chromatography (GLC) on a JEOL JGC-20K apparatus (JEOL, Tokyo) equipped with a glass column (2.0 mm × 2 m) containing 10% Silar 10C on Chromosorb W (60/80 mesh). Column temperature was programmed from 170–220°C at 1°C/min. Duplicate determinations were carried out. Fatty acid methyl esters were identified by comparison of their retention times with those of standards.

## RESULTS

*Fatty acid compositions of diet and colostrum.* The diet for monkeys contained 10.3% total lipids (w/w), of which the major fatty acids were as follows: 37.3% linoleic acid, 36.4% oleic acid, 13.8% palmitic acid, 2.7% linolenic acid and 2.6% stearic acid. Percentages of n-6 and n-3 PUFA in the total lipids were 41.7% and 3.6%, respectively.

Fatty acid compositions of the colostrum from *Macaca fuscata fuscata*, together with the cited data for human samples, are presented in Figure 1. The fatty acid profile in the monkey is similar to that in humans, although the linoleate content is a little higher in the monkey while palmitate is lower than in human colostrum. The total n-3 fatty acid levels in the monkey were similar to those in humans (33). The n-6/n-3 ratio in monkey was 5.9 and slightly higher than in humans. The DHA content in monkey was 2.2% and essentially identical to that in humans (33,34).

*Synthesis of chain elongation-desaturation products by brain microsomes.* Synthesis of chain elongation-desaturation products from linolenate was substantially influenced by age in the monkeys tested (Fig. 2). Judging from the total amount of metabolites produced, the activity in newborns was lower than in older monkeys. The synthesis of 22:6 was highly variable, *i.e.*, the greatest activity was found in a 6.5-year-old, while the activity in newborns was negligible. Similar results were obtained

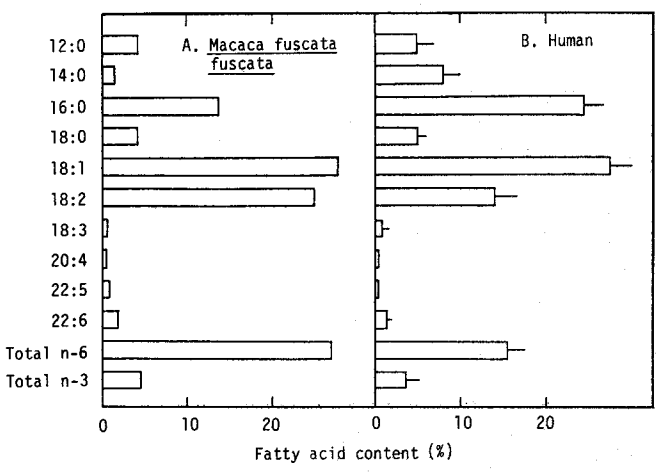


**FIG. 1. Fatty acid composition of breast milk. Comparison between colostrum of *Macaca fuscata fuscata* and human (29). The monkey colostrum was collected from a 10-year-old female four days after delivery.**

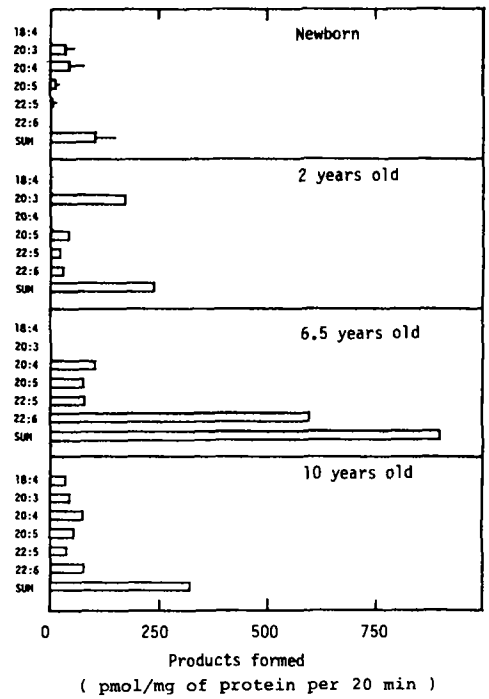


**FIG. 2. Metabolites from 18:3n-3 by brain microsomes from *Macaca fuscata fuscata* of different ages. Sizes of brains are given in parentheses (longitudinal axis × bilateral axis, cm). A female and a male newborn (6.2 × 5.1), a 2-year-old male (6.8 × 5.5), a 6.5-year-old male (7.5 × 6.1) and a 10-year-old male (7.0 × 6.1) were tested.**

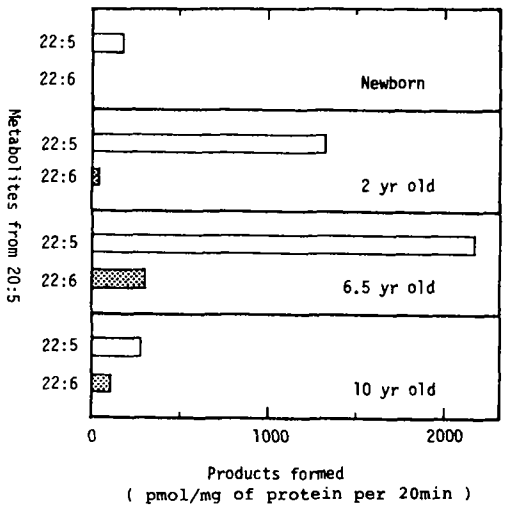


**FIG. 3. Metabolites from 20:5n-3 by brain microsomes from *Macaca fuscata fuscata* of different ages. A female newborn, a 2-year-old male, a 6.5-year-old male, and a 10-year-old male were tested.**

for the metabolism of 20:5 which was elongated and desaturated most extensively in a 6.5-year-old monkey, while the formation of 22:6 in the newborn again was negligible (Fig. 3).

*Regional fatty acid composition and synthesis of long-chain PUFA in cerebral cortex.* The DHA content of total lipids varied considerably between cerebral regions. DHA was found in decreasing abundance in occipital, in temporal and in frontal lobes (Table 1). The reverse was observed for n-6 PUFA, such as 20:4 and 22:4. A similar pattern of PUFA distribution was found in microsomal lipids (Table 2) with DHA being most abundant in the occipital lobe. On the other hand, brain microsomes prepared from frontal and occipital lobes showed almost the same activity for producing DHA from linolenic acid (Fig. 4). Thus, no direct relationship was apparent between long-chain PUFA synthetic activity and PUFA content of the cerebral cortex.

**TABLE 1**

**Regional Variation in Fatty Acid Composition of Grey Matter Lipids of a 10-Year-Old Male *Macaca fuscata fuscata***

| | Frontal | Temporal | Occipital |
|---|---|---|---|
| 16:0 | 19.4% | 20.7% | 21.9% |
| 18:0 | 22.8 | 21.7 | 20.3 |
| 18:1n-9 | 19.9 | 21.6 | 19.1 |
| 18:2n-6 | 2.7 | 2.8 | 3.7 |
| 18:3n-3 | tr | tr | tr |
| 20:4n-6 | 6.8 | 5.8 | 4.1 |
| 20:5n-3 | tr | tr | tr |
| 22:4n-6 | 6.7 | 5.6 | 3.5 |
| 22:5n-3 | 1.2 | 1.0 | 1.1 |
| 22:6n-3 | 11.0 | 13.9 | 16.6 |

**TABLE 2**

**Regional Variation in Fatty Acid Composition of Brain Microsomal Lipids of a 10-Year-Old Male *Macaca fuscata fuscata***

| | Frontal | Temporal | Occipital |
|---|---|---|---|
| 16:0 | 21.4% | 15.8% | 17.3% |
| 18:0 | 22.0 | 18.6 | 19.2 |
| 18:1n-9 | 18.3 | 15.6 | 16.7 |
| 18:2n-6 | 1.0 | 1.5 | 0.8 |
| 18:3n-3 | tr | tr | tr |
| 20:4n-6 | 8.5 | 10.3 | 7.2 |
| 20:5n-3 | tr | tr | tr |
| 22:4n-6 | 1.2 | 1.0 | 1.1 |
| 22:6n-3 | 12.1 | 14.6 | 15.8 |

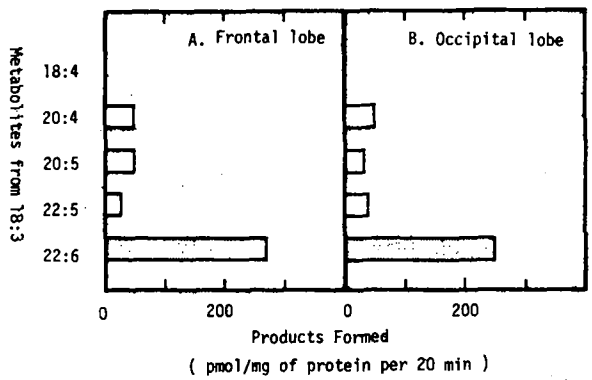


**FIG. 4. Comparison of capacity for chain elongation/desaturation between frontal and occipital lobes from *Macaca fuscata fuscata*. A 6.5-year-old male was tested.**

## DISCUSSION

Although the fatty acid composition of milk of a wide variety of animals has been reported (16–23), the occurrence of DHA in milk was only noted in marine animals which live on a fish diet rich in DHA (16). This does not necessarily rule out the occurrence of trace amounts of DHA in animal milk, because in many of the studies no special attention appears to have been paid to the possible occurrence of small amounts of DHA. In human milk, DHA is always present, although its content varies with dietary habits. It also must be emphasized here that the DHA content in colostrum is highest and that it decreases somewhat in mature milk (33,35,36). This has been used as an argument for the maternal origin of long-chain PUFA needed by human newborns. Because the composition of lipids and fatty acids in the Japanese monkey's colostrum is similar to that in humans, it appears that *Macaca fuscata fuscata* would be a superior model to elucidate PUFA metabolism in neonates relevant to humans.

In the present study, the biosynthetic activity of n-3 long-chain PUFA in monkey brain was assessed using microsomes *in vitro*. Kinetic studies on the metabolic conversion of PUFA have shown that the rate of activation of a fatty acid to its CoA thioester in rat liver microsomes is greater than either elongation or desaturation of the acyl CoA ester (37–39). The incorporation of the metabolic products into lipids is also not rate limiting—the rate of acylation has been found to be of the same order or greater than the rate of activation in rat liver (37). Therefore, the ease of conversion of short-chain PUFA is commonly examined. Our data suggest that, in monkey brain, desaturation steps and not chain-elongation steps are rate-limiting in converting 18:3n-3 to DHA, because among the intermediates of this reaction (Fig. 2), the substrates for the desaturation (20:3, 20:4 and 22:5) were more abundant than the substrates for the chain-elongation (18:4 and 20:5). The ratios of the two were 6.6 for newborns, 4.6 for 2-year-olds, 2.5 for 6.5-year-olds and 2.3 for ten-year-olds.

In the case of rats, Strouvé-Vallet and Pascaud (40) reported that the brain microsomal activity for elongation and desaturation of linoleic acid was high in the first four days after birth, but decreased rapidly thereafter. Studies by Cook (41,42) also confirmed that the highest Δ6 desaturase activity is associated with neonatal rat brain. In liver, desaturase activity is stable up to 30 days (40) or increases slightly after weaning (41,42).

In the case of perinatal piglets, the brain enzyme activity was also greatest in the neonatal period, while the liver enzyme activity was higher in the postnatal period. Δ5 Desaturase activity, in particular, was very low during the neonatal period (25,43).

The importance of the liver in supplying DHA for the nervous system has recently been reported in rat (44). In our study, however, we could not assess the metabolizing activity of liver, because monkeys had been fasted for 24 hr. Under this condition, Δ6 desaturase activity is known to be very low (45,46). In contrast, the activity in brain is quite independent of diet (data not shown).

In the present study, the capacity of the brain for elongation/desaturation was found to be lower in newborn monkeys than in older animals. In particular, Δ4 desaturation and formation of DHA in newborns was negligible (Figs. 3 and 4). The lower chain elongation/desaturation activity in monkey neonates observed in the present study is quite different from the results previously reported for rats (see above). The cause for this is unknown, but differences in the developmental stage of brain neural cells at parturition may be responsible for that, as primates are born at the end of the cell-stretching stage, while rat neonates are at the end of the cell-multiplication stage (47). Therefore, from the point of brain neural cell development, monkey neonates correspond to the stage of rats 10 days after birth, when Δ6 desaturase activity is decreased to very low levels (40). It appears reasonable to assume that the occurrence of DHA in primate colostrum compensates for the low biosynthetic activity in the neonate brain.

Besides the low desaturase activity, another factor responsible for the low capacity of long-chain PUFA biosynthesis may be the competitive compartmentalization of linolenate and its intermediate metabolites between acylation to form glycerophospholipids and desaturation. In Macaque monkey, all neurons have completed cell division during the 120 days of gestation, and after birth only the multiplication of glia can be observed (48). Furthermore, the size of the brain and the number of synapses reach almost steady levels after three years (49). The brain of a 6.5-year-old monkey used in the present study is thought to be mature. In neonatal monkeys, a marked increase of glia cells would occur. Therefore, the accelerated incorporation of PUFA into microsomal membranes of glia cells might result in a low rate of desaturation. However, the lipid analyses of astrocytes and oligodendrocytes in rats showed a very low content of n-3 PUFA other than DHA, similar to neurons (50). This observation again supports the idea of a specific affinity of DHA to neural tissues. Judging from these results, the compartmentalization of 18:3, or intermediate n-3 PUFA, in membrane phospholipids may not be the principal cause for the poor desaturation activity in monkey neonates.

Lowered capacity for long-chain PUFA synthesis in rhesus monkey neonates was also suggested by the observation that the plasma DHA level drastically decreased after birth (6,51). It has been suggested that Δ4-desaturase in humans (52–54) and monkeys (55) is much less predominant than Δ5 and Δ6 desaturase activities, because administration of linolenic acid or 20:5 resulted in accumulation of 22:5n-3 rather than DHA. Although the number of monkeys used in the present studies was quite limited, the data suggest the possibility that Δ4 desaturase activity in primate newborns is too low to produce sufficient DHA for brain development and that a large portion of the long-chain polyunsaturated fatty acids required may depend on maternal supply and/or the metabolites of the liver as source.

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