A Unique transacylase that catalyzes esterification of a short chain ceramide, N-acylsphingosine, was found in Madin-Darby canine kidney cell and mouse tissue homogenates. It esterified the hydroxyl group at the carbon-1 position of the ceramide. The enzyme has a pH optimum of 4.2 and a K_m of 9.4 μM for N-acylsphingosine at pH 4.5. The transacylase activity is independent of free fatty acid or acyl-CoA and instead uses the 2-acyl group of phosphatidylethanolamine or phosphatidylcholine. The transacylase activity in the homogenate was present in the 100,000 × g supernatant, and the lipid extracted from the membranous fraction could function as a donor of the acyl group. When liposomes consisting of 

long chain ceramide, free sphingosine, and a short chain ceramide, [3H]sphingosine formed radioactive polar sphingolipids, as long chain ceramide has received little direct support. Dbaibo et al. (3) reported that NAS was metabolically inert in J urkat lymphoblastic leukemia cells. However, their use of acetyl-labeled NAS limited their ability to detect sphingolipid metabolites since ceramide action would yield unlabeled sphingosine and [3H]acetic acid; the latter would be largely oxidized. In our studies of the lipid changes produced by NAS (4, 5), we found that Madin-Darby canine kidney cells (MDCK) and NIH 3T3 cells responded by producing considerably elevated levels of long chain ceramide and glucosylceramide. This finding appears to indicate that NAS is hydrolyzed in the cells and that the resultant sphingosine is acylated by the fatty acids characteristic of sphingolipids.

The present study was initiated, using N-acetyl-[3-3H]sphingosine as a substrate, to determine how NAS is converted to long chain ceramide in NAS-treated cells. An unknown radioactive nonpolar product was found and identified as 1-O-acetyl NAS. Okabe and Kishimoto (6) previously reported the presence of 1-O-acetyl-ceramide in rat brain. In this case, the amide-linked acyl group had the typical long chain structures. The enzymatic pathways for these acyl ester syntheses have not been elucidated. In the present study, we describe the characteristics of the short chain ceramide 1-O-acylation system and offer evidence that the novel enzyme may regulate not only endogenous ceramide levels but also release of arachidonic acid from phospholipid, particularly from phosphatidylethanolamine.

**EXPERIMENTAL PROCEDURES**

Materials—The reagents and their sources were: o-erythro-[3-3H]sphingosine (22 Ci/mmol) and l-1-palmitoyl-2-[1-14C]arachidonoyl phosphatidylethanolamine (57 Ci/mmol) from DuPont NEN; [9,10-3H]palmitic acid (40–60 Ci/mmol) and l-steaeryl-2-[5,6,8,9,11,12,14,15-3H]arachidonoyl phosphatidylcholine (210 Ci/mmol) from Amersham; [14C]acetic anhydride (50–100 mCi/mmol) from American Radiolabeled Chemicals; steaeryl-CoA, fatty acid-depleted bovine serum albumin, N-ethylmaleimide, Tween 20, ceramide type III from bovine brain sphingomyelin, phosphatidylethanolamine (PE) from bovine brain, phosphatidylinositol from bovine liver, phosphatidylserine from bovine liver; 2-[14C]arachidonoyl phosphatidylethanolamine from bovine liver, fatty acid-depleted bovine serum albumin, N-ethylmaleimide, Tween 20, ceramide type III from bovine brain sphingomyelin, phosphatidylethanolamine (PE) from bovine brain, phosphatidylinositol from bovine liver, phosphatidylserine from bovine liver; and [3H]acetic acid (50–100 mCi/mmol) from American Radiolabeled Chemicals.

**Acknowledgment**

The present study was initiated, using N-acetyl-[3-3H]sphingosine as a substrate, to determine how NAS is converted to long chain ceramide in NAS-treated cells. An unknown radioactive nonpolar product was found and identified as 1-O-acetyl NAS. Okabe and Kishimoto (6) previously reported the presence of 1-O-acetyl-ceramide in rat brain. In this case, the amide-linked acyl group had the typical long chain structures. The enzymatic pathways for these acyl ester syntheses have not been elucidated. In the present study, we describe the characteristics of the short chain ceramide 1-O-acylation system and offer evidence that the novel enzyme may regulate not only endogenous ceramide levels but also release of arachidonic acid from phospholipid, particularly from phosphatidylethanolamine.

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1. The abbreviations used are: NAS, N-acetylsphingosine (C2-ceramide refers to a natural mixture of N-acetylsphingosides); PE, phosphatidylethanolamine; PDMP, 1-phenyl-2-decanoylamino-3-morpholino-1-propanol; MDCK, Madin-Darby canine kidney; TLC, thin-layer chromatography; hRF, 100 × R_f value; DOPC, dioleoylphosphatidylcholine; PC, phosphatidylcholine; PL_A2, phospholipase A_2.
N-Acetylsphingosine—[3H]NAS was incubated with homogenate under a N₂ stream and fractionated by TLC with two solvent systems, chloroform:methanol:water (60:35:8) and chloroform:methanol (2:1). The extracted lipids were washed by partitioning with the fluorogenic reagent, primulin (11). The radioactive spots were visualized with the radioactive products, and the plate was sprayed with the fluorogenic reagent, primulin (11). The radioactive spots were scraped off and analyzed by liquid scintillation. Nonradioactive spots were also visualized by charring with cupric sulfate-phosphoric acid.

Experimental Procedure—

1. Lipid preparation—Brain, D-14384, D-14380, drosphingosine, and no labeled long chain ceramide. The lipids were extracted from the homogenate and dispersed in water or buffer for 8 min in an ice-water bath using a sonicator.

2. Incubation—The incubation mixtures contained buffer (usually citrate, pH 4.5), enzyme solution, or suspension, a ceramide, and a source of fatty acid. Cell homogenates prepared in water served as an enzyme source and acyl donor. Incubations were carried out in a thermostatically controlled ultrasonic bath (10) at 37°C. At the end of the incubation period, 200–500 μl of the reaction mixture were promptly mixed with 3 ml of chloroform:methanol (2:1). The extracted lipids were washed by partitioning against aqueous NaCl, and the resultant lipid extract was dried down under a N₂ stream and fractionated by TLC with two solvent systems, chloroform:methanol:water (60:35:8) and chloroform:methanol:ACOH (90:2.8). In some experiments, the TLC plate was subjected to fluorography at ~80°C. In others, nonlabeled standards were co-chromatographed with the radioactive products, and the plate was sprayed with the fluorogenic reagent, primulin (11). The radioactive spots were scraped off and analyzed by liquid scintillation. Nonradioactive spots were also visualized by charring with cupric sulfate-phosphoric acid.

RESULTS

Ceramidase Activity of MDCK Cell Homogenate toward N-Acetylsphingosine—[3H]NAS was incubated with homogenate in neutral and acidic buffers. The lipoidal contents of the reaction mixtures were separated with two different TLC systems and found to contain very little free radioactive sphingosine, and no labeled long chain ceramide. Unreacted NAS was seen (40% of the amount added to the incubation tubes after a 4-h incubation at low pH and 47% at neutral pH). Also seen was a lipid which migrated faster than NAS on the TLC plates. The hRf values for the two lipids were ~74 and ~92 for NAS and the unknown, respectively, in neutral TLC solvent and ~21 and ~72 in the acidic TLC solvent. The location of the labeled unknown product in the latter solvent system corresponded to that of synthetic 1,2-di-O-palmitoyl NAS. Long chain ceramide mixture appeared at hRf 51–57 in the same system.

More of the unknown product was formed at pH 4.5 than at neutral pH (Fig. 1). The amount found peaked at ~1 h and decreased with time, indicating the presence of the homogenate of both a synthase and a catabolizing enzyme. The NAS spot, on the other hand, showed a corresponding increase with time between 1 and 4 h, suggesting that the catabolic enzyme reconverted the unknown lipid to NAS. In view of the difference in hRf values, the reconversion to NAS, and the similarity in hRf values of the unknown and palmitate ester, it seemed likely that the enzymatic product of NAS was a fatty acyl ester.

The unknown product was not produced by homogenate treated for 10 min at 80°C. NAS labeled in either moiety gave the same results.

Identification of the Unknown Product—The 3H-labeled unknown product was extracted from the TLC plate and then exposed to alkaline methanolyis with chloroform:methanol:20% NaOH for 1 h. TLC of the products showed that the reaction produced disappearance of the radioactive product and an increase in [3H]NAS. This is strong evidence that the ceramide was bound to a carboxylic acid in ester linkage. Most of the radioactivity in the unknown product was recovered in the NAS spot.

To determine the position of the O-acyl group, we treated the 3H-labeled unknown product with 2,3-dichloro-5,6-dicyanobenzoquinone. This reagent oxidizes α,β-unsaturated alcohols to the corresponding ketones. Thus, a free hydroxyl in the C3 position of the sphingosine moiety would be oxidized to a ketone group, and a C3-substituted ester would be inert. Okabe and Kishimoto (6) previously used the reaction to show that the ceramide esters they found in rat brain had the ester linkage at the C1 position of ceramide. We found that most of the 3H in the NAS ester, after 2,3-dichloro-5,6-dicyanobenzoquinone treatment, was recovered in a higher position on the plate (Fig. 2). The location of this product corresponded to that of synthetic 3-keto-O-palmitoyl NAS prepared in the same way. These results support the conclusion that the unknown product produced from NAS by MDCK cell homogenate was 1-O-acyl NAS and that the decrease seen in the ester level later in the incubation (Figs. 1 and 3) was due to the presence of an esterase in the cell homogenate.

Acylation Activity of MDCK Cell Homogenate—Acylation of NAS was measured at pH 4.5 using different concentrations of homogenate: 168, 336, and 673 μg protein/ml. Fig. 3 shows that the rate of synthesis in the most dilute homogenate was constant for ~20 min and then seemed to slow down. Acylation was more rapid with higher amounts of homogenate, but the endogenous hydroxase became important too early to allow a constant rate of synthesis.

To determine the pH optimum of the enzyme, two buffer systems were used: 47 mM sodium citrate and 47 mM Tris maleate. A simple, rather sharp enzyme activity curve with a single peak at pH 4.2 was observed (Fig. 4). Additionally, the esterification activity was measured with different concentrations of [3H]NAS (5–100 μM), using 202 μg/ml of MDCK homo-
N-Acetylphosphatidylethanolamine Acyltransferase

Identification of unknown product. The putative NAS ester isolated by TLC (about 300 cpm) was incubated for 48 h at 37 °C with or without 3% 2,3-dichloro-5,6-dicyanobenzoquinone in 40 μl dimethyl sulfoxide and then dried down using a N2 stream. The dried sample was washed once with 3 ml chloroform:methanol (2:1) plus 0.6 ml of 0.1 N NaOH. The lower layer obtained after brief centrifugation was washed twice with 2 ml of MeOH:0.1 N NaOH (1:1) and twice more with 2 ml of methanol:water (1:1). The lipid in the lower layer was chromatographed with chloroform:methanol:AcOH (90:1:9). The TLC plate was divided into 0.5-cm fractions, starting at the origin, and was examined by liquid scintillation counting.

Transacylase activity of MDCK cell homogenate as a function of time and enzyme amount. The amount of acyl N-acetylsphingosine in the incubated mixtures was measured using different amounts of homogenate. The reaction mixture consisted of 47 mM sodium citrate or 47 mM Tris-maleate, 10 μM N-Ac-[3H]sphingosine, and 202 μg/ml homogenate. The reaction was carried out as in Fig. 1.

Effects of PE on the Transacylation Activity in the Supernatant—Liposomal preparations of potential phospholipid acyl donors were evaluated with [3H]NAS and the supernatant enzyme preparation. DOPC acted as an acyl (oleoyl) donor but did not affect the activity.

To obtain a more sensitive reaction system, possibly containing less endogenous lipid, we examined the distribution of the enzyme in MDCK cell homogenate by centrifuging it for 1 h at 100,000 × g at 4 °C. Neither the supernatant fraction nor the pellet fraction exhibited much activity, but a mixture of the two showed that all of the activity originally seen with the homogenate could be reformed. Heat inactivation of each separate fraction showed that the insoluble portion, even after heat denaturation, acted as the acyl donor and that most of the enzyme was present in the soluble fraction. We also found that the lipids extracted from the pellet could replace the pellet in the esterification reaction. In a separate experiment, the supernatant and membranes showed 25% and 19% of the original activity.

Filteration of the 100,000 × g supernatant through Corning cellulose acetate membranes (0.45-μm pores) lowered the basal activity of the acyltransferase consistent with the removal of endogenous lipids. The activity was restored when tested with membranous lipids. This filtered 100,000 × g supernatant was used as the enzyme source in subsequent studies.

Effects of PE on the Transacylation Activity in the Supernatant—Liposomal preparations of potential phospholipid acyl donors were evaluated with [3H]NAS and the supernatant enzyme preparation. DOPC acted as an acyl (oleoyl) donor but only at the 12.8 μM level (Table I). Inclusion of PE in the liposomes at a 30% mol ratio enhanced the acylation activity, especially at the higher liposomal concentration. Sulfatide in the liposomes produced improved acylation, especially at 128 μM. Since the acyl group in sulfatide is amide linked, it is very probable that the increase in acylation activity represented increased transfer of PC-bound oleic acid rather than N-linked fatty acid in the sulfatide. Inclusion of PE in the DOPC:sulfatide liposomes yielded the highest amount of acyl transfer, raising the possibility that both lecithin and phosphatidylethanolamine were donors for the transacylation reaction.

Transacylation of the Arachidonoyl Group from 1-Palmitoyl-2-arachidonoyl-PE to N-Acetylphosphosine—We incorporated 1-palmitoyl-2-[14C]arachidonoyl-PE into DOPC:sulfatide liposomes and incubated them with the supernatant enzyme. In the absence of NAS, only one major radioactive product was detected, hRF 87 with the acidic TLC solvent; it corresponded to arachidonic acid. When NAS was included in the incubation mixture, two major radioactive products were found. One was...
arachidonic acid, and the other (hRf 69) migrated near the same location as synthetic O-palmitoyl NAS, like the NAS ester formed from endogenous tissue lipids. Extraction of the latter radioactive product from the TLC silica gel and treatment with alkali as before resulted in disappearance of the product and formation of radioactive methyl arachidonate. These results show that arachidonate in the formation of radioactive methyl arachidonate. These results show that arachidonate in the sn-2 position of PE is transacylated to the hydroxyl group at the carbon-1 position of NAS.

Interestingly, the total radioactivity of the two radioactive products produced in the presence of NAS, arachidonic acid, and O-arachidonoyl NAS was similar to the radioactivity of the arachidonic acid produced in the absence of NAS. This suggests that the enzyme that catalyzes transesterification of NAS also caused 30–60% inhibition of both activities. Interestingly, 20 μM d-threo-PDMP, a ceramide-like amine and an inhibitor of glusolylceramide synthase (17), caused 50–60% inhibition of both activities when added to the incubation mixture as a dry residue in the incubation tubes.

Specificity of the Transacylase for Acceptor Sphingolipids—As shown in Table II, N-acetylsphingosine was the best acyl acceptor; N-octanoylsphingosine was considerably less effective. The transacylase activity observed using N-[3H]deoxy-}

arachidonic acid

in the whole homogenate (Figs. 1 and 3) was not present in the supernatant enzyme solution. The two activities, hydrolysis and transacylation, were quite similar.

One millimolar phenylmethylsulfonyl fluoride (an inhibitor of serine proteases and hydrolyses) in the incubation system caused ~30% inhibition of both activities. Interestingly, 20 μM d-threo-PDMP, a ceramide-like amine and an inhibitor of glucosylceramide synthase (17), caused 50–60% inhibition of both activities when added to the incubation mixture as a dry residue in the incubation tubes.

Deacylase and Transacylase Activities—Further evidence for dual activities of a single enzyme was obtained with different concentrations of NAS, using [14C]PE as the acyl source. As shown in Fig. 5, transacylation of arachidonate from PE to NAS increased in a concentration-dependent manner. In contrast, the release of arachidonoyl moiety transferred from PE to water and ceramide by the two reactions was unaffected by the NAS concentration. The addition of 1 μM free nonradioactive arachidonic acid did not affect these reactions.

The high affinity inhibitor of M, 85,000 cPLA₂ could use PE to transacylate lysophosphatidylcholine, producing phosphatidylcholine. When NAS was replaced by lysophosphatidylcholine in the cytosolic reaction mixture containing [14C]PE, little transacylation of arachidonate to form PC was observed, and the release of arachidonic acid was not affected.

Transacylase activity between NAS and PE was slightly increased by EDTA. However, EDTA is a weak chelator of Ca²⁺ at pH 4.5. One millimolar CaCl₂ slightly enhanced the activity. On the other hand, both 10 mM CaCl₂ and 10 mM MgCl₂ slightly reduced the activity. These effects are distinct from the Ca²⁺ requirement observed in presently known types I, II, and III PLA₂, or cPLA₂ (13, 14). An important difference between the new enzyme and other phospholipases A₂ is that the new ceramide transacylase works best at a low pH.

Treatment of the cell homogenate or soluble enzyme with 10 mM N-ethylmaleimide at 0°C for 30 min before assaying the transacylase activity with [3H]NAS did not affect the activity. Evidently, there is no essential accessible thiol group in the enzyme. The detergent, Tween 20 at 2 mg/ml, blocked nearly all of the activity. Fatty acid “free” bovine serum albumin (20 μM) inhibited the enzyme 35–50%. Anandamide (N-arachidonylthanolamine), reported to bind to the cannabinoid receptor (18), had some inhibitory activity with cell homogenates. The ID₅₀ was 200–300 μM.

To test the possibility that the ceramide-acylating activity is simply an aberrant property of an enzyme that is really a known Ca²⁺-independent phospholipase A₂, we examined the lipid products of reaction at two pHs by TLC. The soluble enzyme (108 μg/ml) was incubated 60 min at 37°C with liposomes (90 μM DOPC, 38 μM natural PE, and 18 μM dicetyl phosphate). At pH 4.2, the lipids showed a band corresponding to free fatty acid that was decreased in intensity by the induc-
sion of 10 μM NAS in the incubation tubes. The tubes containing NAS also showed the band for NAS ester. Lipids from incubation at pH 7.1 showed only a trace of fatty acid and no detectable formation of lysoPC. Thus, the supernatant extract contained virtually no enzyme that could hydrolyze PC or PE in the absence of added Ca²⁺ at neutral pH.

Arachidonoyl-lecithin as an Acyl Donor for NAS—Incubations were carried out as before using liposomes containing 1-stearoyl-2-[3H]arachidonoyl PC instead of labeled PE. The 0.5-mL reaction mixture consisted of 47 mM sodium citrate, pH 4.5, 10 μM NAS, 27 μg of cell supernatant protein, and liposomes (64 nmol of phospholipid containing 100,000 cpm of PC per assay tube). The liposomes were made from DOPC:sulfatide, 86:14 or DOPC:brain PE:sulfatide, 61:25:14. Radioactive free arachidonic acid was found, together with arachidonoyl NAS (Table III). In the system lacking NAS, more arachidonic acid was formed. The total amount of labeled lecithin cleavage, as noted before with labeled PE, was the same in both systems (218 cpm/min/mg of protein with NAS versus 228 cpm/min/mg of protein without NAS). The percentage of PC converted to ceramide ester is difficult to compare with PE because there is some competition by the oleic acid in DOPC

and also, in the case of PE-containing liposomes, by fatty acids in the nonradioactive PE. However, from the data for DOPC:sulfatide liposomes (Table III), it would appear that the transacylation was 0.05% with labeled PE under similar conditions, the conversion was 0.60%.

Confirmation of the nature of the reactions was obtained by incubating the enzyme solution with unlabeled DOPC:PE:sulfatide liposomes for 0.5, 1, and 2 h (Fig. 6). Samples incubated without enzyme or NAS showed only the liposomal lipids, indicating their stability in the incubation medium. When enzyme, with or without NAS, was included in the incubations, bands corresponding to lysophosphatidylcholine and free fatty acids could be seen, and the amounts increased with time. LysoPE was also formed, but it comigrated with DOPC and thus could be detected only with ninhydrin. (NAS ester migrated too high on the TLC plate to be shown in this figure.)

Radioactive Lipids Formed from NAS—MDCK cells were cultured for 1, 4, and 10 h with 10 μM [3H]NAS in serum-free medium. As shown in the radioautograph (Fig. 7), it was rapidly incorporated into the cells, and much of it remained unaltered. However, some [3H]NAS was obviously converted to other sphingolipids: sphingosine; long and very long chain ceramides; long and very long chain sphingomyelins; long chain glucosylceramides; and lipids which appeared to be NAS derivatives (N-acetylglicosylsphingosine and N-acetylsphingosylphosphorylcholine). The products were detected within as little as 1 h after introduction of NAS into the medium, and the levels of these metabolites in the cells increased with time.

Based on the specific activity of [3H]NAS, it was estimated that long chain ceramide derived from NAS was 0.71, 2.5, and 5.3 nmol/mg protein at 1, 4, and 10 h, respectively, after NAS addition to the medium. The level of ceramide in untreated MDCK cells has been observed to be ~1 nmol/mg protein (19, 20). Our results suggest that the increase in ceramide level usually observed in NAS-treated cells is due to the conversion of NAS, via free sphingosine, to long chain ceramides. This increase in ceramide is sufficient to produce growth inhibition or apoptosis. Although ceramidase activity toward NAS was very low in homogenized cells, the formation of sphingosine from NAS was confirmed in MDCK cells (Fig. 7). The sphingosine level was 0.16, 0.41, and 0.83 nmol/mg protein at 1, 4, and 10 h, respectively, after the treatment. Thus, sphingosine produced from NAS by ceramidase may be rapidly metabolized to other sphingolipids. Acid ceramidase readily acts as a synthase with appropriate fatty acids and sphingosine (21), but we failed to find incorporation of labeled palmitic acid into ceramide when NAS was present in the cell homogenate.

The intact cells accumulated NAS ester (Fig. 7, right side). Using our knowledge of NAS-specific activity, we could calculate that the concentration of the ester was 0.16, 0.76, and 2.29 nmol/mg protein at 1, 4, and 10 h. This product not only had almost the same mobility as O-palmitoyl NAS in the TLC system but also was degradable to NAS by alkaline. Thus, the

![Fig. 6. Lipid products of PE and PC metabolism by soluble enzyme.](http://www.jbc.org/)

**Table II**

Comparison of different acyl acceptors

| Acyl acceptor         | Reaction products (cpm) | Sphingosine | Arachidonic acid | Total (ester + free acid) |
|-----------------------|-------------------------|-------------|------------------|--------------------------|
| None                  | 0                       | 2500        | 2500             |
| N-Ac-sphingosine      | 1200, 1190              | 1440, 1440  | 2640, 2630       |
| N-Ac-dihydrasphingosine| 650, 680                | 1900, 1990  | 2550, 2670       |
| N-Octanoyl-sphingosine| 120, 100                | 2300, 2310  | 2500, 2410       |
| N-Octanoyl-glucosylsphingosine | 0, 0       | 2650, 2410  | 2650, 2410       |
| Sphingosine           | 0, 0                    | 2620, 2510  | 2620, 2510       |

**Table III**

[3H]Arachidonoyl lecithin as acyl donor for acetylsphingosine

[3H]Arachidonoyl lecithin was included in both kinds of liposomes. Brain PE was included in the DOPC:PE:sulfatide liposomes, lowering the proportion of unlabeled PC in the latter liposomes. Incubation was for 20 min. The data are means and SDs of triplicate incubations.

| Acyl donor liposomes | Reaction products (cpm/min/mg protein) | Acetylsphingosine | Arachidonic acid |
|----------------------|----------------------------------------|-------------------|-----------------|
| DOPC:sulfate (no NAS)| 0                                      | 228 ± 2           |
| DOPC:sulfate (+ 10 μM NAS) | 96 ± 18                  | 122 ± 28          |
| DOPC:PE:sulfate (+ NAS) | 56 ± 22                   | 69 ± 13           |
indeed work with C2-ceramides. However, it is possible, since showed that C2-ceramide does indeed exist in tissues (24). Like attacked by the acyl acceptor molecule or by water.

rate-limiting reaction, may form a transient acyl-enzyme link-
PpH optima differ greatly and the acceptors for transacylation or CoA-independent-transacylases thus far reported, since the 

ENGZHANCED in water, and then centrifuged at 100,000 g. The total lipids were extracted and analyzed as described under “Experimental Procedures.” The plate in the left side of the figure was developed with chloroform:methanol:water (65:30:8), and the plate at the right was developed with chloroform:methanol:AcOH (90:1:9). The GlcCer migrated too high on the former plate to show up clearly. The plates were subjected to fluorography with ENHANCE at –80°C.

transacylation reaction system observed in vitro can also take place in intact cells.

Occurrence of Ceramide Transacylase in Animal Tissues—

Tissues from young male mice were stored at –80°C, homogenized in water, and then centrifuged at 100,000 × g as before. The clarified soluble extract (52–130 μg of protein/ml) was assayed in citrate buffer, pH 4.5, with 10 μM NAS and 64 μM DOPC, 8.6 μM dicetyl phosphate, and 2.65 μM palmitoyl-[14C]arachidonoyl-PE (170,000 cpm) in liposomal form. Incubation was for 20 min, and the NAS arachidonate radioactivity was measured in the usual way.

The observed specific activities (pmol/h/mg protein) were 455 for brain, 281 for spleen, 183 for liver, and 93 for kidney. MDCK cells treated the same way at the same time yielded 2890 pmol/h/mg, a much greater activity. It is likely that the enzyme is widespread and that the differences in specific activity might signify a special role.

**DISCUSSION**

We report that the esterification of NAS to form 1-O-acylceramide occurs via the transacylation of phospholipids, particularly PE. Our findings strongly suggest that both the deacylation and transacylation of arachidonate in the sn-2 position of PE are catalyzed by the same enzyme in the 100,000 × g supernatant. The enzyme is different from the cytosolic PLA2 or CoA-independent-transacylases thus far reported, since the pH optima differ greatly and the acceptors for transacylation are very different (13, 14, 22, 23). The new enzyme, via a rate-limiting reaction, may form a transient acyl-enzyme linkage to a hydroxyl group of a serine residue. The intermediate is attacked by the acyl acceptor molecule or by water.

Curiously, the enzyme is restricted in its activity to very short chain ceramides, supposedly nonexistence in nature. However, a report appearing after the conclusion of this study showed that C2-ceramide does indeed exist in tissues (24). Like the NAS acyl ester, C2-ceramide is formed by a transacylation reaction. In the case of the C2-ceramide, the transfer reaction consists of acetylate from platelet-activating factor to sphingosine. The low Km, found for the NAS-acylating reaction (9.4 μM) is consistent with the belief that the enzyme does indeed work with C2-ceramides. However, it is possible, since long chain ceramide esters do exist in nature, that the same transacylase works with long chain ceramides with the aid of a specific cofactor, in analogy to the role of saposins in sphingo-
lipid hydrolysis.

C2-ceramides can be expected to diffuse out of cells relatively rapidly. Such diffusion has been observed for short chain glu-
cosylceramide and sphingomyelin (for examples, see Ref. 19). Free sphingosine can form rapidly under certain conditions; the ability to acetylate and excrete free sphingosine rapidly could give cells a mechanism for limiting the metabolic influence or damage of sphingosine. The transacylase described in this paper would act to make NAS much less hydrophilic, thus causing the NAS ester to be retained. The membrane-bound esterase that we found to attack O-acyl NAS may have the function of later reforming NAS, which can be slowly hydrolyzed and converted to free sphingosine. In such a system, O-acyl NAS would act as a buffer to store and conserve excess sphingosine until it can be safely used. The system may also regulate the biological activity of free arachidonic acid and the functioning of platelet-activating factor.

Although there are many reports based on the addition of NAS to cell media, it is not clear whether the cell responses were due to NAS itself or to metabolite(s) of NAS. The present results suggest the possibility that the marked increases in long chain ceramide and glucosylceramide, the formation of O-acyl NAS, or the formation of truncated glucosylceramide and sphingomyelin play roles in the reported cellular responses to NAS.

An interesting relationship between PE and NAS has been reported recently (25). The activity of a phospholipase D in intact fibroblasts was stimulated by phorbol ester, and NAS inhibited the cleavage. The activity was evaluated by measuring the amount of phosphatidylethanol formed when ethanol was included in the cell medium. NAS reduced the amount of this transphosphatidylation product, which used primarily PE (not PC). However, it is possible that NAS, a primary alcohol, did not inhibit the enzyme but simply competed with EtOH for the transfer reaction, as in our system.

It is possible that ceramide transacylation normally regulates the cell level of long chain ceramide. The inhibition of transacylase activity by O-threo-PDMP may be one factor in the accumulation of the long chain ceramide that appears in cells treated with this ceramide-like compound (8, 20).

The ability of PDMP to block ceramide transacylase suggests that the inhibitor binds to a site in the enzyme that normally binds ceramide. This explains its ability to block ceramide glucosyltransferase (17), glucosylceramide galactosyltrans-
ferase, lactosylceramide sialyltransferase (26), and two other glycosyl transferases that make globosides (27). This range of inhibitory activities is consistent with structural conservation of the ceramide-binding domains of these diverse enzymes.

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