PHYSICAL STUDIES OF PHOSPHOLIPIDS

III. Electron Microscope Studies of Some Pure Fully Saturated 2,3-Diacyl-DL-Phosphatidyl-Ethanolamines and Phosphatidyl-Cholines

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ABSTRACT

On heating pure, fully saturated 2,3-diacyl-DL-phosphatidyl-ethanolamines and 2,3-diacylphosphatidyl-cholines (lecithins) in water to the transition temperature at which large endothermic heat changes occur, they are observed, by light microscopy, to form myelin figures. This result is discussed in terms of the large difference in the transition temperature for "melting" of the hydrocarbon chains of unsaturated and saturated phospholipids and is illustrated by means of differential thermal analysis (D.T.A.) curves. These structures have been examined by electron microscopy after negative staining and after reaction with osmium tetroxide. Typical phospholipid lamella structures are seen in the phosphatidyl-cholines after negative staining, and in the phosphatidyl-ethanolamines after both negative staining and osmium fixation. The distances across these lamellae have been measured. Some preliminary investigations of the nature of the osmium tetroxide reaction with the phosphatidyl-ethanolamines have been made.

INTRODUCTION

The reaction of lipid membranes with osmium tetroxide has received considerable attention, and discussion is still widespread as to the site of the fixation process. This process was originally thought to take place at the sites of the double bonds in the hydrocarbon chain. Wigglesworth (19) has suggested that osmium can cross-link through the ethylenic double bonds and has given evidence to show the occurrence of an insoluble polymeric complex of lipid and osmium which he considers is the basis of cytological fixation. This observation was considered to be consistent with the fact, noted by many authors, that fully saturated phospholipids, such as the phosphatidyl-ethanolamines, do not react easily with osmium tetroxide at room temperature, although it has been suggested that brominated or hydrogenated phospholipids can take up osmium without fixation occurring. Bahr (1) showed that many amino acids would also react with osmium tetroxide, and so some authors have considered that the dense lines observed in electron micrographs of cell membranes represented protein, whereas the light central line corresponded either to the hydrocarbon chain region of a saturated lipid or to a gap produced by removal of lipid in the preparative stages for electron microscopy (15).

Stoeckenius (16, 17) studied natural lipids after reaction with osmium tetroxide, and concluded that the dark area seen in electron micrographs corresponds to osmium located at the polar groups of the phospholipid molecules. Finean (9), after

an analysis of X-ray data of fixed and unfixed tissue, also considers that the osmium is located between the polar groups of the bimolecular sheets of lipid. Riemersma (14), after chromatographic analysis of the intermediates, formed during osmium fixation of unsaturated lecithins, considers that the initial reaction is with the double bonds, but that there is a subsequent migration of osmium derivatives to the polar groups. Despite this work, some authors are still, nevertheless, of the opinion that the osmium is located at the double bond of the lipids (11).

If, indeed, osmium is located at the polar groups of phospholipids, rather than at the double bonds, we may ask ourselves why it is not possible to react osmium tetroxide with fully saturated phospholipids, such as the phosphatidyl-ethanolamines. Is an initial reaction with a double bond a necessary condition for this to occur? We have recently pointed out that pure phospholipids can undergo thermotropic mesomorphism and that, in the presence of water, at room temperature, there is an important physical difference between fully saturated phospholipids and highly unsaturated phospholipids. The former are in a crystalline condition at this temperature, whilst the latter are already in a liquid crystalline condition (3, 4, 6). We, therefore, pose the following question. Are both myelin figure formation and the osmium tetroxide reaction related to this temperature-dependent difference in physical condition of phospholipids?

In this paper we study the effect of heat on saturated phospholipids when they are in the presence of water. The gross structure of the phospholipids is examined by light microscope techniques, and the fine structure by electron microscopy after both negative staining and reaction with osmium tetroxide. The possibility of hydrolysis occurring is considered, and experiments are described which ascertain the extent of this. Some experiments are described in which osmium is reacted with ethanolamine and ethanolamine phosphate. Some unsaturated phospholipids are also examined in order to compare and contrast their behavior with that of the saturated phospholipids.

MATERIALS AND METHODS

Materials

The phospholipids studied include the following: 2,3-dimyristoyl-, 2,3-dipalmitoyl-, 2,3-distearoyl-, 2,3-dioleoyl-DL-phosphatidyl-ethanolamines and 2,3-distearoyl-DL-phosphatidyl-choline. The above lipids were synthesized in these laboratories by Mr. D. T. Collin and Mr. P. Bird (5).

A sample of pure egg yolk lecithin was a gift from Dr. A. D. Bangham, and lysopalmitoylphosphatidyl-ethanolamine and 2,3-dioctadecylphosphatidyl-ethanolamine were gifts from Dr. T. H. Bevan.

The purity of all synthetic phospholipids examined was confirmed by thin layer chromatography.

Methods

a. Dispersion of the Phospholipids in Water

Efficient dispersion of the fully saturated phospholipids in water proved very difficult. Various methods were tried, including violent shaking, the use of a Teflon pestle and a close-fitting glass mortar, and ultrasonication in a bath-type apparatus. The latter proved the most efficient for quantitative studies. For nonquantitative work, it was possible to obtain dispersion by violent shaking of the sample during the initial heating stages at the temperature at which myelin-figure formation occurs. This heating was carried out in an oil bath maintained at the required temperature by an automatic constant temperature thermometer.

b. Reaction with Osmium Tetroxide

Either a 2% solution of osmium tetroxide in water or a 2% solution in veronal-acetate buffer at pH 7.4 was used. If heating with osmium was necessary, this was carried out as above.

c. Electron Microscopy

Negatively stained samples were prepared as follows: A very dilute suspension of the lipid in water was sprayed on to carbon-coated copper grids using a nebulizer. The grids were then inverted on to a 2% solution of phosphotungstic acid adjusted to pH 6.5 with potassium hydroxide. Excess stain was blotted off and the grids examined when dry.

Material for sectioning after fixation was dehydrated at 4° through a graded alcohol series and then embedded in Araldite (12). Thin sections of the polymerized blocks were cut on a Huxley, Porter-Blum, or LKB ultramicrotone. Grey-silver sections were mounted on carbon-coated copper grids and examined at 50 or 60 kv in a JEM 6 or a Siemen's Elmiskop 1. Criteria of fixation were considered to be the following: blackening with osmium, stability of the reduced osmium-lipid complex in the subsequent dehydration and embedding media, and preservation of the lamella structure under the electron microscope.

d. Chromatography

THIN LAYER CHROMATOGRAPHY: This was carried out on 250-μ layers of silica gel G (Merck &

Co., Darmstadt), made up with either water or N/5 oxalic acid solution on glass plates. Plates were activated at 110°C; if not used within a reasonable time, they were reactivated. Detection agents were iodine vapor, ninhydrin, and Zinzadze spray (20).

PAPER CHROMATOGRAPHY: Paper chromatography was carried out on 6-in. squares of Whatman No. 1 paper.

EXPERIMENTAL AND RESULTS

1. *Differential Thermal Analysis (D.T.A.)*

The D.T.A. curves show, as in Fig. 1, that with pure, fully saturated phosphatidyl-ethanolamines a large endothermic reaction occurs at ~115°C, whereas with egg yolk lecithin a similar transition occurs at ~20°C. A brief discussion of this technique appeared in Chapman and Collin (6). These transitions are for the pure materials. When the saturated phosphatidyl-ethanolamine is dispersed in water, the large endothermic transition occurs at a lower temperature. This is at ~90°C with 2,3-distearoylphosphatidyl-ethanolamine and is accompanied by an exothermic transition at ~80°C.

2. *Formation of Myelin Figures*

Egg yolk lecithin and dioleoylphosphatidyl-ethanolamine on dispersion in distilled water form myelin figures at room temperature. Examples of the structures formed are as in Fig. 2. Neither fully saturated long chain lecithins, nor fully saturated phosphatidyl-ethanolamines normally form myelin figures in water at room temperature. However, if the saturated lipids are heated to the temperatures at which, as indicated by the D.T.A. results, phase changes occur, typical myelin figures are produced as shown in Fig. 2. The relationship of the minimum temperatures required for myelin figure formation and the major peaks of the D.T.A. curves is shown in Table I. Myelin figures have also been formed in water at ~100°C, from an ether-linked saturated derivative—dioctadecyl-phosphatidyl-ethanolamine.

3. *Electron Microscopy*

a. UNSATURATED PHOSPHOLIPIDS: Myelin figures from egg yolk lecithin start to form spontaneously in water at room temperature and, at this temperature, the lecithin is readily fixed by 2% aqueous osmium. After thin sectioning of the fixed phospholipid, and after negative staining of the unfixed material, a typical lamellar structure is seen with the electron microscope. The lamellar spacings are similar to those obtained by Stoeckenius (17) for fixed brain lipid and by Bangham and Horne (2) for negatively stained lecithin.

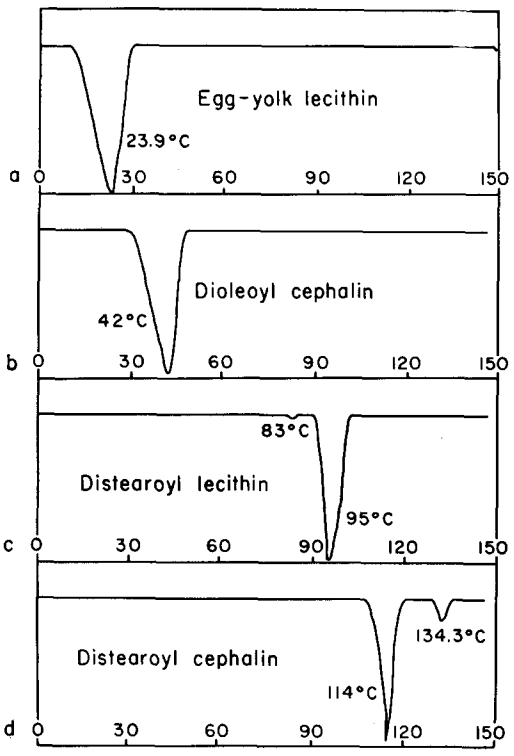

FIGURE 1 D.T.A. curves of pure phospholipids, showing the major endothermic peaks for the following: *a*, Egg yolk lecithin. *b*, 2,3-dioleoyl-DL-phosphatidyl-ethanolamine. *c*, 2,3-distearoyl-DL-phosphatidyl-choline. *d*, 2,3-distearoyl-DL-phosphatidyl-ethanolamine.

b. SATURATED PHOSPHOLIPIDS: The myelin figures observed with 2,3-dipalmitoylphosphatidyl-ethanolamine and 2,3-distearoylphosphatidyl-choline were studied in the electron microscope after negative staining. In each case, the lamellar spacings are similar in order to those obtained by previous workers using unsaturated lecithins. Electron micrographs of these negatively stained preparations are shown in Fig. 3.

Fully saturated phosphatidyl-ethanolamines do not react with osmium tetroxide at room temperature. However, if the temperature is raised to that at which myelin figures form in water, then the material appears to be characteristically fixed and can subsequently be prepared for thin sectioning.

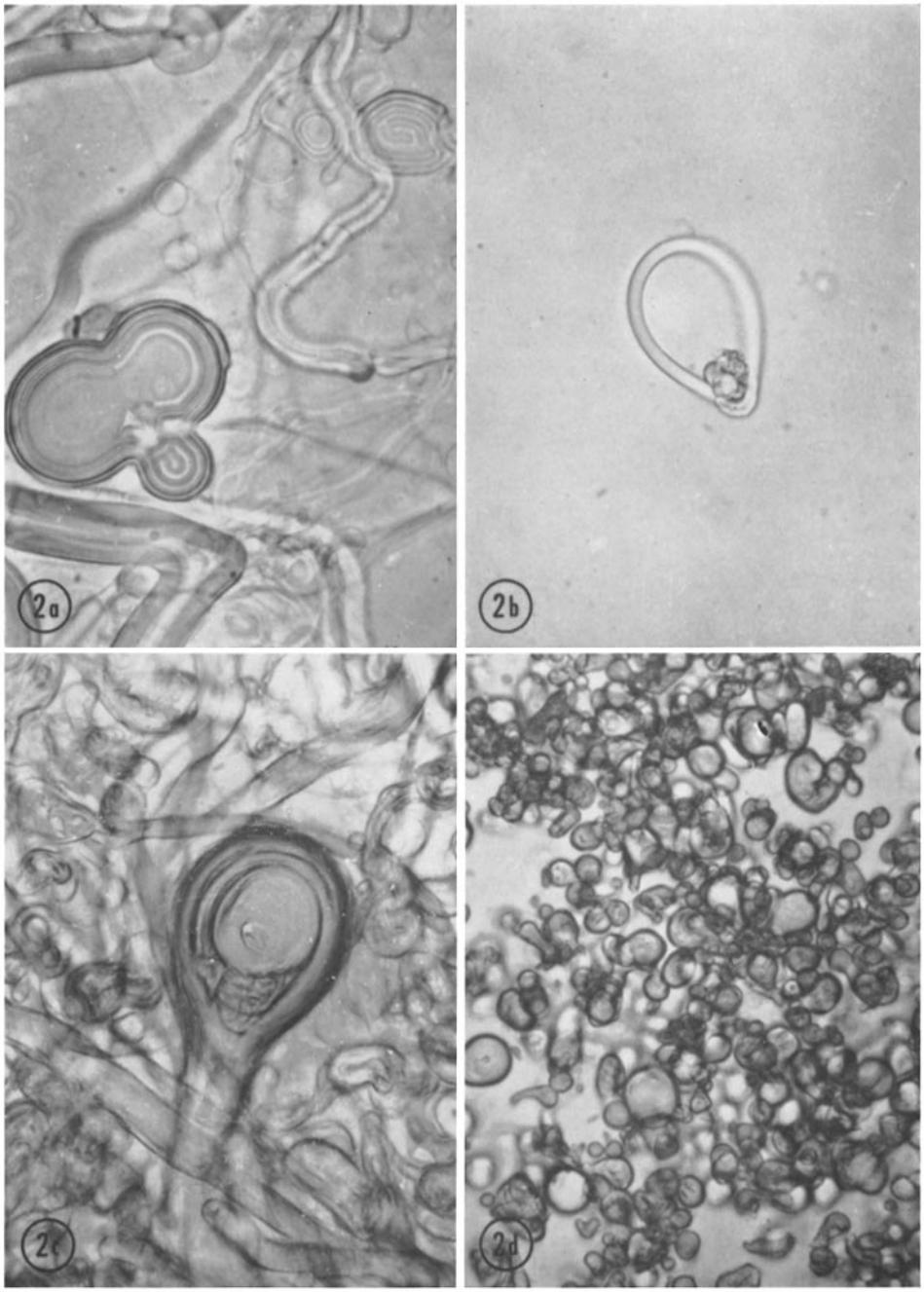

FIGURE 2 Light micrographs of myelin forms of saturated and unsaturated phospholipids. The experimental conditions are as described in the text. *a*, Egg yolk lecithin. *b*, Dioleoylphosphatidyl-ethanolamine. *c*, Distearoyl lecithin. *d*, Distearoylphosphatidyl-ethanolamine. × 580.

No significant differences were observed in the temperature required for fixation of dimyristoyl, dipalmitoyl, or distearoyl phosphatidyl-ethanolamine. However, phosphatidyl-ethanolamines containing lauric acid, particularly dilauroylphosphatidyl-ethanolamine and stearoyllauroylphosphatidyl-ethanolamine, did appear to fix rather more readily than other phospholipids. The results of both temperature and concentration on fixation in aqueous and buffered osmium are shown in Figs. 4 and 5. The lysopalmitoylphosphatidyl-ethanolamine was considered to fix in 10 to 15 min at 80°C and the dioctadecylphosphatidyl-ethanolamine in 5 min at 100°C.

The 2,3-distearoyl-DL-phosphatidyl-choline would not fix in either aqueous or buffered osmium at room temperature, nor after heating at 100°C for up to 2 days. After this time, the phospholipid becomes a grey-blue color, but much of the colored material is partially soluble in the dehydration alcohols and we consider that fixation has not taken place.

Thin Araldite sections of each of the phosphatidyl-ethanolamines examined show similar lamella structures under the electron microscope. Whilst the observed lamellae are rarely as intact as those observed with the unsaturated phospholipids in each case, sufficient "stacks" of lamellae are observed to permit measurement of lamellar spacing. Examples of lamellae systems from these osmium tetroxide–fixed saturated phosphatidyl-ethanolamines are shown in Fig. 6. The dioctadecyl phosphatidyl-ethanolamine behaves like the saturated derivatives as shown in Fig. 6.

The lamellar spacings from both sectioned and negatively stained preparations are summarized in Table II.

TABLE I

Myelin-Figure Formation and its Relationship to the Major Peaks of the D.T.A. Curves

Lipid	Minimum temperature of myelin-figure formation	D.T.A. major peak. Dry
	C	C
Unsaturated		
Egg-yolk lecithin	Room temp.	23°
Dioleoylphosphatidyl-ethanolamine	Room temp.	42°
Saturated		
Distearoyl lecithin	60°	95°
Distearoylphosphatidyl-ethanolamine	80°	117°
Dioctadecylphosphatidyl-ethanolamine	100°	106°

4. *Chromatographic Experiments*

a. AN EXAMINATION OF THE PRODUCTS FORMED ON HEATING SATURATED PHOSPHOLIPIDS WITH WATER: A suspension of 5 mg of saturated phosphatidyl-ethanolamine in 2 ml of distilled water was made up and homogenized until thoroughly dispersed. The dispersion was then heated at 100°C for 45 min (i.e., well past the normal time for myelin figure formation). Part of the sample was then extracted into chloroform; the extract was concentrated and an aliquot chromatographed on silica plates. A sample taken from the unextracted portion was similarly chromatographed. In neither instance were any spots seen other than those of the original phosphatidyl-ethanolamine. This indicates that, in water at 100°C, in the time required for myelin figure formation, there is no significant hydrolysis of the phospholipid. The experiment was repeated, with heating for 3 days at 100°C. Chromatography showed that, although the majority of the material was in the original form, there was, after this time, some free fatty acid and lysophosphatidyl-ethanolamine produced.

15 mg of distearoyllecithin were similarly ultrasonicated in 3.0 ml of distilled water. The dispersion was then heated at 100°C. Samples for chromatography were taken at 1, 2, 3, 9½, 24, and 43 hr. There was no indication of lysolecithin or free fatty acid until 9½ hr. A considerable amount of lecithin was still present after 43 hr.

b. AN EXAMINATION OF THE PRODUCTS FORMED ON HEATING SATURATED PHOSPHOLIPIDS WITH WATER AND WITH OSMIUM TETROXIDE: 1. 10 mg of saturated phosphatidyl-ethanolamine were dispersed ultrasonically in 2 ml of distilled water. The dispersion was heated at 100°C until myelin figure formation took place. An equal volume of 2% aqueous osmium tetroxide was then added and the sample was heated at 80°C. Samples were taken at 5, 10, 15, 30, and 60 min. Thin layer chromatography of aliquots from these samples showed no detectable formation of lysophosphatidyl-ethanolamine or free fatty acid until after 60-min heating.

2. A similar dispersion of saturated phosphatidyl-ethanolamine in water was prepared and was

heated at 100°C until myelin figure formation took place. An equal volume of 2% osmium tetroxide was added and the sample heated at 100°C. Samples were taken at 5, 10, 15, 30, and 60 min. Thin layer chromatography showed that lysophosphatidyl-ethanolamines were present after 10 min and both free fatty acid and lysophosphatidyl-ethanolamines after 15 min. After 30 min,

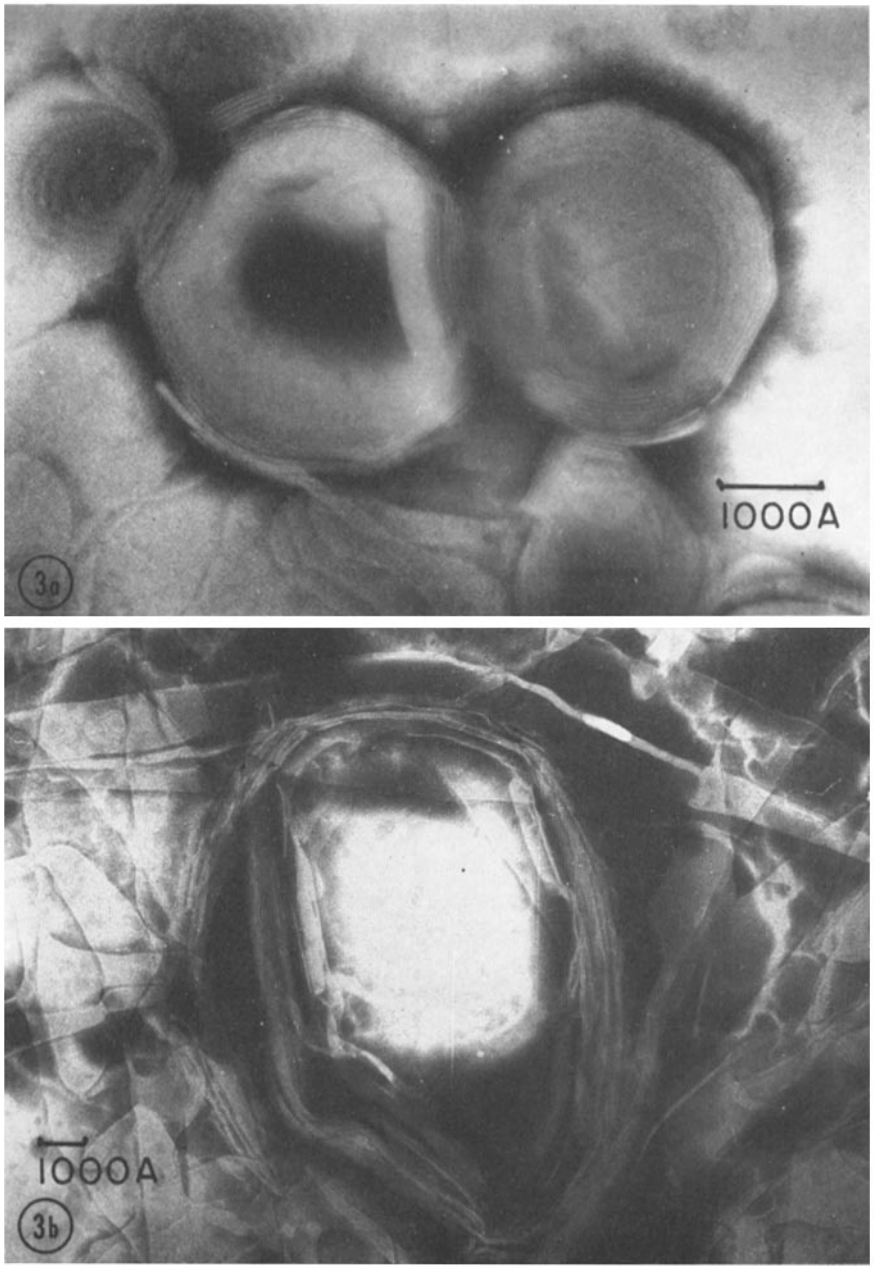

FIGURE 3 Electron micrographs of saturated phospholipids negatively stained with potassium phosphotungstate. Experimental conditions as described in the text. *a*, Dipalmitoylphosphatidyl-ethanolamine. × 125,000. *b*, Distearoyl lecithin. × 51,000.

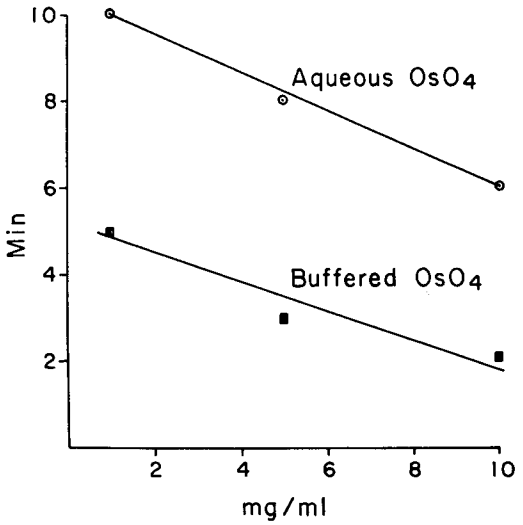

FIGURE 4 The time required for fixation of distearoyl-phosphatidyl-ethanolamine to occur at 100°C at different lipid concentrations in buffered and unbuffered osmium tetroxide.

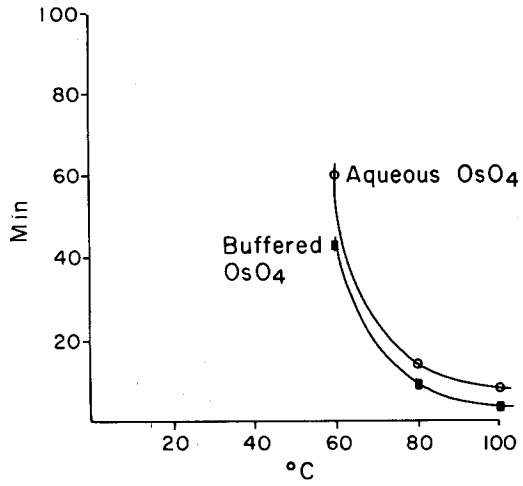

FIGURE 5 The time required for fixation of distearoyl-phosphatidyl-ethanolamine to occur in buffered and unbuffered osmium tetroxide (lipid concentration 5 mg/ml) as the temperature is increased.

the phosphatidyl-ethanolamine spot decreased in size, consistent with an increase of a black base-line spot, presumably of reduced osmium-lipid complex. The lysophosphatidyl-ethanolamine spots did not increase with time, whereas the free fatty acid did so.

3. A similar examination of 2,3-distearoyl-DL-phosphatidyl-choline was carried out. 15 mg were dispersed in 3.0 ml of distilled water and heated at 100°C until myelin figure formation took place. An equal volume of 2% aqueous osmium was added. A trace of lysolecithin was detected by chromatography after heating for 3½ hr. A considerable amount was present after 24 hr.

C. SOME PRELIMINARY INVESTIGATIONS INTO THE NATURE OF THE REACTION OF OSMIUM TETROXIDE WITH FULLY SATURATED PHOSPHATIDYL-ETHANOLAMINES:

1. 60 mg of ethanolamine in 15 ml of 1.3% osmium tetroxide in water blackened at once at room temperature. Paper chromatography of the reaction products in a 2-way system of *t*-butanol-formic acid-water and phenol-ammonia-water showed that the majority of the ethanolamine remained unchanged. The ethanolamine which had reacted remained at the base line.

2. A similar reaction mixture containing phosphoryl ethanolamine instead of ethanolamine showed no indication of reduction of osmium until heated at 60°C. At this temperature, reduction of osmium seemed to be complete in 2 hr. At 80°C the reaction was complete in 30 min, and at 100°C in 10 min.

A 2-way paper chromatographic analysis of the products of these reactions showed a considerable amount of unchanged phosphoryl ethanolamine. Ethanolamine itself was absent. A spot corresponding to an rf value for glycine was observed.

Phosphorus estimations by the vanadate-molybdate method of Webb 1959 (unpublished) showed that 12% of the available phosphate had been released as inorganic orthophosphate.

3. Glycine, when reacted with osmium tetroxide under conditions similar to those used with the ethanolamine and phosphoryl ethanolamine reactions, showed that no reaction with osmium tetroxide occurs at room temperature. A reaction was detectable after 2 hr at 60°C, approximately 30 min at 80°C, and approximately 10 min at 100°C.

Paper chromatography, under conditions similar to those used above, showed that only a trace of unreacted glycine remained. An intense black base line spot was present.

4. Distearoyl phosphatidyl-ethanolamine when reacted in a similar way showed no visible reaction below 60°C after about 2 hr. At 80°C a reaction was observed after 30 min, and at 100°C in less than 10 min. Chromatography, as above,

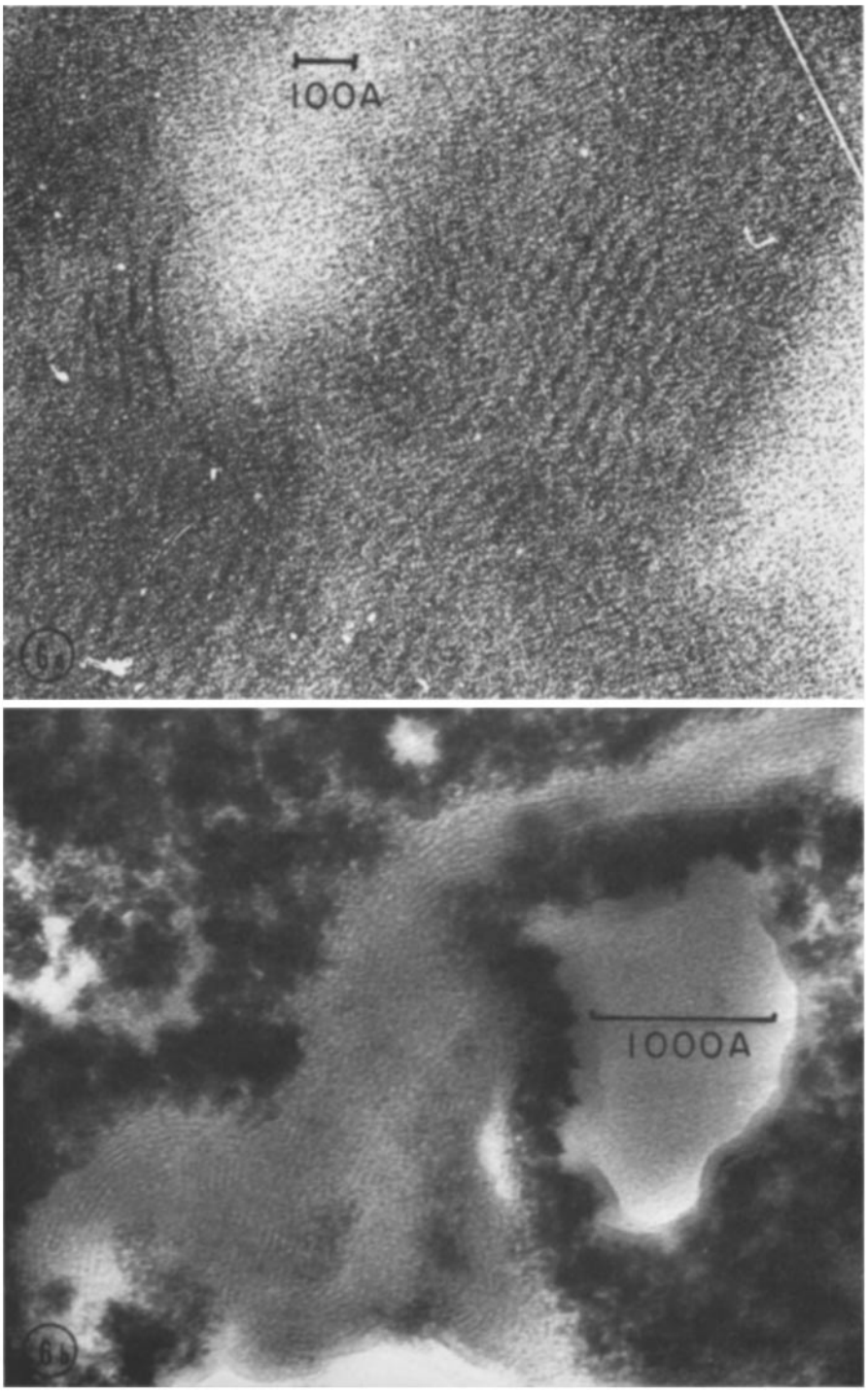

FIGURE 6 Electron micrographs of OsO_4-fixed saturated phospholipids. Experimental conditions as described in the text. *a*, Distearoylphosphatidyl-ethanolamine. × 745,000. *b*, Dioctadecylphosphatidyl-ethanolamine. × 238,000.

TABLE II

Lamellar Spacings of Saturated Phospholipids

	Lamellar spacing			
	OsO_4-fixed and then sectioned	SD	Negative strain	SD
	A	A	A	A
Dimyristoylphosphatidyl-ethanolamine	37	3.05		
Dipalmitoylphosphatidyl-ethanolamine	40	1.73	62	3.4
Distearoylphosphatidyl-ethanolamine	39.2	1.35		
Dioctadecylphosphatidyl-ethanolamine	44	3.1		
Distearoyl lecithin			62	1.9
Dielaidoyl lecithin			60	3.7
Dielaidoyl phosphatidyl-ethanolamine			60	5.5
Egg yolk lecithin			59	2.8

showed very little breakdown of the phospholipid; traces of material at the rf value corresponding to glycine were present. Phosphorus estimations, as above, showed a release of inorganic phosphate corresponding to only about 1% of the starting material.

5. Distearoylphosphatidyl-ethanolamine, when reacted with 1.3% osmium tetroxide in carbon tetrachloride solution, showed no reduction at room temperature. At 60°C, 80°C, and 100°C, blackening occurred in times similar to those necessary for reaction of phosphoryl ethanolamine and glycine with osmium tetroxide in aqueous solution. (Heating of the phospholipid in the carbon tetrachloride solution of osmium tetroxide was carried out under reflux.)

DISCUSSION

The D.T.A. heating curves show the considerable temperature differences which exist between the transition temperatures for unsaturated phospholipids, such as egg-yolk lecithin and 2,3-dioleoyl-DL-phosphatidyl-ethanolamine, and those for the fully saturated phospholipids. We note that, when water is present, the transition temperature is lowered to a certain value, but that the transition temperatures of the fully saturated phospholipids are still considerably greater than room temperature. This transition temperature also varies with the polar grouping present, being lower for the phosphatidyl-cholines than for the phosphatidyl-ethanolamines.

By the use of other physical techniques, such as infrared spectroscopy (3) and nuclear magnetic resonance spectroscopy (7), it has been demonstrated that these endothermic transitions are accompanied by a considerable mobility of the hydrocarbon chains. Luzzati and Husson (13) have also emphasized the "liquid-like" structure of the hydrocarbon chains in liquid crystalline phases obtained with a brain phospholipid extract. The hydrocarbon chains of egg-yolk lecithin are in a fluidlike condition near room temperature, while the hydrocarbon chains of the fully saturated phospholipid are usually in a well packed crystalline condition. At the transition temperature at which melting of the chains occurs, water can penetrate readily into the crystal structure. The phospholipid swells and, as we have demonstrated, myelin-tube formation occurs and the polar groups of the lipid will be orientated towards the aqueous phase. With egg-yolk lecithin this occurs at room temperature, and with the fully saturated phosphatidyl-ethanolamines at ~80°C.

The fact that the temperature at which myelin tube formation occurs depends upon the degree of unsaturation in the hydrocarbon chain, as well as upon the polar group present in the lipid, is clearly of relevance to an understanding of the function of biological membranes. It is known that in a given tissue a variety of polar groupings occur, and a particular distribution of fatty acid residues. The mitochondrial membrane lipids are more unsaturated in fatty acid residues than the myelin lipids (10), which probably indicates greater fluidity of the former membranes, facilitating diffusion processes, and perhaps a greater tendency for them to form membranes. Furthermore, in a particular tissue, to achieve the same resultant fluidity of chains the fatty acid distribution may differ, depending upon whether the fatty acids are associated with a lecithin or a

phosphatidyl ethanolamine. The latter conclusion may depend upon how the different types of phospholipid are arranged in a membrane, either in a random mixture or in specific patterns. There is no information at present available about this organization.

In our opinion, a necessary condition for myelin tube formation to occur is that the hydrocarbon chains of the phospholipid should be in a fluid condition. What, then, is the relevance of the distances observed between the black lines on the electron micrographs? We presume that this is related to the distance between the polar layers when the chains are in a coiled configuration. We have, in a separate investigation (5), examined the X-ray long spacings of the 2,3-dipalmitoyl-DL-phosphatidyl-ethanolamine in the anhydrous condition, below and above the transition melting temperature. The X-ray long spacing falls from 54.8 to 33.2 A as the phospholipid is heated. The latter value is to be compared with the value, deduced from electron micrographs, of approximately 40 A.

Our results with osmium tetroxide show that a fully saturated lecithin does not appear to react and that fixation does not occur. This appears to confirm the ideas of Riemersma (14) that, with lecithins, an initial reaction with a double bond is required before fixation occurs. The fact that the saturated phosphatidyl-ethanolamines are found to react with osmium tetroxide requires a little more consideration.

As there are no double bonds present in the fully saturated phosphatidyl-ethanolamines, the osmium tetroxide must be reacting with the polar head group and the electron microscope appearance must arise from osmium located at the polar head group. In order to obtain the saturated phosphatidyl-ethanolamines in the myelin tube condition, it is necessary to heat the material to fairly high temperatures. Could this reaction with osmium tetroxide occur only because of the high temperature involved? We have examined the possibility that hydrolysis can occur at the high temperature, but, whilst it undoubtedly does take place, we think that it is small enough to be ignored as a possible explanation. Furthermore, the ether-linked phosphatidyl-ethanolamine also shows a reaction with osmium tetroxide, and we think that hydrolysis will not occur with this molecule.

It seems to us that the reaction is, nevertheless, a temperature-dependent one, and the experiments with phosphoryl ethanolamine are pertinent to this. The experiments with the fully saturated phosphatidyl-ethanolamines in carbon tetrachloride solution are also consistent with this, suggesting that the presence of an unsaturated bond is needed for the initial reaction. However, there is a possibility that the polar groups of the phospholipid are less accessible to reaction when in this solvent as compared with the material dispersed in water. It is probable that this reaction is different from the one normally involved, at room temperature, with the unsaturated phosphatidyl-ethanolamines. Further experiments are needed to clarify the nature of this difference.

Recent studies by Stoeckenius and Mahr (18) may be pertinent to this. Experiments were carried out on the mode of action of OsO_4 with a number of lipids. These showed a reaction with the double bond of unsaturated lipids, and possibly further secondary reactions.

CONCLUSIONS

1. There is a relationship between the major endothermic D.T.A. peak and the temperature at which a phospholipid produces myelin forms in water. Phosphatidyl-ethanolamines and phosphatidyl-cholines of the same chain length appear to have different transition temperatures. Saturated phospholipids produce myelin forms after heating above room temperature. Negative staining methods show the existence of lamellae.
2. It is not possible to react OsO_4 appreciably with either a saturated phosphatidyl-ethanolamine or a saturated phosphatidyl-choline at room temperature. If the temperature of a fully saturated phosphatidyl-ethanolamine is raised to $\sim$60°C, a reaction with OsO_4 occurs and, apparently, fixation. No such reaction occurs with a fully saturated phosphatidyl-choline.
3. The reaction observed with phosphatidyl-ethanolamines may be a temperature-sensitive reaction not necessarily related to the reaction, either primary or secondary in type, which occurs with an unsaturated phosphatidyl-ethanolamine.

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