Synthesis of Cerebronic Acid from Lignoceric Acid by Rat Brain Preparation

SOME PROPERTIES AND DISTRIBUTION OF THE α-HYDROXYLATION SYSTEM*

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SUMMARY

The conversion of [1-14C]lignoceric acid (tetracosanoic acid) into cerebronic acid (Z-hydroxytetracosanoic acid) by a cell-free preparation of rat brain was investigated. The α-hydroxylation enzyme required molecular oxygen, Mg++, pyridine nucleotides, and a heat stable water soluble cofactor. The activity had pH optimum of 7.8, and the Kₐ value for lignoceric acid was found to be 4.2 µM. The presence of CO did not inhibit the activity. Except for Mg++, all heavy divalent metal ions were strongly inhibitory. EDTA was also strongly inhibitory. The pyridine nucleotides DPNH and TPNH were equally effective in producing full enzyme activity; their oxidized forms were less effective. The product cerebronic acid was detected only as a component of ceramide or cerebroside and not as free acid or CoA ester. There was no detectable activity in rat brain during the first 6 days after birth, but a rapid increase was noted between the ages of 8 and 21 days. Activity in the brain attained a maximum in rats 21 to 28 days old and then gradually decreased until an age of 85 days was reached. The cerebellum was found to have the greatest activity. No α-hydroxylating activity was detected in any of the extraneural tissues studied.

2-Hydroxy fatty acids, found in small amounts in certain yeasts, bacteria, and mammalian extraneural tissues (1), are uniquely abundant in the mammalian nervous system. The 2-hydroxy acids found in the nervous system consist mainly of both saturated or monounsaturated compounds with a chain length of 22 to 26 carbon atoms. They contain an unusually high concentration of odd-numbered carbon acids. They were detected exclusively in cerebrosides and sulfatides, which are considered characteristic myelin lipids. Their concentration in the brain increase very rapidly during the period of active myelination and more slowly during the later life of the animal (2).

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In vivo experiments (3-5) indicate that the cerebral 2-hydroxy fatty acids are derived directly from the corresponding non-hydroxy fatty acids. The 2-hydroxy acid is subsequently oxidatively decarboxylated to the fatty acid with one less carbon atom. This sequence of in vivo degradation of fatty acids is referred to as one carbon degradation or α-oxidation. Available evidence suggests that cerebroside, a major 2-hydroxy acid of brain sphingolipids, is derived directly from lignoceric acid, a major nonhydroxy acid of these lipids (6).

The cerebral enzymes which convert 2-hydroxy fatty acids to nonhydroxy acids with one less carbon atom have been partially characterized (7-9). The enzyme responsible for 2-hydroxylation of the fatty acids has not yet been characterized, however. In this communication we describe some of the properties of an enzyme system from rat brain which converts lignoceric to cerebroside. The activity of this enzyme, which appears to be limited to the brain, was determined in various areas of the brain at different stages of development.

EXPERIMENTAL PROCEDURE

Materials

[1-14C]Lignoceric acid (51.8 mCi per mmole) was synthesized from K⁴CN (Amersham-Searle, Arlington Heights, Ill.) and triocys bromide according to Morrell et al. (10). The bromide was prepared from 1-tricosanol (Lachat Chemical, Chicago Heights, Ill.) and PBr₃ by a published procedure (11). [1-14C]cerebroside (70 µCi per mmole) was prepared from [1-14C]-lignoceric acid by α-bromination with PBr₃ and Br₂ followed by alkaline hydrolysis in aqueous solution (11). [14C]Ceramide (N-[1-14C]lignoceroyl sphingosine) and [14C]kerasine (N-[1-14C]lignoceroyl psychosine), both with a specific activity of 51.5 mCi per mmole, were synthesized by acylation with [1-14C]-lignoceroyl chloride of D-erythro-3-O-benzoyl sphingosine sulfate and psychosine sulfate, respectively (12). Mild alkaline methanalysis (13) was used to remove the benzoyl group from the 3-O-benzoyl ceramide in the ceramide synthesis. [1-14C]Lignoceroyl chloride was obtained by treating the free acid with thionyl chloride (12), and psychosine sulfate was prepared from beef brain cerebrosides according to a published procedure (14). Nonradioactive ceramide and cerebrosides were similarly prepared.

N. S. Radin and R. C. Arora, private communication.
pared with lignoceric acid or m-cerebronic acid. The hydroxyl group of cerebronic acid was protected by acetylation prior to its use in the synthesis (15). Synthetic N\-3-O-benzoyl sphingosine sulfate was a generous gift of Dr. Julian N. Kanfer. [1-14C]-Stearic acid was purchased from New England Nuclear Corp. (Boston, Mass.). All radioactive lipids were purified by preparative thin layer chromatography prior to use.

Pyridine nucleotides were purchased from either P-L Biochemicals (Milwaukee, Wis.) or Sigma Chemical Co. (St. Louis, Mo.). Antimycin A was obtained from Nutritional Biochemical, Inc. (Cleveland, Ohio). 2-Amino-4-hydroxy-6,7-dimethyl tetrahydropteridine was kindly given by Dr. K. Fukuushima, University of Tokyo, Japan. Most other cofactors and enzymes were purchased from Sigma Chemical Co. Ultrapure sucrose was obtained from Schwarz-Mann (Orangeburg, N.Y.). Detergents were gifts from Rohm and Haas Company, Atlas Chemical Industries, Inc., or Miranol Chemical Company, Inc. Thin layer chromatography plates precoated with 0.25-mm thick Silica Gel G were purchased from Analtech, Inc. (Newark, Del.) and with Silica Gel F254 from Brinkmann Instruments (Westbury, N.Y.). Unisil was obtained from Clarkson Chemical Co. (Williamsport, Pa.). Analytical Filter-Aid grade Celite, purchased from Johns-Manville (Denver, Colo.), was used without further purification.

Methods

Enzyme Preparations—Brains from 3- to 4-week-old Sprague-Dawley rats, killed by decapitation, were washed with ice-cold 0.9% NaCl and then homogenized in 2 volumes of ice-cold 0.15 M NaCl using a Potter-Elvehjem homogenizer fitted with a Teflon pestle. The homogenates were centrifuged at 900 x g for 10 min, and the supernatant solutions (postnuclear fraction) served as the source of the enzyme described in these studies. Protein was determined according to the Lowry method as modified by Hess and Lewin (16).

Determination of Enzyme Activity—Enzyme preparations were assayed for their ability to convert [1-14C]-m-cerebronic acid to cerebronic acid. In the standard assay the following components were incubated with vigorous shaking at 37° for 45 min in a final volume of 0.5 ml: 10 \( \mu \)moles of Bicine buffer at pH 7.8; 0.5 \( \mu \)moles of MgCl\(_2\); 0.2 \( \mu \)moles of DPNH; 2.06 \( \mu \)moles of [1-14C]-m-cerebronic acid (2 \( \times \) 10\(^5\) cpm) coated on 10 mg of Celite (17); and 0.3 ml of enzyme preparation (4.5 to 5.0 mg of protein). The reaction was terminated by addition of 8 ml of CHCl\(_3\)-CH\(_2\)OH 1:1. After mixing and centrifugation, the clear supernatant solution was removed and the residue was washed with an additional 2 ml of the same solvent. The combined CHCl\(_3\)-CH\(_2\)OH extracts were mixed with 5 ml of CHCl\(_3\), washed with 2.5 ml of water, and then dried under a stream of nitrogen.

Cerebronic acid was isolated from the crude lipid residue according to a published procedure (18) with minor modification. The lipid residue was treated with 2.5 ml of propylene glycol containing 2.5 \( \mu \)moles of KOH and 1.25 \( \mu \)g of cerebronic acid carrier for 30 min at 190–200°. The mixture was then cooled to room temperature, and most of the cholesterol was removed by extraction with 3 ml of hexane. The remaining propylene glycol layer was acidified with 4 ml of 1 N HCl and then extracted with 8 ml of CHCl\(_3\). The chloroform layer, containing the free fatty acids, was washed with 6 ml of water, evaporated under a nitrogen stream, and then dried thoroughly over P\(_2\)O\(_5\) under reduced pressure.

The dried residue was dissolved in a small volume of benzene-ether 99:1 and applied to a column containing 0.5 g of Unisil. The column was first rinsed with 25 ml of benzene-ether 99:1 to remove nearly all unreacted lignoceric acid and was then eluted with 15 ml of benzene-ether 99:1 to obtain the 2-hydroxy fatty acids. The residue from the latter eluate was dissolved in 0.3 ml of CHCl\(_3\)-ethanol 2:1, diluted with 1.1 ml of ethanol, and treated with 0.2 ml of 1 M cupric oleate in CHCl\(_3\), at 4° for 30 min. The insoluble copper chelates of the 2-hydroxy acids were collected by centrifugation, washed twice with 2 ml of ethanol-CH\(_2\)Cl\(_2\) 3:1, and then dissolved in 0.2 ml of 0.5 N methanolic HCl by warming. The clear, yellowish solution was transferred to a counting vial with three 5-ml portions of a toluene-based scintillation mixture containing Hyamine hydroxide 10-X (8 ml of 1 M methanolic Hyamine hydroxide dissolved in a liter of the scintillation mixture).

Identification of Product—The isolation procedure described above is specific for 2-hydroxy fatty acids (18). However, further confirmation of the identity of the product was accomplished by chemical degradation (a) and by thin layer chromatography (b). (a) The enzymic reaction products were recovered as free acids from the copper chelates by treatment with 1 N HCl (18). The fatty acids were then oxidized with permanganate (19) to CO\(_2\) and to the nonhydroxy fatty acids with one less carbon atom. 2-Hydroxy fatty acids are oxidized nearly quantitatively under these conditions. The CO\(_2\) was collected in a solution of Hyamine hydroxide and the amount of radioactive label determined. The nonhydroxy fatty acids and small amounts of unreacted 2-hydroxy acids remaining after oxidation were extracted with ether from the reaction mixture. The amount of radioactive label in these compounds was determined after fractionation by Unisil column chromatography. (b) The copper chelate was dissolved with warming in 1 ml of 0.5 N methanolic HCl. The methyl ester thus formed was extracted with hexane-ether 99:1, analyzed by thin layer chromatography on Silica Gel G plate with hexane-ether 6:4 as the developing solvent, and subjected to radioautography.

Identification of Lipids Containing [14C]Cerebronic Acid—Lipids extracted from the enzymic incubation mixture were analyzed by thin layer chromatography-radioautography either directly or after fractionation by Unisil column chromatography. The radioactive spots were separately scraped and subjected to methanalysis (20). The fatty acid methyl esters thus obtained were examined by thin layer chromatography-radioautography.

Determination of Radioactivity—The radioactivity was determined with a Tri-Carb liquid scintillation spectrometer model 3820. Eastman Kodak RP/R54 or NS-54T film was used for the detection of radioactive compounds on thin layer chromatograms.

RESULTS

Validation of Assay Method

[1-14C]Cerebronic acid, 10,000 cpm (0.45 mg), was added to a standard incubation mixture containing heat-inactivated enzyme in the absence of substrate and immediately processed. Most of the original radioactivity (86%) was recovered in the copper chelate of the 2-hydroxy acid. The greatest loss of radioactivity occurred during the saponification procedure. Treatment of [1-14C]-lignoceric acid in a similar manner resulted in recovery of 0.04% of original radioactivity in the copper chelate. When Unisil column chromatography was omitted from the procedure, 1% of the radioactivity from [1-14C]-lignoceric acid contaminated the chelate. Authentic [14C]lactamide was also
of a standard enzyme preparation increased with increasing Tween's, Triton's, Miranol's, Cutscum, and bile acid salts, releasing 0.2 pmole of DPNH; and 0.3 ml of enzyme preparation (4.5 to 5.0 nmol) to cerebronic acid. Radioactive lignoceric acid (2.06 pmol) was coated on the quantities of Celite indicated, and incubated with the brain enzyme. The standard assay system contained 10 μmol of Bicine buffer at pH 7.8; 0.5 μmol of MgCl₂; 0.2 μmol of DPNH; and 0.3 ml of enzyme preparation (4.5 to 5.0 pmol of protein) in a total volume of 0.5 ml.

carried through this procedure, and it was found that complete cleavage of the amide linkage of cerebrosides occurred.

Testing a variety of detergents, including BRIJ's, MYRJ's, Tween's, Triton's, Miranol's, Cutscum, and bile acid salts, revealed none which was able to replace the Celite. The activity of a standard enzyme preparation increased with increasing amounts of Celite up to 5 mg, remained constant until 13 mg were added, and then gradually decreased with larger amounts (Fig. 1). Virtually no α-hydroxylating activity was observed when 100 mg of Celite were used.

CO₂ which might have been produced during the enzymic incubation, was collected and was found to be nonradioactive. Apparently, the enzymes for oxidative decarboxylation of cerebronic acid (9) are not active under these experimental conditions, or the cerebronic acid produced is immediately incorporated into an amide bond.

Identification of Product

Approximately 0.5 mg of 2-hydroxy fatty acid containing 1226 cpm was isolated from a standard incubation product and oxidized with permanganate. As shown in Table I, nearly all of the radioactivity was recovered as CO₂. The products of the enzymic reaction were converted to their methyl esters and were analyzed by thin layer chromatography-radioautography. Radioactivity was detected only at an RF value equal to that of authentic lignocerate. These results, together with the specific method of assaying, indicated that the product was [1-¹⁴C]lignoceric acid.

When the lipids from the enzymic reaction mixture were partitioned by the Folch procedure (21), approximately 1% of the radioactivity was recovered in the upper phase. This was a 10-fold increase over the radioactivity obtained with a heat-inactivated enzyme. None of this radioactivity was attributed to the presence of either free 2-hydroxy acids or 2-hydroxy acids attached to lipids. The radioactivity was most likely due to the formation of a CoO bond with the substrate. When [¹⁴C]-ceramide or [¹⁴C]kerasine was incubated with the brain preparation, no radioactivity was found in this fraction.

The lipids in the lower phase of the Folch procedure from a standard assay mixture were fractionated on a Unisil column. Elution with ether produced free fatty acids and cholesterol, and additional elution with CHCl₃-CH₃OH 1:4 produced polar lipids. These fractions were then examined by combined thin layer chromatography-radioautography (Fig. 2). In addition to nonhydroxy fatty acids (unchanged substrate), many spots corresponding to complex lipids, including ceramides and cerebrosides, were found to be radioactive. No radioactive free 2-hydroxy fatty acid was detected, however. The spot corresponding to ceramides containing nonhydroxy fatty acids was much more radioactive than the one containing ceramides with 2-hydroxy fatty acids as shown in Fig. 2. On the other hand, cerebrosides with nonhydroxy fatty acids were only slightly radioactive, while those with hydroxy fatty acids were quite radioactive. Each radioactive spot was scraped and subjected to methanolysis (22). The methyl esters were analyzed by thin layer chromatography-radioautography (Fig. 3). Radioactive spots corresponding to methyl cerebranate were obtained from ceramides and cerebrosides which contained 2-hydroxy fatty acids. All others gave only one radioactive spot corresponding to methyl lignocerate.

The configuration of the hydroxyl group of the cerebronic acid produced by the enzymic system was determined to be α from the following observations. The lipids extracted from the enzymic reaction mixture were first subjected to a mild alkaline methanolysis procedure to remove most glycerolipids (13). The alkali stable lipids were applied to a Unisil column which was eluted with CHCl₃-CH₃OH 98:2 to obtain ceramides and then with CHCl₃-CH₃OH 3:1 to recover cerebrosides and sulfatides (13). The ceramides were then examined by thin layer chromatography-radioautography. The solvent system, CHCl₃-CH₃OH 95:5, was used to separate ceramides containing α- and L-cerebronic acid as well as those containing lignoceric acid (23). The results indicated that the enzymic product did contain radioactive ceramides with both lignoceric acid and α-cerebronic acid. Chromatographic examination of the cerebrosides required five subsequent developments with CHCl₃-CH₃OH 9:1 to effect such separation. Like the ceramides, the cerebrosides with α-cerebronic acid or lignoceric acid were found to be radioactive. The radioactive ceramide and cerebroside spots which contained α-cerebronic acid were recovered from the plates and subjected to methanolysis. The liberated methyl esters were then examined by thin layer chromatography-radioautography.

| Hydroxy fatty acid                  | Radioactivity | CO₂     | CO₂ + NFA |
|------------------------------------|---------------|---------|-----------|
| [1-¹⁴C]Cerebronic acid (synthetic) | 956           | 13      | 42        | 98.6      |
| Product by enzyme                  | 1009          | 27      | 26        | 97.6      |

Table I

Decarboxylation of 2-hydroxy fatty acids

The enzymatic product and synthetic [1-¹⁴C]cerebronic acid was individually oxidized by permanganate to liberate the carboxyl carbon as CO₂. The nonhydroxy fatty acid (NFA) formed during the oxidation of the 2-hydroxy acid and the unreacted starting material (HFA) were recovered and separated from each other by a column chromatography. See text for the details.

**Fig. 1. Effect of varying the amount of Celite on the conversion of [1-¹⁴C]lignoceric acid (specific activity 97,000 cpm per nmol) to cerebronic acid.**

**Graph: CEREBRONIC ACID FORMED (pmoles/mg protein)**

**CELITE (mg)**

Table: Decarboxylation of 2-hydroxy fatty acids

| Hydroxy fatty acid                  | Radioactivity | CO₂     | CO₂ + NFA |
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FIG. 2 (left). Autoradiogram of a thin layer chromatographic separation of crude lipids extracted from a standard assay reaction mixture. Conditions for the incubation are indicated in the legend of Fig. 1. Developing solvent used was CHCl₃-CH₃OH 9:1. The crude lipid was preliminarily fractionated on a Unisil column. Fraction E was eluted with ether and contained free fatty acids, both nonhydroxy and 2-hydroxy, and cholesterol. Fraction CM was eluted with CHCl₃-CH₃OH 2:1 and contained polar lipids. The radioactive spot in Fraction E corresponds to free lignoceric acid. Cerebronic acid would have remained at the origin under these conditions. In the radioautogram of Fraction CM Spots 1 and 2 correspond to ceramides containing nonhydroxy or 2-hydroxy fatty acids, respectively, and 4 and 5 correspond to cerebrosides containing these fatty acids.

All of the radioactivity present in these lipids were found in cerebroside acid.

Properties of Enzyme

Effect of pH—Maximum activity of the enzyme was reached at a pH of 7.8 with every buffer tested (Fig. 4). Bicine and Tris buffers were the most useful among those tested and phosphate was least effective.

Effects of Metals—Mg²⁺ was required for full activity of the α-hydroxylating enzyme, and could not be replaced by Ca²⁺ and Mn²⁺, which were somewhat inhibitory. Most heavy metal ions, including iron and copper, were strongly inhibitory at 0.1 mM, except for nickel, which was inhibitory only at concentration above 4 mM. EDTA strongly inhibited the enzyme while iron chelators, such as dipyridyl and O-phenanthroline, had no effect. EGTA (ethyleneglycol-bis(β-aminoethyl ether)-N,N′,N′-tetraacetic acid), which binds calcium in preference to Mg²⁺, also had no effect on the enzyme activity (Table II).

Pyridine Nucleotide Requirement—The results shown in Table III indicate that pyridine nucleotides are essential for the enzyme activity. The activity increases linearly with increasing concentration of DPNH up to 0.15 mM, plateaus briefly and then gradually decline with further increases in the concentration (Fig. 5). TPNH was as effective as DPNH at all concentrations employed.

Aerobic Nature of Reaction—The hydroxylation reaction apparently requires O₂ (Table IV) in conjunction with a pyridine nucleotide, a finding which suggests that the conversion may be catalyzed by a “mixed function oxidase.” The presence of cytochrome P-450 in this system was not implicated, since CO did not inhibit the activity. Most common inhibitors of the energy linked electron transfer system strongly inhibited the enzymic hydroxylation as also shown in Table IV. These results suggest that the hydroxylation system is closely related to the respiratory chain and not to the hydroxylation system which depends upon cytochrome P-450.

Effect of Various Other Cofactors—The flavins, FMN and FAD, were both strongly inhibitory at a concentration of 1 mM, while ATP, CoA, pyruvate, and succinate at the same concentration had no effect on the α-hydroxylating activity. 2-Amino-4-hydroxy 6,7-dimethyl,5,6,7,8-tetrahydropteridine was slightly inhibitory at 1 mM and had no effect at 0.1 mM. Imidazole, 1 mM, was slightly stimulative, and cytochrome c was inhibitory even at 0.1 mM. The addition of H₂O₂ generating system (glu-
case and glucose oxidase from Aspergillus niger, Sigma) had a strong inhibitory effect.

**Substrate Specificity**—The brain \( \alpha \)-hydroxylating system appears to be highly specific for free lignoceric acid as indicated in Table V. Stearic acid, which has 18 carbon atoms, was not utilized. The substrate, after enzymic conversion to cerebronic acid, was found solely in ceramides or cerebrosides. It might be argued that the hydroxylation occurs after lignoceric acid is incorporated into ceramides or cerebrosides. However, these sphingolipids had little, if any, effect as precursors, and the

![Graph](image)

**FIG. 4.** \( \alpha \)-Hydroxylating activity as a function of pH of the buffer. The concentration of the buffer was 20 mM. Other conditions are listed in Fig. 1.

**TABLE II**

*Effect of chelating reagents on \( \alpha \)-hydroxylation activity*

| Chelating reagents added | \( \alpha \)-Hydroxylating activity % |
|-------------------------|-----------------------------------|
| None                    | 100                               |
| EDTA, 1 mm              | 1                                 |
| EGTA, 1 mm              | 100                               |
| \( \alpha, \alpha'- \)Dipyridyl, 1 mm | 100     |
| 8-Hydroxyquinoline, 1 mm | 79       |
| \( \alpha \)-Phenanthroline, 1 mm | 110      |
| Ethanol, 5 \( \mu \)l    | 104                               |

**TABLE III**

*Pyridine nucleotide requirements for \( \alpha \)-hydroxylation*

Conditions for the complete system were described in the legend of Fig. 1. The effect of omitting DPNH from the assay system was examined with or without concomitant removal of \( \text{Mg}^{2+} \) and with the addition of other pyridine nucleotides as indicated.

| Incubation mixture | \([\text{I}^4\text{C}]\)Cerebronic acid formed pmoles/mg protein/hr |
|--------------------|---------------------------------------------------------------|
| Complete           | 4.29                                                          |
| Minus MgCl\(_2\) minus DPNH | 0.34              |
| Minus DPNH         | 0.77                                                          |
| Minus DPNH plus 0.4 mm TPNH | 3.90               |
| Minus DPNH plus 0.4 mm DPN | 1.03              |
| Minus DPNH plus 0.4 mm TPN  | 3.21              |

**FIG. 5.** Effect of DPNH on the \( \alpha \)-hydroxylation activity. Other conditions are listed in Fig. 1.

**TABLE IV**

*Oxygen requirement, effect of CO and inhibitors of respiratory chain on \( \alpha \)-hydroxylation activity*

The complete system described in the legend of Fig. 1 was incubated under one of the gas phases listed with or without inhibitor addition.

| Gas phase | Inhibitor added | \( \alpha \)-Hydroxylation activity % |
|-----------|-----------------|-----------------------------------|
| \( \text{O}_2-\text{N}_2\) (1:4) | -a | 100 |
| \( \text{O}_2-\text{N}_2\) (1:4) | KCN, 1 mm | 0 |
| \( \text{O}_2-\text{N}_2\) (1:4) | \( \mu \)-Chloromercuribenzoate, 1 mm | 3 |
| \( \text{O}_2-\text{N}_2\) (1:4) | \( \text{I}_{\text{CH}}\text{COOH}, 1 \text{ mm} \) | 17 |
| \( \text{O}_2-\text{N}_2\) (1:4) | Sodium azide, 1 mm | 68 |
| \( \text{O}_2-\text{N}_2\) (1:4) | 2,4-Dinitrophenol, 1 mm | 2 |
| \( \text{O}_2-\text{N}_2\) (1:4) | Antimycin A, 3.7 \( \mu \)M | 1 |
| \( \text{N}_2\) | -a | 7 |
| \( \text{O}_2-\text{CO}-\text{N}_2\) (2:3:5) | -a | 106 |

*a* No inhibitor added.

**TABLE V**

*Comparison of various lipids as substrates for \( \alpha \)-hydroxylation system*

Each substrate of 200,000 cpm (2 nmoles) was coated on 10 mg of Celite and assayed for its conversion to a 2-hydroxy fatty acid under the standard conditions described in Fig. 1.

| Substrate | \( \text{[I}^{14}\text{C}]\)Stearic acid | \( \text{[I}^{14}\text{C}]\)Lignoceric acid | \( \text{N-[}^{14}\text{C}]\)Lignoceroyl sphingosine | \( \text{N-[}^{14}\text{C}]\)Lignoceroyl-1-O-d-galactosyl sphingosine |
|-----------|-----------------------------------------|-----------------------------------------|------------------------------------------|---------------------------------------------|
| cpm       | 34                                      | 1547                                    | 91                                       | 66                                          |
slight activity detected for them (Table V) could be due to
hydrolysis (15, 24) and utilization of released lignoceric acid for
the hydroxylation.

Other Inhibitors and Stimulators—The effect of several lipids,
cerebroside, 2-keto lignoceric acid, ceramides, and cerebro-
sides which are metabolically related to the enzyme substrate or
product of the α-hydroxylating enzyme were found to be in-
hibitory (Table VI). Sphingosine, which is an acceptor of 2-
hydroxy fatty acid CoA esters (25), had no effect, while psycho-
sine was stimulative. The observed stimulation by tripalmitin
difficult is explain.

Sucrose completely inhibited the enzymic reaction when it was
employed to homogenize the brain tissue. The inhibitory effect
of sucrose was confirmed by adding various amounts of sucrose
to the standard assay system (Fig. 6).

Apparent Michaelis Constant—Fig. 7 indicates the effect of
substrate concentrations on the enzymic reaction. The ap-
parent $K_m$ was found to be 4.2 μM by using Lineweaver-Burk
plots of the data.

Requirement for Unknown Cofactor—Cerebronic acid was not
produced until approximately 2 mg of protein were added.

**Table VI**

| Lipids added       | α-Hydroxylating activity (%) |
|--------------------|-----------------------------|
| None               | 100                         |
| Cerebronic acid    | 43                          |
| α-Keto lignoceric acid | 43                    |
| Sphingosine         | 95                          |
| Psychosine          | 165                         |
| Lignoceroyl sphingosine | 60                    |
| Cerebrolyl sphingosine | 47                  |
| Lignoceroyl psychosine | 69                  |
| Cerebrolyl psychosine | 57                  |
| Tripalmitin        | 250                         |

When various amounts of the postnuclear fraction of rat brain
were incubated with [1-14C]lignoceric acid, product formation in-
creased linearly with protein addition until a plateau was reached
at approximately 4 mg (Fig. 8). However, the rate of formation
of cerebronic acid became a linear function of the amount of
enzyme added between 0 and 4 mg of protein, when boiled post-
nuclear fraction was added so that the total amount of protein
was held constant at 5.5 mg in each tube. Bovine serum albumin
could not replace boiled 900 × g supernatant. This suggests
that there is a cofactor which is required above a certain con-
centration. No change was noted in the clear supernatant when
the boiled enzyme was centrifuged at 1500 rpm. These results
Heat, ed at 60° for 1 min. The amount of cerebronic acid formed
fractions there prepared from the brains of rats of various ages,
indicate that there is an unknown water soluble and heat stable
lone (3.18 pmoles), the cerebral hemispheres (0.88 pmole), and
brain stem (0.57 pmole) all had lower activities.

Change of Enzymic Activity as Function of Age Postnuclear
fractions were prepared from the brains of rats of various ages,
and their activities for α-hydroxylation were assayed under op-
timum conditions. Slight α-hydroxylation activity is found in
7-day-old rat brain, but the specific activity then increases
sharply until the animal reaches 21 days, at which time it plateaus
for 2 weeks and then declines (Fig. 10).

Distribution of α-Hydroxylation Activity—In 30-day-old rats
the postnuclear fraction of the cerebellum had the highest ac-
tivity (7.23 pmoles per mg of protein per hour). The diencepha-
lobe (3.18 pmoles), the cerebral hemispheres (0.88 pmole), and
brain stem (0.57 pmole) all had lower activities.

FIG. 9. Time course for the conversion of [1-14C]lignoceric acid
to cerebronic acid. Other assay conditions are listed in Fig. 1.

FIG. 10. Activity of the enzyme from brain as a function of the
postnatal age of the rats. The reaction mixtures were prepared as
in Fig. 1. Each point represents the mean (± S.D.) value for the
age indicated. Numbers on top of each point indicate the number
of assays performed.

Under the conditions used, no activity for converting lignoceric
acid to cerebronic acid was detected in the postnuclear frac-
tions of liver, kidney, spleen, heart, and testes.

DISCUSSION

All the cerebronic acid formed from lignoceric acid by the
brain enzyme system was recovered in cereamides or cerebrosides
and not as the free acid or its CoA ester (Figs. 2 and 3). The
CoA esters of 2-hydroxy fatty acids are converted to cereamides
(25) and then to cerebrosides in the brain (17). In a study of
fatty acid chain length specificity of the enzymes catalyzing the
synthesis of cerebrosides, Radin (26) suggested that the rate
limiting step for the synthesis of cerebrosides containing 2-hy-
droxy acids is at the step of ceramide formation. For cere-
brosides containing nonhydroxy acids the rate limiting step is
the galactosyltransferase reaction which converts ceramides to
cerebrosides. Our finding that all newly synthesized cerebronic
acid is immediately incorporated into ceramides and cerebrosides
indicates that the α-hydroxylation of fatty acids may be the
rate limiting step for the synthesis of cerebrosides which con-
tain 2-hydroxy acids. Both the developmental studies (Fig. 10)
and the tissue distribution studies suggest a close relationship
between α-hydroxylation activity and both cerebroside ac-
cumulation and myelin formation in brain.

2-Hydroxy fatty acids are converted to nonhydroxy fatty
acids with one less carbon atom by an α-oxidation system in the
brain (3, 4, 7–9). The observation that newly synthesized
2-hydroxy acids are immediately converted into sphingolipids
suggests that the 2-hydroxy fatty acids which serve as substrates
for the α-oxidation system may be those released from cerebro-
sides by cerebroside galactosidase (15) and ceramidase activity
(24).

2-Hydroxy fatty acids in the brain cluster around specific
chain lengths. The most predominant cluster is between carbon
atoms 22 to 26, with cerebronic acid as the major component.
Another group found in much smaller amounts includes C18
and Cl8 (1). Ullman and Radin (25) studied the specificity of
brain acyltransferase in the synthesis of cereamides and found
that the transferase is not a controlling factor in the distribution
of 2-hydroxy acids in cerebrosides. Our finding that the α-hy-
droxylating enzyme is specific for lignoceric acid and is not active
with stearic acid and other fatty acids (Table V) suggests that
this enzyme might control the formation of specific homologs
of cerebrosides containing 2-hydroxy acids.

Many biological hydroxylating systems have been shown to
involve cytochrome P-450. ω- and ω-1-hydroxylation of fatty
acids in mammalian liver (27, 28), kidney (29, 30), some yeasts
(31, 32), and bacteria (33, 34) have been studied and found to
involve a mixed-function mono-oxigenase requiring a reduced
pyridine nucleotide and molecular oxygen. Cytochrome P-450
appears to play a central role in many of these hydroxylating
systems. The hydroxylating enzyme of brain does not appear
to involve cytochrome P-450 because CO did not inhibit the reac-
tion as indicated in Table IV, but it was sensitive to general
inhibitors of the respiratory chain, such as cyanide and antimycin
A (Table IV). The hydroxylating enzyme of brain therefore
appears to be linked to the respiratory chain of the brain based
on these observations.

Enzymes which hydroxylate the α-position of fatty acids
are present in young plant leaves (35, 36), germinating peanut
(37, 38), bacteria (39), and mammalian liver (40). The liver
enzymes are responsible for conversion of phytanic acid to 2-hy-
droxyphytanic acid. This enzyme is missing in patients of
Refsum's disease (41). Unfortunately, most of these enzymes were not separated from the enzyme which affects subsequent oxidative decarboxylation of the 2-hydroxy fatty acids, and it is difficult to make a precise comparison of these enzymic activities with those responsible for the hydroxylation. The enzyme from liver shares main properties with the α-hydroxylation enzyme from brain including the requirement for pyridine nucleotides and O₂. It differs by being strongly stimulated by Fe³⁺ and inhibited by dipyridyli. Moreover, in agreement with Tsai et al. (40), the liver preparation did not hydroxylate lignoceric acid, while the brain preparation was not capable of oxidizing phytanic acid (42). Comparison of the enzyme from brain with that from plants reveals several differences. The enzyme from peanut requires both a DPN and an HzO₂ generating system (37, 38), while the young pea leaf system utilizes DPN and O₂ for the hydroxylation (35, 36). Both enzymes are strongly inhibited by imidazole. The enzyme from brain differs in that it requires DPNH and O₂ and is slightly stimulated by imidazole.

The α-hydroxylation enzyme of brain is very labile, and loss of activity during subfractionation of the postnuclear fraction has been observed. However, we recently have succeeded in restoring some of the activity after combining a crude mitochondrial fraction with the 100,000 x g supernatant. Neither fraction exhibited any activity separately, suggesting that both fractions are necessary for the activity. Heating either fraction resulted in complete loss of activity. These results, combined with the requirement for a heat-stable, unknown factor found in the postnuclear fraction, suggest that there are three components necessary for the α-hydroxylation activity; a mitochondrial fraction, a heat-labile, soluble fraction, and a third heat-stable, soluble fraction. Upon ultrafiltration of the cytosol fraction, the heat-labile factor emerged in fractions corresponding to a molecular weight more than 10,000. Further characterization of these fractions are currently underway in this laboratory.

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