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Studies on the Structure of Sphingomyelin. IV. Configuration of the Double Bond in Sphingomyelin and Related Lipids and a Study of their Infrared Spectra

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RECEIVED SEPTEMBER 30, 1953

The *trans* configuration of the double bond in sphingomyelin, N-lignocerylsphingosine and cerebroside has been established by means of infrared spectroscopy. The *trans* absorption band near 10.3 μ in the latter two non-phosphorus containing lipids was completely eliminated after hydrogenation whereas in the case of sphingomyelin this band was significantly diminished and altered. The spectrum of sphingomyelin was complicated by the finding that the band near 10.3 μ was due in part to the covalent phosphate group. However, since N-lignocerylsphingosine is undoubtedly an intermediate in the enzymatic synthesis and degradation of sphingomyelin and cerebroside, the clear-cut results obtained with this molecule should be valid in establishing, by analogy, the *trans* form of the sphingolipids. Isomerization of the double bond during the preparation of the lipids was minimized by the use of only organic solvents under very mild conditions. The infrared study of a variety of phospholipids is presented. It is proposed that infrared spectroscopy may be used for the qualitative and quantitative analysis of the two major classes of phospholipids, *i.e.*, the sphingolipids and the glycerophospholipids.

In previous communications[2a,b] the authors observed that the absorption band near 10.3 μ in the infrared spectrum of sphingomyelin was significantly diminished and altered after hydroxylation. Since it has been well established[3] that the *trans* double bond (but not the *cis*) is associated with a band near 10.3 μ, it seemed reasonable that the observed alteration of the band in sphingomyelin was due to the abolishment of the double bond after hydroxylation. However, since the amount of diminution was small, other linkages must be involved in the production of this band.

Although the configuration of the double bond in sphingomyelin had not been established, sphingosine and cerebroside have been studied in this regard. Niemann[4] reported that sphingosine sulfate ordinarily isolated from natural sources is isomerized by treatment with alcoholic H_2SO_4, and concluded that the natural form has the *cis* configuration. Conflicting results were obtained by Mislow[5] who studied sphingosine and its derivatives by means of infrared spectroscopy and showed that they possess a band near 10.3 μ. Since a cerebroside sample prepared by Mislow likewise possessed a band in this region, he concluded by analogy that the double bond in both sphingosine and cerebroside has the *trans* configuration. He further stated that in a personal communication to him Dr. H. E. Carter substantiated his findings by demonstrating that the band near 10.3 μ in sphingosine and in cerebron was essentially eliminated after reduction. Recently Fodor[6] presented evidence for the *trans* configuration of sphingosine based on the phenomenon of syncrystallization.

It must be emphasized, however, that experiments performed on sphingosine obtained from natural sources should be cautiously evaluated since the procedure for the isolation of this compound requires drastic chemical treatment which may well alter the configuration of the molecule. Furthermore, since the covalent phosphate group has been shown to absorb in the same region as the *trans* double bond,[7,8] experiments involving cerebroside must demonstrate that it be free from phosphorus impurities.

The elimination of the band near 10.3 μ in cerebron after hydrogenation is in the opinion of the authors the strongest supporting evidence for the *trans* configuration in this molecule. However, since the results of Carter are unpublished, the precautions taken to ensure that no isomerization occurred during its preparation are unknown.

In order to obtain more definitive information regarding the configuration of the double bond in sphingomyelin, the authors chose to isolate from natural sources, the closely related N-lignocerylsphingosine (V) as well as sphingomyelin (I) and cerebroside (III). N-Lignocerylsphingosine offers several advantages. First, it can be easily obtained in a pure form by the use of organic solvents alone under mild conditions. Secondly, it does not contain the phosphorylcholine group, thus interfering absorption due to covalent phosphate is eliminated. Thirdly, this compound may well be an intermediate in the enzymatic synthesis or degradation of both sphingomyelin and cerebroside[9,10]; thus the results obtained on this molecule should be valid in establishing, by analogy, the configuration of the sphingolipids, and in confirming studies made directly on these latter compounds.

Thus pure sphingomyelin (I), cerebroside (III) and N-lignocerylsphingosine (V) were isolated from natural sources and reduced to their corresponding dihydro derivatives (II, IV and VI). The infrared analysis of these compounds clearly demonstrated the complete elimination of the band near 10.3 μ after hydrogenation in the case of cerebroside (Fig. 3) and N-lignocerylsphingosine (Fig. 4), giving strong evidence that the double bond in these lipids has the *trans* configuration. The study of sphingomyelin was not as clear cut. However, a significant diminution in the intensity and an alteration in the shape of the band near 10.3 μ was observed both after reduction (Figs. 1, 2) and hydroxylation.[1,2] In Nujol the band changed from a smooth parabolic curve into a peak showing the ap-

(1) National Science Foundation Fellow.

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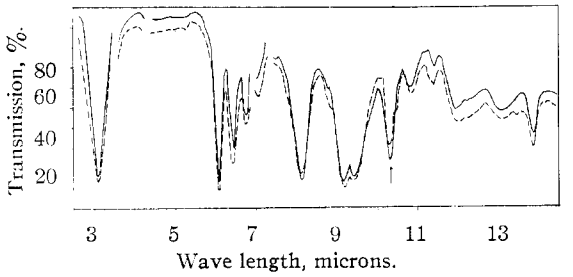

Fig. 1.—Infrared spectra of sphingomyelin (I) (solid line) and dihydrosphingomyelin (II) (dashed line); 5 mg./6 mg. Nujol; cell thickness, 0.025 mm. The absorption peaks are: sphingomyelin (I), 3.10, 6.09, 6.42, 6.75, 8.12, 9.18, 9.44, 10.32, 10.86, 11.41, 12.01, 13.09 and 13.86 μ; dihydrosphingomyelin (II), 3.10, 6.06, 6.42, 6.75, 7.05, 8.12, 9.18, 9.44, 10.29, 10.86, 11.41, 12.01, 13.09 and 13.86 μ.

pearance of a shoulder. Since the molar concentrations in Nujol may vary within a few per cent., the significance of a small diminution (on a quantitative basis) must be taken with caution. This objection can be overcome by comparing other bands in the spectrum. Thus it can be seen (Fig. 1) that the band near 10.3 μ is significantly diminished, whereas the other bands near 3.0, 8.2 and 9.2 μ are either increased or only slightly decreased. This would rule out the argument that the diminution of the 10.3 μ band was due to a slightly smaller concentration of the derivative of sphingomyelin, for if this were so all the bands should show a decrease in absorption.

Another way to minimize the probable error of unequal molar concentrations of compound is to use more solvent, more lipid, and thicker cells, so that errors due to manipulation can be reduced. Thus sphingomyelin (I) and dihydrosphingomyelin (II) were studied in chloroform solution at a concentration of 40 mg. per 1.5 ml. of chloroform in cells 0.251 mm. thick (instead of at a concentration of 5 mg. per 6 mg. of Nujol in cells 0.025 mm. thick). The results (Fig. 2) showed a 4% reduction of the band near 10.3 μ, whereas the band near 9.2 μ was slightly increased by about 1% after reduction. The quantitative changes in these spectra are considered to be significant.

Since N-lignocerylsphingosine differs from sphingomyelin only in that it does not contain the phosphorylcholine group and since its band near 10.3 μ was completely eliminated after reduction, whereas the same band in sphingomyelin was only slightly decreased, it seems evident that the band near 10.3 μ in sphingomyelin is due to both the *trans* double bond and the covalent phosphate group, but that the latter group has the stronger absorption.

Dihydrosphingomyelin (II) and dihydrocerebroside (IV) were hydrolyzed and the dihydrosphingosine bases VII and VIII isolated. In each case a mixture of bases was obtained corresponding possibly to dihydrosphingosine and O-methyldihydrosphingosine. This was substantiated by paper chromatographic analysis. The infrared spectra of the mixtures of these bases (Fig. 5) showed the essential elimination of the band in the region of 10.3 μ. Since the spectra of sphingosine and its derivatives[5] possess a moderately strong band at

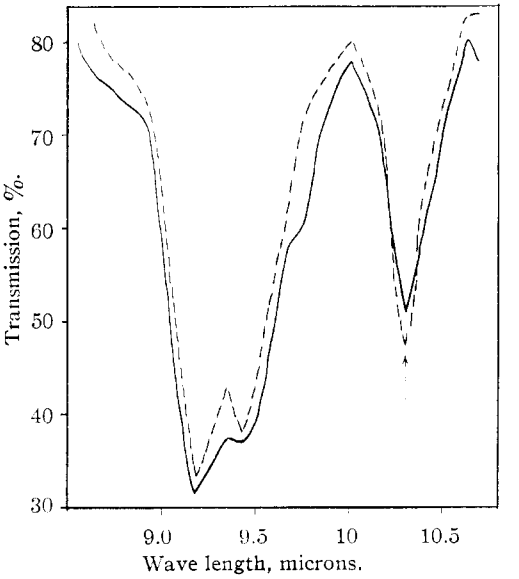

Fig. 2.—Infrared spectra of sphingomyelin (I) (dashed line) and dihydrosphingomyelin (II) (solid line); 40 mg./1.5 ml. chloroform; cell thickness, 0.251 mm.

10.3 μ, the elimination of the band in the dihydro compounds demonstrated that the reduction of sphingomyelin and cerebroside occurred on the double bond of the sphingosine moiety. Quantitative measurement of the hydrogen uptake showed that the theoretical one mole of hydrogen was required for each mole of sphingomyelin reduced.

In order to learn more about the possible atomic linkages which were responsible for the bands in the spectra of sphingomyelin and other phospholipids, the study was made to include lecithin (X), cephalin (IX), glycerophosphorylcholine (XI) and acetal phospholipid (XII). After the work presented in this paper had begun, several publications on the infrared spectroscopy of the phospholipids were reported by other workers. Baer and co-workers have published the spectra of pure synthetic cephalins[11] and lecithins.[12]

The spectra of hydrolecithin and its unsaturated counterpart dipalmitoleyllecithin were reported by Hanahan and Jayko.[13] Schwarz, *et al.*,[14] have applied infrared spectroscopy to the study of tissues both before and after lipid extraction and have made some advance toward the use of this technique for quantitative analysis. They also have given data on the infrared spectra of a few phospholipids. More recently Freeman, *et al.*,[15] have used infrared spectroscopy to study the phospholipids of lipoproteins both on a qualitative and quantitative basis and indicated the limitations and areas for improvement in this technique. It is apparent that the use of infrared spectroscopy to the biochemist is becoming increasingly valuable. Al-

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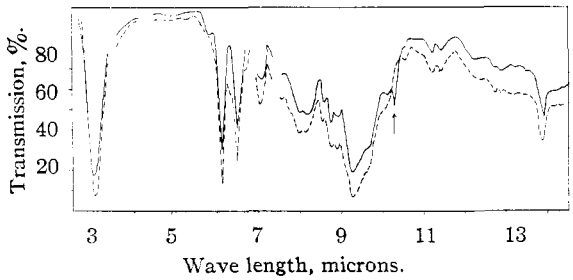

Fig. 3.—Infrared spectra of cerebroside (III) (solid line) and dihydrocerebroside (IV) (dashed line); 4 mg. compound (III) and 5 mg. compound (IV) per 6 mg. Nujol; cell thickness, 0.025 mm. The absorption peaks are: cerebroside (III), 3.01, 6.12, 6.50, 7.05, 7.97, 8.18, 8.55, 8.74, 8.91, 9.26, 9.65, 10.28, 11.18, 11.41, 12.85 and 13.86 μ. Dihydrocerebroside (IV), 3.01, 6.14, 6.50, 7.05, 7.97, 8.17, 8.55, 8.75, 8.91, 9.30, 10.55, 11.18, 11.41, 12.68, 12.85 and 13.86–13.88 μ.

though the spectra of the major phospholipids have been investigated, little has been done to correlate the spectral findings. This paper provides some of this information.

The results of the spectra of the phospholipids, cerebroside and N-lignocerylsphingosine (Figs. 1–10) are summarized below, keeping in mind that some of the structural assignments are tentative.

(1) All possessed a moderate or strong band near 10.3 μ. In the case of the saturated lecithin (Fig. 7) this band is believed due mainly to the covalent phosphate (P–O–C) group; with cerebroside (Fig. 3) and N-lignocerylsphingosine (Fig. 4) it was due entirely to the *trans* double bond, and with sphingomyelin (Fig. 1) it was due to both the *trans* double bond and the covalent phosphate groups. This band was weakest in cerebroside. Acetal phospholipid (Fig. 9) and cephalin (Fig. 6) were unique in that the covalent phosphate band apparently occurred at a longer wave length (10.4–10.9 μ). The band near 10.3 μ in these compounds, which is believed to be due also to covalent phosphate absorption was moderately weak.

(2) All possessed a strong band near 9.2 μ which was in general a rather broad doublet and which is believed due mainly to the covalent phosphate linkage, or in the case of the sphingolipids to an additional absorption by the sphingosine molecule. This postulate is based on the finding that the band occurs both in glycerophosphorylcholine (Fig. 8) and in the non-phosphorus-containing cerebroside, dihydrosphingosine bases and N-lignocerylsphingosine (Figs. 3–5).

(3) All had a strong band near 8.2 μ. In the case of cerebroside (Fig. 3) this band is believed due to the acetal C–O–C linkage, in acetal phospholipid (Fig. 9) to both the acetal C–O–C and covalent phosphate groups; in lecithin (Fig. 7) and cephalin (Fig. 6) to both the ester C–O–C linkage and the covalent phosphate group, and in sphingomyelin (Fig. 1) mainly to the covalent phosphate group. These findings are substantiated by the fact that this band is virtually absent in dihydrosphingosine (Fig. 5) and N-lignocerylsphingosine (Fig. 4), both of which do not contain the above mentioned groups. The P=O linkage may also contribute to this absorption

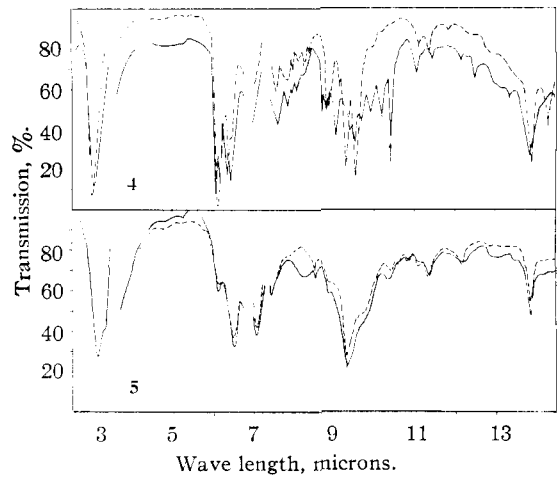

Fig. 4.—Infrared spectra of N-lignocerylsphingosine (V) (solid line) and N-lignoceryldihydrosphingosine (VI) (dashed line); 5 mg./6 mg. Nujol; cell thickness, 0.025 mm. The absorption peaks are: N-lignocerylsphingosine (V), 3.05, 6.07, 6.19, 6.38, 6.45, 7.62, 7.87, 8.07, 8.72, 8.82, 9.07, 9.40, 9.52, 9.67, 9.91, 10.19, 10.40, 11.07, 11.44, 12.15, 12.50, 13.38 and 13.88 μ; N-lignoceryldihydrosphingosine (VI), 2.99, 6.12, 6.34, 6.45, 7.75, 7.82, 7.97, 8.11, 8.26, 8.82, 8.90, 9.15, 9.30, 9.53, 11.13, 13.89 and 14.31 μ.

Fig. 5.—Infrared spectra of a mixture of dihydrosphingosine bases (VII) (dashed line) and (VIII) (solid line) obtained from dihydrosphingomyelin (II) and dihydrocerebroside (IV), respectively; 4 mg./6 mg. Nujol; cell thickness, 0.025 mm. The absorption peaks are: dihydrosphingosine bases (VII), 3.02–3.12, 6.15, 6.50, 7.06, 7.44, 8.51, 9.30, 10.40, 10.83, 11.08, 11.31, 12.15 and 13.86 μ; dihydrosphingosine bases (VIII), 3.02–3.12, 6.15, 6.50, 7.06, 7.44, 8.51, 9.30, 10.32, 10.80, 11.34, 12.21 and 13.86 μ.

band as well as the covalent phosphate (P–O–C) group.

(4) All possessed a long chain hydrocarbon band near 13.86 μ, which is due to the long carbon-to-carbon chains found in the higher fatty acids and in sphingosine. In the case of N-lignocerylsphingosine this band occurred at a slightly longer wave length. Furthermore, it was observed that after reduction of this compound the band shifted to an even longer wave length (Fig. 4). Only in the case of cephalin (Fig. 6) did it appear to exist as a doublet with peaks at 13.72 and 13.90 μ (Fig. 6).

(5) All had a relatively weak band near 11.4 μ (unassigned).

(6) The glycerophospholipids (except acetal phospholipid) possessed a very intense ester C=O stretching band at 5.76–5.78 μ.

(7) The sphingolipids were characterized by the typical strong amide C=O stretching band near 6.1 μ and the amide NH deformation band near 6.45 μ. In addition, these lipids possessed the NH and OH stretching bands in the region 3.0–3.1 μ. The spectrum of N-lignocerylsphingosine (Fig. 4) indicated that the amide bands apparently exist as two components since the usual single peak at 6.10 μ was replaced by a doublet (6.07 and 6.19 μ), and the band at 6.45 μ occurred as two individual peaks at 6.38 and 6.45 μ.

(8) After reduction of N-lignocerylsphingosine

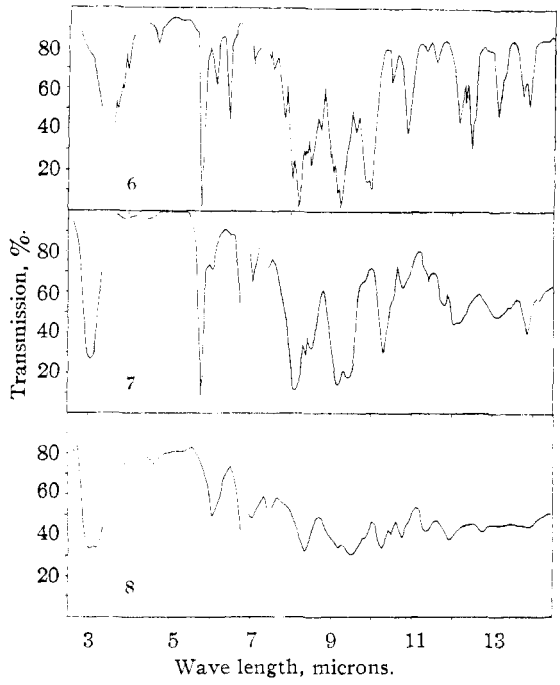

Fig. 6.—Infrared spectrum of synthetic L-α-dimyristoylcephalin (IX); 5 mg./6 mg. Nujol; cell thickness, 0.025 mm. The absorption peaks are: 3.0–3.10 (shoulder), 3.71, 3.82, 3.95, 4.71, 5.76, 6.14, 6.44, 7.07, 7.55, 7.82, 8.01, 8.19, 8.50, 8.74, 9.24, 9.60, 9.83, 9.97, 10.25, 10.89, 11.33, 11.59, 12.16, 12.31, 12.46, 13.13, 13.72 and 13.90 μ.

Fig. 7.—Infrared spectrum of synthetic L-α-dimyristoyllecithin (X); 5 mg./6 mg. Nujol; cell thickness, 0.025 mm. The absorption peaks are: 3.03, 5.78, 6.09, 7.07, 8.10, 8.39, 8.55, 9.18, 9.44, 10.29, 10.78, 11.41, 11.82, 12.01, 13.10 and 13.86 μ.

Fig. 8.—Infrared spectrum of synthetic L-α-glycerophosphorylcholine (XI) ($CdCl_2$ salt); 4 mg./6 mg. Nujol; cell thickness, 0.025 mm. The absorption peaks are: 2.99–3.21, 4.62, 6.05, 7.05, 8.32, 9.18, 9.44, 10.25, 10.74, 11.34, 11.94 and 12.72 μ.

several significant changes were noted. The bands near 9.3 and 9.5 μ (Fig. 4) were intensified remarkably. Furthermore, the OH,NH peak shifted to a shorter wave length, whereas the long chain hydrocarbon band was displaced toward a slightly longer wave length. In addition, a new band appeared at 14.31 μ. The *trans* double bond band which normally occurred as one peak near 10.3 μ, consisted in this compound apparently as three components with peaks at 9.91, 10.19 and 10.40 μ, since all were abolished after reduction. The appearance of the new band at 14.31 μ may possibly indicate that reduction of N-lignocerylsphingosine was not complete and that some of the *trans* isomer was isomerized to the *cis* form. Identical results were obtained with another sample of beef lung ceramide kindly donated by Dr. S. J. Thannhauser. These changes did not occur with sphingomyelin.

(9) The ethanolamine-containing phospholipids (cephalin and acetal phospholipid) possessed a relatively weak band in the region of 4.61–4.71 μ (unassigned).

(10) The bands which occurred in the region

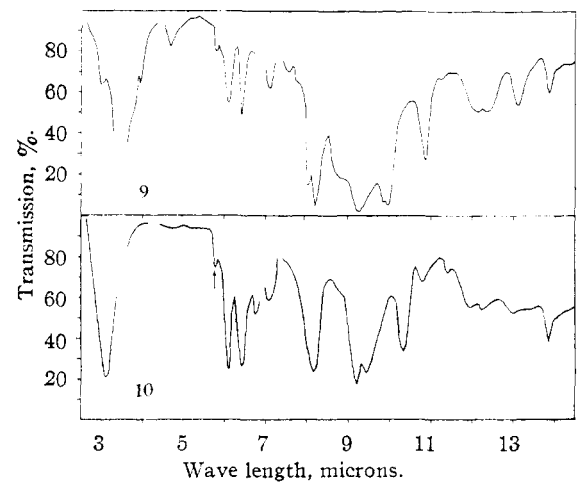

Fig. 9.—Infrared spectrum of acetal phospholipid (XII); 5 mg./6 mg. Nujol; cell thickness, 0.025 mm. The absorption peaks are as follows: 2.99, 3.94, 4.62, 5.80 (believed due to ester impurities), 6.10, 6.42, 7.10, 7.56, 8.03, 8.21, 9.26, 9.84, 9.98, 10.86, 12.15, 12.34, 13.09 and 13.86 μ.

Fig. 10—Infrared spectrum of a 95:5 mixture of sphingomyelin (I) and L-α-dimyristoyllecithin (X); 5 mg./6 mg. Nujol; cell thickness, 0.025 mm.

6.7–7.5 μ are undoubtedly due mainly to CH_3 and CH_2 bending.

(11) The ethanolamine-containing phospholipids were characterized by a strong band at 9.97–9.98 μ which may be useful in identifying these lipids. This band was relatively weak in lecithin and sphingomyelin and is believed due to covalent phosphate absorption.

Since the glycerophosphatides (except acetal phospholipid) possessed a strong ester C=O band near 5.76 μ, this should provide a means for both the qualitative and quantitative analysis of these lipids. The sphingolipids were characterized by the intense amide bands near 6.1 and 6.45 μ which may serve as reference peaks whereby they may be analyzed. It is feasible therefore, that infrared spectroscopy may be used to analyze the two major classes of phospholipids, *i.e.*, the glycerophosphatides and the sphingolipids.

In order to test the feasibility of this proposal, a mixture of sphingomyelin and lecithin (95:5) was studied. Infrared analysis proved that *at least* 5 parts per 100 of lecithin can be detected in such a mixture as evidenced by the appearance of the ester C=O band at 5.76 μ (Fig. 10) which is completely absent in sphingomyelin (Fig. 1). The relative intensities of these bands (ester and amide) should give the ratio of the components in a lipid mixture. If only a study of relative amounts is desired, infrared analysis appears to offer a rapid and simple means of obtaining this information since the cell thickness would not be critical. However, if absolute amounts are desired, then the cell thickness becomes an important factor. The recent development of the pressed KBr disc procedure[16] which is well suited for the microanalysis of solids, should greatly expedite the application of infrared spec-

(16) Perkin-Elmer Instr. News for Science and Industry, Vol. 4, No. 3, Perkin-Elmer Corp., Norwalk, Conn., 1953.

troscopy for the quantitative determination of the lipids.

As is the case in most quantitative determinations, certain precautions are necessary. Thus the examination of the spectra revealed that cephalin and acetal phospholipid (Figs. 6, 9) (and very likely serine phospholipid) possess moderate bands in the same region where the amide group absorbs. Therefore interference by these lipids in the analysis of sphingomyelin must be considered. Furthermore, the proposed procedure would not include acetal phospholipid which does not contain either the ester or amide groups. It appears, therefore, that only the ratio of sphingolipids to lecithin and cephalin can be determined. Since these lipids comprise the major fraction of the total phospholipids and sphingolipids in many tissues, a study based on this procedure should have useful application. When combined with chemical data, infrared spectroscopy should offer a versatile combination for the study of the phospholipids. It must also be noted that this present study did not include the inositol-containing phospholipids. Until these latter compounds can be obtained in a pure form of known structure and until their spectra are determined, it is difficult to predict how they might fit into or modify the above mentioned scheme.

Experimental

Preparation of Sphingomyelin (I).—Crude sphingomyelin was prepared from 10 lb. of beef brain essentially according to the procedure of Carter and co-workers.[17] The product was dissolved in glacial acetic acid at room temperature and filtered in order to remove cerebrosides. The filtrate was brought to dryness *in vacuo* and the residue dissolved in petroleum ether–methanol (9:1) and passed through an Al_2O_3 (Fisher, for chromatographic purposes) column to remove last traces of cerebroside impurities. The process was repeated until the eluate gave a negative test for sugar with the anthrone reagent. Usually two runs were sufficient. The eluate was brought to dryness *in vacuo* and the residue crystallized several times from ethyl acetate–methanol (100:5) to yield 950 mg. of white powder which melted at 205–207° dec., with previous sintering between 165–175°. Tests with the Molisch, anthrone and Bials reagents were completely negative, thus indicating the absence of cerebroside and ganglioside impurities. The infrared spectrum (18) (Fig. 1) was essentially identical to the spectrum of the sphingomyelin obtained from beef brain using the procedure of Thannhauser and Boncoddo[19] and reported previously by the authors.[1,2] The complete lack of the ester carbonyl band in the region 5.7–5.9 μ indicated the absence of glycerophospholipid impurities. The authors felt that the spectral analysis and chemical tests were sufficient criteria to prove the purity of the sample, and consequently C, H, N and P analyses were not made.

Sphingomyelin was found to be very soluble in glacial acetic acid, chloroform, and formic acid at room temperature, but only slightly soluble in pyridine. It was insoluble in acetone and ether.

Dihydrosphingomyelin (II).—In order to measure the hydrogen uptake quantitatively, a microhydrogenation apparatus made according to the design of Hyde and Scherp[20] was used. The instrument was first calibrated against known amounts of hexenoic and oleic acids.

One hundred and sixty milligrams (0.2 mmole) of sphingomyelin was dissolved in 25 ml. of glacial acetic acid, 50 mg. of platinum oxide added and the lipid reduced with hydrogen at atmospheric pressure at 26°. The blank flask contained 50 mg. of platinum oxide and enough glacial acetic acid (approximately 25 ml.) so that the gas phases over each flask were equal in volume. The reaction was complete at the end of 150 minutes and 0.22 mmole of hydrogen was taken up. The mixture was filtered to remove the catalyst and the filtrate brought to dryness at 40° with a stream of nitrogen. The white residue was washed by suspending it three times in 10-ml. portions of acetone and centrifuging. The precipitate was dried *in vacuo* at 56° for 3 hours over NaOH to yield 105 mg. of white powder, m.p. 211–212°, with previous sintering at 185–190°.

The dihydrosphingomyelin (II) and the original sphingomyelin (I) were analyzed in the infrared both as Nujol mulls and chloroform solutions. It was observed that compound II was significantly less soluble in chloroform than compound I. The spectra are given in Figs. 1 and 2.

Another sample of sphingomyelin (I) was reduced with hydrogen in a Paar apparatus at 70° and under 50 lb. pressure for 2 hours using platinum oxide catalyst. The spectrum of the isolated dihydrosphingomyelin was identical to the spectrum of the product II obtained by reduction in acetic acid.

Cerebroside (III).—The acetic acid insoluble residue obtained from the preparation of sphingomyelin was found to consist mainly of cerebrosides. In view of the finding that cerebrosides are insoluble in glacial acetic acid at room temperature, but soluble in hot solution, and that sphingomyelin and glycerophospholipids are very soluble in acetic acid at room temperature, this solvent offered a good means for purifying the product. Therefore the cerebroside was repeatedly crystallized from acetic acid to yield a white crystalline product. However, the spectrum of this compound revealed a moderate band near 5.76 μ indicating ester impurities (lecithin, cephalin, etc.). Therefore the cerebroside was treated with pyridine, in which it was soluble, the solution passed through an Al_2O_3 column and the eluate brought to dryness *in vacuo*. The residue was recrystallized several times from methanol and acetic acid to give 2.4 g. of white crystalline product, m.p. 194–196° dec., with previous sintering. The following chemical analyses were obtained

	N (Nessler), %	Galactose (anthrone), %
Calcd.	1.69–1.73	21.7–22.2
Found	1.70	20.7

The calculated values depend on the fatty acid present, *i.e.*, whether the cerebroside is phrenosin ($C_{48}H_{93}NO_9$) or kerasin ($C_{48}H_{93}NO_8$).

Tests for phosphorus (Fiske–SubbaRow) and for gangliosides (Bials) were negative. The sugar was identified by hydrolyzing the cerebroside with 6 *N* HCl for 15 minutes at 100°, filtering, and analyzing the filtrate by paper chromatography, using collidine saturated with water as solvent system. Known sugars (galactose, glucose, fructose, mannose and xylose) were run at the same time. The cerebroside filtrate gave only one spot due to sugar which moved identically with galactose. The sugars were identified by dipping the chromatograms in Tollens reagent (in ethanol) and heating in an oven at 80° for 5–10 minutes.

Spectral analysis of the final product confirmed the absence of glycerophospholipids because of the essential lack of the ester carbonyl band near 5.76 μ (Fig. 3).

Dihydrocerebroside (IV).—Four hundred mg. of cerebroside (III) was dissolved in warm ethanol and reduced at 70° and 50 lb. pressure, using platinum oxide catalyst. After 2 hours the warm solution was filtered and the filtrate concentrated *in vacuo* and placed in the cold (0°). The dihydrocerebroside which precipitated out of solution was separated by filtration and dried *in vacuo* to give a white powder which melted at 214–216° dec., with previous sintering. Spectral examination showed the complete elimination of the band near 10.3 μ (Fig. 3).

N-Lignocerylsphingosine (V).—This ceramide was prepared according to the procedure of Tropp and Widersheim.[21] Six kg. of beef spleen yielded 135 mg. of pure crystalline product, m.p. 94–95° (reported by the above workers, 94–95°). The compound was readily soluble in

(17) H. E. Carter, W. J. Haines, W. E. Ledyard and W. P. Norris, *J. Biol. Chem.*, **169**, 77 (1947).

(18) The infrared spectra were run by Mr. Carl Whiteman of the Chemistry Department of this University, using a Perkin–Elmer Model 12AB recording spectrometer with an NaCl prism.

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(21) C. Tropp and V. W. Widersheim, *Z. physiol. Chem.*, **222**, 39 (1933).

warm acetone and warm methanol–chloroform (9:1) from which it was repeatedly recrystallized in the form of small rosettes. Tests for cerebroside impurities with the anthrone reagent and for P impurities by the Fiske–SubbaRow method were entirely negative. Spectral analysis confirmed the absence of glycerophospholipid contaminants because of the absence of the ester carbonyl band in the region 5.7–5.9 μ (Fig. 4).

N-Lignoceryldihydrosphingosine (VI).—Fifty mg. of compound (V) was reduced with hydrogen in methanol solution at 60° and 50 lb. pressure for 1.5 hours, using platinum oxide catalyst. The product was easily isolated since it was insoluble in cold methanol. Thus the methanol solution was filtered while hot in order to remove the catalyst, and the filtrate placed in the cold (0°) for several hours. The white precipitate which formed was separated by filtration and dried *in vacuo* at 56° to yield 45 mg. of product, m.p. 99–100°. The infrared spectrum of this compound (Fig. 4) clearly demonstrated the complete elimination of the bands near 10.3 μ.

Hydrolysis of Dihydrosphingomyelin (II) and Dihydrocerebroside (IV).—Ninety mg. of (II) and 200 mg. of (IV) were hydrolyzed with methanolic sulfuric acid according to the procedure of Carter, *et al.*,[18] and the fatty acid methyl esters and the dihydrosphingosine bases isolated. The fatty acid esters (10 mg.) obtained from (II) consisted of a white waxy solid, m.p. 54–58° with softening at 36–38°, which corresponds to a mixture of methyl esters of stearic (m.p. 38–39°) and lignoceric (m.p. 58°) acids. The free fatty acids obtained from (IV) after saponification, melted over the range 73–78°. Comparison with the m.p. of lignoceric acid (81–84°), nervonic acid (40–41°) and cerebronic acid (100–101°), indicated that the mixture contained mainly lignoceric acid with smaller amounts of cerebronic acid. No attempt was made to further characterize these acids since they were not critical in the investigation.

The dihydrosphingosine bases VII from dihydrosphingomyelin (II) were purified by crystallization from hexane to yield a pale yellow solid, m.p. 67–73°. O-Methyldihydrosphingosine is reported to melt at 67–68°,[22] synthetic dihydrosphingosine at 99.5–100.5°[23] and naturally occurring dihydrosphingosine at 83–90°.[18] The naturally occurring free sphingosine and dihydrosphingosine bases are difficult to obtain in a pure state.

Approximately 18 mg. of bases VIII was obtained from the reduced cerebroside (IV). After crystallization from hexane a very pale yellow product was obtained, m.p. 67–73°. This compound turned deeper yellow on standing at room temperature for several days as did the bases VII. The sulfate derivative of these bases melted at 238–245°.

Chromatographic Analysis of Compounds (VII) and (VIII).—The dihydro bases VII and VIII gave strong positive tests with ninhydrin. Paper chromatographic analysis, using *n*-butyl alcohol saturated with water, gave confirmatory evidence that two bases were present in each mixture since two spots, giving a ninhydrin color test, were obtained. The faster moving component, which showed no fluorescence in the ultraviolet, had an R_f value of 0.58. The slower moving component, which exhibited a bright blue fluorescence, had an R_f value of 0.04. In phenol saturated with water the above compounds moved as one spot with R_f value of 0.91.

An attempt was made to isolate free unreduced sphingosine from both sphingomyelin (I) and cerebroside (III) but in each case a gummy oil or semi-solid was obtained which rapidly turned brown on standing at room temperature and finally became a viscous black-brown oil. However, a sufficient amount of material was obtained for chromatographic study. Chromatograms run in *n*-butyl alcohol saturated with water showed two spots which gave positive ninhydrin color tests. The slower moving component had an R_f value of 0.10 whereas the faster moving compound possessed an R_f value of 0.31. In contrast to the dihydro bases VII and VIII, both the bases obtained in this case possessed a bright blue fluorescence. In phenol saturated with water the sphingosine bases moved as one spot with an R_f value of 0.82. All chromatograms were run on unwashed Whatman filter paper No. 1. The spots were developed by spraying the papers with a 0.2% solution of ninhydrin in butyl alcohol saturated with water, and drying at room temperature for 6–24 hours. This treatment gave deep purple-blue spots, whereas if the chromatograms were heated after spraying, reddish-purple spots developed. Brom cresol green indicator also could be used to detect the bases.

Since it was not essential to separate the mixture of dihydro bases, the mixtures were studied in the infrared. The spectra (Fig. 5) demonstrated the essential elimination of the *trans* band near 10.3 μ, thus proving that hydrogenation actually occurred on this molecule. The spectra of sphingosine and its derivatives, reported by Mislow,[5] show a moderately strong band near 10.3 μ.

Cephalin (IX), Lecithin (X) and Glycerophosphorylcholine (IX) (α-GPC).—Pure synthetic samples of L-α-dimyristoylcephalin (IX), L-α-dimyristoyllecithin (X) and L-α-glycerophosphorylcholine (XI) ($CdCl_2$ salt for the latter compound) were kindly furnished by Dr. Erich Baer of the University of Toronto. These compounds were dried *in vacuo* at 56° for 3 hours before determining the infrared spectra (Figs. 6–8). The spectrum of α-GPC was difficult to obtain due to considerable light scattering. This compound was not easily mulled in Nujol. The finding of a strong band in the OH region in the spectrum of lecithin (Fig. 7) would support the open hydrated structure for this lipid as proposed by Baer.[12]

Acetal Phospholipid (XII).—A sample of acetal phospholipid (XII), m.p. 197–200° dec., was generously supplied by Dr. S. J. Thannhauser of the Pratt Diagnostic Institute and the Tufts Medical School, Boston, Mass. This lipid gave the expected Schiff test. The spectrum is given in Fig. 9. The weak band near 5.8 μ is believed due to small amounts of glycerophosphatide impurities.

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