Formaldehyde Brain Lipids

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THE ACTION OF FORMALDEHYDE SOLUTIONS ON HUMAN BRAIN LIPIDS

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The action of formaldehyde solutions on human brain lipids was investigated by extracting and analysing the lipids from normal adult human white matter preserved from 1 year to 24 years in formaldehyde solutions. The human brains were kindly provided by Dr. F. C. Stam, Valeriuskliniek, Amsterdam.

In this way the effect of a prolonged formaldehyde exposure on several lipids could be ascertained. In previous publications (4, 7) it was stated that the action of formaldehyde on the lipids should be divided into a long (more than 1 year) and a short (less than a year) fixation. Formaldehyde reacts very fast with phosphatidylethanolamine (presumably with the $—NH_2$ group); moreover a marked formation of lysophosphatidylethanolamine and lysolecithin was found after two weeks fixation. As unbuffered formaldehyde solutions are acidic, part of the degradation of lipids can be ascribed to slow hydrolysis.

These results are not sufficient to enable us to predict the effect of more prolonged fixation. We chose human brains as substrate for the following reasons. Mammalian nervous tissues always possess a high lipid content. In many institutes normal and pathological brains are nearly always stored in unbuffered formaldehyde solutions. Notwithstanding the fact that detailed information on the effects on the lipid composition by storage in formaldehyde solutions is lacking, many histological, histochemical and neurochemical investigations are carried out on brain specimens preserved for many years by formaldehyde. This concerns particularly brains in which pathological conditions are expected, as this material often is scarce. As the lipids are a main structural element of the myelin sheath the lipid composition of the adult normal brain can be expected to be more constant than that of other organs.

MATERIALS AND METHODS

White matter: Pieces of white matter weighing ca. 15 grams from normal adult human brains were taken from the corpus callosum and adjacent white matter. Care was taken not to include parts of the nuclei of the brain stem or major blood vessels. As this white matter consists mainly of myelinated nervous fibers the composition of the extracted lipids corresponds to a high degree to the lipid composition of the myelin sheath in the central nervous system.

Extraction: The pieces of brain were homogenised after which the lipids were extracted by the method of Folch *et al.* (5) with chloroform:methanol 2:1 v/v, washing the extract with 0.1 *M* KCl. Extracting fresh human brain we found it advisable to boil the homogenised tissue for a few minutes with methanol, after which the required amount of chloroform was added.

After weighing the extracted lipids as described before (4, 7) part of the lipids was dissolved in isoamyl alcohol:benzene 1:1 v/v to a final concentration of 4% w/v. This solution was used for quantitative paper chromatographic analysis. Another part was dissolved in chloroform and used for column chromatographic analysis.

Quantitative paper chromatography: A quantitative paper chromatographic analysis was carried out by applying fixed amounts of the lipids dissolved in isoamyl alcohol:benzene to the chromatographic paper. After chromatography the location of the different spots was determined, the chromatogram was cut in as many strips as spots could be discerned, and the lipids were eluted from the strips (4). In this investigation 11 spots including the starting spot were cut as indicated in figure 1 by the numbers 1–10 and S. Each eluate was evaporated to dryness after which the phosphorus content was determined, allowing the amount of each lipid to be calculated.

Column chromatography: The lipids dissolved in chloroform were applied to a silicic acid column and fractionated by elution with chloroform, chloroform-methanol mixtures, and methanol as described elsewhere (4, 6). The first elution fraction (with pure chloroform) contains quantitatively the following lipids: cholesterol, cholesterol esters, fatty acids, glycerides, and a small fraction of hydrocarbons and pigments (1). The total weight of this fraction is determined by evaporating the chloroform in a stream of nitrogen and weighing the lipids obtained; the various

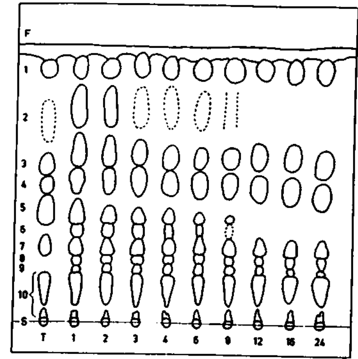

FIG. 1. Chromatograms of total lipid extracts from fresh and formaldehyde fixed human white matter. T = fresh tissue. 1–24 = period of fixation in years. Spot 1 = neutral lipids and fatty acids; 2 = phosphatidic acid; 3 = phosphatidylethanolamine; 4 = phosphatidylserine; 5 = lecithin; 6 = lysophosphatidylethanolamine; 7 = sphingomyelin; 8 = lysophosphatidylserine; 9 = lysolecithin; 10 = phosphoinositides and sulphatides. S = starting spot. F = elution front. For the localisation of the cerebrosides see text. On all chromatograms 400 µg total lipid was applied to the starting spot.

components of this fraction can be separated by the column chromatographic method described by Barron *et al.* (1).

Quantitative determinations:

Phosphorus: Phosphorus was determined according to Chen *et al.* (2).

Cholesterol: Cholesterol was determined by weighing the digitonide precipitate according to Schoenheimer *et al.* (12) in the total lipid extracts and in the first (pure chloroform) fraction of the silicic acid columns.

Galactose: Cerebrosides and sulphatides were determined by their galactose content according to Morris (10).

RESULTS

The analyses of the lipids extracted from pieces of white matter from human brains preserved in toto during 1 year and 2, 3, 4, 6, 8, 12, 16, and 24 years in unbuffered formaldehyde solutions (pH 4.4–4.8) were compared with an identical analysis of fresh white matter. From each year category the piece of white matter was taken from one brain specimen. The data obtained can be represented as follows.

After a very prolonged fixation a more or less stationary situation is reached. Several lipids are completely converted into smaller breakdown products. Some of these products retained their lipid character, others are hydrophilic and could not be detected in the lipid extract.

In the slightly acidic formaldehyde milieu we can expect that e.g. lecithin is broken down via lysolecithin to fatty acids and glycerylphosphorylcholine. As will be seen below we also found an initial increase and subsequent decrease of phosphatidic acid. This indicates that the liberation of the nitrogen base and consequently the formation of glycerophosphoric acid as one of the water soluble products must be postulated. Phosphatidylethanolamine and phosphatidylserine will behave in an analogous way. By this process the amount of fatty acid will increase in the extracted lipids. As the total amount of extractable lipid must be slightly decreased by the loss of small, water soluble, phosphorus containing products, the increase in the amount of fatty acids will appear even more pronounced. Also the content of several other lipids that are not attacked by formaldehyde fixation will appear to increase slightly. The loss of water soluble, phosphorus containing, breakdown products will be reflected in the steadily diminishing phosphorus content of the total lipid extracts.

Visually this process can be followed by observing the chromatograms of the total lipid extracts as represented in figure 1.

Lecithin, phosphatidylethanolamine, and phosphatidylserine disappear from the chromatograms. The corresponding lyso compounds, after an initial increase, also tend to disappear. The cerebrosides, which in our method of chromatography are not separated from phosphatidylethanolamine and phosphatidylserine and are not broken down by formaldehyde fixation become visible as two spots staining with brilliant green after the breakdown of lecithin, phosphatidylethanolamine and phosphadtidylserine. Sphingomyelin and the sulphatides do not show any decrease.

The changes in the lipid composition caused by formaldehyde solutions were investigated quantitatively by column chromatography, phosphorus-, galactose-, and cholesterol determinations in the lipid extracts and in the column fractions; and by quantitative paper chromatography. For the quantitative paper chromatography the chromatograms were divided into 11 strips indicated by the numbers 1–10 and S in figure 1. In each strip the amount of phosphorus was determined. In many cases several identical strips had to be combined owing to the low phosphorus content of these strips. The results of this analysis are represented in table 1 as µg P per 4 mg total lipid after subtraction of the paper blanks, and in the figures 2–7.

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TABLE 1

µg Total P per 4 mg Total Lipid as Determined by Quantitative Paper Chromatography

Spot no	Fraction	Formaldehyde Fixation in Years									
		Fresh tissue	1	2	3	4	6	8	12	16	24
1	Neutral lipids and fatty acids	—	—	—	—	—	—	—	—	—	—
2	Phosphatidic acid	0.3	3.9	3.0	1.4	1.0	0.9	0.7	0.2	—	—
3	Phosphatidyl-ethanolamine	6.8	3.4	1.7	1.4	1.0	0.6	0.4	—	—	—
4	Phosphatidylserine	10.7	6.3	3.0	2.5	1.7	0.8	0.6	—	—	—
5	Lecithin	27.0	14.4	8.5	4.2	2.5	1.6	1.0	—	—	—
6	Lysophospha-tidylethanolamine	—	3.9	2.0	1.4	0.6	0.3	0.4	—	—	—
7	Sphingomyelin	12.0	11.6	11.6	11.8	11.3	12.6	12.2	12.7	13.0	12.1
8	Lysophosphatidylserine	—	2.2	5.2	5.1	4.8	3.4	3.4	3.0	2.4	2.8
9	Lysolecithin	—	4.1	7.0	9.0	9.2	7.7	6.2	5.1	3.3	2.4
10	Phosphoinositides	5.2	5.2	5.5	6.0	6.5	5.8	5.2	6.2	6.5	6.1
S	Starting spot	0.1	0.3	0.4	0.4	0.4	0.3	0.4	0.3	0.5	0.2
	µg total P per 4 mg total lipid	62.1	55.3	47.9	43.2	39.0	34.0	30.5	27.5	25.7	23.6

For the corresponding chromatograms see figure 1. — = no detectable amount of phosphorus.

Total amount of lipid extracted: The total lipid contents of our different specimens varied between 12 and 16% from the wet weights. No relation between the total lipid content and duration of fixation was found. The fluctuations must be ascribed to different water contents or to individual variations. Even a prolonged fixation by formaldehyde did not result in an appreciable decrease of the total amount of extractable lipid. Owing to these fluctuations the comparison of the lipid extracts must be based on the analysis of the lipid composition per gram extracted lipid. Determinations of lipids per gram tissue as often encountered in literature give in the case of fixed tissues an unreliable basis of comparison.

Neutral lipids and fatty acids: By the term "neutral lipids and fatty acids" we indicate the lipids present in the first elution fraction (with pure chloroform) of a silicic acid column. The total amount of this fraction was determined by weighing. In figure 2 the increase of this fraction as % of the total lipid weight is shown. This increase is due to an increase of the amount of fatty acids derived from the breakdown of various phospholipids. Curve 1 represents the percentages actually found.

As we have seen the yield of total lipid suffered a loss due to the formation of water soluble, phosphorus containing, breakdown products. When we correct the determined values represented in curve 1 for this loss we arrive at curve 2,

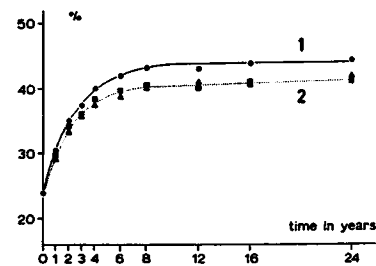

FIG. 2. Increase of the fraction containing the neutral lipids and the fatty acids as % of the total lipid weight. For 1 and 2 see text.

■. The diminution of several phospholipids represented in table 1 enables us to calculate the amount of fatty acids (assuming a mean mol. weight of 275 per fatty acid) liberated by the breakdown of these lipids, and hence the increase to be expected in the first elution fraction (assuming a near constant percentage of this fraction in fresh white matter). By this calculation we also arrive at curve 2, ▲.

Cholesterol: The cholesterol content showed a slight increase of 20.8% in fresh white matter to 23.4% after 24 years of fixation. This increase must be ascribed to the loss of water soluble products indicated above. Our conclusions are in agreement

with Rodnight's (11) that the cholesterol content remains unaffected by storage in formaldehyde solutions.

Cerebrosides and sulphatides: The amount of cerebrosides and sulphatides showed an increase from 35.5% in fresh white matter to 38.6% after 24 years of fixation. We conclude on the same grounds as was the case with the cholesterol content that these lipids remain substantially intact during formaldehyde storage.

That the cerebrosides occur in chromatograms of fresh tissues on the place of the spots 3 and 4 (figure 1) can be demonstrated by chromatography of fresh total lipid extracts on silicic acid impregnated paper (7) impregnated with uranyl nitrate. In that case all phospholipids remain close to the starting spot, while the cerebrosides retain their normal R_F values and remain visible as two spots staining with brilliant green.

The sulphatides are located in the elongated spots in region 10 (figure 1). They stain intensely with brilliant green.

Phospholipids: The breakdown of many phospholipids is shown in the decrease of the phosphorus content of the total lipid extracts as illustrated in figure 3. The slope of this curve indicates that this process is nearly completed after 16 years of storage.

Lecithin and lysolecithin: The fate of these compounds is illustrated in figure 4. It is remarkable that no lecithin can be demonstrated after 12 years of storage, whereas a small amount of lysolecithin is present even after a fixation of 24 years.

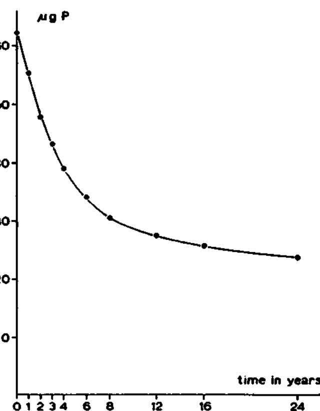

FIG. 3. Phosphorus content of the total lipid extracts as µg P per 4 mg lipid.

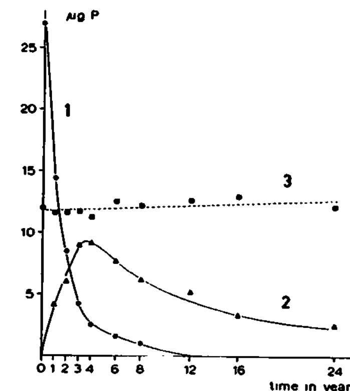

FIG. 4. µg P of lecithin, lysolecithin and sphingomyelin in 4 mg total lipid. Curve 1 = lecithin; curve 2 = lysolecithin; curve 3 was calculated from the relative increase caused by the loss of water soluble components assuming a constant sphingomyelin content corresponding with 11,8 µg sphingomyelin P per 4 mg total lipid. ■ = sphingomyelin phosphorus contents determined.

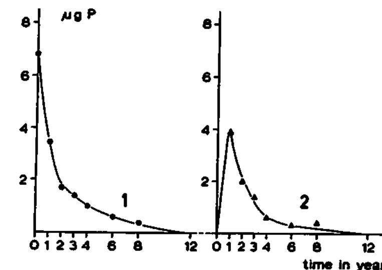

FIG. 5. µg P of phosphatidylethanolamine (curve 1) and lysophosphatidylethanolamine (curve 2) in 4 mg total lipid.

Sphingomyelin: No breakdown of sphingomyelin could be detected. The slight increase in concentration shown in figure 4 can be explained by the loss of water soluble breakdown products from lecithin, phosphatidylethanolamine and phosphatidylserine.

Phosphatidylethanolamine and lysophosphatidylethanolamine: These compounds react very fast with formaldehyde (4). Clearly the initial reaction concerns only the reaction of the $-NH_2$ group with formaldehyde (4). The further slow breakdown illustrated in figure 5 is comparable with that of lecithin and phospha-

tidylserine. According to Davison *et al.* (3) the plasmalogen phosphatidylethanolamine is first attacked.

Phosphatidylserine and lysophosphatidylserine: Phosphatidylserine also is completely broken down; see figure 6. The formation of a lyso compound analogous to lysolecithin and lysophosphatidylethanolamine can be expected. In accordance with data given by Marinetti (9) we identified spot 8 in our chromatograms as lysophosphatidylserine. In the chromatogram of a fresh nervous tissue extract spot 8 cannot be detected.

Phosphatidic acid: The phosphorus content of the elongated spot 2 shows a sharp increase in the first year (figure 7). According to Hübscher *et al.* (8) this compound can be identified as phosphatidic acid. The small amount formed must also be derived from lecithin, phosphatidyl-

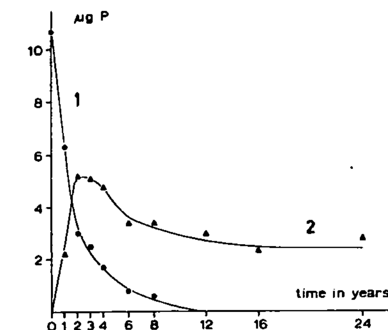

FIG. 6. μg P of phosphatidylserine (curve 1) and lysophosphatidylserine (curve 2) in 4 mg total lipid.

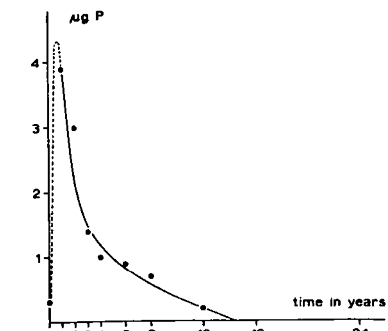

FIG. 7. μg P of phosphatidic acid in 4 mg total lipid.

ethanolamine and phosphatidylserine. Clearly the breakdown of these lipids in acidic formaldehyde solutions follows two pathways: one by the formation of lyso compounds; the other by the liberation of the nitrogen base, phosphatidic acid being formed. The decrease of the phosphatidic acid fraction makes the presence of a small amount of lysophosphatidic acid probable. Chromatographically we expect this compound on the same place as the lecithin spot; however as yet we have been unable to detect this substance.

Phosphoinositides: The amount of phosphoinositides as determined by the phosphorus content of the spot 10 region (monophosphoinositides, diphosphoinositides, and sulphatides) remains unaffected (see table 1).

DISCUSSION

Storage in formaldehyde solutions has a marked influence on the composition of brain lipids. All our results and experiences indicate that even after prolonged (unbuffered) formaldehyde fixation all lipids or parts of lipids remain extractable with chloroform:methanol 2:1 v/v. Several lipids are completely broken down, while the amount of others remains unaffected. The contents of the unaffected lipids can be determined with a reliable precision after prolonged storage in formaldehyde solutions.

In performing and evaluating histochemical reactions for fatty materials on formaldehyde fixed tissues extreme caution should be taken. These reactions should only be performed after a detailed analysis of the lipid extract. The alterations in the composition of tissue lipid can influence the result of histological staining methods on tissue components containing a high lipid content.

The breakdown of phospholipids due to a slow hydrolysis in the mildly acidic formaldehyde solution leads to the expectation that glycerides are, at least partly, converted in fatty acids and glycerol; the influence of formaldehyde on adipose tissues should be investigated.

SUMMARY

The composition of the lipids extracted from fresh normal human brain and from brains preserved up to 24 years in unbuffered formaldehyde solutions was analysed quantitatively using column and paper chromatography, followed by various determinations.

The amounts of cholesterol, cerebrosides, sulphatides, phosphoinositides and sphingomyelin remain unaffected.

Lecithin, phosphatidylethanolamine and phosphatidylserine are broken down to the corresponding lyso compounds, fatty acids, phosphatidic acid and probably lysophosphatidic acid. The lyso compounds are further broken down by the liberation of the second fatty acid. By this process the fatty acid content is markedly increased. The total lipid content decreases slightly by the formation of water soluble phosphoryl compounds. Consequently the phosphorus content of the total lipid extract suffers marked diminution.

Histochemical reactions on lipids in formaldehyde stored tissues should preferably be performed in combination with an analysis of the lipid extract.

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