Glutamate Formed from Lignoceric Acid by Rat Brain Preparation in the Presence of Pyridine Nucleotide and Cytosolic Factors: A Brain-Specific Oxidation of Very Long Chain Fatty Acids†

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ABSTRACT: When [1-^{14}C]lignoceric acid was incubated with a rat brain particulate fraction in the presence of heat-stable and heat-labile factors and NADPH, considerable radioactivity was found in the water-soluble fraction. These conditions were similar to those for the synthesis of cerebronic acid (α-hydroxylation) and ceramide synthesis. The water-soluble product was purified to apparent homogeneity by combining gel filtration, steric exclusion high-performance liquid chromatography, and CM-Sepharose chromatography. The purified material was identified as glutamic acid by an NMR spectrum, high-performance liquid chromatography with three different columns, ninhydrin test, and thin-layer chromatography–radioautography. The incorporation of radioactivity into glutamate from lignoceric acid absolutely required heat-stable and heat-labile factors. NADPH could be replaced by NADP, NADH, or NAD with nearly equal results. α-Cyclodextrin increased the glutamate formation 3–5-fold, but it could not replace the heat-stable factor. CoA and ATP did not increase the activity. CoA had an inhibitory effect. The enzymatic reaction was inhibited by EDTA, respiratory chain inhibitors, and an uncoupler. This glutamate formation was not detected in liver, kidney, and spleen. The radioactivity from [1-^{14}C]palmitic acid was also converted to a water-soluble material. The conversion required the two cytosolic factors but was not stimulated by α-cyclodextrin. Although the extraneural tissues, especially liver, could also produce a water-soluble material, the cytosolic factors were not required for this. These observations suggest that in brain there is a unique type of oxidation which has a high specificity for longer chain fatty acids.

The α-hydroxylation of lignoceric acid (tetracosanoic acid) and its conversion to ceramide require two cytosolic factors from rat brain or calf cerebellum and NADPH, in addition to a particulate fraction from rat brain (Singh & Kishimoto, 1979). We have recently discovered that during incubation considerable radioactivity from [1-^{14}C]lignoceric acid is converted to an unknown water-soluble material, in addition to nonhydroxyceramide, hydroxyceramide, nonhydroxycerebroside, and hydroxycerebroside. This communication documents the identification of this compound as glutamic acid. The formation of glutamic acid requires all the cytosolic factors, NADPH, and the rat brain enzyme preparation. The velocity of this reaction appears to be much greater than that of brain β-oxidation.[1] In addition, this enzymatic reaction appears to be brain specific and favored for the oxidation of longer chain fatty acids. Preliminary results of these findings have been presented (Uda et al., 1980).

Experimental Procedures

Materials. [1-^{14}C]Lignoceric acid (56 mCi/mmol) was synthesized in this laboratory as described previously (Hoshi & Kishimoto, 1973). [1-^{14}C]Palmitic acid (57 mCi/mmol) was obtained from New England Nuclear. D-*erythro*-Sphingosine was purchased from Miles Laboratories, Inc., and further purified in this laboratory as described previously (Shoyama et al., 1978). CoA lithium salt, NADPH, and all other pyridine nucleotides were purchased from P-L Biochemicals. L-Carnitine was a gift from Otsuka Pharmaceutical Co. ATP was purchased from Sigma Chemical Co. Precoated, 0.25-mm-thick silica gel plates (K5) were obtained from Whatman. X-ray films (no-screen, NS-5T) were from Kodak. Bio-Gel P-4 and Bicine were obtained from Bio-Rad; Sephadex G-25 and G-50 and CM-Sepharose CL-6B were purchased from Pharmacia.

Preparation of Enzyme and Factors. The enzyme preparation was essentially similar to that described previously for the α-hydroxylation assay (Singh & Kishimoto, 1979). The brains from 21- to 28-day-old Sprague–Dawley rats (Charles River CD) were homogenized in 2 volumes (w/v) of buffer containing 0.154 M KCl, 40 mM Bicine, pH 7.3, 1 mM $MgCl_2$, and 1 mM EGTA. The homogenate was first centrifuged at 800*g* for 15 min and then at 12000*g* for 30 min. The second sediment was suspended in the same buffer in one-third the volume of the 12000*g* supernatant. Crude heat-stable and heat-labile factors were prepared as described previously (Singh & Kishimoto, 1979). The heat-stable factor was prepared from calf cerebellum by extracting it with water. After removal of the insoluble material by centrifugation, the extract was heated, and the denatured protein was removed by centrifugation. The supernatant was lyophilized, and the residue was dissolved in water in one-tenth the original volume. This solution was mixed with 5 volumes of chloroform–methanol (2:1) and centrifuged. The upper layer was evaporated. Salts and small molecular contaminants were removed by UM-2 Amicon membrane ultrafiltration after the residue was dissolved in a small volume of water. The crude heat-labile factor was also prepared from calf cerebellum by homogenizing it with 2 volumes of buffer containing 0.154 M KCl, 20 mM Bicine, pH 7.3, 1 mM EGTA, and 1 mM $MgCl_2$. The homogenate was centrifuged, and the cytosol supernatant was fractionated by ammonium sulfate precipitation. The precipitate between 50% and 70% saturation was dissolved in 10 mM Bicine buffer, pH 7.3, and dialyzed.

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[1] N. Kawamura and Y. Kishimoto, unpublished results.

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Enzyme Assay. Most of the assay conditions for glutamate formation were similar to those described previously for α-hydroxylation (Singh & Kishimoto, 1979) and ceramide synthesis (Singh & Kishimoto, 1980). The reaction mixture was in a total volume of 0.5 mL and consisted of 4 μM [1-^{14}C]lignoceric acid (56 mCi/mmol) coated on Celite, 0.4 mM NADPH, 1 mM $MgCl_2$, 40 mM Bicine buffer, pH 7.3, crude heat-labile factor containing approximately 1 mg of protein, and crude heat-stable factor containing approximately 70 μg of carbohydrate. The reaction was initiated by adding rat brain particulate fraction containing approximately 1.5–2.5 mg of protein, and it was continued for 45 min at 37 °C. The reaction was stopped by adding 10 mL of chlororform–methanol (1:1) and centrifuging. Chloroform (5 mL) and 2.5 mL of water were mixed with the supernatant and centrifuged. A portion (2 mL) of the upper phase was then evaporated to dryness under a flow of nitrogen. The residue was dissolved in 1.5 mL of water, and the radioactivity was measured after adding 10 mL of Triton X-100 toluene-based scintillation mixture.

The α-hydroxylation activity was assayed as described previously (Akanuma & Kishimoto, 1979). The lower layer of the Folch partition was evaporated to dryness. The residue was methanolyzed by methanolic HCl, and the fatty acid methyl esters obtained were fractionated by thin-layer chromatography on a silica gel G plate. The radioactivity in the spot of methyl cerebronate was measured. The $^{14}CO_2$ produced during the incubation was assayed by absorbing it in KOH as described previously (Murad & Kishimoto, 1975). The recovery of $Na_2{}^{14}CO_2$ was 83.7%.

High-Performance Liquid Chromatography. The basic equipment consisted of two Spectra-Physics pumps, a programmer, a pressure monitor, a sample injector, and a Schoeffel spectromonitor as described previously (Nonaka & Kishimoto, 1979a). A train of steric exclusion columns consisting of one 7.5 mm (i.d.) × 10 cm and three 7.5 mm (i.d.) × 30 cm stainless steel tubes packed with TSK-Gel G3000 SW (Toyo Soda Co.) were used. The conditions for the chromatograph are described in the legend to Figure 1. The effluents for each peak were collected. After evaporation, the residue was dissolved in 1.5 mL of water and 10 mL of Triton X-100 toluene-based scintillation fluid, and the radioactivity was counted.

Other Analytical Procedures. The NMR spectrum was taken with a JOEL FX-100 spectrometer by Dr. Lou-Sing Kan. The sample was repeatedly lyophilized from 99.7% deuterated water (Aldrich Chemicals Co.) and then finally dissolved in 0.4 mL of the same solvent. Protein was measured by the method of Lowry et al. (1951). Carbohydrate was determined by the phenol–sulfuric acid method (Dubois et al., 1956).

Results

Isolation of Glutamic Acid from the Incubation Product. When a 50-μL portion of the water-soluble fraction of the incubation mixture containing 1350 dpm was injected into the high-performance liquid chromatography system equipped with a steric exclusion column, many peaks appeared on the chromatogram as shown in Figure 1. Most of the injected radioactivity was recovered from a relatively small peak, no. 13, as shown in Table I. For isolation of this material, 20 standard incubation mixtures were pooled and centrifuged at 105000*g* for 60 min, and the supernatant was lyophilized. The residue, which contained 116 000–138 000 dpm, was dissolved in a small volume of 2.5 mM Bicine buffer, pH 7.3, and transferred to a column, 2.6 cm (i.d.) × 30 cm, containing

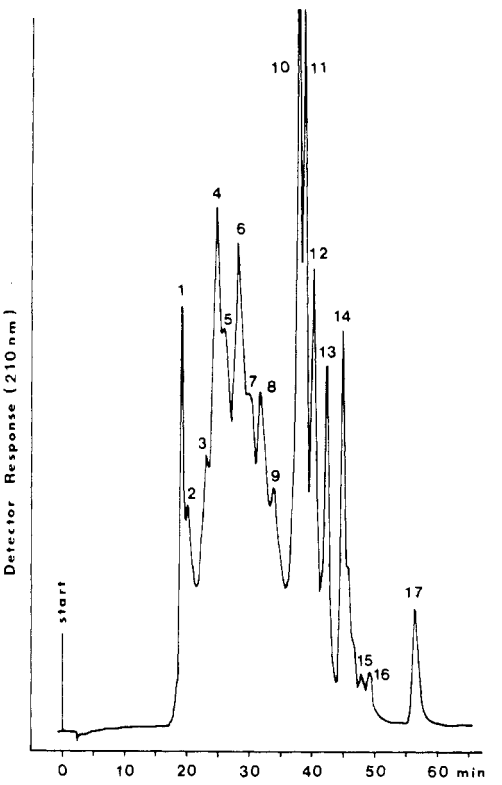


FIGURE 1: A high-performance liquid chromatogram of the water-soluble fraction of the incubation mixture. Spectra-Physics–Schoeffel equipment with a 7.5 mm (i.d.) × 1 m column containing TSK-Gel G3000 SW was used. The fraction (50 μL) containing 1350 dpm was injected, and the column was eluted with 10 mM potassium phosphate buffer, pH 7.2, at a flow rate of 0.85 mL/min. The column effluent was monitored at 210 nm and collected. The radioactivity in each peak is shown in Table I. Information regarding the equipment and column is given in the text.

Table I: Distribution of the Radioactivity in the Water-Soluble Fraction of the Incubation Mixture[a]

peak no.[b]	radioactivity[c] (dpm)	peak no.[b]	radioactivity[c] (dpm)
1	17	10	7
2	5	11	77
3	8	12	35
4	7	13	831
5	27	14	39
6	7	15	5
7	5	16	4
8	19	17	6
9	22		

[a] See legend to Figure 1 for high-performance liquid chromatography separation. [b] Peak numbers correspond to the peak numbers in Figure 1. [c] The total radioactivity injected was 1350 dpm, and the total recovery was 1121 dpm (83%).

Sephadex G-50 packed in the same buffer. The column was eluted with the same buffer at the rate of 30 mL/h, 6-mL fractions were collected, and the radioactivity in each was measured. A small peak of radioactivity appeared immediately after the void volume, and a single peak emerged in fractions 25–30, which contained from 46% to 60% of the radioactivity applied.

The same fractions from three identical runs were pooled and lyophilized. The residue, which contained 179 000 dpm, was further fractionated on a column, 2.6 cm (i.d.) × 40 cm, containing Sephadex G-25 fine. This column was eluted with the same buffer at a flow rate of 25 mL/h, and 3-mL fractions were collected. A single peak of radioactivity was obtained in fractions 53–60, which contained 101 600 dpm, a 74% re-

covery rate. These fractions were pooled and rechromatographed twice on the same Sephadex G-25 column. The same single peak was obtained with a recovery rate of 66% each time. During these gel filtrations, the fractions were monitored for 280-nm absorption. On this basis, most of the UV-absorbing material was removed by the chromatography. In the final gel filtration, only a small amount of 280-nm absorption was associated with the radioactive peak.

A portion of the final product was analyzed by high-performance liquid chromatography as described in the legend of Figure 1. The chromatogram showed two small peaks corresponding to no. 12 and 13 and a large peak corresponding to no. 14 of Figure 1. Ninety-five percent of the radioactivity was recovered in peak 13. This radioactive material was further purified by repeated injections into high-performance liquid chromatography, and peak 13 was collected from each run. From material containing 65 700 dpm, we were able to recover 66% of the radioactivity. The final product of this repeated high-performance liquid chromatography showed a single peak of radioactivity corresponding to a single peak at 210 nm. Finally, this material was desalted by gel filtration on a column, 1.6 cm (i.d.) × 30 cm, containing Bio-Gel P-4. The column was eluted with water, and 3-mL fractions were collected. Eighty percent of the applied radioactivity was recovered in fractions 19–21, which contained 34 500 dpm.

A portion of the radioactive compound containing 25 000 dpm was mixed with 0.5 mL of the crude heat-stable factor, approximately 2 mg of protein, prepared as described above, to obtain larger amounts of this material. The mixture was purified by repeated fractionation on CM-Sepharose and Bio-Gel P-4. Two observations prompted this approach. First, we found that the crude heat-stable factor contains considerable amounts of material corresponding to peak 13. High-performance liquid chromatography analysis disclosed that the crude heat-stable factor consists of four major peaks corresponding to no. 11, 12, 13, and 14 of Figure 1, with peak 12 the most prominant. Second, the high-performance liquid chromatography separation pattern can be reproduced on CM-Sepharose chromatography. One quarter of the above mixture was placed on a column, 2.6 cm (i.d.) × 30 cm, containing CM-Sepharose CL-6B. The column was first eluted isocratically with 200 mL of 10 mM sodium acetate buffer, pH 4.9, and then with a 400-mL linear gradient of the same buffer containing from 0 to 0.25 M NaCl. Fractions of 3 mL were collected, and the radioactivity, absorption at 280 nm, and carbohydrate content were measured. A single radioactive peak containing 18 000 dpm, 75% of the original radioactivity, appeared in fractions 35–37 at approximately 0.03 M NaCl. This was repeated 3 times.

The combined radioactive fractions, containing a total of 60 400 dpm, were applied to the Bio-Gel P-4 column under the conditions described above. Fractions of 2 mL were collected, and the radioactive peak appeared in fractions 23–25, which contained 51 700 dpm, an 85% recovery rate. This material was then further purified by repeating the CM-Sepharose column chromatography and Bio-Gel P-4 gel filtration. The CM-Sepharose chromatography effectively removed the contaminating UV-absorbing material, and the Bio-Gel P-4 gel filtration removed the contaminating carbohydrates. When lyophilized, the final product was a colorless solid with no absorption in the 260–280-nm range and no phenol–sulfuric acid positive material.

Identification of Glutamic Acid. The purified radioactive material described above was identified as glutamic acid as a result of the following observations. (1) NMR spectrum:

Table II: Comparison of the Purified Product and Several Organic and Amino Acids by High-Performance Liquid Chromatography

compounds	retention times (min) G-3000 SW[a]	Ultrasil CX[b]	Ultrasil SX[c]
reaction product	38.39	4.28	5.28
acetyl-CoA	30.51	2.24	
γ-aminobutyric acid	46.26		3.38
L-aspartic acid	38.58	3.52	7.12
citric acid	33.70	2.34	11.18
L-glutamic acid	38.39	4.24	5.24
L-glutamine	45.7	6.01	3.52
succinic acid	35.04	3.25	9.02

[a] Column, 7.5 mm (i.d.) × 1 m, containing TSK-Gel G3000 SW (steric exclusion; Toyo Soda Co.) was eluted with 10 mM phosphate buffer, pH 7.2, at a flow rate of 0.85 mL/min. [b] Column, 4.6 mm (i.d.) × 25 cm, containing Ultrasil CX (cation exchange; Altex Scientific Inc.) was eluted with 10 mM CH_3COOH–CH_3COONa buffer, pH 3.6, at a flow rate of 1 mL/min. [c] Column, 4.6 mm (i.d.) × 25 cm, containing Ultrasil SX (anion exchange; Altex Scientific, Inc.) was eluted with 30 mM KH_2PO_4–HCl buffer, pH 3.5, at a flow rate of 1 mL/min.

Material weighing approximately 2.0 mg was dissolved in deuterated water and its NMR spectrum measured, as described under Experimental Procedures. The spectrum showed an AX_2-type triplet for methine proton at δ 4.05 and two A_2B_2X-type triplets for methylene protons as the only significant signals. This spectrum is almost identical with the published spectrum of glutamic acid (Pouchent & Campbell, 1975). (2) High-performance liquid chromatography retention times: The retention times for the purified material were identical with those of L-glutamic acid with three different columns and conditions (Table II). (3) Thin-layer chromatography–radioautography: Purified material containing approximately 5000 dpm was spotted on a silica gel K-5 plate and developed with phenol–water (3:1 w/v) with standards of L-glutamic acid, L-aspartic acid, L-glutamine, and γ-aminobutyric acid as described previously (Kawamura & Kishimoto, 1981). The plate was then exposed to an X-ray film for 1 week. The radioautogram, subsequent spot elution, and counting indicated that nearly 90% of the radioactivity was associated with the L-glutamic acid spot. Slightly over 5% of the radioactivity was detected in the spot corresponding to glutamine and γ-aminobutyric acid, which were inseparable from one another, and the last 5% was found in the original spot. There was no detectable radioactivity in the spot corresponding to L-aspartic acid. (4) Ninhydrin test: The material gave a positive ninhydrin test.

Characterization of the Enzymatic Conversion. Table III shows that NADPH and heat-stable and heat-labile factors are all essential for the formation of glutamate from [1-^{14}C]lignoceric acid. As in α-hydroxylation (Hoshi & Kishimoto, 1973; Singh & Kishimoto, 1979) and ceramide synthesis (Singh & Kishimoto, 1980), NADPH can be replaced by NADP, NADH, and NAD (Table III); similarly, ATP slightly stimulates and CoA inhibits the reaction, as shown in table III. Also, as shown in Table III, EDTA inhibits the enzyme activity, thus indicating that Mg^{2+} is essential. Sphingosine, which stimulates α-hydroxylation and ceramide synthesis approximately 3-fold, inhibits the glutamate formation. We also observed that respiratory chain inhibitors, such as 1 mM KCN, 0.2 mM rotenone, and 0.2 mM antimycin A, and an uncoupler, 1 mM dinitrophenol, are strongly inhibitory (data not shown).

The right-hand column of Table III shows that when [1-^{14}C]palmitic acid, a typical substrate of regular β-oxidation,

Table III: Cofactor Requirements for the Formation of Glutamate

reaction mixture	glutamate formation (% of control) from [1-^{14}C]-lignoceric acid	[1-^{14}C]-palmitic acid
complete system[a]	100	100
−NADPH	2	3
−NADPH + 0.4 mM NADP	94	84
−NADPH + 0.4 mM NADH	92	153
−NADPH + 0.4 mM NAD	54	147
−heat-stable factor	2	3
−heat-labile factor	3	5
+10 mM ATP	120	130
+1 mM CoA	64	49
+10 mM ATP + 1 mM CoA	107	66
+0.2 mM D-*erythro*-sphingosine	87	66
+1 mM EDTA	2	11

[a] Complete system contained brain particulate fraction, heat-stable factor, heat-labile factor, and NADPH (0.4 mM), as described under Experimental Procedures. The complete system oxidized 17.1 pmol per mg of protein per h and 80.4 pmol per mg of protein per h of [1-^{14}C]lignocerate and [1-^{14}C]palmitate, respectively, after subtracting the blank values. After heat-deactivation of the brain particulate fraction, there were 2.3 and 8.1 pmol per mg of protein per h for each fatty acid, respectively.

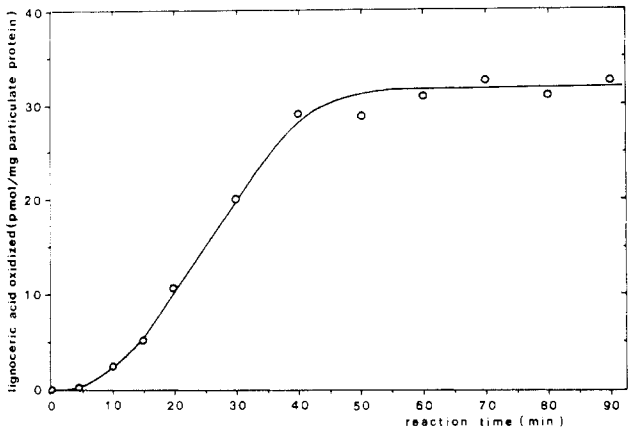


FIGURE 2: Lignoceric acid oxidation time course. The incubation product was measured by determining the radioactivity in the Folch upper layer. See text for details.

was used instead of lignoceric acid it was oxidized by a similar enzyme system. However, there were a few minor differences in the nature of the enzymatic reaction, stimulation by L-carnitine and a higher rate of oxidation by NAD or NADH than by NADP and NADPH.

The enzymatic activity had a pH optimum of 7.3 with a Bicine buffer. As in the case of α-hydroxylation, the radioactivity in the Folch upper layer of the reaction product increased linearly on increasing the amount of crude heat-labile factor to 1.60 mg of protein and crude heat-stable factor to 0.15 mg of protein. The activity then leveled off with further increases in the amounts of either heat-stable or heat-labile factors. There was, however, a lag when up to 0.5 mg of particulate protein was added. This initial lag was observed to a greater extent in α-hydroxylation (Singh & Kishimoto, 1979) and ceramide synthesis (Singh & Kishimoto, 1980). After the lag, the activity increased linearly with up to more than 2 mg of particulate protein. In the time–course study with standard conditions, the activity lagged before it increased linearly from 5 to 45 min and then leveled off (Figure 2).

Further oxidation of glutamate to CO_2 apparently occurs at a much slower rate. From 220000 dpm of [1-^{14}C]lignocerate, 4304 dpm or 2.0% of the lignocerate was recovered as glutamate in the Folch upper layer while only 101 dpm or 0.046% of the lignocerate was collected as CO_2. A similar experiment with 220000 dpm of [1-^{14}C]palmitate yielded 14550 dpm or 6.6% of the palmitate in the glutamate and 576 dpm or 0.26% of the radioactivity in CO_2.

Table IV: Distribution of Fatty Acid Oxidation Activity in Various Rat Tissues

tissue[a]	system	fatty acid oxidized (pmol per mg of particulate protein per h) from [1-^{14}C]-lignoceric acid	[1-^{14}C]-palmitic acid
brain	complete[b]	20.9	52.4
	−HSF[c]	0.20	6.46
	−HLF[c]	0.10	10.1
	−HSF, −HLF	0.00	3.17
liver	complete	0.88	158
	−HSF	1.05	76.2
	−HLF	0.95	203
	−HSF, −HLF	0.33	154
kidney	complete	0.35	5.92
	−HSF	0.23	5.33
	−HLF	0.58	10.2
	−HSF, −HLF	0.41	8.43
spleen	complete	0.18	3.50
	−HSF	0.12	1.26
	−HLF	0.12	9.40
	−HSF, −HLF	0.17	3.23

[a] The various tissues were removed from eight 22-day-old rats and pooled separately. Particulate fractions were prepared from each group of pooled tissue and assayed as described for brain in the text. [b] See footnote *a* of Table III for the complete system. [c] Heat-stable factor (HSF) and heat-labile factor (HLF) were prepared from calf cerebellum as described in the text.

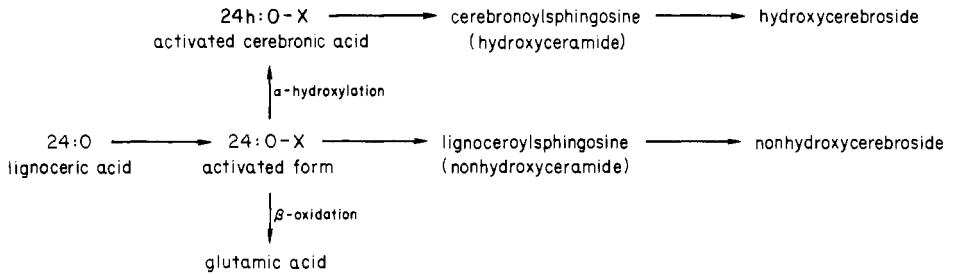
The conversion of lignoceric acid to glutamate is apparently brain specific. Particulate fractions were prepared from liver, kidney, and spleen of eight 22-day-old rats by using the same procedure as for the brain enzyme preparation. Each particulate fraction was assayed in the presence of heat-stable and heat-labile factors for the conversion of lignocerate to material in the Folch upper layer fraction. The assay conditions were identical with those for the brain preparation. The data in Table IV (left-hand column) show that the particulate fractions from extraneural tissues do not significantly oxidize lignoceric acid.

On the other hand, all the extraneural tissues, especially liver, showed considerable palmitate oxidation (Table IV, right-hand column). However, this oxidation may be due to a different enzymatic system since it did not decrease on removal of the cytosolic factors. In fact, removal of the heat-labile factor resulted in a stimulation.

Discussion

Brain characteristically contains large quantities of very long chain fatty acids, C_{22}–C_{26}, as components of cerebrosides and sulfatides which are localized in myelin (Kishimoto & Radin, 1966; Norton & Poduslo, 1973; Nonaka & Kishimoto, 1979b). These fatty acids are degraded by a unique oxidation system called α-oxidation (Hajra & Radin, 1963a,b; Mead & Levis, 1963). In this oxidation scheme, a fatty acid is first hydroxylated on the α carbon (Hoshi & Kishimoto, 1973) and then further degraded by oxidative decarboxylation, giving a fatty acid containing one fewer carbon atom (Davies et al., 1966; Lippel & Mead, 1969). The products of α-oxidation, namely α-hydroxy fatty acids and fatty acids with odd-num-

Scheme I



bered carbon chains, are also abundant in the brain and are components of myelin sphingolipids.

In addition to α-oxidation, however, in vivo experiments give strong evidence indicating the presence of active β-oxidation of very long chain fatty acids in the brain (Hajra & Radin, 1963a,b; Seidel et al., 1975; Akanuma & Kishimoto, 1978). β-Oxidation of fatty acids involves the sequential removal of pairs of carbon atoms from the carboxyl end. Acetyl-CoA, the product of the two-carbon removal, is either oxidized to CO_2 and water by the tricarboxylic acid cycle, utilized in lipogenesis through ketone bodies or de novo synthesis, or converted to amino acids by transamination of the intermediates of the tricarboxylic acid cycle (Bressler, 1970). We have previously shown that the major routes of β-oxidation of palmitic acid in brain, unlike liver, are the second and third pathways (Kawamura & Kishimoto, 1981). Edmond & Popjak (1974) demonstrated a high utilization of ketone bodies for lipogenesis in rat brain.

Our previous investigation of brain β-oxidation indicates that under standard conditions oxidation of lignoceric acid is barely detectable.[1] The specific activity is only 2 pmol of lignoceric acid oxidized per mg of protein per h in developing rat brain. This compares with an activity of over 50 pmol of lignoceric acid oxidized per mg of protein per h in liver; 3 nmol and 150 pmol per mg of protein per h of palmitic acid are oxidized by liver and brain preparations, respectively. Unexpectedly, we found that the oxidation of lignoceric acid by the liver preparation appears to be due to a peroxisome system rather than a mitochondrial system.

This investigation shows that in the brain there is a unique system, different from α-oxidation and regular β-oxidation, which requires heat-stable and heat-labile factors and favorably oxidizes very long chain fatty acids. This system oxidizes approximately 20 pmol of lignoceric acid per mg or protein per h. The same brain preparation shows higher palmitic acid oxidation, and the ratio of lignocerate to palmitate oxidation is only 1:3. As reported elsewhere,[2] lignoceric acid oxidation is stimulated 3–5-fold by the addition of α-cyclodextrin while palmitate oxidation is not affected. α-Cyclodextrin appears to aid solubilization of lignoceric acid. The ratio of lignocerate to palmitate oxidation increases to almost 2:1 in the presence of α-cyclodextrin. This compares well with our previous finding that the ratio of the oxidation of palmitate to that of lignocerate, measured under standard β-oxidation conditions, is 1:75.[1] There was no significant oxidation of lignoceric acid in rat liver in this assay system. The presence of this brain-specific oxidation system may explain the β-oxidation of lignoceric acid observed in in vivo experiments as described above.

The major product of this lignocerate oxidation is glutamate. CO_2 production is less than 10% of the water-soluble products. A similar product distribution is observed in palmitic acid β-oxidation by rat brain postnuclear fraction (Kawamura & Kishimoto, 1981). This oxidation pattern is distinctly different from that of liver oxidation, where most of the acetyl-CoA formed by β-oxidation is used for energy production (Bressler, 1970). Thus, physiological brain β-oxidation may be significant for the synthesis of glutamate rather than for energy production. The synthesis of glutamate from acetyl-CoA requires the trapping of ammonia in α-ketoglutarate. Therefore, it is conceivable that fatty acid β-oxidation in the brain may be important in the removal of ammonia. The enzymatic activities related to the urea cycle, which removes ammonia from extraneural tissues, are very low in the brain. Also, glutamate formation may be involved with the biogenesis of neurotransmitters (Johnson, 1972).

The brain preparation used in the present study also contains the system for the α-hydroxylation and conversion to ceramide of lignoceric acid and other very long chain fatty acids. The requirements for heat-stable and heat-labile factors, pyridine nucleotide, and Mg^{2+} as well as the inhibition by a number of compounds, including respiratory chain inhibitors, are almost identical for all three reactions. This strongly suggests that all these reactions share a common pathway. At present, we think the shared pathway is the activation of lignoceric acid as shown in Scheme I. Since CoA inhibits all these reactions (Hoshi & Kishimoto, 1973; Singh & Kishimoto, 1979), the CoA ester of lignoceric acid is probably not the intermediate. We have also previously concluded that lignoceroyl-CoA is not the direct substrate in α-hydroxylation (Akanuma & Kishimoto, 1978). On the other hand, our recent study indicates that ATP is actively being produced in this system.[3] It is, therefore, likely that the heat-stable and heat-labile factors may be involved in the activation of lignoceric acid. Recently, we discovered that the heat-stable factor is composed of two compounds, one an oligosaccharide and the other apparently a nucleotide.[2] We believe that full characterization of these factors will elucidate the mechanism of activation.

Acknowledgments

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Induction and Expression of Nodule-Specific Host Genes in Effective and Ineffective Root Nodules of Soybean†

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ABSTRACT: Regulation of moderately abundant host sequences in soybean root nodules was studied by using purified complementary deoxyribonucleic acid (cDNA) probes prepared by hydroxylapatite fractionation of total nodule cDNA. A probe for a subset of moderately abundant sequences (M1-cDNA) hybridized with uninfected root polysomal RNA containing poly(adenylic acid) [poly(A)+] to 52% as compared to 77% with homologous (nodule) RNA, suggesting the presence of "nodule-specific" sequences within this RNA population. A nodule-specific cDNA probe (NS-cDNA) with a complexity of 2.3×10^4 nucleotides was then purified following hybridization of moderately abundant cDNA (M-cDNA) with uninfected root mRNA. NS-cDNA hybridized to over 95% with nodule mRNA and to only 15% with uninfected root mRNA. These sequences are of host origin as demonstrated by the hybridization of NS-cDNA with soybean DNA and its lack of hybridization with *Rhizobium* DNA. Hybridization of NS-cDNA with nuclear RNA from uninfected root, hypocotyl, and leaf suggested that these sequences either are not transcribed or are present at exceedingly low levels within these organs. They appear to be regulated at the transcriptional level since NS-cDNA reacts to the same extent with nuclear and polysomal RNAs from uninfected tissue. Their relative concentration increases in parallel with leghemoglobin during nodule development and reaches a pleateau when nitrogen fixation commences. At this stage, they represent about 7.5% of the mass of polyadenyated polysomal RNA in root nodules. The concentration of nodule-specific sequences is reduced to different extents in nodules developed due to the infection with ineffective strains of *Rhizobium*. This study demonstrated the presence of a set of nodule-specific host sequences which are induced following infection of *Rhizobium* and may be involved in symbiotic nitrogen fixation.

The molecular events leading to the successful infection and development of a root nodule symbiosis between legume hosts and *Rhizobium* spp. are largely unknown. The notion is emerging that a precisely timed, intricately coordinated expression of specific plant and bacterial genes is essential for the establishment of an effective, nitrogen-fixing nodule [see Verma (1980a) for review]. Several host genes have been implicated in this process, and some of them have been identified through classical genetic experiments (Nutman, 1956; Holl & LaRue, 1976; Caldwell & Vest, 1977). In addition to leghemoglobin (Lb), which is obligatory for symbiotic nitrogen fixation in legumes, a number of other "nodule specific" host proteins (nodulins), which may be involved in the development of root nodule symbiosis, have been detected (Legocki & Verma, 1980).

Since nodulins are present in relatively low concentration in nodules and are not detected in uninfected roots (Legocki & Verma, 1980), the mRNA sequences encoding these proteins may be absent or present in very low concentrations in roots prior to infection by *Rhizobium*. Comparison of the uninfected root and nodule mRNA populations by total cDNA/mRNA homologous and heterologous hybridizations revealed a marked shift in concentration of the superabundant (Lb) and moderately abundant mRNAs, but detection of a small mass fraction of nodule-specific sequences was beyond

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