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Condensation activity for polyunsaturated fatty acids with malonyl-CoA in rat brain microsomes. Characteristics and developmental change

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Condensation activities for γ-linolenic acid (18:3($n-6$)), octadecatetraenoic acid (18:4($n-3$)) and eicosapentaenoic acid (20:5($n-3$)) with malonyl-CoA were measured and compared with the condensation activities for 16:0-CoA, 18:1-CoA, 18:2($n-6$)-CoA and 18:3($n-3$)-CoA in rat brain microsomes of various ages. The age-dependence of condensation activities for 18:3($n-6$), 18:4($n-3$) and 20:5($n-3$) showed a maximum at 1- to 2-month-old and were still higher at 3-month-old 2- to 3-fold than the activities in microsomes of pups. Conversely, the age-dependence of condensation activity for 16:0-CoA showed a peak around 1 month-old, but decreased at 3-month-old to the level of the activities in pups. The condensation activity for 20:5($n-3$) was inhibited by 18:3($n-6$) or 18:4($n-3$) and the inhibition was not competitive. The condensation of 18:3($n-6$) was also inhibited by 18:4($n-3$) in the same manner. A physiological implication of the inhibition system at the substrate level was discussed.

Introduction

Special attention has been paid to the role of $n-3$ and $n-6$ series of polyunsaturated fatty acids in the brain. Animals fed a $n-3$ fatty acid-deficient diet have a drastic decrease of $n-3$ fatty acids and compensatory increase of $n-6$ fatty acids in the phospholipids of the brain and retina [1–4]. Rats had abnormal electroretinograms [1] by $n-3$ fatty acid-deficient diets and monkeys developed visual impairment by 4 weeks of age [2]. The $n-3$ fatty acid-rich diet was reported to lead to improvement of several diseases [5,6] and of learning ability of animals [1,7,8].

Alternatively, mitochondrial damage is enhanced when mitochondrial membranes are enriched with $n-3$ fatty acids by a fish oil diet [9]. Recently we demonstrated that a fatty acid hydroperoxide inhibited the proton pump activity of cytochrome *c* oxidase and that the peroxide could be catabolized by cytochrome *c*. The balance between $n-6$ and $n-3$ series of polyunsaturated fatty acids may be an important factor for maintaining cell functions.

Normally the level of total polyunsaturated fatty acids remained almost constant in a steady state [10,11], even with the intake of various amounts of $n-6$ or $n-3$ series fatty acid-rich diets. However, some enzyme activities (Na/K-ATPase and desaturases) were affected by $n-6$ or $n-3$ fatty acid-rich diets [1,12].

There are several reports showing that a retroconversion system for very-long-chain polyunsaturated fatty acids is present to produce a more shorter chain fatty acid (e.g., 22:6($n-3$) → 20:5($n-3$) or 22:4($n-6$) → 20:4($n-6$)) in peroxisomes [13,14]. Thus the amount of polyunsaturated fatty acids may be regulated in the brain by: (1) transport of fatty acids from

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Abbreviations: BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid; TLC, thin-layer chromatography.

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blood stream or vessels [15]; (2) desaturation; (3) elongation; (4) retroconversion to a shorter chain or further β-oxidation; and (5) conversion to some biologically active materials from those fatty acids in a short time scale.

Since the desaturation activity in the brain is very low in the adult rat or mouse [16,17], a main portion of polyunsaturated fatty acids in brain may be transported from the blood stream and the regulation of the amount of very-long-chain polyunsaturated fatty acids in membrane phospholipids may be carried out by a fine control of elongation in microsomes and retroconversion (or β-oxidation) in peroxisomes, or other reactions such as acylation and lipase action to maintain the level of total polyunsaturated fatty acids.

The investigation of elongation and its control mechanism may contribute to understanding of the regulation mechanism of the amount of polyunsaturated fatty acids in the brain. Especially the condensation activity for polyunsaturated fatty acids with malonyl-CoA may be useful to estimate the overall elongation activity without effect of desaturation activity, since the condensation process is rate limiting and does not involve the reductant (i.e., NADPH) and thus the following desaturation and further elongations will not proceed. This allows estimation of the elongation activity as an isolated reaction.

Particularly, the measurement of overall elongation activity should be cautious when successive desaturations occurred considerably. The desaturation reaction may affect the precedent elongation reaction [18]; that is, a precedent elongation activity for 16:0-CoA was affected by the following desaturation (Δ^9) of the elongation product (18:0-CoA) and a correct evaluation of the elongation activity may be difficult.

In this report we have demonstrated that the condensation activity for polyunsaturated fatty acid is high in the post-myelination period and that the inhibition of condensation activity for a fatty acid by the other fatty acid may contribute to keep the change of total amount of condensation products as small as possible.

Materials and Methods

Male (Sprague-Dawley) rats were purchased from Iffa-Credo (France) with various ages. [2-^{14}C]Malonyl-CoA and [1-^{14}C]palmitoyl-CoA were purchased from NEN (Dupont, France). γ-Linolenic acid (18:3($n-6$)), octadecatetraenoic acid (18:4($n-3$)), eicosapentaenoic acid (20:5($n-3$)), fatty acid-free bovine serum albumin, adenosine triphosphate disodium salt, malonyl-CoA, linoleoyl-CoA, linolenoyl-CoA, oleoyl-CoA were from Sigma Chemical (St. Louis, MO, U.S.A.). The protein assay reagent using bicinchoninic acid was from Pierce (Rockford, IL, U.S.A.). Hexane, sucrose and other routine chemicals were from Merck (Darmstadt, F.R.G.).

Preparation of rat brain microsomes of various ages

Rats were killed by dislocation of the cervical vertebrae after anesthetization with diethyl ether and the cerebra were excised followed by rinse with ice-cold phosphate-buffered saline and homogenized with a Potter apparatus in 50 mM phosphate buffer (pH 7.4) with 0.32 M sucrose and 1 mM EDTA.

Brain microsomes were prepared principally by the reported method [19]. Briefly, the crude microsomal pellet obtained by centrifugation of the brain homogenate at 105000 $\times g$ for 1 h (or at 250000 $\times g$ for 20 min) was suspended in 0.32 M sucrose-50 mM phosphate buffer (pH 7.4) with 1 mM EDTA and further fractionated. When microsomes were prepared from brains of rats older than 15 days, the microsomal suspension was layered on a sucrose gradient with 0.9 M and 1.25 M sucrose in 50 mM phosphate buffer (pH 7.4), with 0.1 mM EDTA and centrifuged at 100000 $\times$ g for 90 min to avoid the contamination of myelin and other particles. The fraction between 0.9 M and 1.25 M sucrose phases was used as a microsomal fraction.

For 3- and 7-day-old rat brains the main microsomal fraction appeared in the sucrose gradient between 0.8 M and 0.9 M sucrose (containing 50 mM phosphate buffer (pH 7.4) and 0.1 mM EDTA) and a small fraction between 0.7 M and 0.8 M sucrose. Both fractions and the mixture of these fractions showed a similar specific activity of condensation for palmitoyl-CoA. Finally the mixture of both fractions was used as a microsomal fraction for 3- and 7-day-old rat brains.

In any cases, the microsomal fraction was then washed by 0.15 M KCl in 50 mM Hepes buffer (pH 7.4) with 0.1 mM EDTA and finally suspended in 0.32 M sucrose in 50 mM Hepes buffer (pH 7.4) with 0.1 mM EDTA and stored at -80°C until use. Protein concentration was measured by the BCA method [20] using bovine serum albumin (Pierce) as a standard.

Condensation assay

Condensation activities were measured principally according to the reported method [21] except the final detection using a liquid scintillation counter.

The assay procedures using free fatty acid or acyl-CoA as a substrate were different. For free fatty acid, the microsomes were washed with the fatty acid-free BSA to remove endogenous free fatty acids before assay of condensation as follows.

Microsomes (0.16 mg protein/ml) in 0.15 M KCl with 50 mM Hepes buffer (pH 7.4) were mixed with BSA (0.65 mg/ml) and sonicated in a bath-type sonicator with a maximum output (Ultrasonik 300; NEY, CA, USA) for 2 min. After incubation at room temperature for 30 min, the microsome-BSA mixture was layered on

0.32 M sucrose in 50 mM Hepes buffer (pH 7.4) with 0.1 mM EDTA and centrifuged at 327000 × *g* for 20 min. The resultant microsomal pellet was suspended in 0.32 M sucrose-Hepes buffer ('BSA-washed microsomes').

Various amounts of free fatty acids in ethanol were dried by flushing with argon gas and mixed with argon-saturated phosphate buffer (0.1 M) containing BSA-washed microsomes (0.1 mg protein).

The mixture was sonicated for 2 min and allowed to stand at 26°C for 30 min. The reaction was started by the addition of 1 mM ATP to the reaction mixture containing 0.1 mg BSA-washed microsomes, 1 mM $MgCl_2$ and 52 μM [^{14}C]malonyl-CoA (0.08 μC_i) in a total volume of 0.5 ml, continued for 20 min at 37°C and terminated by the addition of 0.5 ml of 15% KOH in methanol. After saponification at 65°C for 30 min, followed by neutralization by phosphoric acid, the products were extracted in hexane. The radioactivity was measured using Beckman HP scintillation cocktail and a liquid scintillation counter. Radioactivity of the control (in the absence of ATP) was subtracted from that of the sample. The time-course of condensation under this condition was linear until 20 min. Addition of free CoA up to 10 μM did not change the condensation activity, but rather inhibited the activity at more than 50 μM.

This shows that the amount of free CoA in the assay system is sufficient to progress the condensation reaction for free fatty acid through synthesis of acyl-CoA and the step of acyl-CoA formation does not affect the condensation step kinetically.

For condensation of acyl-CoA substrates, the reaction mixture contained 0.1 M phosphate buffer (pH 7.4), 0.3 mg of fatty acid-free BSA/ml, 12.8 μM acyl-CoA, 52 μM [^{14}C]malonyl-CoA (0.08 μC_i). The condensation was started by the addition of 0.1 mg of microsomal protein without washing by BSA. The mixture did not include ATP and $MgCl_2$. The remaining procedure was the same as that described above and background radioactivity determined in the absence of microsomes was subtracted from that of the sample.

For acyl-CoAs, the condensation activity was optimum at around 13 μM and at more than 20 μM the activity was decreased steeply. The maximum activity for acyl-CoA is almost identical to the activity for saturable amount of the same fatty acid substrate under the present assay condition (data not shown).

Thin-layer chromatography

TLC plates were prepared according to the reported procedure [17] especially designed to separate polyunsaturated fatty acid methyl esters on silicagel 60 plates (Merck).

The initial condensation product (3-keto compound) was decarboxylated by saponification to form methyl ketone [21], i.e., 3-keto-20:3 → 19:3-2-one (methyl ketone). The methyl ketone product could be separated, similar to the methyl ester compounds, using a solvent mixture (petroleum benzine (b.p. 40–60°C)/diethyl ether; 1:1, v/v) on a silica gel plate. The methyl ketone product is referred to 'condensation product' in this paper.

The products extracted in hexane were mixed with carrier fatty acid methyl esters [17]. The labelled compounds were detected by a linear analyzer (Berthold, F.R.G.), setting 15 min for scanning of one sample lane.

The bands of TLC plates were scraped and the products were extracted in chloroform/methanol (2:1, v/v) mixture. The extracts were counted by a liquid scintillation counter to confirm the reliability for linearity of the TLC radioanalyzer.

For condensation assays used for TLC analysis, the reaction mixture contained 0.4 mg microsomal protein and 28 μM [^{14}C]malonyl-CoA (0.1 μC_i) and the assay procedure was the same as that described above.

Results

Condensation activities for polyunsaturated fatty acids

We first optimized the assay condition to measure the condensation activity for exogenous free fatty acid substrates with a liquid scintillation counter. It was essential to decrease the condensation activity for endogenous fatty acids (mainly 16:0 and 20:4) as much as possible using a 'BSA-washed' microsomes.

In Fig. 1, the peak intensities of condensation products observed in TLC were summarized with various amounts of 18:3($n-6$). When 18:3($n-6$) was added

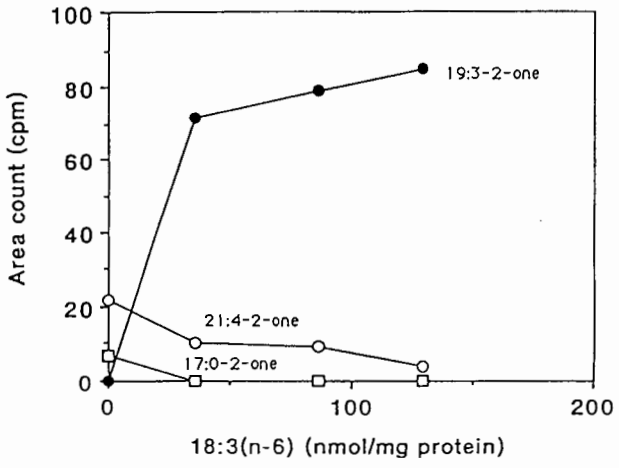

Fig. 1. Dependence of condensation activity for 18:3($n-6$) acid and endogenous fatty acids on the amount of substrate 18:3($n-6$) in BSA-washed microsomes detected by a linear analyzer. When 18:3($n-6$) substrate was used at 130 nmol/mg protein, the condensation activities for endogenous substrates were decreased to 14% of those without the exogenous substrate. In this assay 0.4 mg microsomal protein was used after washing with BSA.

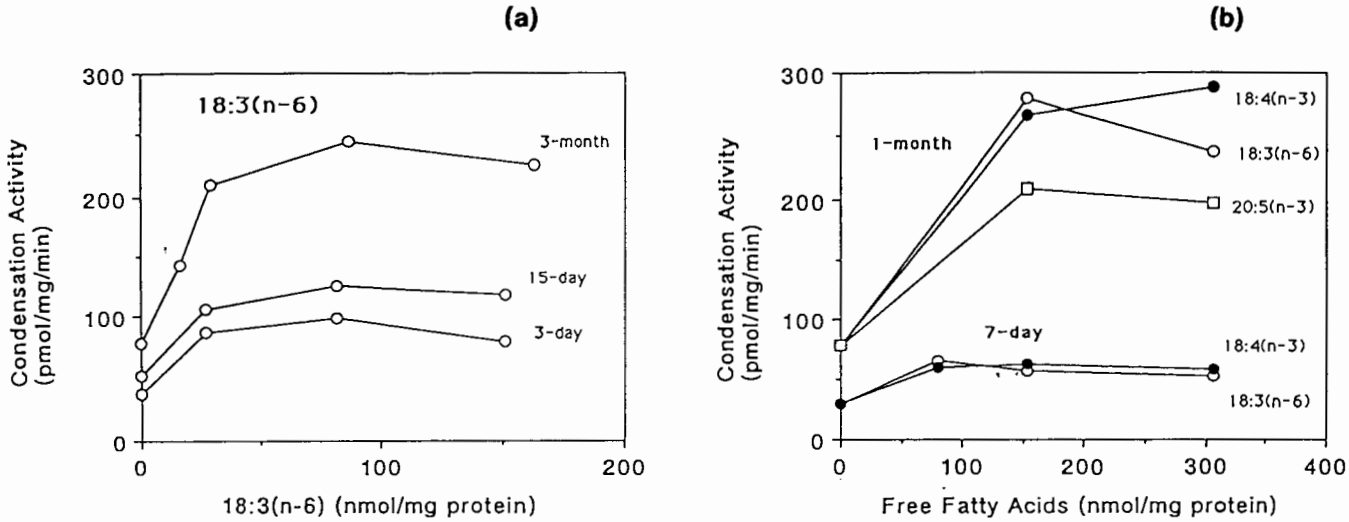

Fig. 2. Dependence of condensation activity for various fatty acids on concentration of each substrate in rat brain microsomes at various ages. Each data point is the mean value of duplicate determinations for one preparation. (a) 18:3($n-6$) condensation at ages of 3-day-, 15-day- and 3-month-old. (b) condensations for 18:3($n-6$), 18:4($n-3$) and 20:5($n-3$) at ages of 7-day- and 1-month-old.

to microsomes at 150 nmol/mg protein, the condensation activity for 18:3($n-6$) was nearly maximum and the activities for endogenous substrates became negligible (below 5% of the total radioactivity).

The dependence of condensation activity on concentration of exogenous fatty acids showed saturation curves in brain microsomes from rats of various ages as shown in Fig. 2(a) and 2(b) when the concentration of exogenous fatty acid was below 300 nmol/mg protein. In Fig. 2(a), the dependence of activity on the relatively low concentration of 18:3($n-6$) was shown for microsomes from 3-, 15-day-, and 2-month-old rat brains. The patterns are similar with each other, but there are large differences in maximum activities of condensation in brain microsomes from pups and young adult rats.

In Fig. 3(a) and 3(b), we summarized the age-dependent optimal condensation activities for polyunsaturated fatty acids (in (a)), poly-, mono-unsaturated and saturated acyl-CoAs (in (b)). In this case a saturated amount of exogenous fatty acid was used to minimize the contribution of activity for endogenous fatty acids. The age-dependence of 16:0-CoA condensation showed that the activity was the highest at 1 month-old, but only 40–50% higher than that of pups, and the activity at 3 month-old was nearly at the level of pups.

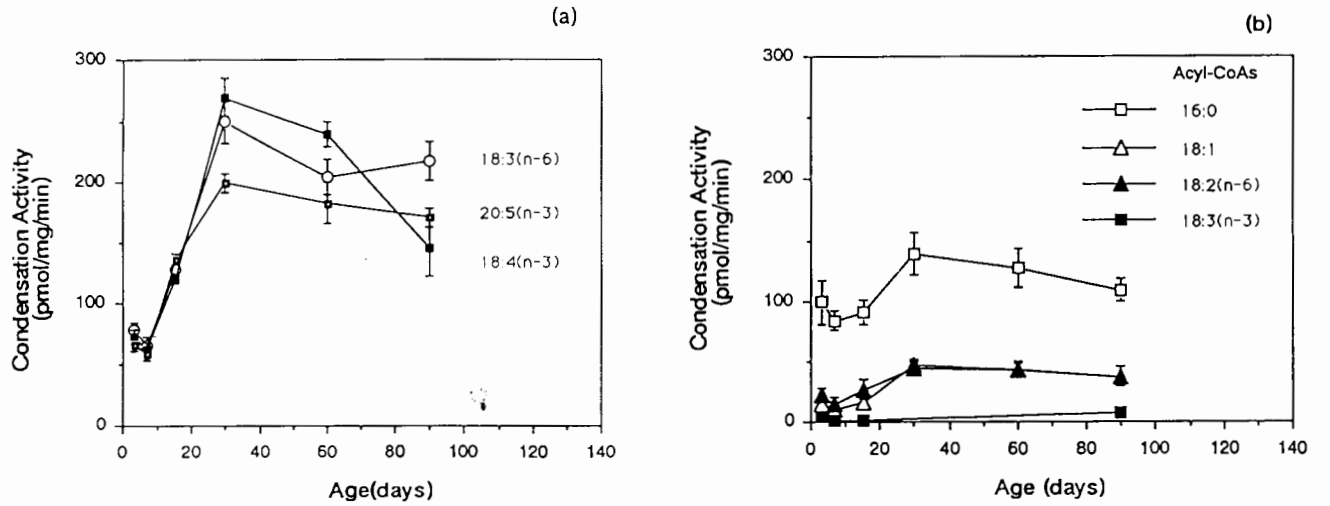

Fig. 3. Age-dependence of condensation activity for various fatty acids in rat brain microsomes. (a) Condensations for 18:3($n-6$), 18:4($n-3$) and 20:5($n-3$). Values are the mean of two sets of experiments (with duplicate determinations) using two preparations of microsomes of various ages and the standard errors are presented by error bars. (b) Condensations for 16:0, 18:1, 18:2($n-6$) and 18:3($n-3$) in CoA ester forms at 12.8 μM.

This pattern is similar to the overall elongation of 16:0-CoA which showed a peak at the age of 20–30 days, the active myelination period [22] in rat brain.

On the contrary, the pattern for polyunsaturated fatty acids, especially for 18:3($n-6$), 18:4($n-3$) and 20:5($n-3$), showed that the activities were the highest at the age of 1 month and were kept high at 2- to 3-month-old, a post-myelination period. The activity for 18:4($n-3$) at 3 month-old was decreased more than those for 18:3($n-6$) and 20:5($n-3$), but that for 18:4($n-3$) at 1- to 2-month-old was 4 to 4.5-times higher than that at 7-day-old. The activities for 18:3($n-6$) and 20:5($n-3$) were almost constant at 2- to 3-month-old and nearly 3-times higher than that for pups.

The activities for 18:2($n-6$)-CoA at 1–3 month-old were higher than those for pups by nearly 2-times. The activity for 18:3($n-3$)-CoA was very low at all ages tested. The pattern of age-dependence of 18:1-CoA condensation was similar to that of 18:2-CoA, but the activities for 18:1-CoA at 1–3-month-old were nearly 4-times the activity at 7-day-old.

The condensation activities for 18:3($n-6$), 18:4($n-3$) and 20:5($n-3$) in the post-myelination period were much higher than those at pups and than that for saturated and monounsaturated fatty acids and even than that of 18:2($n-6$)- and 18:3($n-3$)-CoA.

We compared the optimal activities for those substrates and apparently there was no difference in the optimal activity between 18:2($n-6$)-CoA and 18:2($n-6$) acid (data not shown). The difference between condensation activities for polyunsaturated fatty acids (Fig. 3a) and acyl-CoAs (Fig. 3b) may not be caused by the type of substrate (i.e., free acid or acyl-CoA), but by the configuration of fatty acid.

These differences in condensation activity for various unsaturated fatty acids may suggest that the unsaturated fatty acids with double-bonds more nearer than the 6th position from the carboxy terminal had much higher activity of condensation than other unsaturated fatty acids examined as shown in Fig. 4. The optimal condensation activity for 20:4($n-6$) was higher than that for 16:0-CoA and nearly 70% of that for 18:3($n-6$) (data not shown).

It may be most probable that the three-dimensional configuration of the fatty acid in microsomal membrane is a critical factor for the substrate specificity of condensing enzyme. However, the information of exact configuration of the fatty acid in membrane is not available at present. Graphically the position of the first double bond seems more critical for the fatty acid configuration necessary to the optimal condensation activity than that of the last double bond.

Inhibition of condensation activity for 20:5 by 18:3 or 18:4

In the synthesis of 20:4($n-6$), the main product from 18:2($n-6$), there is one elongation step (18:3($n-6$) → 20:3) and in the synthesis of 22:6($n-3$) from 18:3($n-3$), there are at least two elongation steps (18:4($n-3$) → 20:4 and 20:5($n-3$) → 22:5). We checked the mutual inhibition pattern of condensation activities in the presence of multiple substrates to estimate the presence of multiple condensing enzymes like the case for long- and very-long-chain saturated and monounsaturated fatty acid [23].

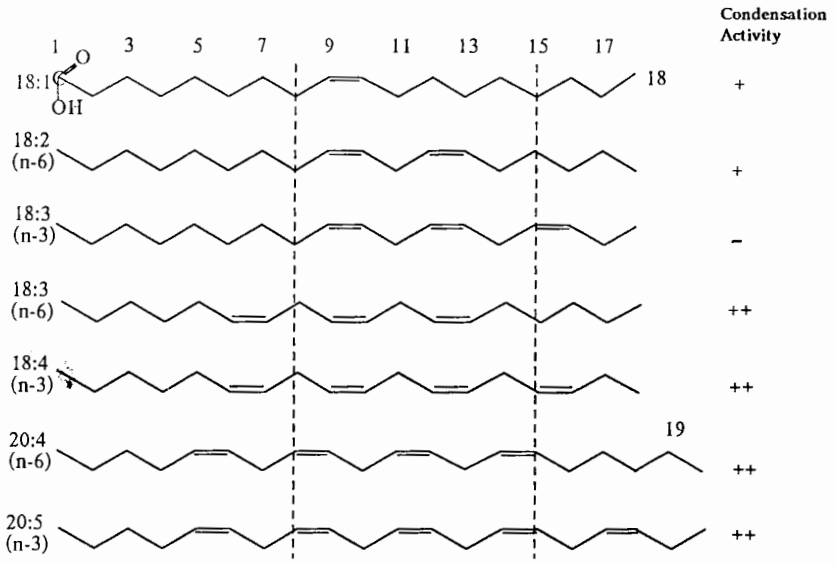

Fig. 4. Relationship between structures of fatty acids and condensation activities. The condensation activity for 18:3($n-3$) is very small and those for 18:2($n-6$) and 18:1($n-9$) are in the middle class, those for 18:3($n-6$), 18:4($n-3$), 20:4($n-6$) and 20:5($n-3$) are in the top class.

The dependence of condensation activity for 20:5($n-3$) on the concentration of the substrate showed a typical Michaelis-Menten type. As shown in Fig. 5(a) the condensation activity for 20:5 was inhibited by 18:3($n-6$) and 18:4($n-3$) nearly in the same manner and the maximum activity for 20:5 was decreased by those inhibitor fatty acids. This result indicates that the inhibition by those fatty acids is not competitive practically in an enzymological sense.

When a model of non-competitive or un-competitive inhibition was applied to these data, the non-linear least-square regression analysis of the data with and without the inhibitor showed $K_m = 14 \pm 4$ nmol/mg (%CV = 25.5), $V_{max} = 111 \pm 5$ cpm/20 min (%CV = 4.1), $K_i = 178 \pm 19$ nmol/mg (%CV = 10.9) for the non-competitive model, and $K_m = 16 \pm 3$ nmol/mg (%CV = 19.8), $V_{max} = 114 \pm 4$ cpm/20 min (%CV = 3.5), $K_i = 148 \pm 14$ nmol/mg (%CV = 9.1) for the un-competitive model. Both types of inhibition could not be discriminated concerning to the present data.

It is not likely to explain this inhibition profile just by the competition with malonyl-CoA substrate, be-

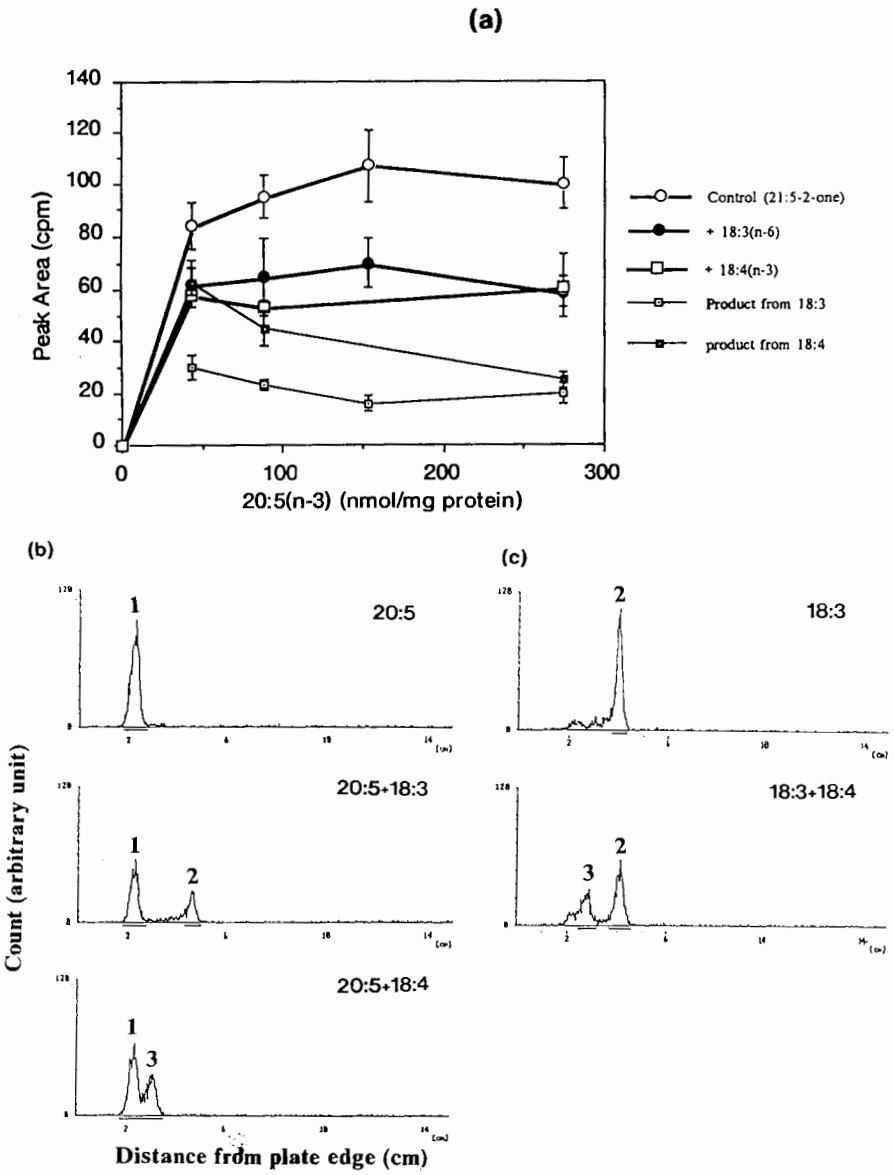

Fig. 5. Analysis of condensation activity for 20:5($n-3$) and 18:3($n-6$) by TLC method and the inhibition by various fatty acids. (a) Dependence of condensation on the concentration of 20:5($n-3$). The maximum activity of condensation of 20:5 was decreased by addition of 18:3($n-6$) or 18:4($n-3$) at 80 nmol/mg protein. The condensation products from 18:3 and 18:4 were decreased also by 20:5. The product was quantitated by measuring the peak area in the TLC radioanalyzer and denoted in a unit of cpm. Each value is the mean of triplicate determinations for one preparation. (b) Scanning profile for the condensation product (peak 1) from 20:5($n-3$) at 300 nmol/mg protein in the absence and presence of 18:3($n-6$) (peak 2) or 18:4($n-3$) (peak 3) at 80 nmol/mg protein. (c) Scanning profile for 18:3 condensation product (peak 2) in the absence and presence of 18:4($n-3$) (peak 3) at 80 nmol/mg protein.

cause a nearly saturable amount of malonyl-CoA was used for this experiment. Since the rate of acyl-CoA synthesis is much more rapid than the condensation rate and the addition of CoASH does not increase the condensation activity for fatty acid substrates with a saturable amount, but rather inhibits (data not shown), the effect of change of acyl-CoA synthesis step on the condensation activity may be negligible.

In Fig. 5(b), the scanning profiles of condensation products by the TLC radioanalyzer were shown for 20:5($n-3$) at 274 nmol/mg protein (top), for 20:5 with 18:3(80 nmol/mg protein)(middle) and for 20:5 with 18:4(80 nmol/mg protein)(bottom), on silver nitrate-impregnated silica gel plates.

In Fig. 5(c), we examined the inhibition of 18:3($n-6$) condensation by 18:4($n-3$) and the scanning profiles of condensation products were shown. In the top figure, the product from the saturable amount (306 nmol/mg protein) of 18:3($n-6$) was observed and in the bottom the condensation products were seen from 18:3($n-6$) and 18:4($n-3$). The maximum activity for 18:3($n-6$) was largely decreased by the addition of 18:4($n-3$) at 80 nmol/mg protein in a similar fashion to Fig. 5(b). This result also shows that the inhibition of 18:3 condensation by 18:4 was not competitive in an enzymological sense.

Although the present data can not be fit to the competitive inhibition model, the exact inhibition type may not be identified at present. We may not exclude a possibility that the inhibition is a mixed type of both competitive (but small contribution, if present) and un- or non-competitive (practically major contribution) inhibitions. The important point, however, is that the present data is practically fit to the non-competitive inhibition model with a large change of maximum velocity and this model may be sufficient to analyze the actual behavior of a system containing multiple substrates for multiple condensing enzymes.

A non-specific disturbance of free fatty acid substrate to the condensing enzyme may be small because the condensation in the presence of excess amount (100–300 nmol/mg) of substrate is still active as shown in Fig. 2 and 5. In contrast, acyl-CoA inhibited strongly its own condensing enzyme at more than 100 nmol/mg (using 0.1 mg microsomal protein) (data not shown, or Ref. 24) and this inhibition may be ascribed to a non-specific detergent effect of the amphiphilic acyl-CoA.

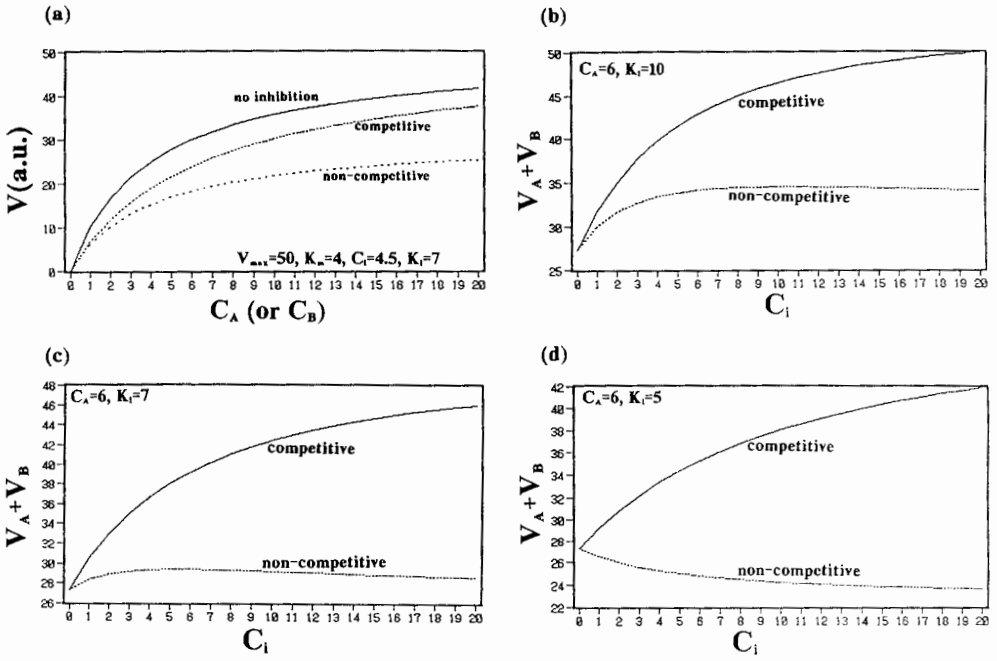

Fig. 6. Simulation analysis of change of total reaction products from two substrates with a competitive or non-competitive mutual inhibition under various conditions. Formulas for these simulation analysis used are;

$V = V_{max} \cdot C_s / \{K_m \cdot (1 + C_i / K_i) + C_s\}$ for competitive inhibition,

$V = V_{max} \cdot C_s / \{(K_m + C_s) \cdot (1 + C_i / K_i)\}$ for non-competitive one,

where C_s is a substrate concentration. (a) C_A, concentration of substrate A; C_B, concentration of substrate B; V, activity; C_i, concentration of inhibitor (substrate A against the activity for B); K_i, inhibition constant. In this case, as an example, values for V_{max}, K_m, C_i and K_i are 50, 4, 4.5 and 7 in an arbitrary unit, respectively. (b) Sum of products ($V_A + V_B$) when $K_i = 10$. (c) $V_A + V_B$ when $K_i = 7$, (d) $V_A + V_B$ when $K_i = 5$.

In order to realize the implication of the presence of non-competitive inhibition system for condensing enzymes in elongation of various polyunsaturated fatty acids, we consider a system containing two kinds of fatty acids, for simplicity, which would be elongated with the same kinetic parameters and simulate a case where one of the fatty acids inhibits the elongation of the other fatty acid with a competitive or a non-competitive manner. By this simulation we could estimate which type of inhibition would be useful to keep the change minimum for the total products from both fatty acid substrates when the amount of one of the substrates was changed.

In Fig. 6, the results of such simulations were summarized for a simple system with two kinds of substrates. It is clear that the change of the amount of total products from fatty acids (A and B), which occurred with changing the amount of fatty acid B and maintaining the amount of fatty acid A, can be minimized only when the inhibition type is a non-competitive one. For this analysis, the basic formula $V = V_{max} \cdot C_s/\{(K_m + C_s)*(1 + C_i/K_i)\}$ was used, where C_s, C_i, K_i, V, V_{max} and K_m were the substrate concentration, inhibitor concentration, inhibition constant, velocity, maximum velocity and Michaelis constant, respectively. For two-substrates system, the total activity, $V_A + V_B$, is described by a non-competitive inhibition model as:

$$V_A + V_B = V_{max} \cdot C_A / \{(K_m + C_A) \cdot (1 + C_B / K_i)\} + V_{max} \cdot C_B / \{(K_m + C_B) \cdot (1 + C_A / K_i)\},$$

and for actual simulation the constant values of C_A, K_m, V_{max} were used and $C_B (= C_i)$ was varied with a certain value of K_i. Simply this graph indicates the change of total products from substrates A and B when the inhibitor (substrate B) was changed. When the un-competitive inhibition model was applied, similar results were obtained.

When the inhibition type is competitive, the amount of total products is increased inevitably when increasing the amount of the other substrate. For this model, the formula $V = V_{max} \cdot C_s/\{K_m \cdot (1 + C_i/K_i) + C_s\}$ was used.

This simulation data showed that the non- (or un)-competitive type of inhibition contributed to maintain the total products more effectively than the competitive type inhibition. There was a case where the total products were decreased when increasing the amount of inhibitor substrate with a small value of K_i in a non-competitive inhibition model.

Discussion

In this report, we have shown that: (1) the condensation activities for 18:3($n-6$), 18:4($n-3$) and 20:5($n-3$) were much higher in the post-myelination period than those in the pups and this pattern was different from that for 16:0-CoA; and (2) the inhibition of condensing enzyme for 20:5($n-3$) by 18:3($n-6$) or 18:4($n-3$) and for 18:3($n-6$) by 18:4($n-3$) was not a competitive type.

Age-dependence of condensation activity

Overall elongations of polyunsaturated fatty acids were studied by Cook [24], although data for elongation activities in the post-myelination period were not presented sufficiently. He only pointed out that changes in elongation activity for polyunsaturated fatty acids during early brain development indicated increased microsomal activity during the suckling period with a decline in specific activity in adult brain and that the pattern of age-dependence of the enzyme activity corresponded apparently with that of active myelination.

However, the detailed analysis of age-dependence, as we reported in this paper, demonstrated that the condensation activity, a rate-limiting step of elongation reaction, for polyunsaturated fatty acids during development was different from that for palmitoyl-CoA which showed the maximum activity around 1 month-old and declined to the level of pups in adult rats. The elongation activity for polyunsaturated fatty acid may not only correspond to the active myelination, but also to the post-myelination period.

The age-dependence of various desaturation activities in the brain has been well studied [16,17] and the desaturation activity was the greatest in the neonatal brains with a steep decrease in the activity during the developmental period in the brain. From this reason most of the polyunsaturated fatty acids in the brain such as docosahexaenoic acid were supplied from blood and the liver might be the most important organ for this supply [25].

On the other hand it was reported that there was a retroconversion system for very-long-chain polyunsaturated fatty acids in peroxisomes in several types of cells to shorten C22 or C20 fatty acids to C20 or C18 [26,27]. It is likely to speculate that the microsomal elongation system for polyunsaturated fatty acids in the brain may be high enough in the post myelination period to compensate for lack of the desaturation activity and for the retroconversion system to regulate the amount of polyunsaturated fatty acids in membranes.

Inhibition of condensation activity by fatty acids

It is important to note that the inhibition of 20:5($n-3$) condensation by 18:3($n-6$) or 18:4($n-3$) could not be overcome by increasing the amount of 20:5 as described in the result section and this result can be fit to the non-(or un-) competitive inhibition model and strongly suggests that several enzymes are concerned in condensation for fatty acids with various chain lengths

and various degrees of unsaturation. But the presence of separate enzymes for 20:5, 18:3 and 18:4 is not convincing from the kinetic data alone. Irrespective of this difficulty, the kinetic inhibition pattern (Fig. 5 and 6) may help us to speculate suitably the regulation mechanism of elongation of many polyunsaturated fatty acids at the substrate level.

It has been reported that several kinds of condensing enzymes are also involved in elongations of 16:0, 16:1 and 18:3($n-6$) [23]. Several kinds of condensing enzymes and 3-keto acyl-CoA reductases were involved in elongations of fatty acids (16:0, 20:0 and 20:4) [28,29].

It was speculated [23] that a separate condensing enzyme would lead to self-regulation by each substrate and product and this may be useful to maintain a high level of each fatty acid in the membrane. Actually the level of total polyunsaturated fatty acids in phospholipids for both $n-6$ and $n-3$ series in the brain [2] or plasma [11] or other tissues in rat [10] is maintained almost constant irrespective of intake of various amounts of $n-6$ or $n-3$ series fatty acids (i.e., safflower oil or fish oil) from diets.

Lands et al. [10] reported that the 20-carbon highly unsaturated fatty acids of the $n-3$, $n-6$ and $n-9$ types were maintained in a competitive hyperbolic relationship in many rat tissues and they illustrated an underlying principle of competition among fatty acids for a limited number of esterification sites.

In general the level of a particular fatty acid in phospholipids may be regulated by a steady state balance in transport, synthesis (elongation and desaturation), degradation (retroconversion, β-oxidation) and esterification of fatty acids. There is no unique specificity for esterification of any of the different types of fatty acids and it helps to emphasize the vital importance of the initial selection of the proportions of the acids [10].

It is reported that almost all β-oxidation enzymes in peroxisomes act on the whole range of substrates from short-chain to long-chain acyl-CoAs [30] and this suggests that there is no unique specificity for β-oxidation of any of the different types of polyunsaturated fatty acids in peroxisomes.

On the other hand, a report represented the existence of a distinct very-long-chain acyl-CoA synthetase which might function in the activation of very-long-chain saturated and polyunsaturated fatty acids [31]. Especially it was reported that inhibition of docosahexaenoyl-CoA synthetase by arachidonic acid or other fatty acids was not a competitive type [32]. However, the step of acyl-CoA synthesis may not be a rate-limiting for the overall synthesis of polyunsaturated fatty acids.

A fine tuning of elongation and degradation of polyunsaturated fatty acids would be necessary to maintain the level of polyunsaturated fatty acids in cell membranes. A separate enzyme system for elongation of a distinct polyunsaturated fatty acid would be useful for this purpose and this system would lead to a non-competitive inhibition by the other fatty acids.

The simulation study as shown in Fig. 6 suggests that a non-competitive inhibition system by multiple fatty acid substrates may be useful for an 'enzymological strategy' of a cell to maintain the amount of total polyunsaturated fatty acid products in phospholipids when various kinds and amounts of fatty acids are incorporated into the brain from various diets. In another words, the non-competitive inhibition affects the velocity largely and eventually the system with multiple substrates can get easily a homeostatic state.

Very recently it was reported by Rosenthal et al. [33] that the ratio of C22 and C20 polyunsaturated fatty acids in human cells is regulated by the relative rates of retroconversion and chain elongation and they also pointed out a possibility that the apparent Δ^4 desaturation (22:5($n-3$) → 22:6) may be the result of reactions involving chain-elongations (20:5 → 22:5 → 24:5), Δ^6 desaturation (24:5 → 24:6) and subsequent retroconversion (24:6 → 22:6). We showed previously that the successive elongation pathway from 20:4($n-6$) to 24:4 was present in brain microsomes [19].

Cook and Spence [34] have proposed a model using cultured cell lines to show that the desaturation, chain elongation and esterification of the exogenous essential fatty acids may involve a concerted or closely coordinated system, especially in the formation of arachidonic acid.

Although the regulation mechanism of amount of polyunsaturated fatty acids in cell membrane phospholipids is not fully elucidated, the present results thus provide useful informations for understanding the mechanism.

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