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Enzymatic Hydrolysis of Sphingolipids. VIII. Further Purification and Properties of Rat Brain Ceramidase*

Ephraim Yavin and Shimon Gatt

ABSTRACT: Rat brain ceramidase, the enzyme that catalyzes both the hydrolysis and synthesis of ceramide (*N*-acylsphingosine), was purified over 200-fold. Advantage was taken of the fact that the enzyme withstands prolonged treatment with trypsin and chymotrypsin. This treatment digests 80% of the protein and decreases the molecular weight of the enzyme as determined by gel filtration through Bio-Gel but does not impair the enzymatic activity. Evidence is presented that fatty acyl coenzyme A is not a direct substrate for ceramide synthesis.

The acyl portion can be utilized only after hydrolysis by an accompanying hydrolase to coenzyme A and a free fatty acid. Trials were made to separate the hydrolytic and synthetic activities by subjecting the enzyme to ammonium sulfate fractionation, sonic irradiation, adjustment to acid pH, heating, treatment with proteolytic enzymes or with SH reagents, and chromatography on TEAE-cellulose. None of these procedures resulted in a separation of the two activities from each other. Reaction mixtures were taken to an apparent equilibrium, starting with either ceramide or a mixture of sphingosine and fatty acid. The calculated equilibrium constant, defined as K_{equil} = (sphingosine) × (fatty acid)/(ceramide), depended upon the substrate employed. It was about 10^{-4} M when determined in the direction of ceramide synthesis, but only 5×10^{-6} M when measured in the direction of hydrolysis. The meaning and possible significance of these findings are discussed.

Ceramide is the trivial name of the *N*-acyl derivative of sphingosine or a related long-chain base. All sphingolipids can be considered as derivatives of ceramide; *e.g.*, sphingomyelin is ceramide phosphorylcholine and cerebroside is galactosylceramide. The biosynthesis and degradation of this compound are therefore important steps in the metabolism of all sphingolipids. An enzyme that catalyzes the hydrolysis of ceramide to sphingosine and fatty acid was purified from rat brain (Gatt, 1963, 1966). This same enzyme also catalyzes the reverse reaction, namely, the synthesis of ceramide from sphingosine and a free fatty acid.

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In order to gain further insight into this reaction, in which the amide bond between the sphingosine base and the fatty acid is both hydrolyzed and synthesized, the enzyme was further purified and several of its properties, including the equilibrium of the reaction, were investigated. A preliminary report has appeared (Yavin and Gatt, 1967).

Experimental Procedure

Substrates. *N*-[1-^{14}C]Palmitoylsphingosine was prepared according to Gatt (1966); *N*-[9,10-di-^{3}H]oleylsphingosine was prepared using a similar procedure. ^{14}C-Labeled fatty acids were purchased from the Radiochemical Centre, Amersham, England. Sphingosine was isolated from hydrolysates of spinal cord lipids by chromatography on silica gel columns (Barenholz and Gatt, 1968). [9,10-Di-^{3}H]oleyl coenzyme A was prepared by the following modification of the method of Kornberg and Pricer (1953). An incubation mixture in a volume of 22 ml contained 1500 μmoles of potassium phosphate (pH 7.3), 300 μmoles of cysteine, 225 μmoles of $MgCl_2$, 90 mμmoles of potassium fluoride, 20 μmoles of [9,10-di-^{3}H]oleic acid, previously neutralized with KOH, and 75 mg of lyophilized rat liver microsomes. After incubating for 90 min at 37° under nitrogen the reaction was terminated with 22 ml of 1% perchloric acid. The precipitate was collected by centrifuging for 10 min at 15,000*g* and was washed twice, successively, with 15 ml each of 1% perchloric acid and three times with 9 ml each of 80% ethanol. The residual sediment was extracted three times with 1 ml of a mixture of equal volumes of pyridine, water, and isopropyl alcohol. The pooled extracts were evaporated to dryness, dried *in vacuo* over H_2SO_4, and the residue was dissolved in 2 ml of 5 mM Tris (pH 7.4). The product (4.5 μmoles of oleyl coenzyme A, 2.5×10^6 dpm/μmole) had a maximum absorption at 259 mμ and a minimum at 230 mμ (ratio 260/230 = 1.92). When the compound was partitioned in the biphasic system of Dole (1956), only 5% of the radioactivity was found in the heptane phase, indicating a contamination of about 5% or less with free fatty acid.

Chemicals. Triton X-100 was obtained from Rohm and Haas, sodium cholate from Mann, Sephadex from Pharmacia, Bio-Gel from Bio-Rad, and trypsin and chymotrypsin (Grade II) from Sigma. Soybean trypsin inhibitor (prepared according to Birk *et al.*, 1963) was a gift of Mr. A. M. Konijn of the Laboratory of Nutrition.

Assay of the Reaction. To facilitate homogeneous dispersion of substrates and detergents (*i.e.*, cholate, which has a poor solubility at an acid pH), the assays were performed at pH 7.4 rather than at a more acid pH value.

Hydrolysis. The reaction (in a volume of 0.2 ml) was terminated with 3 ml of a mixture of isopropyl alcohol–heptane–1 N NaOH (400:100:10, v/v) (modification of Dole, 1956). Heptane (1.8 ml) and water (1.6 ml) were added, the mixture was shaken, centrifuged, and the upper, heptane phase was discarded. The lower phase was washed twice with 2 ml each of heptane; 1 ml of 1 N H_2SO_4 was added and the fatty acid was partitioned into 2.4 ml of heptane. The upper heptane layer was transferred to a counting vial. Scintillation fluid (10 ml) (5 g of 2,5-diphenyloxazole and 130 mg of 1,4-[bis-2-(4-methyl-5-phenyloxazolyl)]benzene in 1 l. of toluene) were added and the samples were counted in a scintillation spectrometer.

Synthesis. The reaction was terminated as above. The upper heptane phase was collected and washed with two 2-ml portions of the lower phase of a mixture of isopropyl alcohol–heptane–0.03 N NaOH (4:4:3, v/v). It was then transferred to a counting vial and counted as above.

Methods. Suspensions of the substrates were prepared according to method b of Gatt (1966). The ultracentrifugation of the enzyme was performed in a Beckman-Spinco analytical ultracentrifuge by Mr. P. Yanai of the Department of Biological Chemistry.

Results

Further Purification of the Enzyme. The enzyme was formerly purified by Gatt (1963). Methods were sought to further purify and increase the yield of the enzyme. Such purification could be achieved by heating the enzyme at 58°, by adjusting the pH to 4.1 (where most of enzymatic activity remains in solution), by chromatography on DEAE- or TEAE-cellulose, and by treatment with trypsin and chymotrypsin. After comparing these methods, the following procedure was adopted (Table I).

Rats (16-day old) were decapitated and their brains were homogenized with nine volumes of a solution containing 0.25 M sucrose and 1 mM EDTA (pH 7.4). Debris was sedimented by centrifuging for 10 min at 800*g* and washed twice with sucrose–EDTA (3 ml/g of brain). The combined supernatants were centrifuged for 30 min at 27,000*g* and the sediment ("particles") was suspended in sucrose–EDTA (4 ml/g of brain). This suspension was subjected to sonic irradiation for 3 min at 10 kc in a Raytheon sonic oscillator and centrifuged for 30 min at 27,000*g*. The sediment was suspended in a solution of 0.5% sodium cholate in sucrose–EDTA (0.5 ml/g of brain) and the mixture was stirred for 2 hr at 0°. It was then centrifuged for 45 min at 100,000*g* and the supernatant was dialyzed against two changes of 4 l. each of 5 mM Tris (pH 7.4). The content of the dialysis bag ("cholate extract") was clarified by centrifuging for 10 min at 27,000*g* and fractionated with solid ammonium sulfate. The protein that precipitated between 30 and 60% ammonium sulfate saturation was collected and dialyzed against 5 mM Tris (pH 7.4).

The dialyzed enzyme (70 mg) was incubated (in a final volume of 6 ml) with 1 mg each of trypsin and chymotrypsin in 0.1 M Tris-HCl (pH 7.4). After 2 hr at 37° the reaction was stopped by the addition of 0.25 mg of soybean trypsin-inhibitor. The mixture was filtered through a column of Sephadex G-150 (35 × 4.2 cm), previously equilibrated with 5 mM of Tris-HCl (pH 7.4), and the protein was eluted in 4-ml fractions, with the same buffer. The elution pattern is shown in Figure 1. About 80% of the protein (as determined by

TABLE I: Purification of Rat Brain Ceramidase.[a]

Fraction	Vol (ml)	Protein Total (mg)	Protein Recov (%)	Act. Total (units)	Act. Recov (%)	Sp Act. (units/mg)	Purifcn-fold
Homogenate	870	20,000		71,100		3.56	
Particles	406	12,000	60	40,500	57	3.4	0.95
Cholate extract	96	215	1.08	9,200	12.9	42.7	12
Ammonium sulfate 30–60% fraction	6.6	90.5	0.45	8,340	11.7	92	26
Sephadex effluent							
Fractions 22–26	20	11.9	0.06	4,830	6.8	405	114
Fractions 27–32	24	4.1	0.02	3,060	4.3	750	210

[a] Incubation mixtures in volumes of 0.2 ml contained 30 μmoles of Tris-HCl buffer (pH 7.4), 0.02 μmole of [*N*-9,10-di-^{3}H]oleylsphingosine (4.5×10^6 dpm/μmole), 0.1 mg of Triton X-100, 0.2 mg of sodium cholate, and appropriate amounts (1–30 μg) of enzyme. They were incubated for 30 min at 37° and fatty acid was isolated as described in Experimental Procedure. One unit is defined as that amount of enzyme catalyzing the hydrolysis of 1 mμmole of substrate/hr. The protein content was determined by the method of Lowry *et al.* (1951) or Warburg and Christian (1941).

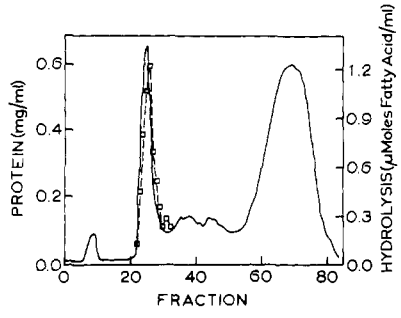

FIGURE 1: Gel filtration of proteinase-treated enzyme through Sephadex G-150. For details, see text. Solid line: protein; broken line: degree of hydrolysis.

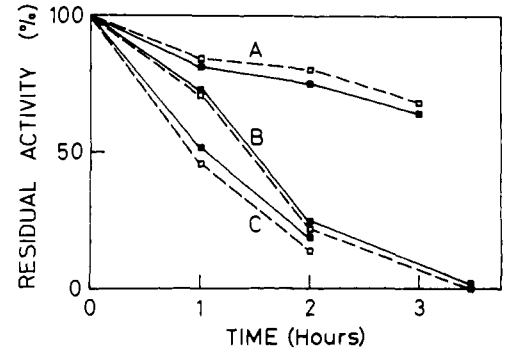

FIGURE 2: Effect of heating on enzymatic activity. Incubation mixtures in volumes of 0.2 ml contained 0.02 μmole of [*N*-9,10-di-^{3}H]oleylsphingosine (for hydrolysis) or 0.08 μmole of sphingosine and 0.02 μmole of [9,10-di^3-H]-oleic acid (for synthesis), 0.1 mg of Triton X-100, 0.2 mg of sodium cholate, enzyme, and buffer as specified below. The mixtures were incubated at 58°; at the specified times aliquots were removed and fatty acid or ceramide was isolated according to Experimental Procedure. Expt A: 8 μg of cholate extract and 10 μmoles of sodium phosphate (pH 8). Expt B: 10 μg of a supernatant obtained by adjusting a cholate extract to pH 4.1 and removing the precipitate formed; 10 μmoles of sodium phosphate (pH 8). Expt C: 4 μg of fraction 25 (Figure 1) and 30 μmoles of Tris-HCl (pH 7.4). (■—■) Hydrolysis of ceramide; (□---□) synthesis of ceramide.

the absorption at 280 mμ) was eluted in fractions 55–80 as low molecular weight compounds suggesting that a large portion of the protein was digested by the proteolytic enzymes. This procedure did not, however, impair ceramidase activity which was collected in fractions 22–32. The over-all purification (Table I) was over 200-fold, resulting in a yield of about 4 mg of enzyme from 80 rat brains. Other animals, such as pig, calf, or ox were tested as source for larger enzyme quantities. Because of very low specific activities of the enzyme in homogenates of brains of these animals, purification was not attempted.

Stability of the Enzyme. STORAGE. Dialyzed cholate extracts retained considerable enzymatic activity for at least 4 years when stored at −20°. The purified fractions, which had been treated with the proteolytic enzymes, lost up to 50% of their activity after 6 months at −20°.

HEAT STABILITY. Only little loss of activity was observed when the enzyme (a fraction obtained at 30–60% saturation with ammonium sulfate) was heated for 5 min at temperatures up to 55°. Figure 2 shows the effect of heating at 58° on the enzyme at three purification stages. The preparation employed in the experiments shown in curves A–C were purified 12-, 39-, and 112-fold, respectively. It is clear that the more purified preparations are less stable when heated at the above temperature.

STABILITY TO PROTEOLYSIS. The enzyme withstands prolonged treatment with either trypsin, chymotrypsin, or with a mixture of both these proteases. When incu-

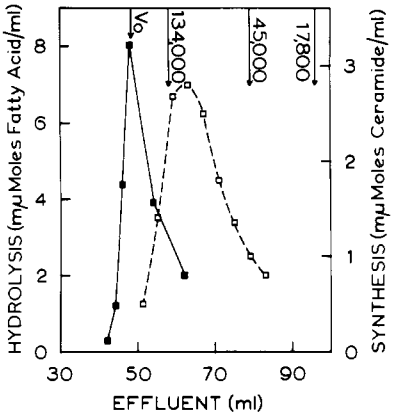

FIGURE 3: Determination of the molecular weight of the enzyme on Bio-Gel-P-150. Enzyme solutions were filtered through a column of Bio-Gel P-150 (45 × 3 cm), previously equilibrated with 50 mM $NaHCO_3$. The column was eluted with the same solution, 2-ml fractions were collected, and the enzyme was assayed for hydrolysis or synthesis of ceramide. The column was standardized using the following proteins: Bovine serum albumin (dimer) mol wt 134,000, ovalbumin mol wt 45,000, and myoglobin mol wt 17,800. (■—■) Ammonium sulfate 30–60% fraction. The activity was measured by hydrolysis of ceramide. (□—□) Enzyme after proteolysis and gel filtration through Sephadex G-150. The activity was measured by synthesis of ceramide.

bated with both trypsin and chymotrypsin, the enzyme lost only little activity within 2 hr; after 24 hr, a decrease of only 25% was obtained.

It was of interest to test whether the proteases changes the molecular size of the enzyme. The purified enzyme, after proteolysis, migrated in the analytical ultracentrifuge as a single peak of 3.3 S. However, the molecular weight could not be determined since sufficient protein was not available to determine the diffusion constant. The molecular weight was therefore estimated by gel filtration through Bio-Gel. The elution pattern from a column of Bio-Gel P-150 is shown in Figure 3. The enzyme after proteolysis was eluted at 1.3 times the void volume, suggesting a molecular weight of about 100,000. The enzyme before proteolysis was eluted at the void volume, indicating a molecular weight of 150,000 or more. This suggests that the proteolytic treatment decreased the molecular size of the enzyme, without consequent loss in activity. The possibility should not however be overlooked that proteolysis does not change the size of the enzyme, but releases it from high molecular weight association with lipids or other proteins.

Studies on the Reversible Nature of the Reaction Catalyzed by the Enzyme. FREE FATTY ACID AS SUBSTRATE FOR SYNTHESIS OF CERAMIDE. Gatt (1963, 1966) has shown that the enzyme catalyzes the synthesis of ceramide, utilizing a free fatty acid and either sphingosine or dihydrosphingosine (phytosphingosine has now been tried and also served as a substrate, except that the rates of synthesis were only 25% of those obtained with sphingosine). Gatt (1966) also found that palmitoyl coenzyme A could replace the free fatty acid as substrate for synthesis of ceramide.

TABLE II: Comparison of Oleic Acid and Oleyl Coenzyme A as Substrates for Ceramide Synthesis.[a]

Fraction	Ceramide Synthesized: From Oleic Acid (mμmoles)	Ceramide Synthesized: From Oleyl–CoA (mμmoles)	Fatty Acid Released from Oleyl–CoA (mμmoles)
40–70	2.0	2.3	11.0
17	0.5	0.03	0.4

[a] Incubation mixtures in volumes of 0.2 ml contained 30 μmoles of Tris-HCl (pH 7.4), 0.08 μmole of sphingosine, 0.02 μmole of either [9,10-di-^{3}H]oleic acid or [9,10-di-^{3}H]oleyl coenzyme A, 0.1 mg of Triton X-100, 0.2 mg of sodium cholate, and enzyme (30 μg of fraction 40–70 and 4 μg of fraction 17). The mixtures were incubated for 1 hr at 37°. For determination of the degree of hydrolysis of oleyl coenzyme A, the appropriate incubation mixtures had the same components with the exception of sphingosine. The reaction was terminated according to Dole (1956) and the phases were separated. The upper heptane phase was shaken with 4 ml of 0.1 N NaOH in 50% ethanol and the phases were separated. The upper layer which had the ceramide was transferred into counting vials. The lower phase was acidified and the fatty acid was partitioned into heptane and counted.

An investigation of the enzyme at the purification stage used by Gatt (1966), revealed the presence of an enzyme, which hydrolyzed the acyl–CoA to coenzyme A and a free fatty acid. The rate of hydrolysis of oleyl coenzyme A was about five times greater than that of ceramide synthesis. This suggested that the palmitoyl–CoA probably was not the direct substrate for ceramide synthesis, but might have been first split by the hydrolase; the free fatty acid thus released subsequently condensed with sphingosine.

Table II shows an experiment designed to clarify this point. Two separate ceramidase preparations were used. The first (fraction 40–70) had not been subjected to proteolysis; it hydrolyzed oleyl–CoA at a five times greater rate than that of ceramide synthesis. The second (fraction 17) had been treated with trypsin and chymotrypsin and then filtered through Sephadex G-150; it hydrolyzed oleyl–CoA at a rate similar to that of ceramide synthesis.

Using fraction 40–70, about equal rates of ceramide synthesis were obtained with sphingosine and either oleic acid or oleyl–CoA. The formation of ceramide from oleyl coenzyme A could be accounted for by the 11 mμmoles of fatty acid released from this compound. This conclusion is substantiated by the fact that, with fraction 17, where only 0.4 mμmole of fatty acid was released from oleyl–CoA, 15 times more ceramide was formed from free oleic acid than from the coenzyme A derivative. These results suggest that acyl coenzyme A is not a direct substrate for ceramide synthesis but donates its acyl portion only after hydrolysis to a free

TABLE III: Equilibrium Constant of Enzyme-Catalyzed Reaction.[a]

	Substrate	Products		
Expt	Ceramide (μM)	Sphingosine (μM)	Fatty Acid (μM)	K_{equil} (M $\times$ 10^6)
H_1	10	4.2	4.2	3.0
H_2	20	7.0	7.0	3.76
H_3	30	12.0	12.0	8.0
H_4	50	16.0	16.0	7.52
H_5	100	17.6	17.6	3.75
	Substrates		Product	
	Sphingosine (μM)	Fatty Acid (μM)	Ceramide (μM)	K_{equil} (M $\times$ 10^4)
S_1	10	40	6.75	0.16
S_2	30	29	4.5	1.38
S_3	19	38	2.5	2.34
S_4	95	100	23.0	2.41

[a] Incubation mixtures in 1-ml volume contained 150 μmoles of Tris-HCl (pH 7.4), 1 mg of Triton X-100, 1 mg of sodium cholate, 100 μg of enzyme, and varying substrate in amounts specified in the table. At various times 0.1-ml aliquots were removed and the respective products were determined as described in Experimental Procedure.

fatty acid. This conclusion is strengthened by the finding that addition of ATP and coenzyme A did not enhance the synthesis of ceramide from sphingosine and a free fatty acid (Gatt, 1966).

TRIALS TO SEPARATE THE HYDROLYTIC AND SYNTHETIC ACTIVITIES. The possibility was considered that the hydrolysis and synthesis of ceramide might be catalyzed not by the same, but by two separate enzymes or by one protein having two independent active sites. The following experiments were performed to test this possibility; in each, the rates of synthesis and hydrolysis were determined before and after treatment of the enzyme.

Ammonium Sulfate Fractionation. Solid ammonium sulfate was added to a cholate extract (see purification of the enzyme) and the protein precipitating at 0–30, 30–40, 40–50, and 50–60% saturation was collected. Each fraction was taken up in 5 mM Tris (pH 7.4), dialyzed against the same buffer, and its activity was determined. The ratio of synthesis to hydrolysis was practically the same in all fractions.

Proteolysis and Gel Filtration through Sephadex. The enzyme was incubated with trypsin and chymotrypsin as previously described. The reaction was terminated with soybean trypsin inhibitor and filtered through a column of Sephadex G-150. The protein eluted was collected in 3-ml fractions and the activity of each fraction was determined. The ratio of synthesis to hydrolysis was constant in all fractions collected.

Heat Treatment. The enzyme was heated for 5 min at temperatures ranging from 37 to 80°. After each heating step the enzyme was centrifuged and the activity of the supernatant was measured. Alternatively, the enzyme was incubated without substrate at 58° under the conditions described in Figure 2 and aliquots were collected at the times specified in the figure. Each sample was centrifuged, the sediment was discarded, and the supernatant was taken for measurement of enzymatic activity. The ratio of synthesis to hydrolysis remained constant throughout the whole procedure.

Sonic Irradiation of Particles. Particles (see enzyme purification) were suspended in sucrose–EDTA and subjected to sonic irradiation at 10 kc for 1–15 min. Aliquots were removed, centrifuged at 27,000g, and the supernatant was collected. The sediment was extracted with 0.5% sodium cholate and the detergent was removed by dialysis. Both solutions were assayed for enzymatic activity. Beyond 6-min sonication there was a decline of activity of the enzyme, however, the ratio of synthesis to hydrolysis was constant between zero and 15-min sonic irradiation.

Adjustment of the pH to 4.1. Aliquots of cholate extract were adjusted stepwise with dilute HCl or phosphoric acid to pH 6.5, 6.0, 5.5, 5.0, and 4.1. The protein which sedimented at each pH value was collected, suspended in 2 mM phosphate buffer (pH 7.6), and dialyzed against the same buffer. The contents of the dialysis bags were centrifuged and the activity of the supernatants (including the supernatant remaining after adjustment of the pH to 4.1) was measured. About 5–10% of the total activity sedimented at each pH value. The ratio of synthesis to hydrolysis was constant in all fractions tested.

Effect of SH Inhibitors. Purified enzyme was preincubated for 1 hr at room temperature with *p*-hydroxymercuribenzoate. Substrate was then added and the enzymatic activity was determined; 50% inhibition was obtained at 1 mM and total inhibition at 3–5 mM of the reagent. The ratio of synthesis to hydrolysis remained constant at all inhibitor concentrations. Addition of an excess of cysteine reversed the inhibition and restored both synthetic and hydrolytic activities.

N-Ethylmaleimide and iodoacetamide even at 10^{-2} M had no effect on either the synthetic or hydrolytic activities.

Chromatography on TEAE-cellulose. A cholate extract was chromatographed on a column of TEAE-cellulose, previously equilibrated with 2 mM phosphate buffer (pH 7.6). The protein was eluted with a linear gradient of increasing concentrations of phosphate buffer at the same pH; the enzyme was eluted at about 0.075 M phosphate buffer. The ratio of synthesis to hydrolysis was constant throughout all the fractions eluted from the column.

Equilibrium of the Reaction. The above experiments show that under a variety of conditions, separation of the synthetic and hydrolytic activities could not be achieved. This suggested that both activities are catalyzed by the same protein. Experiments were then conducted to measure the equilibrium constant of the reaction.

Reaction mixtures were incubated either with ceramide as substrate or with a mixture of sphingosine and fatty acid and aliquots were removed periodically. Incubations were continued until the reaction reached an apparent equilibrium. These values were used to calculate an equilibrium constant for the reaction, ceramide + H_2O = sphingosine + fatty acid, as defined as K_{equil} = (sphingosine)(fatty acid)/ceramide.

Table III shows the experimental data and the values for the calculated equilibrium constant. It is clear that, disregarding fluctuations in each set of experiments, the constant, calculated from the data obtained when ceramide was employed as substrate, was lower than the corresponding value obtained with sphingosine and fatty acid. Thus, when the reaction was measured in the direction of ceramide hydrolysis, the equilibrium constant equaled 4–8 $\times$ 10^{-6} M; when measured in the direction of synthesis it equaled about 2 $\times$ 10^{-4} M.

Activators of Ceramide Synthesis. Several compounds affected the initial rate of synthesis of ceramide, while having no effect on the hydrolysis. These included compounds having an SH group, such as dithiothreitol and cysteine (75–125% increase in the rate of synthesis at 0.5 mM of affector), EDTA (40–60% increase at 0.02–5 mM), and hydrazine (40–60% increase at 0.5–5 mM). Several amines had a less pronounced effect (10–20%) on the reaction.

Discussion

Ceramidase catalyzes a reversible reaction in which ceramide (*N*-acylsphingosine) is both hydrolyzed and synthesized. Evidence is presented that the free fatty acid rather than the coenzyme A derivative is the substrate for synthesis of ceramide. Sribney (1966) has shown that chicken liver microsomes catalyze the synthesis of ceramide from palmitoyl coenzyme A. In those experiments, [1-^{14}C]palmitic acid was incorporated into ceramide only after addition of ATP and coenzyme A. He suggested that this represents an alternative pathway to that here described, *i.e.*, synthesis of ceramide utilizing an activated rather than a free fatty acid. For evaluation of this suggestion it must be remembered that microsomes have an active thiol ester hydrolase. The possibility must be ruled out that the acyl coenzyme A derivative formed by the microsomal thiokinase is split, releasing a free fatty acid which then condenses with sphingosine. In unpublished experiments, Gatt and Barenholz have observed that rat liver microsomes converted phytosphingosine into the corresponding *N*-acylated derivative. This synthesis of ceramide did not require ATP or coenzyme A and was enhanced by the addition of hydroxylamine to the reaction mixture. This suggests that the ceramide-synthesizing enzyme in rat liver microsomes utilizes a free rather than an activated fatty acid for the synthesis of ceramide.

Numerous experiments were conducted with the purpose of separating the enzyme into two individual entities, one which hydrolyzes and the other which synthesizes ceramide. Since such separation was not achieved, it was assumed that the same enzyme catalyzes a reversible reaction, *i.e.*, both hydrolysis and synthesis. The equilibrium constant of the reaction, ceramide + $H_2O \rightleftarrows$ sphingosine + fatty acid, defined as K_{equil} = (sphingosine) $\times$ (fatty acid)/ceramide, was then determined. When the equilibrium of the reaction was measured using sphingosine and oleic acid as substrates, the calculated constant equaled about 2 $\times$ 10^{-4} M. When the equilibrium was measured in the direction of hydrolysis, using oleylsphingosine, a lower value of about 4–8 $\times$ 10^{-6} M was obtained. These results are somewhat surprising as they show that the quantities of reactants and products at equilibrium were not the same when measured from either direction. Furthermore, the values calculated from the equilibrium constant suggest that hydrolysis of ceramide is an endergonic reaction with a standard free energy of 5000–7000 cal/mole.

Several remarks are noteworthy in this connection. The concentrations of the reactants and products, used to calculate the equilibrium constant, were those measured by chemical or radiochemical means. These however are not the actual concentrations available to the enzyme. The reaction is conducted in the presence of the detergents, Triton X-100 and sodium cholate. It is not clear whether the enzyme acts on the substrates in micellar form or on monomers, released from these micelles. In either case the actual (or "active") concentrations of the reaction components, available to the enzyme, are but a fraction of the total amount added. Calculation on the basis of the total quantities yields an "apparent" rather than true equilibrium constant. This might also explain the different calculated values of the constant when measuring the reaction in the direction of synthesis or hydrolysis. The reason for this discrepancy might be a different degree of dispersion (*i.e.*, different "activity") of the product(s), formed in situ during incubation, than that of the same component(s), added as mixed micelles of substrate(s) and detergents. Also, part of the reactants or products may be bound to nonspecific proteins and thus not available to the enzyme.

Calculation of the "true" equilibrium constant is further complicated by the fact that while ceramide is

a neutral compound, both the sphingosine base and fatty acid are ionizable, *i.e.*, the hydrogen ion concentration must be added to the equation. Also, the concentration of water was taken as "unit activity" and its concentration was therefore not introduced into the formula employed to calculate the equilibrium constant. The possibility must be considered that only a fraction of the water concentration should be employed in calculating the equilibrium constant. This fraction includes only those water molecules actually participating in the reaction. They are "compartmentalized" at the point of interaction of the enzyme with the substrate micelles. Their concentration might be very small, *i.e.*, of the order of magnitude of the "activity" of the substrate. Introducing this low value into the denominator of the equilibrium equation might raise the constant to a higher value. The standard free energy corresponding to this value might more reasonably fit a reversible reaction.

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Studies on Nuclear Exoribonucleases. III. Isolation and Properties of the Enzyme from Normal and Malignant Tissues of the Mouse*

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ABSTRACT: An exoribonuclease, which degrades single-stranded ribonucleic acid to nucleoside 5′-monophosphates, has been isolated from the nuclei of mouse liver, kidney, embryo, mammary tumor, and Ehrlich ascites tumor. The enzyme is inactivated by heating at 50° and treatment with 3.2 M urea. The enzyme is specific for polyribonucleotides; it will not hydrolyze deoxyribonucleic acid, pTpT, or thymidine 5′-*p*-nitrophenylphosphate. Studies on the mechanism of attack on polyribonucleotides show that: (1) the enzyme (which degrades from the 3′-OH end) catalyzes attack on the 5′-phosphorus atom of the termina nucleotide, with resultant P–O splitting to yield a nucleoside 5′-phosphate; (2) a single polynucleotide molecule stays bound to the enzyme until that polynucleotide molecule is degraded to completion, in a manner similar to the mechanism for degradation of polynucleotides by bacterial polynucleotide phosphorylase and ribonuclease II; and (3) the enzyme is strongly inhibited by the presence of terminal 3′-phosphate or 2′,3′-cyclic phosphate groups on a potential substrate molecule.

The control of nuclear RNA metabolism is one of the central problems in cell biology. It is now apparent that gene function in the nucleus may be regulated by the control of degradation of nuclear RNA (Harris, 1963; Georgiev, 1967; Shearer and McCarthy, 1967; Church and McCarthy, 1967; Soerio *et al.*, 1968; Stewart and Farber, 1968) as well as by control of synthesis of nuclear RNA. It is thus important to define the various nuclear enzymes which degrade RNA and to establish their functional role in the cell.

The present series of papers deals with an exoribonuclease found in a wide variety of cell nuclei; this enzyme degrades single-stranded RNA to nucleoside 5′-monophosphates. Two previous papers in this series (Lazarus and Sporn, 1967; Lazarus *et al.*, 1968) have described the isolation of the enzyme from Ehrlich ascites tumor cell nuclei and a kinetic model for degradation of polynucleotides by the enzyme. The present paper and a following one (H. M. Lazarus and M. B. Sporn, in preparation) will describe the

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