Anhydrocerebrin from Baker's Yeast: Further Confirmation of Its Structure and Unusual Opening of Its Tetrahydrofuran Ring†

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ABSTRACT: A small amount of anhydrocerebrin was obtained from baker's yeast without a hydrolytic procedure and its structure was confirmed as a series of 2-hydroxy fatty acid amides of 1,4-anhydrophytosphingosine and its C_{20} homolog. The major fatty acid, 2-hydroxyhexacosanoic acid, was accompanied by much smaller amounts of C_{24}–C_{28} 2-hydroxy fatty acids with saturated, straight chains. The ratio of the C_{18} and C_{20} homologs of the sphingosine base was approximately 1:1. *N*-Dinitrophenyl-1,4-anhydrophytosphingosine was synthesized from *N*-dinitrophenylphytosphingosine by treatment with tosyl chloride and base. *N*-DL-Cerebronoyl-1,4-anhydrobase was synthesized by condensation of the anhydrobase which has been obtained by hydrolysis of the yeast cerebrin with DL-2-acetoxylignoceric acid chloride. An unusual opening of the tetrahydrofuran ring of the anhydrobase was observed when the anhydrobase was permethylated with sodium hydride and methyl iodide and then subjected to methanolysis. Evidence which suggests that N-methylation of the anhydrocerebrin is essential for the ring opening is presented.

Reindel and his colleagues (Reindel, 1930; Reindel *et al.*, 1940) first observed that the cerebrin from a yeast was converted to an anhydro derivative (anhydrocerebrin) by refluxing with very dilute (0.06%) methanolic H_2SO_4 for 20 hr. Upon further hydrolysis of the anhydrocerebrin with stronger conditions, they obtained, in addition to 2-hydroxyhexacosanoic acid, an anhydro product of an amino alcohol which had been obtained by hydrolysis of intact yeast cerebrin. This amino alcohol was later identified as a C_{18}- and C_{20}-phytosphingosine (Oda, 1952; Carter *et al.*, 1954; Oda and Kamiya, 1958; Prostenik and Stanacev, 1958).

Carter and his coworkers (1954) isolated C_{18} homolog of this anhydrobase as its *N*-benzoyl derivative from crude inositol lipids of corn. From their observation that this *N*-benzoyl anhydrobase did not consume periodate and did not contain a double bond, Carter *et al.* deduced that the anhydrobase must have a tetrahydrofuran ring, and, therefore, the anhydrobase was 1,4-anhydrophytosphingosine. They also suggested that the anhydrobase was formed from phytosphingosine during the hydrolysis procedure in a way analogous to the ring closure of 1,4-glycols. Subsequently, O'Connell and Tsien (1959) confirmed this conclusion mainly from elemental analysis and periodate oxidation of the free anhydrobase obtained from both a hydrolysate of a phosphatide fraction of corn and a lecithin fraction of peanut.

The presence of a tetrahydrofuran ring in the yeast anhydrobase was confirmed by Prostenik and his colleagues (1960) by studying infrared spectra of the base as well as its various derivatives. Subsequently, they reported a partial synthesis of the yeast anhydrocerebrin by condensing 2-hydroxyhexacosanoic acid, which had been obtained from the yeast cerebrin and converted to the 2-acetyl acid chloride, and the anhydrobase, which had been obtained from the yeast anhydrocerebrin, in the presence of quinoline. The synthetic anhydrocerebrin which resulted was identical with the natural anhydrocerebrin (Prostenik and Ries-Lesic, 1960). Another anhydrobase-containing cerebrin, ergocerebrin, was isolated from ergot and was found to be a 2-hydroxy fatty acyl amide of a base which appears to be identical with 1,4-anhydrophytosphingosine (ApSimon *et al.*, 1965). Cerebronic acid (2-hydroxytetracosanoic acid) was the principal fatty acid. From its isolation procedure, which involved minimal exposure to mineral acid under very mild conditions, ApSimon *et al.* (1965) suggested that ergocerebrin is not an artifact of the isolation procedure but appeared to be a true natural product.

While studying the structure of 2,3-dihydroxy fatty acids of yeast cerebrin phosphate (Hoshi *et al.*, 1973), we obtained a compound which is identical with the yeast anhydrocerebrin, without any exposure to mineral acid, as a by-product of the cerebrin phosphate. Using modern techniques available in these laboratories we obtained further conclusive evidence for the structure of the anhydrocerebrin. During the course of this study, we observed an anomalous opening of the tetrahydrofuran ring of the anhydrocerebrin.

Experimental Section

Materials. Phytosphingosine was purchased from Aldrich Chemical Co., Inc. (Milwaukee, Wis.), sodium hydride (50% oil suspension) from Ventron Corp. Alfa Products (Beverly, Mass.), and 1-fluoro-2,4-dinitrobenzene from Eastman Kodak Company (Rochester, N. Y.). Thin-layer chromatographic plates precoated with 0.25-mm thick silica gel G were obtained from Analtech, Inc., Newark, Del. Unisil (silica gel 100–200 mesh) was purchased from Clarkson Chemical Co., Williamsport, Pa. and silica gel 60 extra pure (70–230 mesh; catalog no. 7754) from EM Laboratories Inc., Elmsford, N. Y.

Isolation of the Anhydrocerebrin. This cerebrin was obtained as a by-product during isolation of cerebrin phosphate (Hoshi *et al.*, 1973). In short, 10 lbs. of baker's yeast was subjected to autolysis, and the lipids were extracted with boiling ethanol. Precipitates which were deposited from the etha-

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nol extract after cooling were collected and relatively nonpolar materials were removed with ether by Soxlet extraction. The ether-insoluble materials were then repeatedly recrystallized from acetic acid and then from ethanol to obtain cerebrin phosphate. Mother liquors of the ethanol recrystallization were pooled and evaporated to dryness *in vacuo*. The dried residue, weighing 135 mg, was fractionated by column chromatography on 8 g of Unisil. The residue was dissolved in warm chloroform and transferred onto the column, and the column was eluted with 200 ml of chloroform. A white solid material, weighing 38 mg, was obtained from the eluate after removal of the solvent *in vacuo* and was recrystallized once from 4 ml of chloroform–methanol (2:1); the yield was 25 mg (white solid; mp 111–115° uncorr.). Further elution of the Unisil column with chloroform–methanol in various ratios yielded a mixture of more polar materials.

Permethylation of the Cerebrin with Methyl Iodide and Sodium Hydride. The procedure of Diner *et al.* (1960) was followed with minor modification. The anhydrocerebrin (10 mg) was mixed with 50 mg of sodium hydride (prewashed with dry ether to remove oil), 1 ml of dimethoxyethane (dried over Dri-Na, J. T. Baker Chemical Co., Phillipsburg, N. J., and redistilled), and 0.5 ml of methyl iodide in a Teflon-lined screw cap test tube; and this mixture was shaken vigorously with a Rotary Evapo-Mix (Buchler Instruments, New York, N. Y.) at 37° for 8 hr. The shaking was occasionally stopped, and the hydrogen which had evolved was released by momentarily loosening the cap. The product was centrifuged, and precipitates were extracted twice with 2 ml of dry ether, then twice with 2 ml of dry benzene. The pooled supernatant and extracts were then washed with water and evaporated to dryness. The residue was purified by column chromatography on 1 g of Unisil: the column was first rinsed with 30 ml of benzene and was then eluted with chloroform. Evaporation of the solvent from the latter fraction produced permethylated cerebrin, yield 12.2 mg.

Permethylation of the Cerebrin with Methyl Iodide and Silver Oxide. The method previously reported for permethylation of cerebroside fatty acid esters (Kishimoto *et al.*, 1968) was slightly modified; 15 mg of the cerebrin was heated with 300 mg of silver oxide and 1.5 ml of methyl iodide for 16 hr at 43°; 1 ml of ether was added and the mixture was stirred and then centrifuged to remove silver oxide. The supernatant was collected, and the precipitate was washed twice with warm ether. This ether solution was combined with the above supernatant and evaporated to dryness with a nitrogen flow. The residue was then purified by column chromatography on 1 g of Unisil. The column was first eluted with 30 ml of benzene and then with 30 ml of benzene–ethyl acetate (1:1). The latter fraction, weighing 15.1 mg, was further purified by preparative thin-layer chromatography using a silica gel G plate with chloroform–methanol (99:1). The plate was sprayed with Bromothymol Blue solution and dried, and the scraped powder from the major band was eluted with ether. Evaporation of the ether produced the permethylated cerebrin, yield 9.5 mg.

Preparation of the Anhydrobase. A mixture of 1,4-anhydrophytosphingosine and its C_{20} homolog was prepared by methanolysis of 50 mg of a by-product of the isolation of cerebrin phosphate (presumably a mixture of cerebrins which contained mostly anhydrobase as the long chain base). The methanolysis product was fractionated to yield fatty acid methyl esters and sphingosine bases (Hoshi *et al.*, 1973). The latter fraction was further fractionated by the chromatographic procedure described by Morell *et al.* (1970). Silica gel 60 extra pure (8.6 g) was packed in 0.8 cm × 46 cm columns with chloroform–methanol (1:1) and washed with 10 ml of chloroform. The base fraction was dissolved in 4 ml of a mixture of chloroform–methanol–2 N NH_4OH, 90:10:1, transferred to the column, and rinsed with 4.8 ml of the same solvent. The column was then eluted with 60 ml of chloroform–methanol–2 N NH_4OH, 75:25:2.5, and 3-ml portions were collected separately. The anhydrobase appeared in fractions 9–12, which were then combined, and the solvent was removed. The residue was a white solid and weighed 10.3 mg. Further elution of the column with chloroform–methanol 1:4 yielded 3.3 mg of phytosphingosine.

Synthesis of N-Cerebronoyl Anhydrobase. The procedure was essentially the same as the synthesis of ceramides described previously (Hoshi and Kishimoto 1973); 10 mg of *O*-acetylcerebronic acid (DL-2-acetoxytetracosanoic acid) was converted into the acid chloride and treated with 5 mg of the above anhydrobase in a mixture of 0.4 ml of tetrahydrofuran and 0.5 ml of 50% aqueous sodium acetate. Examination of the product by thin-layer chromatography on silica gel G with chloroform–methanol (98:2) revealed two closely placed major spots; the R_F of the upper spot was 0.33 and lower was 0.24. The product was therefore fractionated on three similar plates under the same conditions. The bands were located by spraying with Bromothymol Blue and both bands were scraped separately and eluted with ether. Removal of the ether left 2.79 mg from the upper band and 2.75 mg from the lower band; both were colorless solids. Treatment of these products with chloroform–methanol–NaOH (Hoshi and Kishimoto 1973) yielded deacetylated products, 2.07 mg (mp 114–115°, uncorr.) from the upper band acetate and 2.09 mg (mp 108–109° uncorr.) from the lower band product.

Synthesis of N-Dinitrophenyl-1,4-anhydrophytosphingosine. Phytosphingosine (2 mg) was converted to a *N*-dinitrophenyl derivative according to the procedure of Karlsson (1970) using a 0.2 M potassium borate buffer. One mg of the dinitrophenyl derivative and 30 mg of *p*-toluenesulfonyl chloride were dissolved in 50 μl of ether in a small, conical, screw cap test tube; 50 μl of KOH solution (15 g in 50 ml water) was added while the mixture was magnetically stirred, and the resulting solution was heated at 100° in an oven for 10 min. The mixture was cooled to room temperature and extracted three times with 1 ml each of ether. The pooled ether extract was washed with water until neutral and then evaporated under nitrogen. The above dehydration was a modification of the procedure by Wendler and Slates (1958).

Spectrometries. High-resolution mass spectra were obtained with a CEC-21-110B high-resolution spectrometer (Dupont Instruments, Monrovia, Calif.). Low-resolution mass spectra were obtained with a gas chromatograph–mass spectrometer system (DuPont Model 21-491) using a 3% SE-30 column. Infrared spectra were taken either as KBr disks or as solutions in CCl_4. A Beckman IR-33 infrared spectrometer (Beckman Instruments, Fullerton, Calif.) was used for this purpose. A nuclear magnetic resonance (nmr) spectrum of the *N*-dinitrophenyl derivative of the anhydrobase was taken by Dr. Brian Sykes at the Department of Chemistry, Harvard University, using a Varian XL-100 (Varian Associates, Palo Alto, Calif.). The sample (1 mg) was dissolved in 0.5 ml of $CDCl_3$–CD_3OD (2:1), allowed to stand at room temperature for 10 min, and then evaporated to dryness. The residue was dissolved in 1 ml of $CDCl_3$, and the spectrum was measured. The nmr spectra of permethylated cerebrins were taken in $CDCl_3$ (approximately 3 mg in a 30-μl microcell) using a Varian T60 (Varian Associates, Palo Alto, Calif.). Optical rotatory dispersion spectra were taken with a Cary 60 spectropolarimeter (Cary Instruments, Monrovia, Calif.).

Gas Chromatography. An F&M Model 7624A (Hewlett-

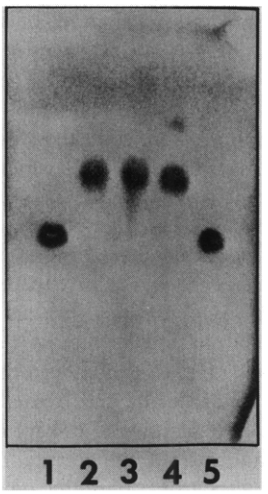


FIGURE 1: Thin-layer chromatogram of cerebrins. Lane 1 and 5, *N*-palmitoylsphingosine; lane 2, a diastereomer of *N*-cerebronoylanhydrobase obtained from the monoacetate that had a higher R_F value; lane 3, anhydrocerebrin of yeast; lane 4, a diastereomer of *N*-cerebronoyl anhydrobase obtained from the monoacetate that had a lower R_F value. Silica gel G with chloroform–methanol (95:5) was used for this chromatography.

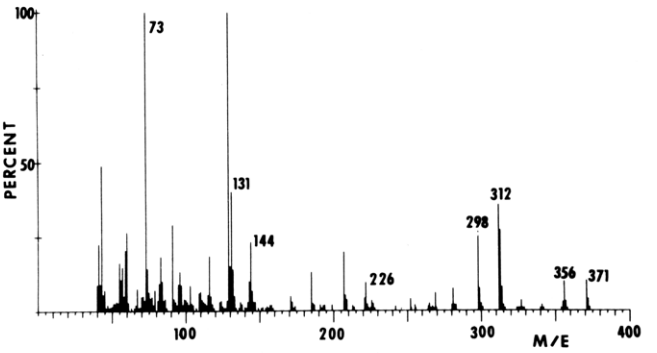


FIGURE 2: Mass spectrum of 1,4-anhydrosphingosine trimethylsilyl ether obtained from the methanolysis product of yeast cerebrin.

Packard, Avondale, Pa.) with a 3 mm × 2 m glass column packed with 3% OV-1 or 1.5% OV-210 was used with a flame ionization detector and an integrator (Model 3370B Hewlett-Packard).

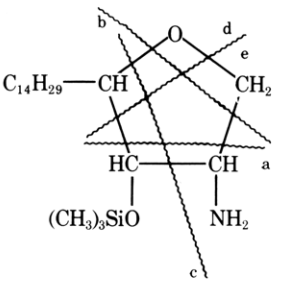
Preparation of Trimethylsilyl Ether. Sphingosine bases were converted to their trimethylsilyl ethers using pyridine–hexamethyldisilazane–trimethylchlorosilane (1.3:1.0:0.8) according to the method of Carter and Gaver (1967), and trimethylsilyl ethers of 2-hydroxy fatty acid methyl esters were prepared with the same reagents in a ratio of 9:3:1 (Hoshi *et al.*, 1973).

Results

Structure of the Anhydrocerebrin. The cerebrin isolated from the yeast yielded an infrared spectrum which is essentially identical with the published spectrum (Prostenik and Ries-Lesic, 1960). Thin-layer chromatography revealed only one spot which moved slightly faster than that of *N*-palmitoylsphingosine (Figure 1). Methanolysis of 5.4 mg of the cerebrin by 0.5 N methanolic HCl for 16 hr at 75° (Kishimoto and Radin, 1965) yielded 2.2 mg of methyl esters of 2-hydroxy fatty acids and 1.6 mg of anhydrobases.

Gas–liquid chromatography of the esters as their trimethylsilyl ethers on an OV-1 column indicated that the fatty acid composition was: 2-hydroxytetracosanoate 7.6%, 2-hydroxypentacosanoate 12.4%, 2-hydroxyhexacosanoate 76.4%, 2-hydroxyheptacosanoate 3.0%, and 2-hydroxyoctacosanoate 0.6%. The configuration of the mixture of 2-hydroxy fatty acids was found to be D, in agreement with previous reports (Prostenik, 1956), by measuring its optical rotatory dispersion in heptane. The spectrum showed a positive curve identical with that of D-(−)-lactic acid (Craig and Roy, 1965). In agreement with this finding, the gas chromatogram of menthyloxycarbonyl esters of these methyl esters of 2-hydroxy acids on an OV-210 column (Annett and Stumpf, 1972) showed major peaks which agreed with those of the D series.

The anhydrobase obtained by methanolysis of the yeast cerebrin produced a single spot upon examination by thin-layer chromatography. Its R_F values on silica gel G plates with two different systems, one with chloroform–methanol–water (24:7:1) and another with chloroform–methanol–2 N NH_4OH (40:10:1), were almost equivalent to those of 3-*O*-methylsphingosine (Sambasivarao and McCluer, 1963; Taketomi and Kawamura, 1972) which had been obtained by methanolysis of calf brain cerebrosides with anhydrous methanolic HCl. A trimethylsilyl ether of the anhydrobase was prepared and analyzed by gas–liquid chromatography on a 3% OV-1 column at 200°. The gas chromatogram showed two peaks, one at R_t 6.2 min and another at 12.2 min (the R_t of the trimethylsilyl ether of sphingosine was 7.4 min under the same conditions). The ratio of these two peaks was approximately 1:1. A mass spectrum of the earlier peak (R_t 6.2 min) obtained by a gas chromatograph–mass spectrometer system is shown in Figure 2. The spectrum contained most of the ions anticipated for the straightforward fragmentation of the structure of the anhydrobase (1,4-anhydrophytosphingosine) as noted below. An abundant molecular ion at *m/e* 371 and ions at $M^+ - 15$ (*m/e* 356) and $M^+ - Me_3Si$ (*m/e* 298) were observed. Also, fragments a (*m/e* 131), b − $C_{14}H_{29}$ (*m/e* 144), c (*m/e* 312), d (*m/e* 226), and e − H (*m/e* 144) were clearly observed. Similarly, the mass spectrum of the second peak (R_t 12.2 min) indicated that this compound was the trimethylsilyl ether of the C_{20} homolog of 1,4-anhydrophytosphingosine.



High resolution mass spectral analysis

	m/e Found	Calcd for
molecular ion for C_{18} homolog	371.3195	$C_{21}H_{45}NO_2Si$, 371.3219
molecular ion for C_{20} homolog	399.3531	$C_{23}H_{49}NO_2Si$, 399.3532

An acetyl derivative was prepared from 1.14 mg of the anhydrobase by reaction with pyridine–acetic anhydride (3:1). A high-resolution mass spectrum of the product, which confirmed its identification as *N*-acetyl-*O*-acetyl-1,4-anhydrophytosphingosine (Prostenik *et al.*, 1960), yielded the following ions.

	m/e Found	Calcd for
M^+ (molecular ion)	383.3003	$C_{22}H_{41}NO_4$, 383.3035
$M^+ - CH_3COOH$	323.2834	$C_{20}H_{37}NO_2$, 323.2843
$M^+ - CH_3CONH_2$	324.2673	$C_{20}H_{36}O_3$, 324.2665
$M^+ - (CH_3COOH + CH_3CONH_2)$	264.2443	$C_{18}H_{32}O$, 264.2453

Although the molecular ion of the C_{20} homolog was not detected, abundant $M^+ - CH_3COOH$ at *m/e* 351.3125 and M^+

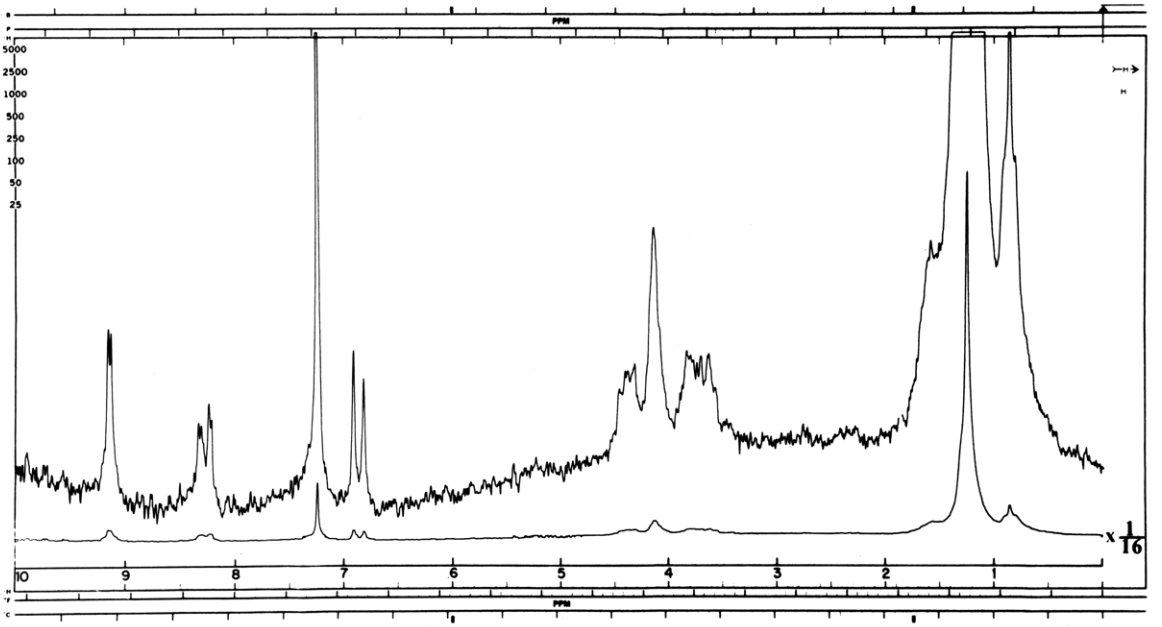


FIGURE 3: The 100-MHz nuclear magnetic resonance spectrum of the *N*-dinitrophenyl derivative of the anhydrobase obtained from yeast cerebrin phosphate. $CDCl_3$ was the solvent (see Experimental Section for details of the derivative preparation).

$-$ (CH_3COOH + CH_3CONH_2) at *m/e* 292.2757 were present in the spectrum.

The *N*-dinitrophenyl derivative of the anhydrobase was prepared and purified by preparative thin-layer chromatography. A high-resolution mass spectrum of this product was determined which exhibited the accurate masses of the molecular ions shown below.

	m/e Found	Calcd for
molecular ion for C_{18} homolog	465.2851	$C_{24}H_{39}N_3O_6$, 465.2839
molecular ion for C_{20} homolog	493.3164	$C_{26}H_{43}N_3O_6$, 493.3152

A nuclear magnetic resonance spectrum of this product is shown in Figure 3. All signals agreed with those expected for *N*-dinitrophenyl-1,4-anhydrophytosphingosine: 0.95 ppm is from CH_3, 1.25 is CH_2 in the alkyl chain, 1.55 is CH_2 at C_5, 3.5–4.55 are from the protons at C_1–C_4, and 6.9, 8.2, and 9.1 are from protons on the dinitrophenyl group.

The trimethylsilyl ethers of the dinitrophenyl derivatives exhibited the following molecular ions by high-resolution mass spectroscopy.

	m/e Found	Calcd for
molecular ion for C_{18} homolog	537.3223	$C_{27}H_{47}N_3O_6Si$, 537.3234
molecular ion for C_{20} homolog	565.3532	$C_{29}H_{51}N_3O_6Si$, 565.3547

N-Dinitrophenyl-1,4-anhydrophytosphingosine was synthesized by reacting *N*-dinitrophenylphytosphingosine with *p*-toluenesulfonyl chloride under alkaline conditions. Under these conditions, the 1,4-diol is converted to the corresponding cyclic oxide (Wendler and Slates, 1958). When the reaction product was examined by thin-layer chromatography on silica gel G with chloroform–methanol (98:2) as the developing solvent, three yellow spots were observed. A spot at R_F 0.55 was the most intense, followed by a spot at R_F 0.30 and then by a spot at R_F 0.26. These three fractions then were separated by preparative thin-layer chromatography under the same conditions, the bands were scraped and eluted with ether, and the residues were tentatively called DNP-A, -B, and -C from the top to the bottom. The R_F of the *N*-dinitrophenyl derivative of anhydrobase described above was identical with that of DNP-B (Figure 4).

A high resolution mass spectrum of DNP-B yielded the molecular ion: *m/e* 465.2858 (calcd for $C_{24}H_{39}N_3O_6$, 465.2839).

A low-resolution mass spectrum of the trimethylsilyl ether of DNP-B was also identical with that of the trimethylsilyl ether of the natural product, except the latter had additional fragments due to the C_{20} homolog. Abundant ions in these spectra included the molecular ion at *m/e* 537 (plus *m/e* 565 for C_{20} homolog in the natural product), $M^+ - 15$ and $M^+ - 30$ ions.

DNP-A, which is the main product obtained from the reaction of *N*-dinitrophenylphytosphingosine with *p*-toluenesulfonyl chloride, was identified from its mass spectrum as the dehydrated product of DNP-B. Characteristic fragmentations included the molecular ion *m/e* at 447, $M^+ - 17$ at *m/e* 430, $M^+ - NO$ at *m/e* 417, and $M^+ - NO_2$ at *m/e* 401. In addition, a compound identical with DNP-A was obtained when 0.5 mg of the dinitrophenyl derivative of the natural anhydrobase was treated with *p*-toluenesulfonyl chloride under the same conditions used for *N*-dinitrophenylphytosphingosine (Figure 4).

Identification of DNP-C was not attempted. However, DNP-C was partially converted to DNP-B when the former was eluted from the thin-layer chromatographic plate. Therefore, it is reasonable to assume that DNP-C is the 3-*O*-tosyl

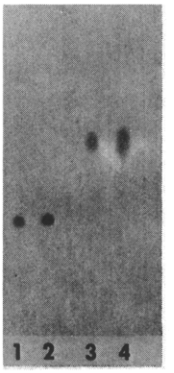


FIGURE 4: Thin-layer chromatogram of the *N*-dinitrophenyl derivative of 1,4-anhydrophytosphingosine and its dehydrated product. Lane 1, *N*-dinitrophenyl derivative of the anhydrobase obtained from yeast cerebrin; lane 2, *N*-dinitrophenyl-1,4-anhydrophytosphingosine synthesized from phytosphingosine (DNP-B); lane 3, dehydrated product of the compound in lane 1; lane 4, dehydrated product of the compound in lane 2 (DNP-A). Silica gel G with chloroform–methanol (95:5) as the developing solvent was used for this chromatography.

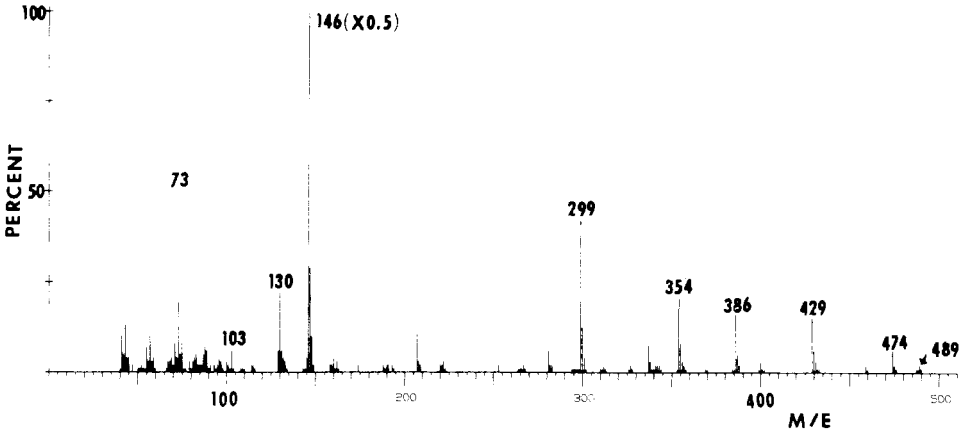


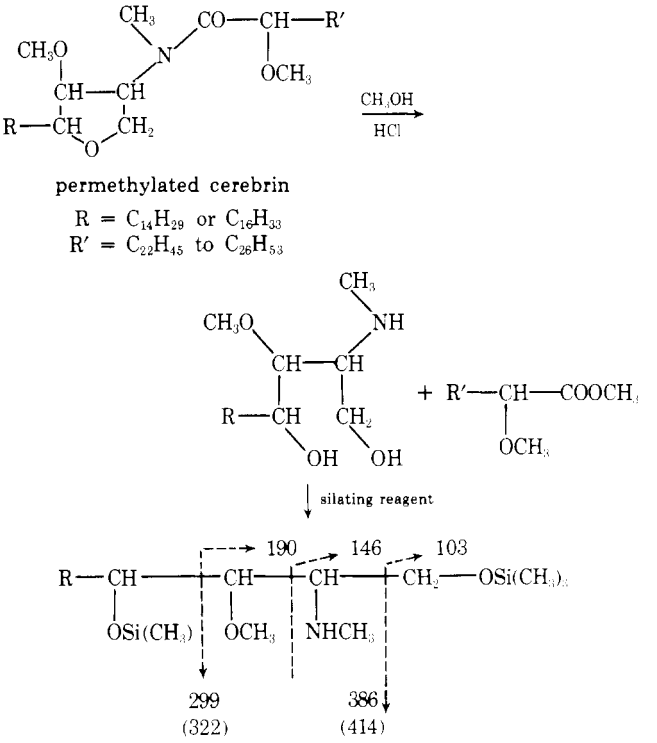
FIGURE 5: Mass spectrum of the base obtained by methanolysis of permethylated yeast anhydrocerebrin (prepared with NaH and CH_3I).

ester of DNP-B (*N*-dinitrophenyl-1,4-anhydrophytosphingosine).

N-DL-Cerebronoyl-1,4-anhydrobases were synthesized from synthetic *O*-acetyl-DL-cerebronic acid and the anhydrobases obtained from yeast. The condensation product, *N*-(*O*-acetyl-DL-cerebronoyl)anhydrobases, gave two equally intense spots at R_F 0.33 and R_F 0.24, respectively, on thin-layer chromatography (see Experimental Section). Presumably, one spot is the D-2-acetoxy fatty acid amide and the other is the L-2-acetoxy fatty acid amide. Such a separation of diastereoisomers was reported on *N*-2-hydroxy fatty acyl sphingosines (a ceramide) (Hammarström, 1971; Karlsson and Pascher, 1971; Ullman and Radin, 1972) although the isomers of *N*-2-acetoxy fatty acyl sphingosine were reported to be inseparable in the system which was investigated (Arora *et al.*, 1973). Deacetylation of the *O*-acetylcerebrins and subsequent separation by preparative thin-layer chromatography resulted in products having identical R_F values, which were also identical with the natural yeast cerebrin, on silica gel G using two different solvent systems, one chloroform–methanol (95:5) and the other benzene–methanol (95:5) (Figure 1).

An infrared spectrum of the synthetic cerebrin (obtained from *O*-acetylcerebrin which gave the higher R_F on the thin-layer chromatogram) was identical with that of natural yeast cerebrin, while that of the synthetic cerebrin which *O*-acetyl derivative had the lower R_F was slightly different. Similar differences were observed in the two diastereoisomers of synthetic ceramides (Hammarström, 1971).

Anomalous Ring Opening of Permethylated Anhydrocerebrin. Ten milligrams of yeast anhydrocerebrin was completely methylated with sodium hydride and methyl iodide, and the permethylated cerebrin was subjected to methanolysis with 0.5 N methanolic HCl at 75° for 15 hr. Gas–liquid chromatography (3% OV-1, 200°) of the trimethylsilyl ether of the base obtained by the methanolysis produced two peaks, one of which had a retention time of 9.6 min and the other one 18.2 min. R_t of 1,4-anhydrophytosphingosine trimethylsilyl ether was 6.1 min at the same conditions. Unexpectedly, the mass spectra of these peaks (the spectrum of the first peak is shown in Figure 5) indicated that the tetrahydrofuran ring of the anhydrobase was opened during the above reactions. The mass spectrum of the first peak, shown in Figure 5, clearly indicated the molecular ion at *m/e* 489, $M^+ - 15$ ion at *m/e* 474, and all other fragments which are illustrated in the above structure. In addition, *m/e* 354, formed by loss of Me_3SiOH and OCH_3 or CH_3OH and OMe_3Si, was one of the major ions observed. Similarly, mass spectrum of the second peak yielded all corresponding



ions for the C_{20} homologs (shown in parentheses in the above formula).

A high-resolution spectrum of the above trimethylsilyl ether mixture did not exhibit molecular ions. Abundant ions corresponding to the loss of a methyl group were obtained for both compounds.

	m/e Found	Calcd for
C_{18} homolog ($M^+ - 15$)	474.3757	$C_{25}H_{56}NO_3Si$, 474.3799
C_{20} homolog ($M^+ - 15$)	502.4135	$C_{27}H_{60}NO_3Si_2$, 502.4112

In contrast to this observation, the permethylated anhydrocerebrin which was prepared by the reaction with Ag_2O and methyl iodide yielded 2-methoxy fatty acid methyl esters and a mixture of homologs of *O*-methylanhydrobase. Gas–liquid chromatography on a 3% OV-1 column of the base as its trimethylsilyl ether revealed two peaks, one at R_t 4.8 min and another at R_t 9.1 min. (R_t of 1,4-anhydrophytosphingosine trimethylsilyl ether was 6.1 min at the same conditions.) The structure of the base component was confirmed by gas–liquid chromatography–mass spectroscopy. The mass spectrum (Fig-

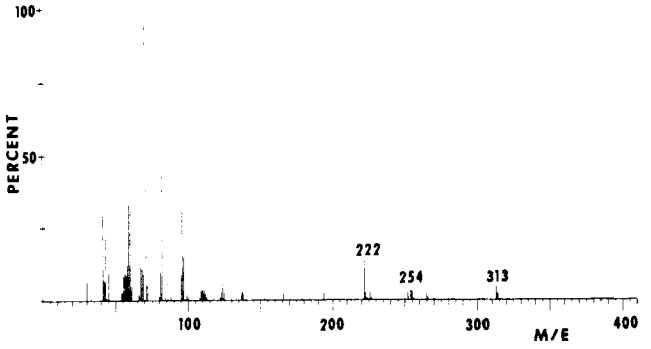
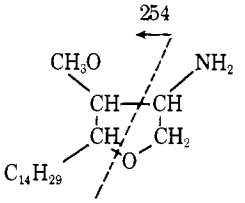


FIGURE 6: Mass spectrum of the base obtained by methanolysis of permethylated yeast anhydrocerebrin (prepared with Ag_2O and CH_3I).

ure 6) of the C_{18} homolog (the peak with shorter R_t) clearly showed the molecular ion at *m/e* 313 and a fragment at *m/e* 254 which is apparently formed by the following cleavage. An



abundant fragment at *m/e* 222 must be derived from the *m/e* 254 fragment by the removal of methanol. Similarly, the molecular ion at *m/e* 341, the $M^+ - 15$ ion at *m/e* 326 and fragments at *m/e* 282 and 250 (corresponding to the ions at *m/e* 254 and 222 of the C_{18} homolog) were present in the mass spectrum of the C_{20} homolog (the peak with R_t 9.1 min). Evidence confirming the identification of the molecular ions and fragments was provided by a high-resolution mass spectrum of the base component.

	m/e Found	Calcd for
M^+ (molecular ion) of C_{18} homolog	313.2962	$C_{19}H_{39}O_2N$, 313.2981
M^+ of C_{20} homolog	341.3279	$C_{21}H_{43}O_2N$, 341.3293

In order to find at which stage (permethylation or methanolysis) the tetrahydrofuran ring of the permethylated anhydrobase opened, the infrared and nuclear magnetic resonance spectra of both the permethylated cerebrin prepared with NaH and CH_3I and the permethylated cerebrin prepared with Ag_2O and CH_3I were recorded and compared. Both permethylated products had identical R_F's by thin-layer chromatography on silica gel G with chloroform or chloroform–methanol (98:2). The spectra of the two permethylated cerebrins showed differences including those noted below in both the infrared (Figure 7) and nmr (Figure 8) spectra.

The product prepared with NaH showed no evidence for either hydroxyl or NH groups in its infrared spectrum (Figure 7A; absence of these groups was also confirmed by taking the spectrum in carbon tetrachloride), while that prepared with Ag_2O showed the presence of an NH group as indicated by absorptions at 3300 and 3430 cm^{-1} (NH stretching) and 1540 cm^{-1} (amide II) (Figure 7B).

In nuclear magnetic resonance spectrum of the permethylated product prepared with NaH (Figure 8) all signals indicated the structure was *N*-methyl-*N*-[2-methoxy fatty acyl]-1,4-anhydrobase 3-methyl ether. The spectrum was expected to have an average of 74 protons in the methylene region of the fatty acid (average chain length as C_{26}) and anhydrobase (average chain length as C_{19}); 6 protons from two methyl groups at the end of aliphatic chains (at 0.9 ppm); 6 protons from two

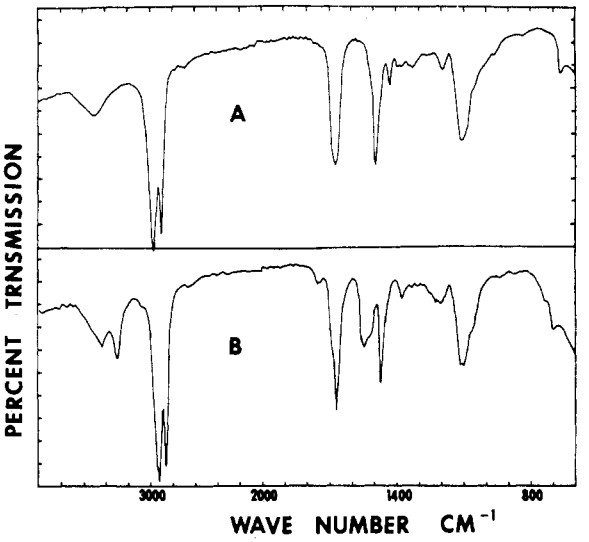


FIGURE 7: Infrared spectra of the permethylated yeast cerebrin which was prepared with NaH and CH_3I (A) and permethylated yeast cerebrin which was prepared with Ag_2O and CH_3I (B). Both spectra were taken as KBr disks. The KBr which was used contained a small amount of water which is responsible for the absorption at 3500 cm^{-1} in spectra A and B; the approximate intensity of the absorption due to water is that observed in spectrum A.

methoxyl groups (at 3.35 ppm); 3 protons from the *N*-methyl group (at 3.15 ppm); 5 protons (at 4.0 ppm) from the furan ring; and 1 proton attached to the α carbon of fatty acid moiety (at 3.75 ppm). The signal due to the proton in the amide group was not observed, but the intensity of all other signals agreed quite well with the above number of protons. The nmr spectrum of the permethylated cerebrin which was prepared with Ag_2O is shown in Figure 8b. When it is compared with that of Figure 8a, it is clear that this cerebrin was not methylated at the amide group. This conclusion was drawn from the absence of the signal for the *N*-methyl group (at 3.15 ppm), the appearance of the signal for the amide proton (6.95 ppm), and the shifting of the signals for protons in the tetrahydrofuran ring to lower ppm regions.

These data therefore indicate that the NaH reaction results in N-methylation of the amide group, and the Ag_2O reaction does not. The presence of the methyl group therefore must be responsible for the ring opening reaction. To confirm this unusual ring opening reaction, *N,O*-diacetylanhydrobase, which was prepared as described above, was methylated with sodium hydride and methyl iodide. A high-resolution mass spectrum indicated the product to be a mixture of *N,O*-dimethyl-1,4-anhydrophytosphingosine *N*-acetate and its C_{20} homolog.

	m/e Found	Calcd for
molecular ion of C_{18} homolog	369.3219	$C_{22}H_{43}NO_3$, 369.3243
$M^+ - 15$ of C_{18} homolog	354.3009	$C_{21}H_{40}NO_3$, 354.3008
molecular ion of C_{20} homolog	397.3539	$C_{24}H_{47}NO_3$, 397.3556
$M^+ - 15$ of C_{20} homolog	382.3323	$C_{23}H_{44}NO_3$, 382.3322

This permethylated product was subject to 0.5 N methanolic HCl at 75° for 16 hr, and the base component was isolated. Gas chromatography–mass spectrometry analysis of the trimethylsilyl ether of the base revealed two peaks which were identical with those of the base obtained by methanolysis of the permethylated cerebrin, and which were identified as the open chained bases.

Discussion

The present communication unequivocally confirmed the structures of yeast anhydrocerebrin as well as its long chain

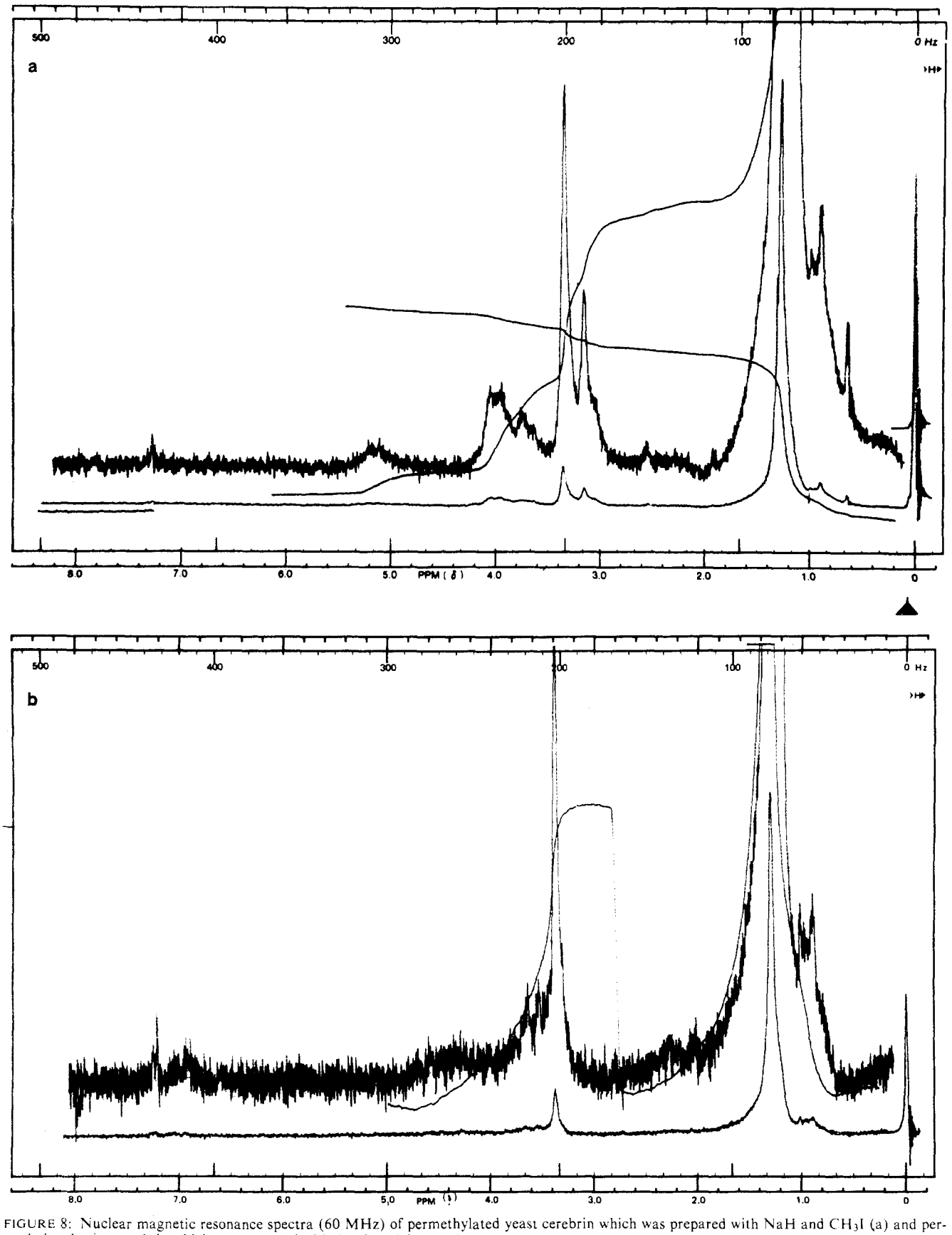


FIGURE 8: Nuclear magnetic resonance spectra (60 MHz) of permethylated yeast cerebrin which was prepared with NaH and CH_3I (a) and permethylated yeast cerebrin which was prepared with Ag_2O and CH_3I (b). Both spectra were taken as $CDCl_3$ solutions.

base component, anhydrobase, as those proposed by previous workers (Carter *et al.*, 1954; Prostenik *et al.*, 1960; Prostenik and Ries-Lesic, 1960). The yeast anhydrocerebrin was obtained previously only after treatment of the cerebrin with diluted methanolic acid at high temperature for prolonged time periods (Reindel *et al.*, 1940), while our preparation was obtained without such a hydrolytic procedure. Whether the anhydrocerebrin isolated by us occurs in nature or is an artifact of

the isolation procedure remains open to question; however, the possibilities that either autolysis or recrystallization from glacial acetic acid cause the dehydration cannot be excluded. ApSimon *et al.* (1965) claimed ergocerebrin, the homologs of anhydrocerebrin, occurs in nature despite its minimal exposure to low concentrations of mineral acids under very mild conditions. O'Connell and Tsien (1955) were not able to convert phytosphingosine or its *N*-benzoyl derivative into the anhydrobase either by refluxing with aqueous or alcoholic sulfuric acid or with aqueous copper sulfate in absolute alcohol.

Cleavage of the tetrahydrofuran ring of the anhydrobase by permethylation with NaH and methyl iodide followed by methanolysis was unexpected and no report of this type of anomalous reaction could be found. The tetrahydrofuran ring is known to be chemically very stable, and only a few specific reactions, such as that with diborane and iodine (Long and Freeguard, 1965), have been reported for opening the ring. The fact that the tetrahydrofuran ring of the cerebrin which was methylated with methyl iodide and silver oxide was not opened by methanolysis strongly suggests that N-methylation is the key step to opening of the tetrahydrofuran ring. The nmr spectrum of the permethylated cerebrin (Figure 8), the mass spectrum of the permethylated diacetylanhydrobase, and the mass spectrum of the partially methylated base obtained by methanolysis of these lipids confirmed that the permethylated cerebrin which was prepared with NaH resulted in N-methylation and that which was prepared by Ag_2O had the NH group of its amide linkage intact.

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