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# Substrate-specificities of acid and alkaline ceramidases in fibroblasts from patients with Farber disease and controls

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The specific activity of acid ceramidase (*N*-acylsphingosine deacylase, EC 3.5.1.23) was measured at pH 4.5 in normal fibroblasts and in fibroblasts from patients with Farber disease and obligate heterozygotes. Greater activity was found when the synthetically made ceramide substrates contained shorter-chain fatty acids or higher content of double bonds. Acid ceramidase activities towards *N*-lauroyl- ($C_{12:0}$), *N*-myristoyl- ($C_{14:0}$) and *N*-palmitoyl- ($C_{16:0}$) sphingosine ($C_{18:1}$) were respectively about 38, 26 and 6 times higher than the activity towards the *N*-stearoyl ($C_{18:0}$) substrate. The activity towards *N*-linolenoylsphingosine ($C_{18:3}/C_{18:1}$), *N*-linoleoylsphingosine ($C_{18:2}/C_{18:1}$) and *N*-oleoylsphingosine ($C_{18:1}/C_{18:1}$) were respectively about 5, 4 and 3 times higher than the activity towards *N*-stearoylsphingosine ($C_{18:0}/C_{18:1}$). The activity towards *N*-stearoyldihydrosphingosine ($C_{18:0}/C_{18:0}$) was about 40% of that towards *N*-stearoylsphingosine. Fibroblast alkaline ceramidase possessed significant activity only towards ceramides of unsaturated fatty acids, with a pH optimum of about 9.0. Deficiency of acid ceramidase activity in fibroblasts from patients with Farber disease and intermediate activities in obligate heterozygotes were demonstrated with all ceramides examined except for *N*-hexanoylsphingosine ($C_{6:0}/C_{18:1}$), whereas alkaline ceramidase activity was unaffected. Comparative kinetic studies of acid ceramidase activity with *N*-lauroylsphingosine and *N*-oleoylsphingosine demonstrated about 5 (2–12)-fold and 7 (4–17)-fold higher $K_m$ values in fibroblasts from patients with Farber disease as compared with normal controls. *N*-Lauroylsphingosine, towards which acid ceramidase activity in control fibroblasts was about 10 times higher than that towards *N*-oleoylsphingosine, may serve as a better substrate for enzymic diagnosis of Farber disease as well as for further characterization of the catalytically defective acid ceramidase.

Ceramide is composed of a long-chain base with a fatty acid attached to the amino group at the C-2 position of the long-chain base. It is an important component of sphingolipids such as cerebrosides, sulphatides, phosphatides (sphingomyelins), gangliosides and other glycolipids. The compositions of the long-chain bases and fatty acids of ceramides were found to be variable in different sphingolipids and different tissues examined, and more than 80 different long-chain bases are known to exist in various tissues. Among them, 80–90% are the $C_{18}$ long-chain base known as D-*erythro-trans*-2-amino-1,3-dihydroxyoctadec-4-ene (sphingosine) (Moser, 1978). This is also the case in the ceramides accumulated in tissues from patients with Farber disease, except for the presence of significant amounts of 2-hydroxy fatty acids in kidney, cerebellum and liver from such patients. In normal tissues, at least outside the nervous system, ceramides contain no or minimal amounts of 2-hydroxy fatty acids (Moser *et al.*, 1969; Sugita *et al.*, 1973; Moser, 1978).

Farber disease (lipogranulomatosis) is a lysosomal storage disease inherited in an autosomal recessive manner (Moser, 1978). Sugita *et al.* (1972) has reported the deficiency of the enzyme ceramidase (*N*-acylsphingosine deacylase, EC 3.5.1.23) in kidney and cerebellum from a patient who died of Farber disease. Acid (pH 4.0) and alkaline (pH 9.0) hydrolytic activities were shown in normal cerebellum (Sugita *et al.*, 1975), fibroblasts and leucocytes (Dulaney & Moser, 1977). How-



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ever, only acid ceramidase activity could be detected in kidney (Sugita *et al.*, 1975). In Farber disease, the acid ceramidase activity is deficient, whereas the alkaline one is preserved. Deficient activity of acid ceramidase and accumulation of ceramides in tissues from patients with Farber disease (Moser *et al.*, 1969; Samuelsson & Zetterström, 1971) and increased excretion of ceramides into patients' urine (Iwamori & Moser, 1975) indicate that the basic defect in this disease is the deficiency of acid ceramidase activity. Incorporation and accumulation of ceramides into lysosomes of fibroblasts from patients with Farber disease, as well as normal fibroblasts overloaded with ceramide in culture medium, were observed (Rutsaert *et al.*, 1977; Chen *et al.*, 1981). The deficiency of acid ceramidase activity in fibroblasts has made possible the prenatal diagnosis of the disease (Dulaney *et al.*, 1976; Fensom *et al.*, 1979).

For the assay of both acid and alkaline ceramidases in human tissues, *N*-palmitoylsphingosine, *N*-stearoylsphingosine, *N*-oleoylsphingosine and the respective dihydrosphingosine (sphinganine) derivatives have been used (Sugita *et al.*, 1975). Among these substrates examined, *N*-oleoylsphingosine was cleaved most rapidly by the acid ceramidase in the crude homogenates of cerebellum and kidney, and *N*-stearoyldihydrosphingosine was cleaved least rapidly. No substrate-specificities have been reported on the acid and alkaline ceramidases in other tissues, including fibroblasts.

In the present paper we describe the substrate-specificities of both acid and alkaline ceramidases in fibroblasts from patients with Farber disease, obligate heterozygotes of Farber disease and normal controls, with special reference to the effect of chain length and the number of double bonds in the fatty acid moiety of ceramides.

## Experimental

### *Materials*

1-$^{14}$C-radiolabelled hexanoic acid ($C_{6:0}$), lauric acid ($C_{12:0}$), myristic acid ($_{14:0}$), palmitic acid ($C_{16:0}$), stearic acid ($C_{18:0}$), oleic acid ($C_{18:1}$), linoleic acid ($C_{18:2}$) and linolenic acid ($C_{18:3}$) were purchased from Research Products International (Elk Grove Village, IL, U.S.A.). Unlabelled fatty acids were the products of Eastman Kodak (Rochester, NY, U.S.A.) and Sigma Chemical Co. (St. Louis, MO, U.S.A.). D-*erythro-trans*-2-amino-1,3-dihydroxyoctadec-4-ene (sphingosine) and D-*erythro*-2-amino-1,3-dihydroxyoctadecane (dihydrosphingosine) were obtained from Calbiochem–Behring (La Jolla, CA, U.S.A.) and Miles–Yeda (Elkhart, IN, U.S.A.) respectively.

### *Synthesis of ceramides*

Radiolabelled and non-radiolabelled ceramides were synthesized by the direct coupling of fatty acid and long-chain base with the use of 1-(3-dimethylaminopropyl)-3-ethylcarbodi-imide hydrochloride (Sigma Chemical Co.) by the method of Hammarström (1971). After silicic acid column chromatography, the synthesized ceramides were purified by preparative t.l.c. on silica gel G (Analtech, Newark, DE, U.S.A.) with a solvent system of chloroform/methanol/acetic acid (94:1:5 or 104:1:5, by vol.). Chemical purity of the product was examined by g.l.c. (Varian gas chromatograph) by analysing the methyl esters of fatty acid moieties and trimethylsilyl derivatives of long-chain bases after acid hydrolysis [3% SE-30 on Chromosorb W-P, 80–100 mesh (Alltech, Deerfield, IL, U.S.A.); 0.2cm × 180cm glass column]. The trimethylsilyl derivatives of intact ceramides were also examined by g.l.c. [1% OV-1 on Gas-Chrom Q, 60–80 mesh (Alltech); 0.2cm × 180cm glass column]. Specific radioactivities of ceramides were adjusted to 650–1200c.p.m./nmol.

### *Fibroblast culture*

Cultured skin fibroblasts from three patients with Farber disease and three obligate heterozygotes were kindly provided by Dr. Hugo W. Moser, Baltimore, MD, U.S.A. Fibroblast cell lines from a patient with Farber disease (GM 2315) and an obligate heterozygote (GM 2317) were obtained from the Human Genetic Mutant Cell Repository, Institute for Medical Research, Camden, NJ, U.S.A. Control fibroblasts were obtained by skin biopsy from individuals apparently unaffected with any known metabolic disorders.

Skin fibroblasts were cultured in Eagle's Minimum Essential Medium supplemented with 10% (v/v) foetal bovine serum and antibiotics (GIBCO, Grand Island, NY, U.S.A.). At confluency, cells were harvested by trypsinization, washed twice with saline (0.9% NaCl) solution, resuspended in 1mM-EDTA/0.25M-sucrose solution (for enzyme stabilization) and sonicated for 10s in an ice bath at 50W (Ultrasonics model W 185 Cell Disrupter) with a microtip. For other lysosomal enzyme assays, harvested cells were sonicated in water instead of 1mM-EDTA/0.25M-sucrose solution. Crude sonicated preparation was used as an enzyme source.

### *Enzyme assays*

Ceramidase activities in fibroblasts were assayed by the method of Dulaney *et al.* (1976), with some modifications. The substrate was sonicated in 0.25M-citrate/phosphate buffer, pH3.0–7.0, or 0.25M-Bicine [*NN*-bis-(2-hydroxymethyl)glycine] (Sigma Chemical Co.) buffer, pH8.0–9.5, in the presence of Triton X-100, Tween 20 and sodium

cholate. The reaction mixture consisted of 50nmol of ceramide, 0.25mg of Triton X-100, 0.1mg of Tween 20, 0.4mg of sodium cholate and sonicated fibroblast preparation containing 0.25–2.5mg of protein in a final volume of 200$\mu$l with a buffer concentration of 0.125M. After incubation at 37°C for up to 5h, the reaction was stopped by adding 1ml of chloroform/methanol (2:1, v/v), containing 50$\mu$g of carrier fatty acid. In some enzyme assays the reaction mixture was decreased to one-quarter of the above mentioned volume, and 150$\mu$l of cold distilled water was added after the addition of 1ml of chloroform/methanol at the end of reaction. The tubes were vigorously mixed. After centrifugation at 1000*g* for 1min, the lower phase was chromatographed on silica gel G t.l.c. plates (20cm × 20cm) with a solvent system of chloroform/methanol/acetic acid (94:1:5 or 104:1:5, by vol.). Free fatty acid and ceramide were located by using $I_2$ vapour, and the corresponding areas were scraped off from the t.l.c. plate. The radioactivities of uncleaved ceramide and released free fatty acid were counted in 10ml of Sintrex liquid scintillator (J. T. Baker, Phillipsburg, NH, U.S.A.). Enzyme activity was expressed as nmol of fatty acid released/h per mg of protein.

Other lysosomal enzymes, e.g. $\beta$-galactosidase, hexosaminidase, $\alpha$-mannosidase and $\alpha$-galactosidase, were assayed with 4-methylumbelliferyl derivatives (Research Products International) at a final substrate concentration of 1mM in 0.25ml of citrate/phosphate buffer (0.1M-citric acid/0.2M-$Na_2HPO_4$), pH4.3, containing 50mM-NaCl. The reaction was stopped by addition of 0.75ml of 0.2M-glycine/carbonate buffer, pH10.2, and the liberated 4-methylumbelliferone was determined by using an Aminco spectrofluorimeter with excitation wavelength at 365nm and emission at 450nm, with 4-methylumbelliferone (Sigma Chemical Co.) as standard.

Acid lipase activity was determined by the method of Cortner *et al.* (1976), with 4-methylumbelliferyl oleate (Vega Biochemicals, Tucson, AZ, U.S.A.) as substrate.

The protein concentration of the enzyme solution was measured by the dye (Bio-Rad Laboratories, Richmond, CA, U.S.A.)-binding method, with bovine $\gamma$-globulin (Bio-Rad Laboratories) as standard (Bradford, 1976).

## Results

The yields of ceramides were 25–50% with respect to the amount of the long-chain base that was used in the synthesis. The amount of free fatty acid in the synthesized ceramides did not exceed 0.5% as shown by preparative t.l.c. of radiolabelled products. G.l.c. analyses of fatty acid methyl esters and trimethylsilyl derivatives of long-chain bases after acid hydrolysis of the synthesized ceramides demonstrated retention times identical with those of the authentic reference materials. G.l.c. chromatograms of trimethylsilyl derivatives of the synthesized ceramides before hydrolysis showed the individually distinct peaks expected, although no reference materials were available.

Fig. 1 shows the pH–activity curves of fibroblast ceramidase activities towards *N*-lauroylsphingosine and *N*-oleoylsphingosine. Fibroblast ceramidase activity towards *N*-oleoylsphingosine had two distinct optimal pH values, one acidic (pH4.5) and the other alkaline (pH9.0), whereas the activity towards *N*-lauroylsphingosine had only the acidic pH optimum. Fibroblasts from a patient with Farber disease showed deficient acid ceramidase activities towards both substrates, but the alkaline ceramidase activity towards *N*-oleoylsphingosine was unaffected. At pH4.5, and final reaction volume of 50$\mu$l, fibroblast ceramidase activities towards *N*-lauroylsphingosine and *N*-oleoylsphingosine were linear at protein concentrations of up to 430 and 580$\mu$g respectively. The concentration of product

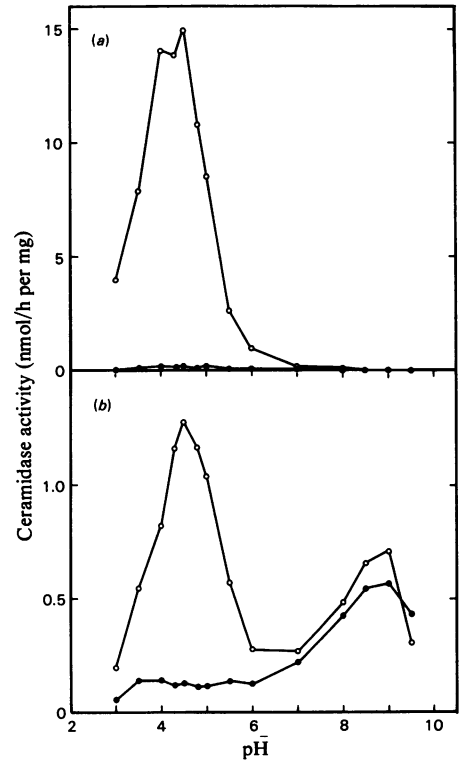


Fig. 1. *pH profile of ceramidase activity towards N-lauroylsphingosine (a) and N-oleoylsphingosine (b) in fibroblasts from a patient with Farber disease* (●) *and a control* (○)
For experimental details see the text.

increased continuously, although non-linearly, for at least 2h with *N*-lauroylsphingosine and for 5h with *N*-oleoylsphingosine. An incubation time of 30min was usually used for *N*-lauroylsphingosine and *N*-myristoylsphingosine and of 2h for the other substrates.

The acid *N*-lauroylsphingosine deacylase and the acid and alkaline *N*-oleoylsphingosine deacylase activities in fibroblasts from four patients with Farber disease, four obligate heterozygotes and control specimens are shown in Table 1. Acid ceramidase activities against *N*-lauroylsphingosine in control fibroblasts and in fibroblasts from heterozygotes and homozygotes for Farber disease were respectively about 10, 7.5 and 3.5 times as high as those towards *N*-oleoylsphingosine. Acid ceramidase activities in fibroblasts from patients with Farber disease were markedly diminished towards both substrates (2–9% of control), and the fibroblasts from obligate heterozygotes showed intermediate activities between those in patients and controls (16–46% of controls). There were no significant differences in alkaline ceramidase activities towards *N*-oleoylsphingosine among the various fibroblast cell lines.

Fig. 2 represents typical results of ceramidase assays with various ceramides with saturated fatty acids of different chain length. Acid ceramidase activity in normal and obligate heterozygote fibroblasts as well as the residual activity in fibroblasts from a patient with Farber disease were highest towards the $C_{12}$ fatty acid derivative (*N*-lauroylsphingosine) and decreased gradually with elongation of the hydrocarbon chain in the fatty acid moiety. Acid ceramidase activity towards the $C_6$ fatty acid derivative (*N*-hexanoylsphingosine) was very low, and no significant differences were found among the patient, heterozygote and control. Fibroblasts from the patient with Farber disease showed markedly decreased acid ceramidase activities for all substrates used (3–7% of control) except for *N*-hexanoylsphingosine, and the enzyme activities in fibroblasts from the obligate heterozygote for Farber disease were between those of the patient and control (13–45% of control).

No significant alkaline ceramidase activities were observed towards substrates containing saturated fatty acid moieties (Fig. 2). The activities of other lysosomal enzymes such as β-galactosidase, hexosaminidase, α-mannosidase, α-galactosidase and acid lipase were within normal ranges in all cell lines examined.

The effect of double bonds in $C_{18}$ fatty acid or long-chain base moieties on ceramidase activities are shown in Fig. 3. Among the ceramides with a $C_{18}$ fatty acid moiety, higher acid ceramidase activity was determined as the number of double bonds increased. The lowest activity was determined towards *N*-stearoyldihydrosphingosine, which possesses no double bonds, either in the fatty acid or in the long-chain base moiety. In control fibroblasts, the acid ceramidase activities towards *N*-stearoylsphingosine ($C_{18:0}/C_{18:1}$), *N*-oleoylsphingosine ($C_{18:1}/C_{18:1}$), *N*-linoleoylsphingosine ($C_{18:2}/C_{18:1}$) and *N*-linolenoylsphingosine ($C_{18:3}/C_{18:1}$) were respectively about 2.4, 6.4, 9.6 and 12 times higher than the activity towards *N*-stearoyldihydrosphingosine ($C_{18:0}/C_{18:0}$). A similar pattern was found for the respective residual acid ceramidase activities in fibroblasts from the patients with Farber disease (3–10% of control) and the obligate heterozygote (14–52% of control). In contrast with the very low alkaline ceramidase activities towards acylsphingosines containing saturated fatty acid moieties, significant alkaline ceramidase activities were observed towards substrates containing un-

Table 1. *Ceramidase activities towards N-lauroylsphingosine and N-oleoylsphingosine in fibroblasts from patients with Farber disease, obligate heterozygotes and controls*

For experimental details see the text. Control values are expressed as means ± S.D.

| | Ceramidase activity (nmol/h per mg of protein) | | |
|---|---|---|---|
| | | *N*-Oleoylsphingosine | |
| Cell type Substrate ... | *N*-Lauroylsphingosine pH4.5 | pH4.5 | pH9.0 |
| Farber disease | | | |
| Homozygotes 1 | 0.199 | 0.088 | 0.279 |
| 2 | 0.452 | 0.099 | 0.295 |
| 3 | 0.387 | 0.095 | 0.344 |
| 4 | 0.278 | 0.096 | 0.460 |
| Heterozygotes 1 | 2.10 | 0.330 | 0.384 |
| 2 | 2.43 | 0.328 | 0.301 |
| 3 | 2.31 | 0.168 | 0.312 |
| 4 | 3.01 | 0.491 | 0.318 |
| Control ($n$ =5) | 10.6 ± 3.3 | 1.06 ± 0.030 | 0.424 ± 0.125 |

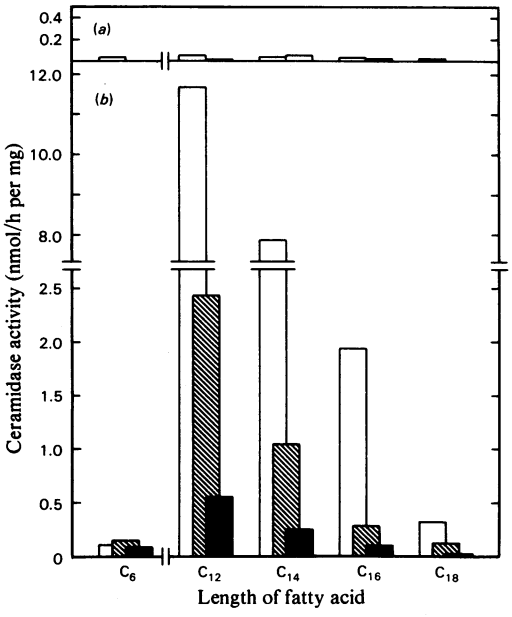


Fig. 2. *Alkaline (a) and acid (b) ceramidase activities towards ceramides with sphingosine ($C_{18:1}$) and saturated fatty acids of different hydrocarbon chain length in fibroblasts from single cell lines of a control (□), an obligate heterozygote (▨) and a patient with Farber disease (■)*
For experimental details see the text.

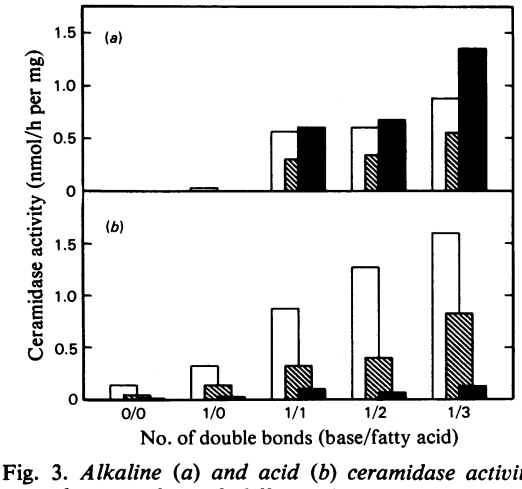


Fig. 3. *Alkaline (a) and acid (b) ceramidase activities towards ceramides with different degrees of saturation of long-chain base and $C_{18}$ fatty acid in fibroblasts from single cell lines of a control (□), an obligate heterozygote (▨) and a patient with Farber disease (■)*
For experimental details see the text.

saturated fatty acid moieties. Alkaline ceramidase activities were only slightly increased with increasing number of double bonds in the fatty acid moiety, and those of the patient with Farber disease and the obligate heterozygote did not differ significantly from those of the control.

Table 2 summarizes the apparent $K_m$ and $V_{max.}$ values obtained for ceramidase activities towards *N*-lauroylsphingosine and *N*-oleoylsphingosine in fibroblasts from the patients with Farber disease and control cell lines. The apparent $K_m$ value for ceramidase activity towards *N*-lauroylsphingosine in the patient's group was about 5 (2–12)-fold higher, and the $V_{max.}$ value was about 9 (5–16)-fold lower, than the respective values in the control group. The apparent $K_m$ value for ceramidase activity towards *N*-oleoylsphingosine in fibroblasts from patients with Farber disease was similarly increased [about 7 (4–17)-fold] as compared with controls, but the $V_{max.}$ of the mutant enzyme was only slightly decreased and did not differ significantly from that of the control group. It should be noted, however, that the highest substrate concentrations used in the kinetic studies (1 mM) was about 4 times the normal $K_m$ value for ceramidase activity towards *N*-lauroylsphingosine but only at about the normal $K_m$ value for ceramidase activity towards *N*-oleoylsphingosine. The $V_{max.}$ of the normal enzyme was only about twice that of the routine assay (substrate concentration of 0.25 mM) for *N*-lauroylsphingosine but about 6 times higher than the enzyme assay velocity when *N*-oleoylsphingosine was employed (Tables 1 and 2).

## Discussion

The findings of the present study indicate that the same acid ceramidase catalyses the hydrolysis of a variety of ceramide substrates, and, although the rate of hydrolysis is affected by the length of the hydrocarbon chain of the fatty acid moiety as well as the content of double bonds in both the fatty acid and the long-chain base moieties, the activity towards the various ceramides in patients with Farber disease appears to be deficient to the same extent with the exception of the activity towards *N*-hexanoylsphingosine. On the basis of this genetic evidence, the mutant enzyme appears to have the same substrate-specificity as the normal enzyme.

Our study also shows that the sonicated fibroblast preparation possesses ceramide-cleaving activity at the alkaline pH of 9.0, which seems to be due to the same enzyme as that found in human cerebellum (Sugita *et al.*, 1975). Another form of ceramide-cleaving activity was found in human duodenal contents, with an optimal pH of 7.6, and was regarded as of intestinal mucosal origin (Nilsson, 1969). Stoffel & Melzner (1980) also reported neutral (pH 7.4) ceramidase activity in the microsomal fraction of rat liver. No neutral activity was detected with the use of our extraction procedure and assay conditions for ceramidase activities in

Table 2. *Apparent $K_m$ and $V_{max.}$ values for ceramidase activities towards N-lauroylsphingosine and N-oleoylsphingosine in fibroblasts from patients with Farber disease and controls*

For experimental details see the text. N.D., Not determined.

| | | *N*-Lauroylsphingosine | | *N*-Oleoylsphingosine | |
|---|---|---|---|---|---|
| Cell type | | $K_m$ (mM) | $V_{max.}$ (nmol/h per mg) | $K_m$ (mM) | $V_{max.}$ (nmol/h per mg) |
| Farber disease | 1 | 0.820 | 1.32 | 6.67 | 2.86 |
| | 2 | N.D. | N.D. | 6.70 | 3.33 |
| | 3 | 1.54 | 3.33 | 6.67 | 4.00 |
| | 4 | 0.935 | 2.00 | 14.3 | 8.10 |
| Mean ± S.D. | ... | 1.10 ± 0.39 | 2.22 ± 1.02 | 8.58 ± 3.81 | 4.57 ± 2.40 |
| Controls | 1 | 0.200 | 20.0 | 1.25 | 4.00 |
| | 2 | 0.129 | 16.8 | N.D. | N.D. |
| | 3 | 0.196 | 20.8 | 1.05 | 4.57 |
| | 4 | 0.417 | 19.2 | 1.54 | 8.39 |
| | 5 | N.D. | N.D. | 0.833 | 5.88 |
| | 6 | N.D. | N.D. | 1.25 | 8.33 |
| Mean ± S.D. | ... | 0.236 ± 0.125 | 19.2 ± 1.73 | 1.18 ± 0.263 | 6.23 ± 2.06 |

fibroblasts. It appears, however, that three distinct forms of ceramidase occur in human tissue: a lysosomal acid form (pH 4.0–4.5), a membranous neutral form (pH 7.4–7.6) and a cytosolic alkaline one (pH 9.0). In Farber disease only the acid ceramidase is deficient.

The acid and alkaline enzyme forms studied in the present work, as well as the neutral enzyme form (Nilsson, 1969; Stoffel & Melzner, 1980), have distinct substrate-specificities, as shown in the assays performed *in vitro*; however, their physiological significance is unclear. As shown in Figs. 2 and 3, acid ceramidase can cleave various ceramides. Among the ceramides that we examined, the only exception is the short-chain fatty acid derivative, *N*-hexanoylsphingosine. Acid ceramidase activity towards this substrate is relatively low as compared with other ceramide-cleaving activities, and is apparently unaffected in patients with Farber disease. This activity may reflect a different enzyme with specificity towards ceramides containing short-chain fatty acids, whereas the enzyme deficient in Farber disease is specific towards *N*-acylsphingosines containing longer-chain fatty acid moieties. Gatt (1966) reported that acid ceramidases in rat brain could not cleave *N*-acetylsphingosine and *N*-lignoceroyldihydrosphingosine. Lignoceric acid is relatively abundant in ceramides from various tissues, so that hydrolysis of this very-long-chain fatty acid acylsphingosine must have physiological importance. In this context, the study by Normann *et al.* (1981) on the substrate-specificities of rat liver microsomal acyl-CoA synthetase, an enzyme distinct from ceramidase, is noteworthy because of the similar distributions of substrate-specificities with respect to carbon chain length of fatty acids. In their study, the highest activity was observed towards lauric acid, and both the addition and subtraction of carbon atoms gradually decreased maximum activities of acyl-CoA synthetase. It is difficult to explain these observations only by different solubility of substrates in the assay condition *in vitro*, as the authors suggested, since a more-soluble acid such as butyric acid was a poor substrate, as well as a less-soluble acid such as lignoceric acid. It is likely that physiological substrate-specificities may be distorted in the presence of detergents, since mixed micelle formation with detergents may be different for each substrate (Morell & Braun, 1972).

Compared with the acid ceramidase in fibroblasts, the substrate-specificities of alkaline ceramidase in fibroblasts are more characteristic. For the reaction *in vitro* with alkaline ceramidase, the presence of a double bond in the fatty acid moiety of the ceramide seems to be essential, and there is a tendency that the highly unsaturated ceramide (*N*-linolenoylsphingosine) is a better substrate for the enzyme, at least among ceramides containing $C_{18}$ fatty acid moieties (Fig. 3). Alkaline ceramidase activities are not affected in fibroblasts from patients with Farber disease, and this observation is consistent with the findings in cerebellum (Sugita *et al.*, 1975).

The neutral ceramidase reported by Stoffel & Melzner (1980) possesses broad substrate-specificities; rat liver microsomal ceramidase could cleave short-chain (butyric acid and acetic acid), medium-chain (octanoic acid and lauric acid) and long-chain (palmitic acid and lignoceric acid) fatty acid acylsphingosines at pH 7.4. The highest activity was observed towards *N*-acetylsphingosine.

The acid ceramidase partially purified from rat brain (Gatt, 1966; Yavin & Gatt, 1969) also catalysed the reverse reaction, the synthesis of

ceramide from long-chain base and free fatty acid. It is noteworthy that the substrate-specificities in the reverse reaction with respect to fatty acid chain length (Gatt, 1966) were similar to our findings for fibroblast acid ceramidase (Fig. 2). Stoffel *et al.* (1980) re-investigated the reverse reaction of ceramidase and showed that the reaction product of long-chain base and free fatty acid in the previous studies was *N*-acylethanolamine instead of *N*-acylsphingosine. Ethanolamine was derived from one of the buffer components and the reaction without ethanolamine did not produce any acyl derivatives at pH 5.0 or 8.0. They concluded that ceramide biosynthesis only occurred by the reaction of long-chain base with fatty acyl-CoA. However, the study by Sugita *et al.* (1975) showed synthesis of ceramide by reaction of long-chain base and free fatty acid at acidic (kidney) and both acidic and alkaline (cerebellum) conditions without use of ethanolamine in the buffer. In addition, this ceramide-synthetic activity at acidic pH was lacking in tissues from patients with Farber disease, whereas alkaline ceramide-synthetic activity was unaffected. Nilsson (1969) also observed ceramide synthesis from oleic acid and long-chain base with human duodenal contents as an enzyme source, which also possessed ceramide-cleaving activity at the same optimal pH of 7.6 with Tris/HCl or Tris/malate as buffer. Although it is likely that free-fatty acid-dependent ceramide synthesis catalysed by ceramidase is physiologically less important than acyl-CoA-dependent ceramide synthesis catalysed by acyl-CoA:sphingosine *N*-acyltransferase (Sugita *et al.*, 1975; Stoffel *et al.*, 1980), the reverse reaction of ceramidase should be evaluated in further studies.

In our present study, the acid ceramidase activities towards *N*-oleoylsphingosine and *N*-lauroylsphingosine are not linear with respect to incubation time, the reason for which may in part be the low effective substrate concentrations as judged from the apparent $K_m$ values. Inhibition by reaction products (fatty acid and long-chain base) and the reverse reaction to synthesize ceramide from cleaved fatty acid and long-chain base might also contribute to the non-linearity of ceramide-cleaving reaction. However, our findings of altered apparent $K_m$ values of acid ceramidase in fibroblasts from patients with Farber disease with *N*-oleoylsphingosine and *N*-lauroylsphingosine as substrates indicate that catalytically defective acid ceramidase is the basic defect in Farber disease. *N*-Lauroylsphingosine, towards which acidic ceramidase activities in control fibroblasts were 10 times higher than those towards *N*-oleoylsphingosine, may be a better substrate for further characterization of the defective acid ceramidase.

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