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The Synthesis and Hydrolysis of Long-Chain Fatty Acyl-Coenzyme A Thioesters by Soluble and Microsomal Fractions from the Brain of the Developing Rat

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1. The specific activities of long-chain fatty acid–CoA ligase (EC 6.2.1.3) and of long-chain fatty acyl-CoA hydrolase (EC 3.1.2.2) were measured in soluble and microsomal fractions from rat brain. 2. In the presence of either palmitic acid or stearic acid, the specific activity of the ligase increased during development; the specific activity of this enzyme with arachidic acid or behenic acid was considerably lower. 3. The specific activities of palmitoyl-CoA hydrolase and of stearoyl-CoA hydrolase in the microsomal fraction decreased markedly (75%) between 6 and 20 days after birth; by contrast, the corresponding specific activities in the soluble fraction showed no decline. 4. Stearoyl-CoA hydrolase in the microsomal fraction is inhibited (99%) by bovine serum albumin; this is in contrast with the microsomal fatty acid-chain-elongation system, which is stimulated 3.9-fold by albumin. Inhibition of stearoyl-CoA hydrolase does not stimulate stearoyl-CoA chain elongation. Therefore it does not appear likely that the decline in the specific activity of hydrolase during myelogenesis is responsible for the increased rate of fatty acid chain elongation. 5. It is suggested that the decline in specific activity of the microsomal hydrolase and to a lesser extent the increase in the specific activity of the ligase is directly related to the increased demand for long-chain acyl-CoA esters during myelogenesis as substrates in the biosynthesis of myelin lipids.

Sphingolipids are major constituents of myelin (approx. 25%, w/w) and their acyl components have characteristically long chains ($C_{24:0}$, $C_{24:1}$) (O'Brien, 1965). The formation of these fatty acids by elongation of C_{16}-CoA and C_{18}-CoA by microsomal fractions from rat brain increases dramatically at the onset of myelogenesis (Brophy & Vance, 1975). The biochemical basis for the rapid increase in the rate of elongation of fatty acyl-CoA esters is not known. One possibility that we considered was a change in the supply of acyl-CoA available for chain elongation. Two enzymes that are responsible for the synthesis and hydrolysis of C_{16}-CoA and C_{18}-CoA esters in the microsomal fraction are long-chain fatty acid–CoA ligase (EC 6.2.1.3) and long-chain fatty acyl-CoA hydrolase (EC 3.1.2.2) respectively. We have measured the specific activities of these two enzymes during myelogenesis to determine whether changes in the relative rates of synthesis and hydrolysis of these acyl-CoA esters could account for the observed increase in elongation activity. A preliminary account of this work has already appeared (Brophy & Vance, 1976).

Materials

The animals and some of the chemicals have been described (Brophy & Vance, 1975). All fatty acids, as well as bovine serum albumin (fatty acid-depleted) and 5,5′-dithiobis-(2-nitrobenzoic acid), were from Sigma Chemical Co., St. Louis, MO, U.S.A. [G-^{3}H]CoA (specific radioactivity 800 mCi/mmol) and [1-^{14}C]stearoyl-CoA (specific radioactivity 62 mCi/mmol) were from New England Nuclear, Boston, MA, U.S.A. [U-^{14}C]Behenic acid (docosanoic acid) (specific radioactivity 770 mCi/mmol) was from Applied Science Laboratories, State College, PA, U.S.A. Millipore filters (25 mm, type HAWP, 0.45 μm, white), were from Millipore Corp., Bedford, MA, U.S.A., and Triton WR-1339 was from Ruger Chemical Co., Irvington, NJ, U.S.A.

Methods

Preparation of subcellular fractions

This was as described by Brophy & Vance (1975) except that dithiothreitol was omitted from all buffers when fractions were prepared in which hydrolase activity was measured by the spectrophotometric method. [U-^{14}C]Behenyl-CoA (0.5 mCi/mmol) was synthesized by the method of Al-Arif & Blecher (1969). The product was purified by preparative chromatography on Whatman 3MM paper developed in butan-1-ol/acetic acid/water (5:2:3, by vol.).

Enzyme assays

Long-chain fatty acid–CoA ligase. This activity was measured by the rate of incorporation of [G-^{3}H]CoA into acyl-CoA as described by Polokoff & Bell (1975) except that the assay temperature was 37°C. Each assay contained 400mM-Tris/HCl buffer, pH7.4, 8mM-$MgCl_2$, 10mM-ATP, 0.5mM-dithiothreitol, 50μM-[G-^{3}H]CoA (10mCi/mmol), 1mg of Triton WR-1339/ml and an optimum concentration of fatty acid (30μM-palmitic acid, 30μM-stearic acid, 30μM-arachidic acid or 15μM-behenic acid) in a total volume of 200μl. Reactions were started by adding microsomal protein and stopped after 10min with fatty acid-depleted bovine serum albumin (25μg) and 2.0ml of ice-cold 0.3M-trichloroacetic acid. The incubation mixtures were kept at 0°C for 5min before filtration through Millipore filters (0.45μm pore size). The filters were washed with 3×2.0ml of cold 0.3M-trichloroacetic acid and the radioactivity was measured after dissolution in 10ml of Tritosol (Fricke, 1975). The incorporation of radioactivity into acyl-CoA was linear with respect both to time (up to 20min) and protein concentration (up to 15μg). Routine controls included incubations that contained either boiled microsomal fraction or no fatty acid.

Palmitoyl-CoA hydrolase activity. This was measured at 37°C as described by Bonner & Bloch (1972). Each assay contained 0.1M-potassium phosphate buffer, pH7.4, 1mM-5,5′-dithiobis-(2-nitrobenzoic acid) and 80μM-palmitoyl-CoA in a total volume of 1.0ml. Reactions were started by adding the microsomal or soluble fraction and the change in E_{412} was measured. Control incubations were performed with either no palmitoyl-CoA or no enzyme. The rate of hydrolysis was linear with respect to both time (up to 6min) and protein concentration (up to 60μg).

Stearoyl-CoA hydrolase activity. This was determined as described by Vance *et al.* (1973). The optimum concentration (80μM) of [1-^{14}C]stearoyl-CoA (1mCi/mmol) was incubated with 0.1M-potassium phosphate buffer, pH7.4, and microsomal or soluble fraction in a total volume of 0.5ml for 10min at 37°C. The reactions were stopped by adding 1M-HCl (100μl) and immediately chilled to 0°C. [1-^{14}C]Stearic acid was extracted from the incubations with 2×5ml of light petroleum (b.p. 30–60°C). The solvent was evaporated and the radioactivity of the residue determined as described previously (Brophy & Vance, 1975). Incubations that contained no enzyme were used as routine controls; the incorporation was linear with respect to both time (up to 20min) and protein (up to 50μg).

Behenyl-CoA hydrolase activity. This was measured as described for stearoyl-CoA hydrolase except that the substrate was [U-^{14}C]behenyl-CoA (0.5mCi/mmol). The rate of hydrolysis was linear with respect to both time (up to 20min) and protein concentration (up to 50μg).

Fatty acid chain elongation. This was measured by the rate of incorporation of [2-^{14}C]malonyl-CoA (10.3mCi/mmol) into long-chain fatty acids in the presence of stearoyl-CoA as described by Brophy & Vance (1975).

Measurements of radioactivity and protein have been described by Brophy & Vance (1975).

Results and Discussion

Changes in the microsomal synthesis of long-chain acyl-CoA during development

Microsomal fractions from brains were prepared from three groups of rats at each age; each group consisted of three animals. Incubation mixtures contained between 4 and 10μg of microsomal protein. The rate of incorporation of [G-^{3}H]CoA (10mCi/mmol) into acyl-CoA was measured in the presence of either palmitic acid (30μM), stearic acid (30μM), arachidic acid (30μM) or behenic acid (15μM) as described in the Methods section. Fig. 1 shows that the activation of both palmitic acid and stearic acid exhibit similar developmental patterns. Both activities are significantly higher at 28 as compared with 6 days after birth ($P<0.02$, by a paired t test); however, there is no large increase in specific

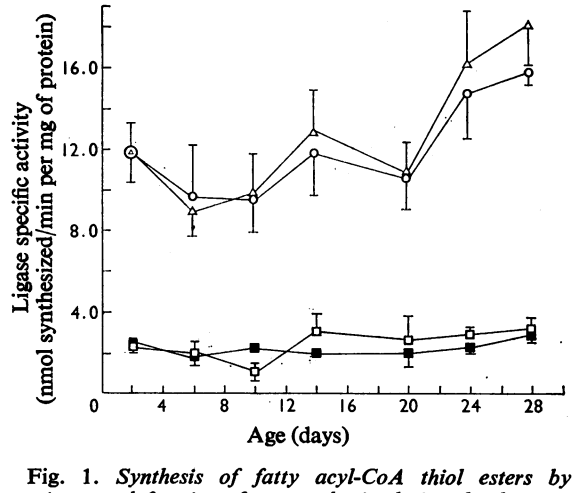

Fig. 1. *Synthesis of fatty acyl-CoA thiol esters by microsomal fractions from rat brain during development*

Microsomal fractions (4–10μg of protein) prepared from three pooled brains were incubated with [G-^{3}H]CoA (10mCi/mmol) as described in the Methods section in the presence of 30μM-palmitic acid (○), 30μM-stearic acid (△), 30μM-arachidic acid (□) or 15μM-behenic acid (■). Ligase specific activity is expressed as the mean±S.D. for three different microsomal preparations.

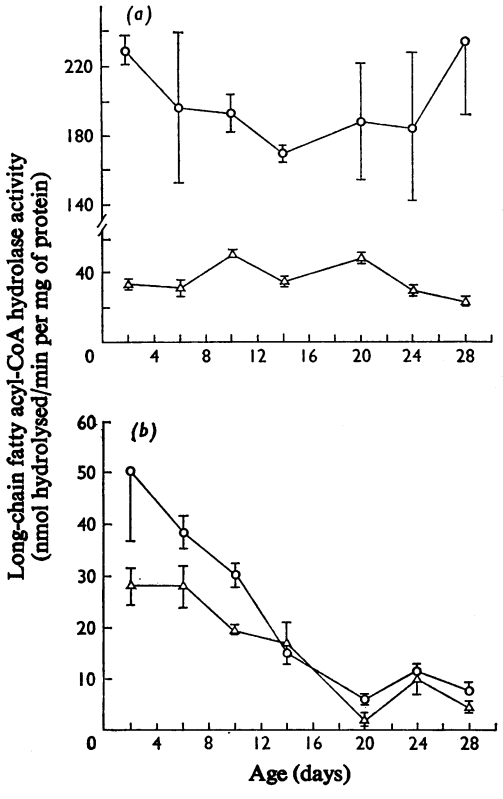

Fig. 2. *Hydrolysis of acyl-CoA thiol esters during development by (a) soluble fraction and (b) microsomal fraction*

Subcellular preparations (30–45 μg of protein) from the pooled brains of three rats were incubated with 80 μM-palmitoyl-CoA (○) or 80 μM-[1-^{14}C]stearoyl-CoA (△) as described in the Methods section. The specific activity of acyl-CoA hydrolase is shown as the mean±S.D. for three different microsomal preparations at each age.

activity at the onset of myelination, 10 days after birth. The chain elongation of fatty acids in rat brain has been shown to increase between 10 and 20 days after birth both *in vitro* (Brophy & Vance, 1975) and *in vivo* (Sinclair & Crawford, 1972). Hence from our results it appears unlikely that changes in the rate of activation of fatty acids to their CoA thiol esters are responsible for the large increase in specific activity of fatty acyl-CoA elongation. This is in contrast with the synthesis of palmitoyl-CoA by rabbit brain microsomal fraction, in which significant increases in specific activity during the early stages of myelogenesis were observed by Cantrill & Carey (1975).

The rates of synthesis of arachidyl-CoA and of behenyl-CoA from the corresponding fatty acids were about 25% of those rates observed for formation of palmitoyl-CoA and stearoyl-CoA (Fig. 1); these rates did not increase significantly between days 6 and 28 ($0.1 > P > 0.05$, by a paired t test). Hence there may be two distinct long-chain fatty acid–CoA ligases in the microsomal fraction (one with a specificity for C_{16} and C_{18} fatty acids, the other with a chain-length specificity for C_{16} and C_{18}, or possibly there is a single enzyme with a substrate specificity for C_{16} and C_{18} fatty acids.

No appreciable long-chain fatty acid–CoA ligase activity was found in the soluble fraction.

Changes in long-chain acyl-CoA hydrolase during development

There was a sharp decrease in the specific activities of both palmitoyl-CoA hydrolase and stearoyl-CoA hydrolase in the microsomal fraction during development, as shown in Fig. 2(*b*). The specific activity at 28 days after birth was significantly lower than that at 6 days ($P > 0.01$). This is in marked contrast with the developmental pattern observed in the soluble fraction [Fig. 2(*a*), where no significant changes were observed during a similar period ($P > 0.5$, by a paired t test)]. Further, the microsomal hydrolase appears to have a similar specificity for palmitoyl-CoA and stearoyl-CoA. It is unlikely that the microsomal hydrolase activity is a contaminant from the soluble fraction, since it displays a different developmental pattern from the soluble activity. In addition, the microsomal preparations were highly purified from soluble contaminants, i.e. less than 2% of the cytosolic fatty acid synthetase could be detected in the microsomal fraction (Brophy & Vance, 1975).

We then tested the microsomal fractions for behenyl-CoA hydrolase activity, because long-chain acyl-CoA esters are potent inhibitors of many enzymes involved in fatty acid synthesis (Hsu & Powell, 1975), for example the chain-elongation activity of the soluble fatty acid synthetase from Mycobacteria (Vance *et al.*, 1973). Thus the increase in chain-elongation activity during the onset of myelogenesis in rat brain (Brophy & Vance, 1975) might be due to an increase in C_{22}- or C_{24}-CoA hydrolysis, which would relieve feedback inhibition of elongation activity by C_{22}- and C_{24}-CoA. The behenyl-CoA hydrolase specific activity was found to be low (1 nmol/min per mg of protein) and did not appear to change during development; this suggested that the hydrolase did not play a role in the control of fatty acid chain elongation.

Most enzymes concerned with cerebral lipid metabolism increase in specific activity during development and especially during myelogenesis. Therefore it is probably significant in the overall regulation of lipid biosynthesis that specific activities of the microsomal C_{16}-CoA hydrolase and C_{18}-CoA

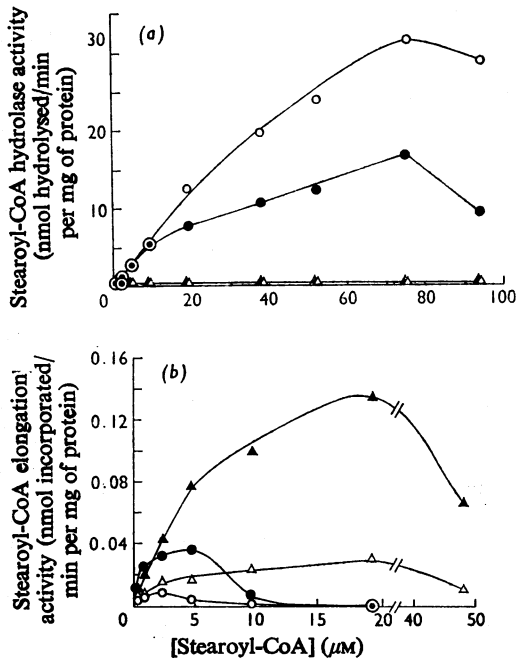

Fig. 3. *Effect of bovine serum albumin on the specific activities of (a) stearoyl-CoA hydrolase and (b) stearoyl-CoA elongation in microsomal fractions from 5- and 19-day-old rats*

Each incubation mixture contained 30μg of microsomal protein derived from four rats at each age and the assays were conducted as described in the Methods section. The specific activities of stearoyl-CoA hydrolase and stearoyl-CoA elongation were measured in microsomal fractions from 5- (○, without albumin; △, with albumin) and 19-day-old rats (●, without albumin; ▲, with albumin) as described in the Methods section.

hydrolase decrease during the active phase of myelogenesis. As a result, it is possible that more C_{16}-CoA and C_{18}-CoA are available as substrates for chain elongation and this might be responsible for the observed increase in the rate of fatty acid elongation. Hence we tested whether or not a decrease in the specific activity of the hydrolase would result in an increased rate of fatty acid chain elongation.

Effect of bovine serum albumin on the elongation and hydrolysis of stearoyl-CoA

We tested the effect of bovine serum albumin on the specific activity of stearoyl-CoA hydrolase (Fig. 3*a*) and of stearoyl-CoA elongation (Fig. 3*b*) by using brain microsomal fractions prepared from 5- and 19-day-old rats. Fig. 3(*a*) shows that microsomal fractions from 5-day-old rats have substantially more stearoyl-CoA hydrolase activity than microsomal fractions prepared from 19-day-old animals. The addition of 3mg of bovine serum albumin before the assay resulted in virtually complete inhibition of the stearoyl-CoA hydrolase activity from both brain preparations.

Since the hydrolysis of stearoyl-CoA was inhibited with albumin, we could measure the effect of decreased specific activity of the hydrolase on the elongation of stearoyl-CoA by microsomal fractions from 5- and 19-day-old rat brains. Fig. 3(*b*) shows that, in agreement with previous results (Brophy & Vance, 1975), the specific activity of stearoyl-CoA elongation in the absence of albumin is higher at 19 than at 5 days. We initially speculated that this increased elongation activity at 19 days might have resulted solely from a decrease in stearoyl-CoA hydrolase specific activity at 19 days without an increased amount of elongation enzyme. If this were true, inhibition of the hydrolase from 5-day-old rats by albumin should have resulted in an elongation activity similar to that observed for the 19-day-old rat microsomal fractions. The results in Fig. 3(*b*) show that, in the presence of albumin, the enzyme specific activities in both preparations are stimulated 3.9-fold and the optimum concentration of stearoyl-CoA for elongation activity increases from approx. 3 to 20μM. Since there are still large differences in the elongation activities of microsomal preparations from 5- and 19-day-old animals, the decrease in specific activity of stearoyl-CoA hydrolase during myelogenesis appears to be unrelated to the large increase in the specific activity of fatty acid elongation.

The interpretation of our results is complicated by the observed stimulation of the chain elongation at both ages by albumin. One explanation for the stimulation is that the elongation enzyme prefers stearoyl-CoA as a monomer and this activity is inhibited by micelles of stearoyl-CoA. The albumin would bind stearoyl-CoA and effectively provide more stearoyl-CoA in a protein-bound monomeric form for chain elongation. This hypothesis is supported by the data in Fig. 3(*b*). Maximum elongation activity without albumin occurs at about 3μM-stearoyl-CoA, which is in the range of the critical micellar concentration for this substrate (Barden & Cleland, 1969). When albumin is added there is no significant stimulation of chain elongation at low concentrations of stearoyl-CoA; however, above 2μM-stearoyl-CoA the elongation activity in the presence of albumin continues to increase. Hence the albumin prevents the apparent micellar inhibition of the fatty acid elongation system.

In contrast with the stimulation of chain elongation by albumin, the microsomal hydrolase [and the soluble hydrolase from rat brain (Anderson & Erwin, 1971)] is dramatically inhibited by albumin. Barden & Cleland (1969) have attributed a similar effect by albumin on the palmitoyl-CoA hydrolase in rat liver

microsomal fractions to a preference for substrate in the micellar form.

Conclusions

The sharp decline in the specific activity of microsomal palmitoyl-CoA hydrolase and stearoyl-CoA hydrolase during the most active phase of myelogenesis is the most striking observation from the present work. This change in specific activity together with the increase in the rate of fatty acid activation during development will result in an increased availability of fatty acyl-CoA esters for phospholipid and sphingolipid biosynthesis. By inhibition of the microsomal hydrolase with bovine serum albumin, we were able to show that this hydrolase does not seem to control the rate of fatty acid chain elongation during myelogenesis. It now appears likely that increased chain-elongation activity results from increased protein synthesis of the enzymes involved.

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