Separation of long-chain bases by thin-layer chromatography; instability of sphingosine

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SUMMARY Purified sphingosine was resolved into dihydrosphingosine, sphingosine, and a fast-moving component by thin-layer chromatography on Silica Gel G. The fast-moving component was generated by the adsorbent, and consisted of a mixture of the cationic forms of dihydrosphingosine and sphingosine. Other sphingosine derivatives gave rise to similar artifacts.

Sphingosine formed two degradation products when stored in solution or on thin-layer plates; related long-chain bases with some of their reactive sites blocked did not deteriorate when stored in solution.

KEY WORDS thin-layer chromatography · sphingosine · dihydrosphingosine · *O*-methyl sphingosines · *N*-acyl sphingosines · hydrochlorides · cationic forms · instability · tribenzoyl sphingosines

IN STUDIES with C^{14}- and H^3-labeled sphingosine, low yields of formic acid were obtained after degradation of the base with periodic acid (1). It was thought that transformations of the base during storage to compounds of varying susceptibility to periodate oxidation were partially responsible. Therefore, characterization of the radioactive mixed base fraction was undertaken by means of thin-layer chromatography (TLC). The excellent separations achieved with this technique prompted an examination of other long-chain bases both for their purity and stability under various conditions.

As this work was in progress, Fujino and Zabin (2) reported the separation of *threo*- and *erythro*-sphingosines by TLC, and Sambasivarao and McCluer (3) separated a variety of long-chain bases with similar techniques using a system containing NH_4OH. In this communication, we will report the identity of the fast-moving polar components from sphingosine and related bases, the instability of sphingosine under various conditions, and some factors affecting the separation of long-chain bases by the thin-layer procedure.

Materials. Sphingosine[1] was isolated either from fresh rat brains or beef spinal cord sphingolipids (4) as the sulfate salt according to the procedures of Carter et al. (5–7). The free base was purified by several precipitations from petroleum ether, bp 60–70°. A typical sample from beef spinal cord gave 86% of the theoretical uptake of hydrogen and showed no methoxyl content. The hydrochlorides of *O*-methyl sphingosines[1] I and II were obtained from hydrolysates of beef spinal cord sphingolipids (8). These compounds were of interest because they are formed from sphingosine, during hydrolysis of sphingolipids with CH_3OH–H_2SO_4, as a result of an allylic rearrangement. Dihydro compounds were

[1] Unless otherwise stated, the sphingosine employed in this investigation contained about 11–14% dihydrosphingosine. *O*-Methyl sphingosines I and II each consist of approximately 30% 3-*O*-methyl sphingosine and 70% 1-hydroxy-2-amino-5-methoxy-3-octadecene; the configurations at carbon atom 5 of the 5-methoxy isomer in bases I and II are D and L, respectively (9).

TABLE 1 R_F VALUES OF LONG-CHAIN BASES AND DERIVATIVES

Compound	R_F Value*
O-Methyl dihydrosphingosine II	0.26
DL-*threo*-Dihydrosphingosine	0.36
D-*erythro*-Dihydrosphingosine	0.45
DL-*threo*-*trans*-Sphingosine	0.50
O-Methyl sphingosine II	0.56
D-*erythro*-Sphingosine	0.59
N-Stearoyl dihydrosphingosine	0.95
N-Stearoyl sphingosine	0.95
Triacetyl sphingosine	0.95
D-*erythro*-Dihydrosphingosine·HCl	0.96
D-*erythro*-Sphingosine·HCl	0.96
O-Methylsphingosine I·HCl	0.96
O-Methylsphingosine II·HCl	0.96
Free bases + metal ions	0.96
Component X	0.96

* Determined by ascending chromatography on Silica Gel G with chloroform–methanol–water 49:49:2.

prepared by reduction of the corresponding unsaturated material in ethanol over platinum or palladium. *N*-Stearoylsphingosine was prepared as described previously (10). The hydrochlorides of sphingosine and dihydrosphingosine were prepared by saturation of a solution of the base in ether with dry HCl; the salts were isolated by removal of the solvent and crystallized from ethanol–petroleum ether 1:2 (v/v). Sphingosine hydrochloride, mp 65–71°. Calcd. for $C_{18}H_{38}O_2NCl$ (335.8): C, 64.32; H, 11.41. Found: C, 63.93; H, 11.45. Dihydrosphingosine hydrochloride, mp 92–93°. Calcd. for $C_{18}H_{40}O_2NCl$ (337.8): C, 63.94; H, 11.63. Found: C, 63.46; H, 11.61. Samples of DL-*threo*-*trans*-sphingosine and DL-*threo*-dihydrosphingosine were furnished by Ciba Ltd., Basel, Switzerland, and the Upjohn Company, Kalamazoo, Mich. Equipment and adsorbents were purchased from Brinkmann Instruments, Great Neck, N.Y.

Methods. TLC was conducted on Silica Gel G using chloroform–methanol–water 49:49:2. Free bases were detected with Brinkmann's ninhydrin reagent; amides of sphingosine were made visible by spraying with *tert*-butyl hypochlorite, starch–KI reagent (11).

In several experiments components were eluted from the adsorbent after TLC with four volumes of chloroform–methanol 2:1 (suspension followed by centrifugation). The supernatant fluids containing each component were concentrated under nitrogen, and the content of long-chain bases was determined (12).

TABLE 2 RECOVERY OF LONG-CHAIN BASES AFTER CHROMATOGRAPHY

Purified Sphingosine Applied	Long-Chain Base Recovered			Recovery
	Dihydrosphingosine	Sphingosine	Component X	
μg	μg	μg	μg	%
551.7	45.0	239.2	129.6	75
551.7	46.7	241.1	137.2	77

Gas–liquid chromatography (GLC) of the long-chain aldehydes was performed as described previously (13) after degradation of the bases with periodic acid.

Characterization of Components. Three components with R_F values of 0.45, 0.59, and 0.96 (Table 1) were obtained when sphingosine was promptly chromatographed on Silica Gel G. After application of 340 mg of the base to 34 plates, the components were isolated and converted to tribenzoyl derivatives; in these experiments the adsorbent was washed with chloroform–methanol 2:1 before use to remove contaminating oils. The products from the bases of R_F 0.45 and 0.59 corresponded to tribenzoyl dihydrosphingosine, mp 142–143°, yield 21.0 mg, and tribenzoyl sphingosine, mp 121–123°, yield 96.0 mg, respectively. The derivative of component X, R_F 0.96 (Table 1), melted at 121–123°, yield 36.0 mg.

Rechromatography of component X gave a single spot without change in R_F. If component X was treated with dilute alkali and washed with water before chromatography, three components corresponding to the original chromatogram were obtained. This suggested strongly that component X consists of a mixture of dihydrosphingosine and sphingosine in cationic form, even though its benzoyl derivative had the melting point of pure tribenzoyl sphingosine. Tests showed that up to 10% of tribenzoyl dihydrosphingosine does not depress this melting point. Further evidence for the identification of component X as an artifact produced by acid conditions either in TLC or in other procedures is as follows.

When samples from the isolated components of sphingosine were rechromatographed, both dihydrosphingosine and sphingosine (now presumably pure) gave two spots, one corresponding to the parent base and the other to component X. This procedure could be repeated, each time with formation of new component X, as long as material from the parent base was available.

GLC of the long-chain aldehydes from sphingosine degraded with periodate showed peaks corresponding to dihydrosphingosine and sphingosine; no peak, whose size would be between that of dihydrosphingosine and sphingosine (Table 2), corresponding to component X was found. Only a single component was observed with the aldehydes from dihydrosphingosine and *O*-methyl sphingosine II.

Separation of Other Compounds. Dihydrosphingosine, *O*-methyl sphingosine II, *O*-methyl dihydrosphingosine II, DL-*threo*-*trans*-sphingosine, and DL-*threo*-dihydrosphingosine (Table 1) also showed the presence of a fast-moving component of the same R_F as that of component X from sphingosine. The hydrochlorides of the bases studied gave single spots with the same R_F values

as that of component X (Table 1). Rechromatography of the fast-moving component, after alkali treatment, from dihydrosphingosine and *O*-methyl sphingosine II gave a spot of the same R_F along with one corresponding to the parent base.

When dihydrosphingosine was rehydrogenated, the proportion of the fast-moving polar component increased, with a concomitant decrease in the main component. This was due to reaction of the base with the acid catalyst; the melting point and elementary analysis of the product agreed with that of dihydrosphingosine hydrochloride.

The compounds *N*-stearoyl sphingosine, *N*-stearoyl dihydrosphingosine, and triacetyl sphingosine have R_F values similar to each other and to the hydrochlorides of the bases in the solvent system used (Table 1).

Effect of Metal Ions. An alternative explanation for the formation of component X was considered, namely that it was due not to the acidity but to the metal ion content of the adsorbent (since bases can function as chelating agents). Sphingosine, dihydrosphingosine, *O*-methyl sphingosine II, and *O*-methyl dihydrosphingosine II were chromatographed with an equal amount of either $CoCl_2 \cdot 6H_2O$, $CuCl_2 \cdot 2H_2O$, $MnSO_4 \cdot 4H_2O$, or $FeCl_3 \cdot 6H_2O$ in methanol. In each case only one spot was observed (Table 1). A spectrophotometric analysis of the various sphingosine–metal ion solutions indicated no complex formation (14); instead, the effect was due to the reduced pH of the solution since a sphingosine sample, in the absence of metal ion, acidified to the same pH (approximately 2.0) with dilute HCl gave a single component of similar R_F.

Chromatography of sphingosine on silicic acid without binder revealed only two components, dihydrosphingosine and sphingosine. Since all of the bases in their cationic form give the same R_F values, as shown by their hydrochlorides and by their solutions containing metal ions (Table 1), it is concluded that residual acidity, perhaps produced by the $CaSO_4$ binder in the Silica Gel G (pH about 7.0), is responsible for the formation of the fast-moving polar component in every case.

Effect of Storage. When sphingosine was stored in chloroform–methanol 2:1 for 8 days at room temperature, new compounds appeared at the origin and solvent front; the concentrations of these compounds increased with longer periods of standing (21 days). No other compounds were observed after standing 61 days; the final chromatogram consisted of five components. The same effect was obtained when the preparation had stood in other organic solvents. A crude rat brain sphingosine-H^3 sample, stored several weeks in methanol at 4°, exhibited the same pattern of deterioration.

When sphingosine was allowed to remain on a Silica Gel G coated plate for 48 hr at room temperature in air before chromatographing, a pattern of five components appeared, identical with that found after storage for a longer period in solution. DL-*threo-trans*-Sphingosine showed the same behavior. As expected, sphingosine stored in the solid state at room temperature in air eventually gave rise to the same transformation pattern. When the components of the sphingosine sample were separated by chromatography in one dimension, and the plate was stored for 48 hr before development in the second dimension with the same solvent, deterioration of the base still occurred.

When dihydrosphingosine, *O*-methyl sphingosine II, *O*-methyl dihydrosphingosine II, DL-*threo*-dihydrosphingosine, and *N*-acyl bases remained in solution for 3 weeks at room temperature, their chromatograms showed little or no change. This greater stability indicates that both the allylic center and amino group are involved in the transformations.

Fujino and Zabin (2) noted the decomposition of sphingosine-C^{14} preparations (loss in counts) during their investigations. The present studies show that sphingosine cannot be stored as the free base either in solution or as the solid without deterioration. Several unidentified spots in the sphingosine chromatograms of Sambasivarao and McCluer (3) may be due to base decomposition. The role of oxygen and moisture as causative agents of sphingosine decay has not been established conclusively; however, it appears that either or both of these agents may be involved because of the more rapid breakdown of the base during storage on Silica Gel G than in solution. The chemistry and mechanism of formation of the compounds which appear at the origin and solvent front in aging samples of sphingosine remain to be determined.

Recovery of Bases. Several experiments were done to determine whether the long-chain bases were quantitatively affected by the Silica Gel G in the course of analysis by TLC; contact with the adsorbent from the time of application to removal was about 24 hr. Recovery of the bases was approximately 75%; losses were probably due mostly to adsorption and to manipulative procedures. Component X comprised about 30% of the bases (Table 2). The dihydrosphingosine content of the base is 11% (Table 2) and is in reasonable agreement with the value of 14% obtained by quantitative hydrogenation of the total base. The similar values in the two determinations suggested no measurable destruction of the bases by adsorbent during the experiment, since it is unlikely that partial destruction of both bases would occur to the same extent.

The authors are grateful to Dr. P. W. O'Connell of The Upjohn Company, and to Drs. E. F. Jenny and J. Druey of Ciba, Ltd., for samples of DL-*threo-trans*-sphingosine and DL-*threo*-dihydrosphingosine. Thanks are due to Mrs. S. Braun for the long-

chain base determinations, to Dr. H. L. Meltzer for discussions, and to Dr. Warren M. Sperry, in whose laboratory this work was done, for many suggestions in the preparation of this manuscript.

This investigation was supported in part by PHS Research Grant 03191-03 from the Institute of Neurological Diseases and Blindness of the National Institutes of Health, U.S. Public Health Service.

Manuscript received July 20, 1964; accepted October 5, 1964.

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