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From the Institute for Epilepsy Research, "Meer en Bosch," Heemstede,
(Med. Dir. Dr. A. M. Lorentz de Haas) and the Department of Propedeutic Chemistry,
University of Amsterdam, The Netherlands

Revised Procedure for the Determination of "Sphingomyelin" in Fresh and Formaldehyde-Preserved Tissue*

By
G. W. F. Edgar, M. Mazure, and G. Smits

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Brante (1949) showed that the alkali-stable phospholipid fraction determined according to the method of Schmidt et al. (1946) contains not only sphingomyelin, but also a non-choline containing lipid, which he named "cephalin B". Dawson (1954) confirmed this finding and observed that the quantitity of non-sphingomyelin phosphorous in the alkali-stable fraction is reduced when the tissue is treated with 10% trichloracetic acid (TCA) before the lipids are extracted. In this regard ice-cold TCA appeared to be less effective than TCA at room temperature. Both authors suggested calculating sphingomyelin from the quantity of alkali-stable lipid choline instead of from the quantity of alkali-stable lipid phosphorus. Using the periodide method for determination of choline, Edgar and Smits (1959) followed this suggestion but, in order to obtain reproducible results, it was necessary to dry tissue samples prior to lipid extraction.

This limits the range of application of this method; moreover, the determination of choline is less suited to routine use than determination of phosphorus. For these reasons an attempt was made to ensure optimal elemination of non-sphingomyelin phosphorus from the alkali-stable lipid fraction by using TCA-treatment more intensive than that in Schmidt's original procedure.

Methods

The total quantity of alkali-stable phospholipids was determined as previously described (Edgar 1956). In this procedure alkaline hydrolysis is followed by treatment with 5% TCA at room temperature for 2 hrs. In the modification now proposed, the effect of TCA was intensified by increasing the concentration to 10%, by carrying out the procedure at 37°C and by prolonging TCA treatment to 3 days. The temperature of 37°C was chosen because alkaline hydrolysis is also carried out at this temperature; consequently the procedure can be performed in the same water bath. The quantity of choline-containing phospholipids was determined as described by Edgar and Smits (1959). In this method, choline is determined according to the method of Smits (1957), which is a combination of the method of Appleton et al. (1953) and that of Kushner (1956).

Results and Discussion

The mean value for alkali- and TCA-stable phospholipids obtained by the modified method in 7 independent estimations, was significantly lower than the mean value for the total quantity of alkali-stable phospholipids obtained in 12 estimations on the same sample by the method previously described ($p = > 0.01$).

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The quantity of alkali-stable lipid phosphorus was not further reduced by prolonging TCA treatment to 6 days.

The quantity of choline in the alkali-stable fraction remains unchanged during TCA treatment during 6 days. It can be assumed, therefore, that choline containing alkali-stable phospholipids (mainly sphingomyelin) are not hydrolysed by intensified TCA treatment as used in this investigation.

In order to investigate the degree of elimination of "non-sphingomyelin" phosphorus, "sphingomyelin" values calculated from alkali-stable lipid choline, were compared with those calculated from the quantity of alkali-stable lipid phosphorus, which resists intensified TCA-treatment (further indicated as alkali- and TCA-stable lipid phosphorus). Different types of tissue were used in this study. The Table shows close agreement of "sphingomyelin" values obtained by applying

Table. *Comparison of "sphingomyelin" values calculated from alkali- and TCA-stable phosphorus (present method) and from alkali- and TCA-stable choline (Brante's method) determined in the same sample*

All figures represent percentages of dry weight. Each figure represents the mean of 3 independent determinations unless stated otherwise (see below)

	Alkali- and TCA-stable P (×25)	Alkali- and TCA-stable choline (×6.4)
Human brain. Normal		
formalin-fixed		
White matter, 5 month old child	2.9	2.8
White matter, 7 years old child	3.8	3.5
Total hemisphere, adult	2.3	2.4
unfixed		
Grey matter, 2 year old child	1.5	1.6
White matter, 2 year old child	2.6	2.9
Cerebellar white matter, adult	3.5	3.6
Total hemisphere, adult	2.5	2.8
Amaurotic idiocy		
formalin-fixed		
Grey matter, case I	2.5	2.6
Grey matter, case II	1.9	1.8
5 rabbits, 3—18 months old		
Total hemisphere	1.7[1] (1.5—1.8)[2]	1.8[1] (1.3—2.4)[2]

[1] Mean value calculated from the mean of 3 determinations made in each of the 5 rabbits.
[2] Range of the observations.

both methods of calculation to the same sample, regardless of whether it had been taken from formaldehyde-preserved or from fresh tissue. In addition, freeze-dried samples taken from a homogenized brain hemisphere, which had been fixed for 1 year in 10% formalin (without neutralization), showed "sphingomyelin" values similar to those obtained from the homogenized contra-lateral hemisphere, which had not been preserved in a fixative (2.3% of dry weight and 2.5% of dry weight, respectively). Unlike the periodide method for the determination of choline, the procedure proposed in this paper does not require drying of tissue samples, since identical "sphingomyelin" values were measured in freeze-dried and in wet samples

from the homogenate of the fresh human hemisphere (2.5% of dry weight and 2.3% of dry weight, respectively).

The date presented here justify substituting the quantity of alkali- and TCA-stable lipid phosphorus for the quantity of alkali-stable lipid choline as a measure of the "sphingomyelin" content of human and animal tissue. The effect of intensified hydrolysis with TCA might be explained by assuming, with Schmidt et al. (1959) and with Davenport and Dawson (1962), that the alkali-stable phospholipid fraction comprises plasmalogens, which are not hydrolysed during the brief treatment with 5% at room temperature used in Schmidt's original procedure (1946).

Finally it should be stated that the alkali- and TCA-stable phospholipid fraction determined by the method proposed here, is not necessarily pure sphingomyelin. It may include, for instance, choline-containing plasmalogens such as described by Gottfried and Rapport (1962), if these compounds were TCA-resistant by nature or because of structural alterations induced by the hydrolysis (Davenport and Dawson 1962). This contamination, however, will also be included in the values for alkali-stable phospholipids calculated from the choline content of the alkali-stable fraction.

There are other phospholipids, moreover, which can be assumed to be alkali- and TCA-resistant because of their ether linkage. This type of compound was isolated for the first time from the alkali-stable phospholipid fraction of egg yolk (Carter et al. 1958). Later it was found to occur in small quantities in erythrocytes (Hanahan and Watts 1961) and in nerve tissue (Svennerholm and Thorin 1960).

In normal tissue the quantity of choline-containing acetal phospholipids and ether phospholipids is so small as to justify the assumption that the quantity of alkali- and TCA-stable phospholipid is an approximate measurement of true sphingomyelin. In pathological conditions, however, the presence of abnormal quantities of ether phospholipids and/or plasmalogens should be excluded before alterations in the alkali- and TCA-resistant phospholipid fraction are attributed to the sphingomyelin fraction. Therefore, we have put to the term "sphingomyelin" in quotation marks when it is used to designate the alkali- and TCA-stable phospholipid fraction measured by the method proposed.

Summary

Almost all phospholipids are hydrolysed by treatment with N KOH at 37° for 16 hrs followed by 10% trichloracetic acid at 37° over a 3-day-period. After this treatment there remains only sphingomyelin and probably certain phospholipids of a type which occurs only in exceptionally small quantities in the CNS. This method is proposed for the estimation of the "sphingomyelin" content. This term is to be set between quotation marks to draw special attention to the small quantity of phospholipids apart from sphingomyelin.

Résumé

Si un mélange de phospholipides est traité durant seize heures à 37° avec KOH N et ensuite pendant trois jours à 37° avec 10% d'acide trichloracetique, presque tous les phospholipides seront hydrolysés. Ne résisteront à ce traitement que la sphingomyéline et peut-être quelques phospholipides qui ne sont présentes que

dans des quantités minimes dans le système nerveux normal. Les auteurs proposent ce procédure pour estimer le taux de «sphingomyéline».

Ce terme a été mis entre guillemets pour attirer l'attention sur la présence de petites quantités de phospholipides autres que shpingomyéline.

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Dr. G. W. F. Edgar, Research Laboratory, Institute for Epilepsy Research "Meer en Bosch", Heemstede, The Netherlands.