A spectrophotometric determination of sphingosine

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[Received for publication September 26, 1960]

» Studies on the biosynthesis of sphingolipids depend to a great extent on the determination of the amount or the specific activities of the constituent building blocks of these complex lipids. The available methods for the estimation of sphingosine appear to be complex, time consuming, and of limited sensitivity. An attempt was made to develop a rapid and sensitive procedure for the determination of this compound. Of the methods more widely used, that of McKibbin and Taylor (1) depends on the determination of the sphingosine nitrogen,while that of Robins *et al.* (2) utilizes the reaction of primary amines with dinitrofluorobenzene for the determination of the long-chain base. A different principle was elaborated by Carter and co-workers (3) based on the oxidation of sphingosine by periodic acid, a method modified by Brady and Burton (4) to determine formaldehyde liberated by the periodate oxidation of sphingosine. Sakagami (5) estimates sphingosine by employing the plasmal reaction for fatty acid aldehydes, using the Schiff reagent. The present method depends on the colorimetric estimation of the complex formed between sphingosine and methyl orange (sodium p-dimethylaminoazobenzene-sulfonate).

PROCEDURE

A sample containing from 0.01 to 0.10 μmoles of sphingosine was evaporated to dryness under a stream of

nitrogen. The residue was dissolved with 0.01 N HCl, made alkaline with NaOH, and the volume adjusted to 2 ml with water. Sphingosine samples obtained by acid hydrolysis or methanolysis were made alkaline and the volume adjusted similarly. The final aqueous alkaline solution should not contain more than 20% methanol. Sphingosine was then extracted from the alkaline solution into 5 ml of ethyl acetate. After centrifugation, the aqueous phase was removed and the ethyl acetate phase washed twice with 2 ml of water. The water was removed. Two milliliters of 0.01M acetate buffer pH 3.65 (previously washed with ethyl acetate) and then 0.1 ml of methyl orange reagent (500 mg methyl orange dissolved in 100 ml of warm water and washed several times with chloroform) were added. The mixture was shaken for 1 minute and centrifuged. The sphingosine-methyl orange complex in the supernatant ethyl acetate phase was measured spectrophotometrically at 415 mμ against a blank carried through from the neutralization step.

The sensitivity of the method can be increased by extracting 2 to 4 ml of the supernatant ethyl acetate with 0.5 to 2 ml of 1 N sulfuric acid. The color concentrated in the acid extract is measured spectrophotometrically at 515 mμ against a blank treated as above.

All sphingosine determinations in this study were based on a DL-erythro-*trans*-sphingosine standard obtained from Ciba Ltd., Basle, Switzerland. Identical results were obtained using a DL-threo-*trans*-sphingosine standard, also obtained from Ciba Ltd.

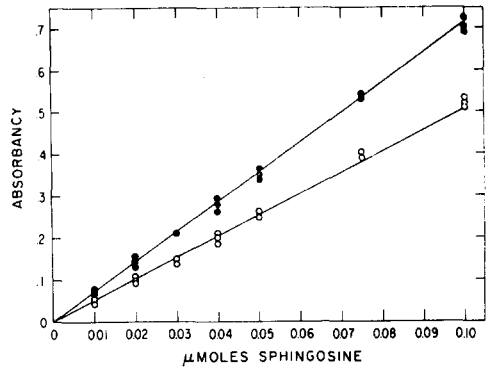

FIG. 1. Standard curves: O—O = ethyl acetate phase at 415 mμ, ratio (μmoles/absorbancy) = 0.199 ± 0.008 (Standard Deviation); ●—● = acid phase at 515 mμ after extraction of 2 ml of ethyl acetate phase into 2 ml of 1 N sulfuric acid, ratio (μmoles/absorbancy) = 0.141 ± 0.004 (Standard Deviation).

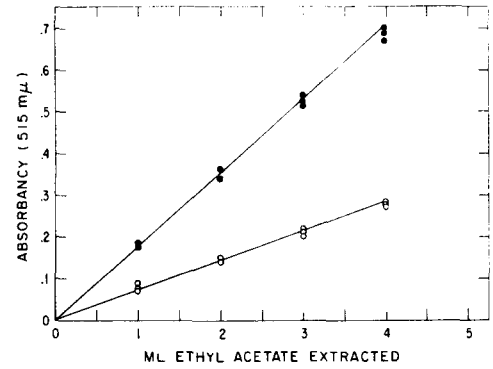

FIG. 2. Linearity between absorbancy of acid phase and volumes of ethyl acetate phase extracted into 2 ml of 1 N sulfuric acid; O—O = sample concentration of 0.02 μ moles sphingosine/5 ml ethyl acetate phase; ●—● = sample concentration of 0.05 μ moles spingosine/5 ml ethyl acetate phase.

RESULTS

For pure sphingosine, the color formation obeys Beer's law within the range specified as shown in Figure 1. The color complex extracted into the acid phase was found to be proportional to the volume of ethyl acetate solution extracted as shown in Figure 2. These data represent triplicate determinations of a pure sphingosine standard carried through the complete procedure.

The sphingosine content in several sphingolipid preparations was estimated as shown in Table 1. The lipids examined had been obtained in varying states of purity as indicated by the analyses for total nitrogen or phosphorus content, and the yield of sphingosine varied accordingly.

In order to estimate the recovery of sphingosine from a hydrolysis mixture, a cerebroside preparation was hydrolyzed alone and also with the addition of a known amount of pure sphingosine. The over-all yields of sphingosine were determined by the methyl orange procedure and compared with the yields determined by the dinitrofluorobenzene method of Robins *et al.* (2). The results are shown in Table 2.

Products such as choline, galactosamine, or sialic acids, which can be obtained from the hydrolysis or methanolysis of lipids, do not interfere with the determination of sphingosine by the present method. Since the method utilizes the relatively nonspecific reaction of a lipophilic base with the methyl orange reagent to yield a colored complex extractable into an organic phase (6, 7), caution should be exercised in the interpretation of data obtained with impure preparations of sphingolipids. Another potential advantage of the present method is its adaptability to isotope incorporation studies and the determination of the specific activity of the sphingosine, after the extraction and isolation of the sphingosine-methyl orange complex.

TABLE 1. SPHINGOSINE YIELD OF PARTIALLY PURIFIED SPHINGOLIPID PREPARATIONS

Substrate	Nitrogen (μmoles/mg)	Phosphorus (μmoles/mg)	Sphingosine (μmoles/mg)	Hydrolysis Conditions
Cerebroside (beef spinal cord)	Calcd: 1.21 Found: 1.48	<0.01	1.21 1.46	(1)*
Cerebroside (calf brain)	Calcd: 1.21 Found: 1.43	0.87	1.21 1.15	(2)†
Cerebroside sulfate (beef brain)	Calcd: 1.10 Found: 1.09	0.35	1.10 0.99	(1)
Ceramides (beef brain)	Calcd: 1.54 Found: 1.72	0.60	1.54 1.52	(1)
Psychosine sulfate	Calcd: 1.84 Found: 1.37	0.14	1.84 1.29	(1)

* (1) = 4 N hydrochloric acid in methanol for 4 hours at 105° in a sealed tube.

† (2) = 4 N hydrochloric acid in methanol for 4 hours under reflux.

TABLE 2. YIELD AND RECOVERY OF SPHINGOSINE FROM MIXTURES OF CEREBROSIDES AND SPHINGOSINE FOLLOWING HYDROLYSIS

Substrate Added		Sphingosine Recovered		
Cerebrosides	Sphingosine	Calculated	Observed	Method
mg	μmoles	μmoles/sample*		
0.05	—	—	0.057	M†
0.05	0.016	0.073	0.076	M
0.05	0.031	0.088	0.092	M
0.05	0.062	0.119	0.125	M
0.20	—	—	0.219	D‡
0.20	0.062	0.281	0.290	D
0.20	0.125	0.344	0.360	D
0.20	0.250	0.469	0.481	D

* Micromoles of sphingosine recovered were calculated from a sphingosine standard carried through the entire procedure including the hydrolysis.

† M = Methyl orange.

‡ D = Dinitrofluorobenzene.

REFERENCES

1. McKibbin, J. M., and W. E. Taylor. *J. Biol. Chem.* **178:** 29, 1949.
2. Robins, E., O. H. Lowry, K. M. Eydt, and R. E. McCaman. *J. Biol. Chem.* **220:** 661, 1956.
3. Carter, H. E., F. J. Glick, W. P. Norris, and G. E. Phillips. *J. Biol. Chem.* **170:** 285, 1947.
4. Brady, R. O., and R. M. Burton. *J. Neurochem.* **1:** 18, 1956.
5. Sakagami, T. *J. Biochem. (Tokyo)* **45:** 313, 1958.
6. Brodie, B. B., and S. Udenfriend. *J. Biol. Chem.* **158:** 705, 1945.
7. Axelrod, J., L. Aronow, and B. B. Brodie. *J. Pharmacol. Exptl. Therap.* **106:** 166, 1952.

Erratum

The Note on Methodology by C. J. Lauter and E. G. Trams, entitled "A spectrophotometric determination of sphingosine," appearing in the January 1962 issue of this Journal, contains an error on page 137, line 11. This line should read:

"Two milliliters of 0.1 M acetate buffer pH 3.65 . . . ."