papers and notes on methodology

Effect of cyclodextrins on the solubilization of lignoceric acid, ceramide, and cerebroside, and on the enzymatic reactions involving these compounds[1]

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Abstract α-Cyclodextrin at concentrations of 1–8 mM helps dissolve, in aqueous solution, fatty acids such as lignoceric, stearic, and palmitic, and complex lipids such as ceramide and cerebroside that contain these acids. Formation of an inclusion complex was indicated on examination of the solution by gel filtration. α-Cyclodextrin strikingly increased synthesis of ceramide from sphingosine and either free lignoceric or stearic acid by rat brain preparations. These results suggest the further use of α-cyclodextrin in lipid enzymology, especially in relation to sphingolipid metabolism.—**Singh, I., and Y. Kishimoto.** Effect of cyclodextrins on the solubilization of lignoceric acid, ceramide, and cerebroside, and on the enzymatic reactions involving these compounds. *J. Lipid Res.* 1983. **24:** 662–665.

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Cyclodextrins are cylindrical polymers composed of either six, seven, or eight glucose moieties linked by an α-1,4 bond and are called α-, β-, and γ-cyclodextrin, respectively. They are prepared from starch by the action of bacterial amylase. Space-filling models and X-ray diffraction patterns of these compounds have revealed the exterior surface to be largely hydrophilic due to hydroxyl groups, and the interior surface to be mostly hydrophobic due to CH groups (1). The internal diameters of these three isomers were calculated to be 5, 6, and 7Å, respectively. A hydrophobic compound inserted into these compounds can be made water-soluble.

This laboratory has been studying the enzymatic reactions of lignoceric acid and its derivatives, ceramide and cerebroside (2–4). An intrinsic problem faced by groups working on lipid enzymology is the fact that most lipids are practically insoluble in water. This insolubility makes in vitro substrate-enzyme contact difficult. Many methods have been devised to circumvent this problem. These include the use of organic solvents, such as ethanol and acetones, detergents, coating on Celite (5, 6) or on the subcellular particles that contain the enzyme (7, 8), and inclusion in liposomes (9). Although these techniques have proved to be useful in many enzymatic investigations, they are not free of adverse effects, especially on kinetic studies (10, 11).

We have recently used α-cyclodextrin to assay the oxidation of lignoceric acid by rat brain preparation and found that it significantly stimulated the formation of cerebronic acid (by α-hydroxylation) and glutamic acid (presumably by β-oxidation) (12). In addition, lignoceric acid in an α-cyclodextrin solution was incorporated into brain lipids when added to an organotypic culture of mouse cerebellum (13). We have further examined the mechanism of α-cyclodextrin solubilization of various fatty acids as well as ceramide and cerebroside and also its effect on enzymatic activities of these compounds.

[1] Trivial names used are: lignoceric acid, n-tetracosanoic acid; stearic acid, n-octadecanoic acid; palmitic acid, n-hexacosanoic acid; ceramide, fatty acyl amide of sphingoid base; cerebroside, ceramide-1-β-galactoside.

EXPERIMENTAL PROCEDURES

Materials

α-, β-, and γ-cyclodextrins were purchased from Sigma Chemical Co. Bio-gel p-2 was obtained from Bio-Rad. [1-^{14}C]Lignoceric acid (56 mCi/mmol), lignoceroyl [3-^{3}H]sphingosine (34 mCi/mmol), and lignoceroyl [3-^{3}H]psychosine (34 mCi/mmol) were synthesized as described previously (6, 14). [1-^{14}C]Palmitic acid (57 mCi/mmol) and [1-^{14}C]stearic acid (50 mCi/mmol) were purchased from New England Nuclear. Sphingosine was obtained from Miles and purified as described previously (14).

Enzymatic Assay

The synthesis of ceramide from free fatty acid and sphingosine (4) was assayed as described previously.

Other analytical procedures

Protein was measured according to Lowry et al. (15) and carbohydrate was determined with phenol-sulfuric acid (16). Radioactivity was measured by a liquid scintillation counter.

RESULTS

Fig. 1 shows the effect of α-cyclodextrin on the solubilization of various fatty acids. The solubility of lignoceric acid increased almost linearly on increasing the α-cyclodextrin concentration up to 2.5 mM before it leveled off. At saturation, approximately 42% of the 6 nmoles of lignoceric acid was dissolved yielding about a 5 μM solution. The effect of α-cyclodextrin was even greater with shorter chain fatty acids. Without α-cyclodextrin, only 3 and 10% of the 6 nmoles of stearic and palmitic acids, respectively, were in solution. The solubility increased greatly on adding 0.5 mM α-cyclodextrin, and nearly all the acids were in solution (about 10–11 μM) with 1.5 mM α-cyclodextrin. On the other hand, β- and γ-cyclodextrin had very little effect on solubilization.

In order to determine whether the fatty acids were solubilized by forming a complex with α-cyclodextrin, the lignoceric acid solution was fractionated by gel filtration on Bio-Gel p-2. As shown in **Fig. 2,** all the radioactivity appeared as a sharp peak in the region of relatively small molecular compounds. When a solution of the potassium salt of lignoceric acid without added α-cyclodextrin was similarly fractionated, the peak of the radioactivity was distinctly retarded as compared to the complex. When α-cyclodextrin alone was fractionated, the peak appeared immediately behind the complex peak. These results strongly indicate that lignoceric acid and α-cyclodextrin form an inclusion complex. Unfortunately, the peak of the complex could not be separated from the α-cyclodextrin peak, and therefore the amount of α-cyclodextrin in the complex could not be calculated.

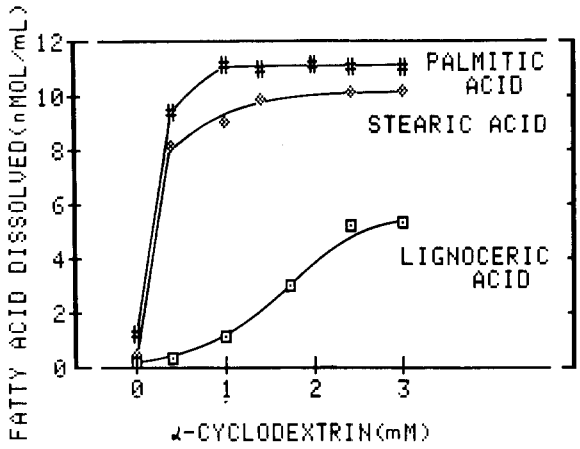

Fig. 1. The solubilization of lignoceric acid, stearic acid, and palmitic acid as a function of the α-cyclodextrin concentration. Six nmol each of [1-^{14}C]lignoceric acid, [1-^{14}C]stearic acid, and [1-^{14}C]palmitic acid were coated on 10 mg of Celite and incubated for 1 hr at 37°C in 0.5 ml of 40 mM Bicine buffer, pH 7.3. Various amounts of α-cyclodextrin were added as indicated. After incubation, the tubes were centrifuged at 5,000 rpm for 40 min and the radioactivity in a portion of the supernatant was counted with Triton X-100/toluene 1:2-based scintillation cocktail.

α-Cyclodextrin had a similar effect on the solubilization of lignoceroyl sphingosine (ceramide) and lignoceroyl psychosine (cerebroside) as shown in **Fig. 3.** The solubility of both lipids increased nearly linearly on increasing the concentration of α-cyclodextrin up to 8 mM. At this concentration, the level of both lipids was 0.25 μM. It is interesting to observe that the molar amounts of ceramide and cerebroside solubilized were almost identical at any given concentration of α-cyclodextrin.

α-Cyclodextrin increased the enzymatic activity for the synthesis of ceramide. As shown in **Fig. 4,** the addition of α-cyclodextrin strikingly increased the synthesis of ceramide from lignoceric acid and sphingosine by rat brain preparation. A similar effect on the synthesis of ceramide from stearic acid was also observed (Fig. 5).

DISCUSSION

Progress in the study of lipid enzymology has been hampered by the insolubility of lipid substrates in assay media. Efforts to circumvent this difficulty have included, as described above, the addition of substrate in

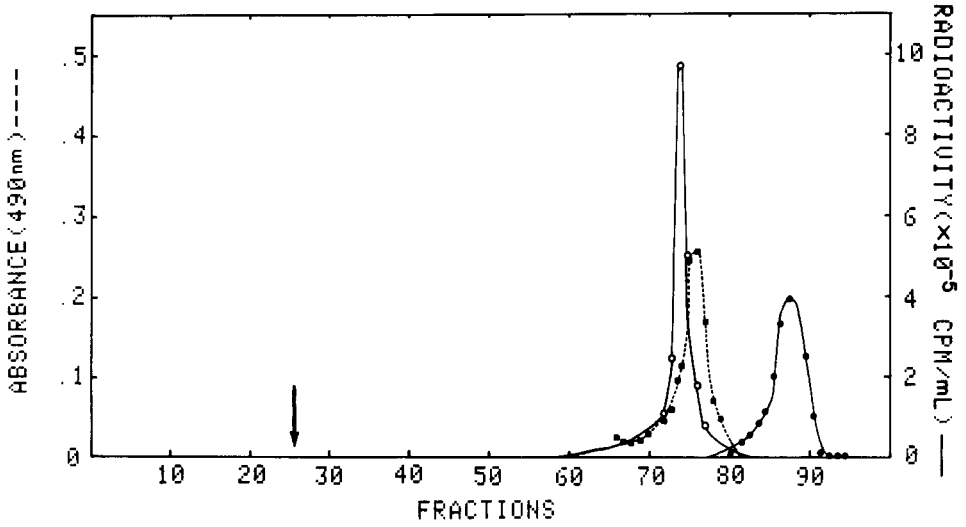

Fig. 2. The gel-filtration of a solution of lignoceric acid and α-cyclodextrin. Six nmol of [1-^{14}C]lignoceric acid (56 mCi/mmol) was incubated with 2 mg of α-cyclodextrin in 1 ml of buffer as described in the legend to Fig. 1, and a portion (0.1 ml) of the supernatant was applied to a column, 1.6 cm i.d. × 65 cm, containing Bio-gel p-2. The column was eluted with 20 mM phosphate buffer, pH 7.0. Fractions of 1.2 ml each were collected, and the radioactivity (shown by open circle with solid line) and hexose content (shown by solid square with broken line) were measured. The data shown by solid circles with solid lines represent the radioactivity when the addition of α-cyclodextrin was omitted. Void volume is indicated by an arrow.

an organic solvent, in detergent micelles, as a thin coat on particles, and by inclusion in liposomes. On the other hand, most of the enzymes involved in metabolism are membrane-bound. Therefore, the enzymic reactions usually have to be performed in three phases: first, a membrane preparation containing the enzyme; second, liposome, micelle, or substrate-coated particles; and finally, the assay medium. As a result, kinetic studies of lipid metabolism have been difficult, and anomalous results often are obtained (10, 11). These problems are more severe in the study of sphingolipid metabolism, because these lipids contain longer chain fatty acids and are more lipophilic than other lipids, such as phospholipids.

The present investigation disclosed that α-cyclodextrin can help solubilize fatty acids, including very long-chain fatty acids, and their derivative sphingolipids in aqueous medium by forming an inclusion complex. Presumably due to the increased solubility, more lipid substrate is made available to the enzyme system, and, con-

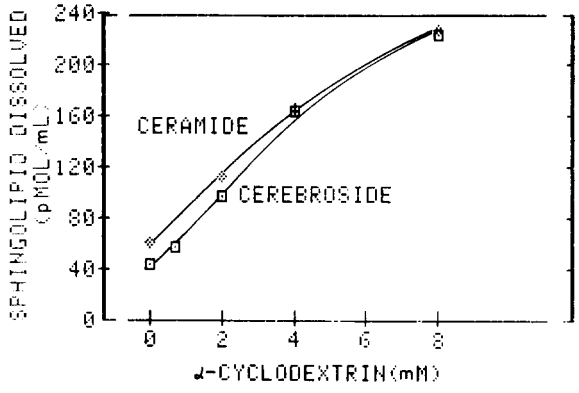

Fig. 3. The effect of α-cyclodextrin on the solubilization of ceramide and cerebroside. Lignoceroyl [3-^{3}H]sphingosine containing 222,225 dpm or lignoceroyl [3-^{3}H]psychosine containing 152,580 dpm was incubated with various amounts of α-cyclodextrin as described in the legend to Fig. 1, and the radioactivity in a portion of the supernatant was measured.

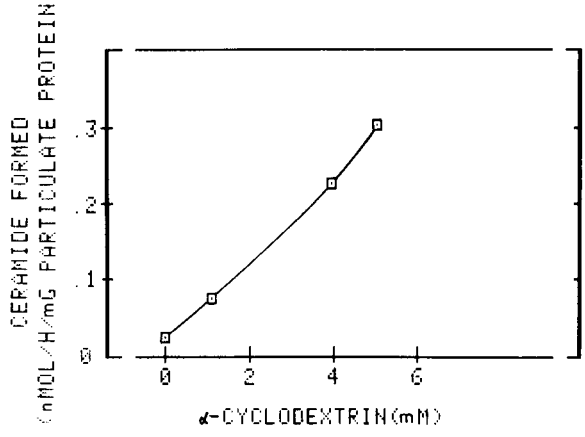

Fig. 4. The effect of α-cyclodextrin on the synthesis of ceramide from lignoceric acid. Six nmol of [1-^{14}C]lignoceric acid (56 mCi/mmol was incubated as described previously (4). The incubation mixture in 0.5 ml contained a rat brain particulate fraction (1 mg of protein), heat-labile factor (1 mg of protein), and 1 mM NADPH. The nonhydroxyceramide formed was separated by thin-layer chromatography and the radioactivity was measured.

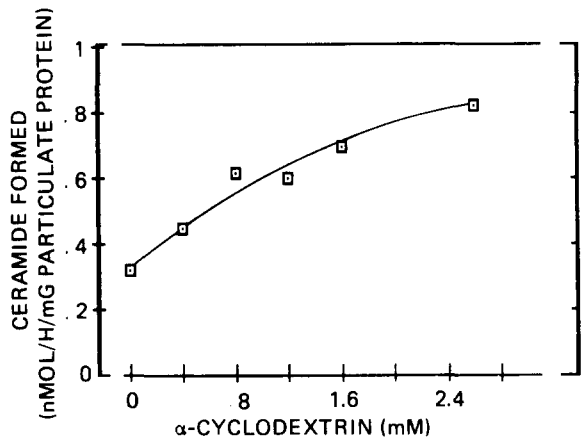

Fig. 5. The effect of α-cyclodextrin on the synthesis of ceramide from stearic acid. Six nmol of [1-^{14}C]stearic acid (50.0 mCi/mmol) was incubated as described in the legend of Fig. 4.

sequently, the apparent velocity of the enzyme reactions increases as shown in the synthesis of ceramides (Figs. 4 and 5). In addition to the enzymatic reactions shown in this communication, we have already demonstrated that the synthesis of cerebronic acid by α-hydroxylation and the formation of glutamic acid presumably by β-oxidation of lignoceric acid increased several-fold on the addition of α-cyclodextrin (12). In addition, we also showed that lignoceric acid in solution with α-cyclodextrin can be taken up and metabolized by brain tissue in an organ culture environment (17). These effects of α-cyclodextrin suggest the possibility of its wide use in in vitro studies of lipid metabolism and help in delineating anomalous enzymatic kinetics encountered in lipid enzymology studies.

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