A CHROMATOGRAPHIC AND SPECTROPHOTOMETRIC STUDY OF THE PRODUCTS OF THE REACTION OF OSMIUM TETROXIDE WITH UNSATURATED LIPIDS

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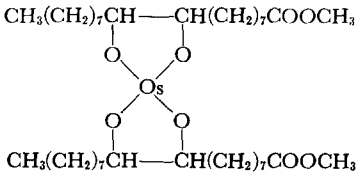
ABSTRACT

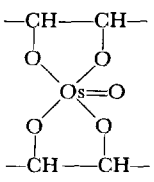
Thin films of methyl oleate, oleic acid, and di-octadecenoyl phosphatidylcholine were reacted with a 2% solution of OsO_4 in water for 1 hr at 0°. As controls, methyl 9,10-dihydroxystearate and 9,10-dihydroxystearic acid were reacted with OsO_4 in 0.25 N NaOH in methanol for 1 hr at room temperature. The reaction products were isolated, purified, and analyzed by thin-layer chromatography, gas-liquid chromatography, and infrared and visible spectroscopy. In all cases, the products were identified as diesters of osmic acid in which two molecules of fatty acids are linked through 1 molecule of osmic acid.

INTRODUCTION

I have recently presented evidence (1) that the product of reaction of a thin film of methyl oleate and aqueous osmium tetroxide is bis(methyl 9,10-dihydroxystearate)osmate. On the basis of C, H, and O analyses, infrared, visible, and nuclear magnetic resonance spectroscopy, and molecular weight determination, the following structure was suggested:



An apparently identical compound was isolated by transmethylation of the lipids of *Acanthamoeba* after fixation in Millonig's osmium tetroxide (2). The studies have now been extended to include the reactions of oleic acid and di-11-octadecenoyl phosphatidylcholine with aqueous osmium tetroxide. The products of these reactions have been compared chromatographically and by infrared and visible spectroscopy to each other and to the products of reaction of methyl 9,10-dihydroxystearate and 9,10-dihydroxystearic acid with osmium tetroxide in methanolic NaOH. The data indicate that, in all cases, the olefins and the glycols are converted into diesters which probably have the common structure:



This formulation differs from that proposed previously in possessing a hexavalent rather than a tetravalent Os and is in keeping with the structures proposed by Criegee (3, 4) for diesters formed from glycols and tetramethyl dipotassium osmate.

EXPERIMENTAL

MATERIALS: Methyl oleate and oleic acid were purchased from Applied Science Laboratories, Inc., State College, Pa. Oleic acid-1-^{14}C was purchased from New England Nuclear Corp., Boston, and purified of any contaminating short chain fatty acids and neutral impurities by partitioning procedures previously described (5). Methyl oleate-1-^{14}C was synthesized by reaction of oleic acid-1-^{14}C with diazomethane in ether. Threo-9, 10-dihydroxystearic acid was purchased from K & K Laboratories, Inc., Plainview, N. Y., and converted to its methyl ester by reaction with diazomethane. The methyl 9,10-dihydroxystearate was then purified by fractional crystallization from heptane. Purified 9,10-dihydroxystearic acid was prepared by saponification of the purified methyl 9,10-dihydroxystearate. The purity of all fatty acids and methyl esters was checked by thin-layer chromatography on silicic acid and by gas-liquid chromatography of the methyl esters on ethylene glycolsuccinate and SE-30.

Phosphatidylcholine was isolated from large cultures of *Agrobacterium tumefaciens* (6). The packed cells (750 g) were extracted with 14 liters of chloroform: methanol (2:1) overnight, and the filtrate was evaporated to dryness. The dried residue was extracted with chloroform which yielded 27 g of lipid. One-half of the lipids was fractionated on a column of 500 g of silicic acid eluting sequentially with 10 liters each of chloroform, 25% methanol in chloroform, 50% methanol in chloroform, and 100% methanol. The eluted fractions were analyzed by thin-layer chromatography, and the 50% methanol fraction was found to contain most of the phosphatidylcholine. This fraction was purified by chromatography on a smaller column of silicic acid. The fraction that was eluted by 40% methanol in chloroform but not by 35% methanol was found to be pure phosphatidylcholine by thin-layer chromatography. Fatty acid analyses (see Results) showed the material to be essentially di-11-octadecenoyl phosphatidylcholine.

CHROMATOGRAPHY: Thin-layer chromatography of the reaction products of osmium tetroxide and methyl oleate, oleic acid, methyl 9,10-dihydroxystearate, and 9,10-dihydroxystearic acid was carried out on silicic acid with a solvent system of heptane: diethyl ether:glacial acetic acid, 50:50:1. The soluble reaction products of osmium tetroxide and phosphatidylcholine were chromatographed on silicic acid with a large number of solvents in none of which did the osmium derivative move from the origin. Phosphatidylcholine was, therefore, readily separable from its osmium derivative. The osmium derivative of the phospholipid did migrate on thin-layer plates of cellulose with chloroform or methanol as the developing solvent, but not when heptane or diethyl ether were used. Compounds were usually eluted from the silicic acid or cellulose with anhydrous methanol. Spots that contained osmium were brown and gave a positive reaction when sprayed with the benzidine reagent described by Riemersma (7). Colorless lipids were visualized under ultraviolet light after the plates were sprayed with 0.1% dichlorofluorescein in 50% ethanol. In the experiments with ^{14}C-compounds, all areas of the plates were eluted with methanol and counted.

Fatty acids were analyzed as their methyl esters by gas-liquid chromatography on both 17% ethylene glycol succinate at 190° and on SE-30 at 205°. The columns measured 6 ft × 5 mm, the inlet pressure was maintained at 10 lb/in^2, and an argon ionization detector was used. The detector was calibrated with standards at known concentrations. Details of these procedures as applied to the compounds of interest in this paper have been published (1, 2).

ANALYSES: Phosphorus was analyzed by a modification (8) of the procedure of Fiske and SubbaRow (9). For determination of infrared spectra, the compounds were dissolved in CCl_4, or made into mulls in Nujol. A Perkin-Elmer 21 spectrophotometer and a Beckman IR 7 spectrophotometer were used. Visible spectra were obtained with a Cary recording spectrophotometer. Radioactivity was measured in a scintillation spectrometer with the use of 0.4% diphenyloxazole in toluene as scintillator-solvent. Quench corrections were made by the channels ratio method.

RESULTS

Synthesis of Osmium Derivatives

Tetramethyl dipotassium osmate was synthesized exactly as described by Criegee et al. (4). Osmium tetroxide (1.02 g) was dissolved in 10 ml of methanol, added to 25 ml of 1 N KOH in methanol, and the solution warmed for a few minutes. The deep green crystals that formed after cooling the solution were collected, washed, and dried.

Methyl oleate-1-^{14}C (1 mmole, 3.6 × 10^6 cpm) was reacted with 1.05 mmole of OsO_4 (as a 2% solution in water) for 1 hr at 0°. The black tarry oil that formed was washed with water and extracted into heptane, leaving behind a black residue. The insoluble material (presumably OsO_2) weighed 167.5 mg. The dark purple-brown heptane solution contained 85% of the initial radioactivity. The soluble product was chromato-

graphed on silicic acid plates, 94% of the radioactivity was recovered in a brown, osmium-positive spot with an Rf[1] identical to that previously found (1) for bis(methyl 9,10-dihydroxystearate)osmate. No radioactivity was present on the thin-layer plate in the areas in which methyl oleate and methyl 9,10-dihydroxystearate would have been. Gas-liquid chromatographic analysis (1) revealed only bis(methyl 9,10-dihydroxystearate)osmate. Both unreacted methyl oleate and methyl 9,10-dihydroxystearate are easily detected by this method. The product was converted quantitatively to methyl 9,10-dihydroxystearate when heated at 80° for 1 hr in 1 N HCl in 50% methanol (1). The specific radioactivity of the osmium derivative after purification by thin-layer chromatography was 8.3×10^3 cpm/mg. The theoretical specific radioactivity for bis(methyl 9,10-dihydroxystearate)osmate synthesized from methyl oleate (3.6×10^6 cpm/mole) is 8.2×10^3 cpm/mg. This agrees with the analytical data published previously (1) which indicated that the product contains 2 moles of fatty acid and 1 mole of osmium. The product purified by thin-layer chromatography was used for the spectral analyses.

Methyl 9,10-dihydroxystearate (0.2 moles) was reacted at room temperature with 0.15 mmoles of OsO_4 for 1 hr in 4 ml of 0.25 N NaOH. The deep yellow-orange solution was acidified, whereupon a dark brown precipitate formed. An equal volume of water was added to the mixture, and it was extracted with heptane. The heptane was evaporated to give 85 mg of a dark purple-brown oil. This product was identical to bis(methyl 9,10-dihydroxystearate)osmate synthesized from methyl oleate by gas-liquid and thin-layer chromatographic analyses. No unreacted methyl 9,10-dihydroxystearate was detected. The osmium derivative could be converted to methyl 9,10-dihydroxystearate by hydrolysis. The product was used for spectral analyses after purification by thin-layer chromatography.

Oleic acid-1-^{14}C (1 mmole, 4×10^6 cpm) was reacted with 1.05 mmole of OsO_4 (2% water) for 1 hr at 0°. The black precipitate that formed was washed with water and was then extracted with heptane:ether, 1:1. The extract contained 95% of the radioactivity. A nonradioactive black residue (113 mg), presumably OsO_2, remained. The extract was analyzed by thin-layer chromatography, which revealed one dark brown, osmium-positive spot with an Rf of 0.39. This material contained 55% of the radioactivity that was applied to the plate. The remainder of the radioactivity was present in the area corresponding to unreacted oleic acid. No radioactivity was detected in the area that would have contained 9,10-dihydroxystearic acid if it were present. The product was converted quantitatively to 9,10-dihydroxystearic acid by hydrolysis (1). The specific radioactivity of the purified osmium derivative was 8.9×10^3 cpm/mg, which is in reasonable agreement with the theoretical specific radioactivity for bis(9,10 - dihydroxystearic acid)osmate synthesized from oleic acid (4×10^6 cpm/mmole), i.e., 9.6×10^3 cpm/mg. The purified material was used for the spectral analyses.

9,10-Dihydroxystearic acid (0.09 mmoles) was reacted with 0.07 mmoles OsO_4 in 4 ml of 0.25 N NaOH at room temperature for 1 hr. The color of the solution progressed from yellow-orange through brown-yellow, green-brown, light purple, and, finally, to a deep violet after 25 min. The color changes are related to the reduction of OsO_4 to tetramethyl dipotassium osmate (3, 4). The solution was cooled and acidified which resulted in a brown solution to which an equal volume of water was added. The product was extracted into heptane:ether, 1:1. The solvent was evaporated, and 34.2 mg of a dark brown oil was recovered. When chromatographed on a thin-layer of silicic acid, one major brown, osmium-positive spot was found with the same Rf as the product formed from oleic acid-1-^{14}C and OsO_4. Nothing was present in the area on the chromatogram corresponding to 9,10-dihydroxystearic acid. The osmium derivative was converted quantitatively to the glycol acid by hydrolysis in 1 N HCl. Spectral analyses were performed on material purified by silicic acid chromatography.

Phosphatidylcholine (136 mg) was reacted with 25 ml of 2% OsO_4 in water for 1 hr at 0°. The black precipitate that formed was washed with water and then extracted several times with methanol. The red-brown methanol solution was evaporated which yielded 101 mg of the methanol-soluble product. The methanol-insoluble material was washed with ether and air-dried to give 83 mg of product. The starting material contained 160 μeq of phosphorus, the methanol-soluble product 83 μeq of phosphorus and the methanol-insoluble fraction 75 μeq of phosphorus. The minimal molec-

[1] Rf = relative to the solvent front.

ular weight of the methanol-soluble product calculated for one equivalent of phosphorus is 1150 (101 mg/0.083 mmoles = 1150 mg/mmole). This value agrees very closely with the theoretical molecular weight for the addition product containing 1 mole of phosphatidylcholine and 1 mole of OsO_4 (850 + 254 = 1104). By analogy to the reactions with methyl oleate and oleic acid, it may be assumed that the methanol-insoluble material contained OsO_2 in addition to the compound that contained the phosphorus.

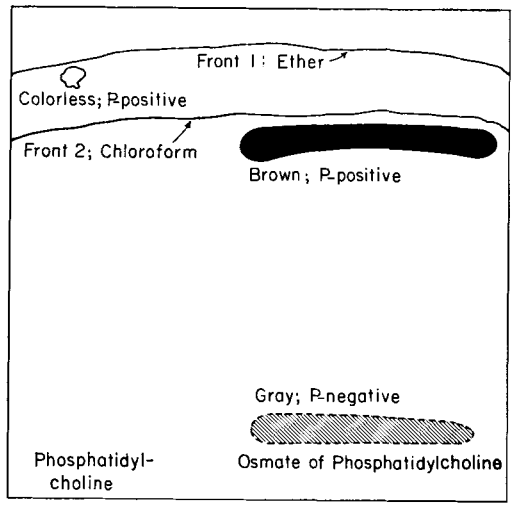


FIGURE 1 Thin-layer chromatogram of the methanol-soluble osmium derivative of di-11-octadecenoyl phosphatidylcholine. The osmium derivative and a sample of the unreacted phospholipid were applied in narrow bands. The plate was developed first with diethyl ether, then air-dried, and redeveloped with chloroform. The unreacted phospholipid migrated with the ether front, while the osmium derivative remained at the origin. In chloroform the osmium derivative migrated with the front, leaving a faint gray, phosphorus-negative band at the origin. The brown band at the chloroform front was phosphorus-positive and osmium-positive.

The methanol-soluble osmium derivative did not move from the origin of silicic acid thin-layer plates in any of the solvents used. Unreacted phospholipid was not present. When chromatography on thin-layers of cellulose was done, phosphatidylcholine was found to move with the solvent front in diethyl ether (Fig. 1), but the methanol-soluble osmium derivative remained at the origin. The osmium derivative migrated with the front when the same plate was redeveloped with chloroform (Fig. 1). The spot was osmium-positive. The only other material detected was a faint gray band at the origin.

The fatty acids of the phosphatidylcholine and its methanol-soluble osmium derivative were converted to methyl esters by methanolysis in 0.5 N sodium methoxide and analyzed by gas-liquid chromatography (1, 2). The original phosphatidylcholine had the composition: 4% hexadecanoate; 2% 9-hexadecenoate; 94% 11-octadecenoate; 2% 11,12-methylene octadecanoate, and can, therefore, be considered to be essentially a di-11-octadecenoyl phosphatidylcholine. The methanol-soluble osmium derivative had the composition: 9.3% hexadecanoate; 87% bis(methyl 11,12-dihydroxystearate)osmate; and 3% 11,12-methylene octadecanoate. It contained no dihydroxyste-

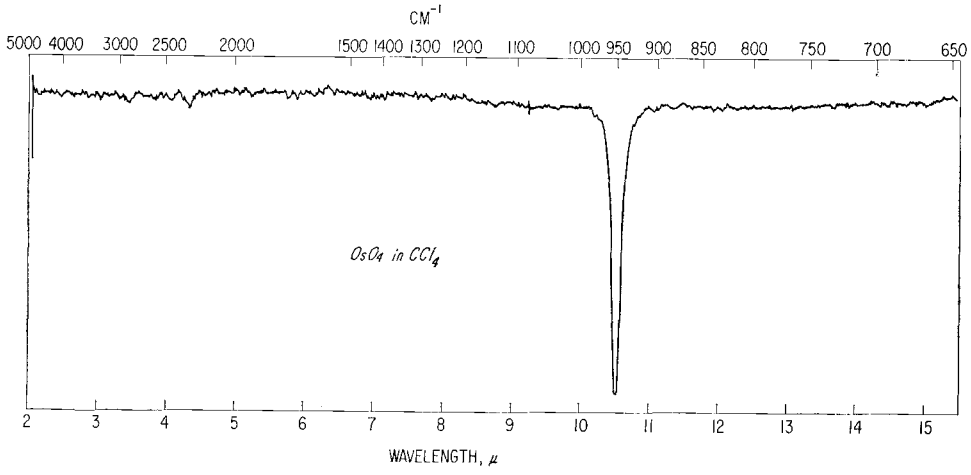


FIGURE 2 Infrared spectrum of OsO_4 in CCl_4.

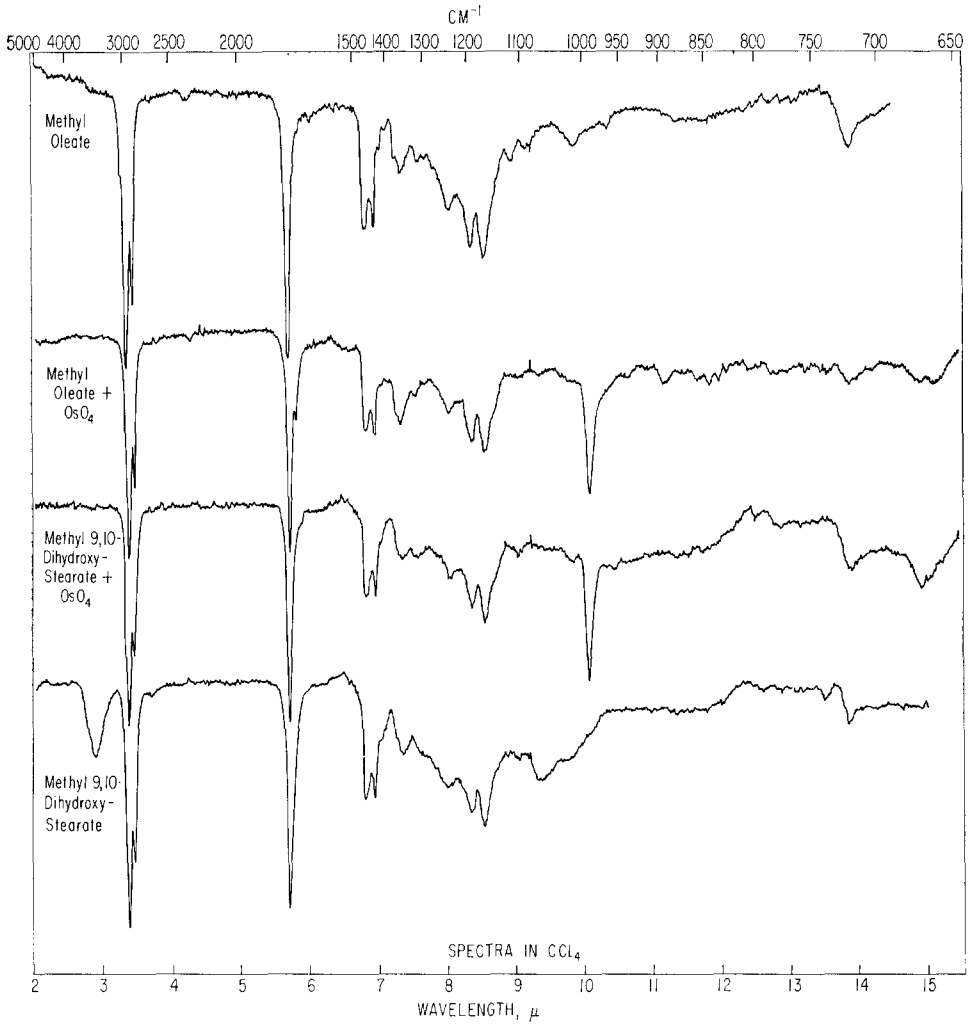


FIGURE 3 Infrared spectra of CCl_4 solutions of methyl oleate, methyl 9,10-dihydroxystearate, and the purified products of the reactions of OsO_4 with methyl oleate and methyl 9,10-dihydroxystearate. The spectra of the two osmium derivatives are essentially identical and differ markedly from the spectrum of methyl 9,10-dihydroxystearate.

arate or olefinic fatty acids. When the osmium derivative was hydrolyzed in 1 N HCl at 80° for 1 hr, the fatty acid analysis revealed: 9.1% hexadecanoate; 14.7% bis(methyl 11,12-dihydroxystearate)osmate; and 76% 11,12-dihydroxystearate.

Infrared Spectral Data

The infrared spectrum of OsO_4 is shown in Fig. 2. The spectrum has one sharp absorption band at 10.53 μ. No absorption bands were found for OsO_2, and tetramethyl dipotassium osmate had no absorption in the region of 10.5 μ. It, of course, does not have an Os=O group. In Fig. 3, the infrared spectra of the osmium derivatives of methyl oleate and methyl 9,10-dihydroxystearate dissolved in CCl_4 are compared to the spectra of authentic methyl oleate and methyl 9,10-dihydroxystearate. The spectra of the two osmium derivatives are nearly identical and show only two significant differences from the spectrum of methyl oleate. One difference is the absence of the absorption band at 3.3 μ attributable to the *cis*-double bond of methyl oleate; the other is the presence of a new absorption band at 10.1 μ. This

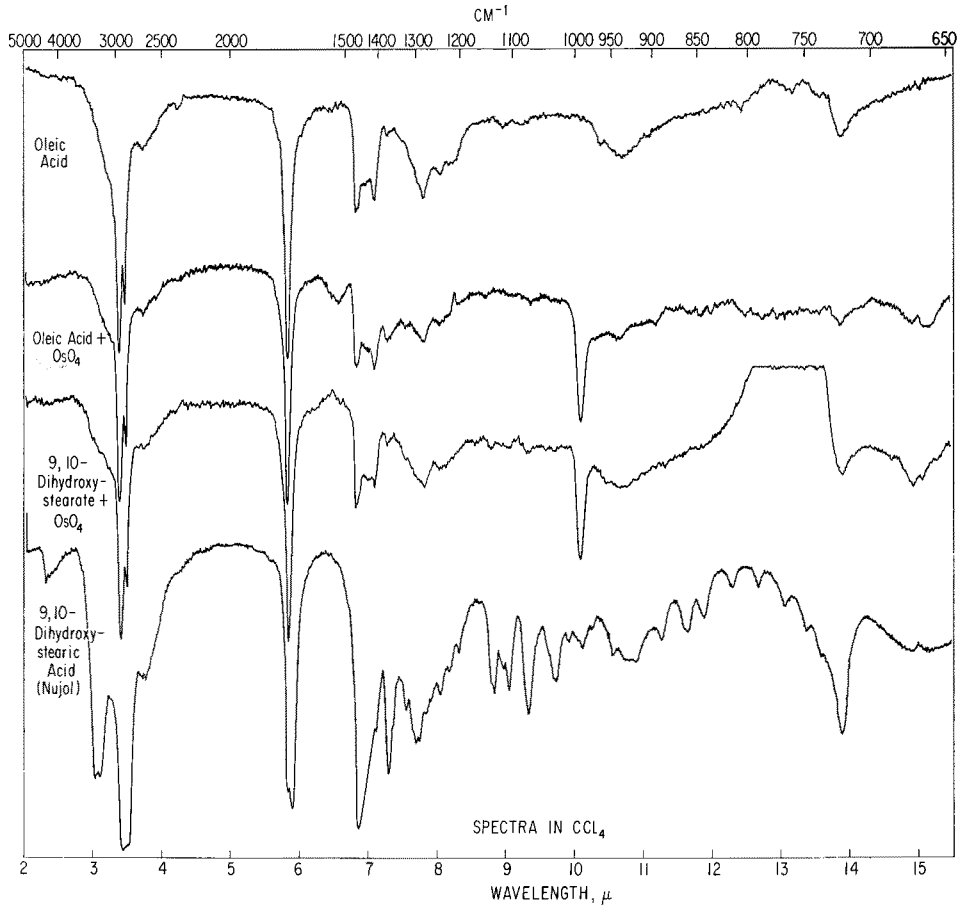


FIGURE 4 The infrared spectra of CCl_4 solutions of oleic acid and the purified products of the reactions of OsO_4 with oleic acid and 9,10-dihydroxystearic acid. The spectrum of 9,10-dihydroxystearic acid was obtained as a mull in Nujol because of its insolubility in CCl_4. The spectra of the two osmium derivatives are essentially identical and differ markedly from the spectrum of 9,10-dihydroxystearic acid.

new band is undoubtedly due to the Os=O function, as suggested also by Stoeckenius and Mahr (10), although its position is somewhat different from the corresponding absorption band of OsO_4. Neither the osmium derivative prepared from methyl oleate nor the osmium derivative of methyl 9,10-dihydroxystearate has an absorption band at 2.9 μ, that is characteristic of hydroxyl groups. That band is, of course, present in the spectrum of authentic methyl 9,10-dihydroxystearate. This confirms the absence of methyl 9,10-dihydroxystearate as indicated by thin-layer and gas-liquid chromatography.

Similar spectra are shown in Fig. 4 for the osmium derivatives of oleic acid and 9,10-dihydroxystearic acid as compared to authentic oleic acid and 9,10-dihydroxystearic acid. Again, the spectra of the two osmium derivatives are nearly identical. The band at 10.1 μ attributable to the Os=O group is present; the band at 3.3 μ due to the *cis*-olefin is missing; and there is no absorption in the region of 3 μ where hydroxyl groups would absorb if they were present. The hydroxyl absorption band is clearly evident in the spectrum of authentic 9,10-dihydroxystearic acid. The broad absorption between 3–4 μ underlying the sharp bands is a result of the COOH group and is present in the spectra of all four acids. The product of reaction of oleic acid and OsO_4 shows a small band at 6.5 μ similar to the band Stoeckenius and Mahr (10) attributed to heavy metal soaps. In the present experiments, it seems unlikely that much

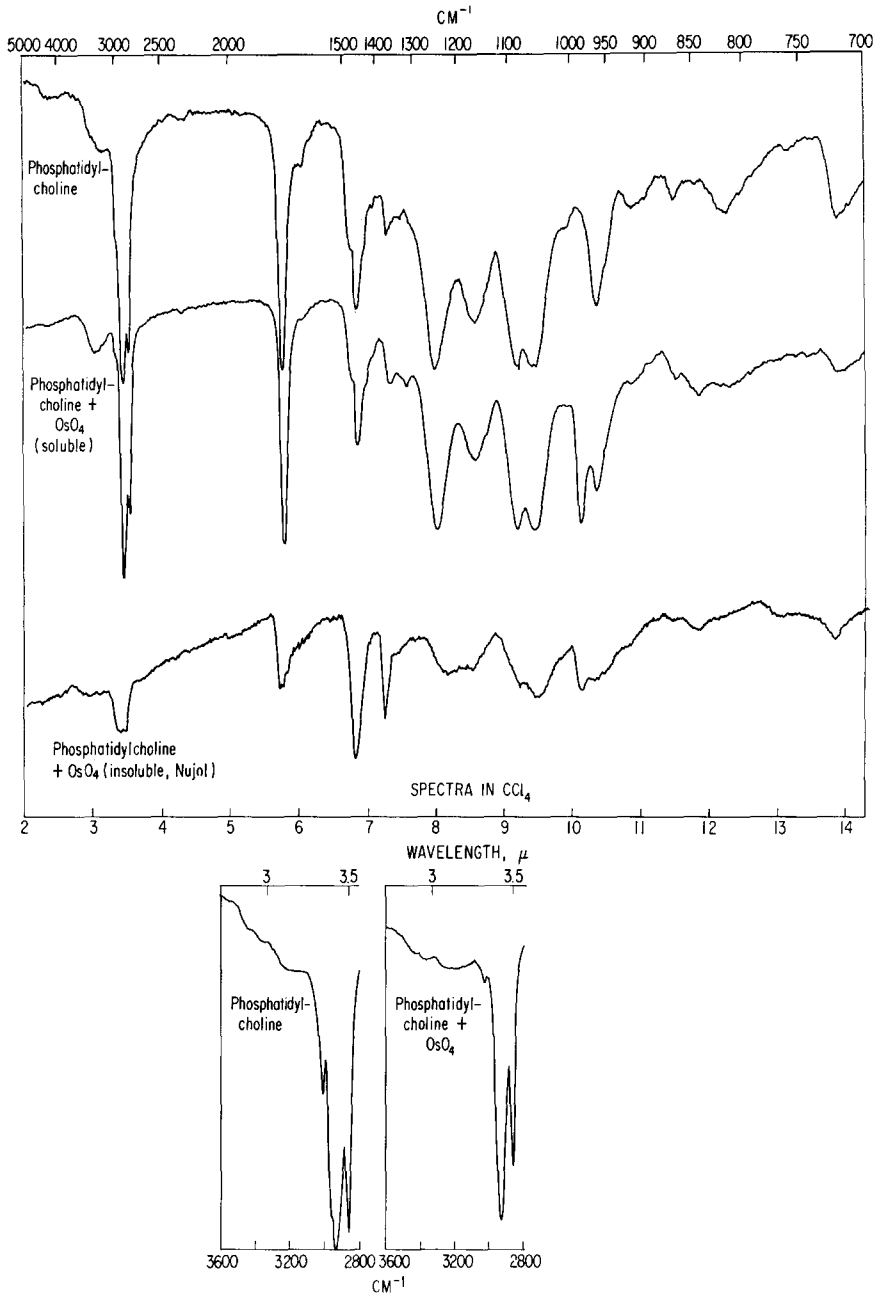


FIGURE 5 Infrared spectra of CCl_4 solutions of di-11-octadecenoyl phosphatidylcholine and its methanol-soluble osmium derivative. The spectrum of the methanol-insoluble osmium derivative was obtained as a mull in Nujol. The osmium derivatives show no increase in absorption in the hydroxyl region (3 μ) (See Table I). The phospholipids were dried in vacuo over P_2O_5 to remove water of hydration.

of the product could be in this form because of its solubility and chromatographic properties, but the possibility cannot be entirely eliminated.

The spectra of the methanol-soluble and methanol-insoluble osmium derivatives of di-octadecenoyl phosphatidylcholine are compared with the spectrum of the original phospholipid in Fig. 5. The major differences between the spectra of

TABLE I

Relative Absorption in the Hydroxyl Region of Phosphatidylcholine, Methanol-Soluble Osmium Derivative of Phosphatidylcholine and Methyl 9,10-Dihydroxystearate

	Phosphatidylcholine	Osmium derivative of Phosphatidylcholine	Methyl 9,10-Dihydroxystearate
$D_{3.1\,\mu}/D_{3.45\,\mu}$	0.099	0.094	0.196
$D_{3.1\,\mu}/D_{5.8\,\mu}$	0.13	0.12	0.23
$D_{5.8\,\mu}/D_{3.45\,\mu}$	0.76	0.78	0.85

$D_{3.1\,\mu}$ is due to hydroxyl groups; $D_{3.45\,\mu}$ is due to methylene groups; $D_{5.8\,\mu}$ is due to ester carbonyl groups.

the methanol-soluble osmium derivative and the starting material are the absence of the absorption band at 3.3 μ (*cis*-double bond) and the presence of a new band at 10.1 μ (Os=O). There is no increase in the absorption in the hydroxyl region of the spectrum. This is more clearly shown in the expanded spectra of that region. One can compare quantitatively (11) the absorption of the hydroxyl groups (3.1 μ) in the two compounds by relating these absorptions to those of the methylene groups (3.45 μ) and to those of the carbonyl groups (5.8 μ). As shown in Table I, the methanol-soluble osmium derivative has relatively less absorption in the hydroxyl region than does the original phosphatidylcholine. This absorption may be due to residual water, which is very difficult to remove

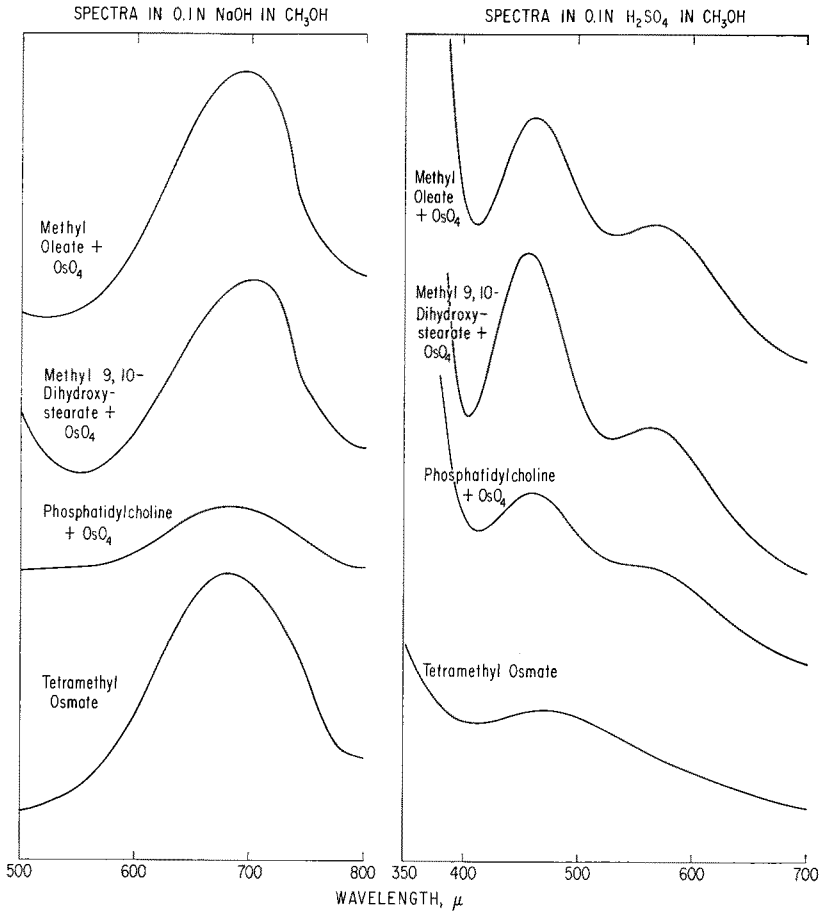


FIGURE 6 Visible spectra in acidic and alkaline methanol of tetramethyl dipotassium osmate and the osmium derivatives of methyl oleate, methyl 9,10-dihydroxystearate, and di-11-octadecenoyl phosphatidylcholine.

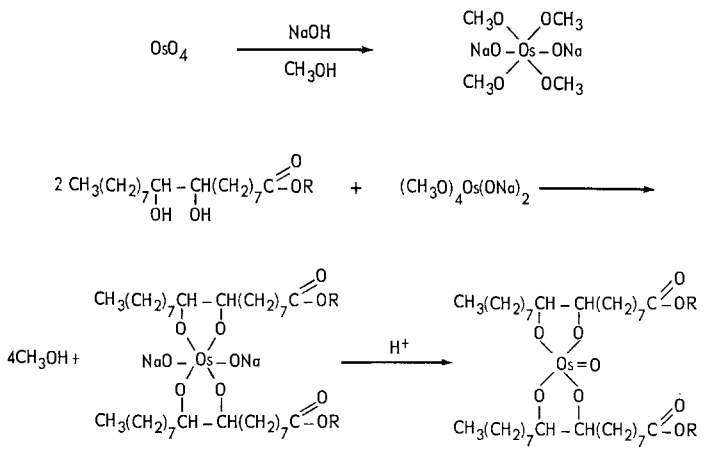


FIGURE 7 Proposed mechanism for the synthesis of bis (methyl 9,10-dihydroxystearate)osmate or bis (9,10-dihydroxystearic acid)osmate from OsO_4 and methyl 9,10-dihydroxystearate or 9,10-dihydroxystearic acid. This mechanism is based on the work of Criegee (3, 4).

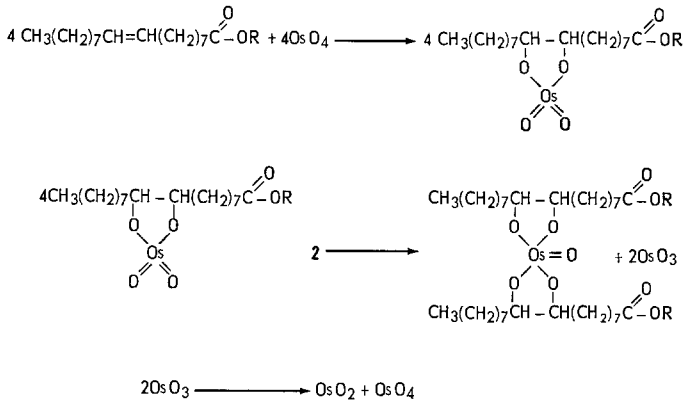


FIGURE 8 Proposed mechanism for the synthesis of bis (methyl 9,10-dihydroxystearate)osmate or bis (9,10-dihydroxystearic acid)osmate from OsO_4 and methyl oleate or oleic acid. This mechanism is very similar to one proposed by Becker (12) for the analogous reaction with acenapthylene.

from phospholipids, to—P—OH groups, or to hydroxyl groups neutralizing the quaternary nitrogen.

The infrared spectrum of the methanol-insoluble osmium derivative of di-octadecenoyl phosphatidylcholine could not be obtained in solution in CCl_4. As a mull in Mujol, its spectrum is very similar to that of the soluble osmium derivative with the characteristic 10.1-μ band from Os=O.

Visible Spectral Data

The visible spectra of the osmium derivatives of methyl oleate, methyl 9,10-dihydroxystearate, and di-octadecenoyl phosphatidylcholine are very similar (Fig. 6). All show a maximum at about 700 mμ when the compounds are dissolved in 0.1 N NaOH in methanol, and all show a change to 2 maxima at about 465 and 565 mμ when the compounds are dissolved in 0.1 N H_2SO_4 in methanol. Similar spectra were obtained for tetramethyl dipotassium osmate, except that only 1 maximum was obtained in acidic methanol. Similar results were also found for the osmium derivatives of oleic acid and 9,10-dihydroxystearic acid, but because of their low solubility these compounds give opalescent solutions and, therefore, poor spectra.

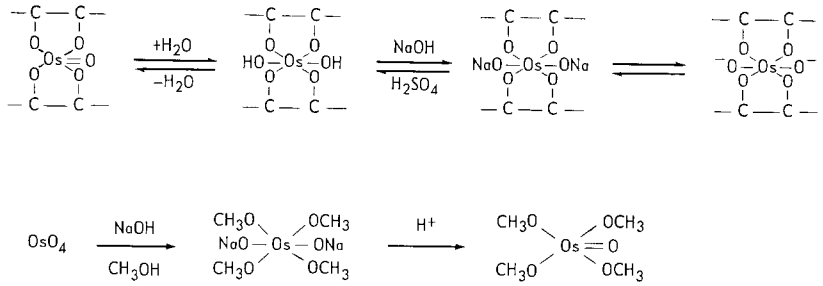


FIGURE 9 Proposed mechanism for the effect of changes in pH on the visible spectra of diesters of osmic acid.

DISCUSSION

All of the data in this and the previous papers (1, 2) are in agreement with the conclusion that the product of the reaction between thin films of oleic acid, or its methyl ester, and aqueous solutions of OsO_4 is a stable diester containing one molecule of osmic acid and two molecules of the glycol formed by oxidation of the olefin. This conclusion is supported by elemental analyses, molecular weight determination, nuclear magnetic resonance spectra, gas-liquid and thin-layer chromatography, the specific radioactivity of the products synthesized from radioactive precursors, and infrared and visible spectral data. Additional support for this identification is given by the fact that indistinguishable compounds are formed from the reaction of 9,10-dihydroxystearic acid, or its methyl ester, with OsO_4 in alkaline methanol. These model reactions are analogous to those that were studied by Criegee (3, 4). Criegee showed that OsO_4 is reduced by alkaline methanol to tetramethyl dipotassium osmate and that this compound reacts with two molecules of glycol in a transesterification reaction as illustrated in Fig. 7.

A plausible mechanism for the synthesis of these products starting from the olefinic acid (or its methyl ester) is shown in Fig. 8. In this case, the reaction is envisaged as occurring in several steps, although intermediates may not accumulate in detectable amounts. Oleic acid (or its methyl ester) would react with OsO_4 in an oxidation-reduction reaction to form the osmic acid ester of 9,10-dihydroxystearic acid. Reactions of this kind have been well characterized by Criegee (3, 4) for other olefins. Two molecules of the monoester could then react forming the diester and OsO_3 which could be expected to be unstable and, therefore, dismutate to OsO_2 and OsO_4. This proposed reaction scheme accounts for all the products that have been found: Bis(9,10 - dihydroxystearic acid)osmate and OsO_2.

In Criegee's research, only monoesters were found as the products of reaction of olefins and OsO_4 in anhydrous organic solvents, but recently Becker (12) has found that acenapthylene forms a diester with osmic acid when reacted with OsO_4 in aqueous dioxane. The reaction mechanism suggested by Becker is almost identical to the scheme shown in Fig. 8, except that he proposes the free glycol as an intermediate. The glycol is not included in the reaction sequence in Fig. 8 because its presence could not be detected by the sensitive methods of gas-liquid chromatography, thin-layer chromatography, and infrared spectroscopy. Riemersma (7) has presented indirect evidence for the formation of glycols during the reaction of phospholipids and OsO_4 in aqueous *t*-butanol. Stoeckenius and Mahr (10) noted an increase in the absorption in the hydroxyl region of infrared spectra of unsaturated lipids after reaction with OsO_4 in anhydrous chloroform. This observation is difficult to evaluate because, under anhydrous conditions, there is no source for hydroxyl groups. It may be that the absorption band was due to water present in the KBr pellets used in the infrared spectroscopy. All of the compounds synthesized in the present study showed an artifactual "hydroxyl" absorption band when the spectra were obtained as KBr pellets.

The structure of the diester in Fig. 8 differs slightly from that proposed in the earlier papers (1, 2). There are two reasons for suggesting that the product contains a hexavalent osmium atom. First, the same product is formed from the olefin and the glycol, and the most plausible explanation are reaction mechanisms that lead to a hexavalent osmium. Second, the acid-base shift in the visible spectra of the osmium derivatives is evidence for a

titratable group attached to the osmium such as a "carbonyl" type oxygen that can undergo "ketol" transformation (Fig. 9).

The fatty acid and phosphorus analyses and the infrared and visible spectra leave little doubt that the products of the reaction between OsO_4 and di-11-octadecenoyl phosphatidylcholine are directly analogous to the products formed from oleic acid and methyl oleate. A reasonable structure for, and mechanism of synthesis of, the methanol-soluble osmium derivative is shown in Fig. 10. In a reaction identical to that proposed in Fig. 8 for oleic acid, OsO_4 would react with two molecules of a monounsaturated fatty acid to form a diester of osmic acid. In this instance, however, the two monounsaturated fatty acids are on the same phospholipid molecule, giving an "internal" diester. The methanol-insoluble osmium derivative of the phospholipid might result when the osmic acid diester links fatty acids of different phospholipid molecules, thus leading to the formation of a polymer. One possible configuration of such a polymer is shown in Fig. 11.

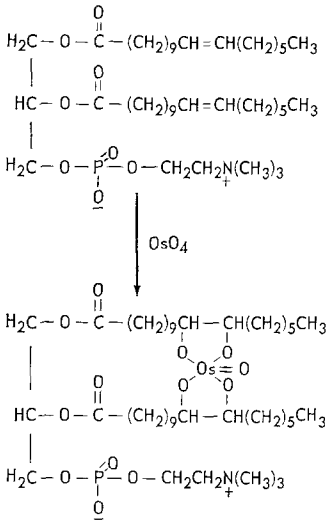


FIGURE 10 Proposed mechanism of synthesis, and structure, of the methanol-soluble osmium derivative of di-11-octadecenoyl phosphatidylcholine.

The data presented in this paper, therefore, give some direct experimental support to the hypotheses of Wigglesworth (13), Baker (14), and Stoeckenius and Mahr (10) that the fixation of biological material by OsO_4 may, in part, be due to the linkage of the double bonds of fatty acids through stable diesters. If more than one unsaturated fatty acid, or one polyunsaturated fatty acid, occurs in the lipids, polymerization is possible. Stoeckenius has interpreted electron micrographs of lipid-water systems fixed with OsO_4 (15, 16) and the infrared spectra of the products of reaction of olefinic lipids and OsO_4 in chloroform (10) to indicate the accumulation of osmium at the polar end of lipid molecules. He suggests that, after an initial stoichiometric reaction of OsO_4 with the double band, the osmic ester might break down, releasing the osmium which could then migrate to the polar

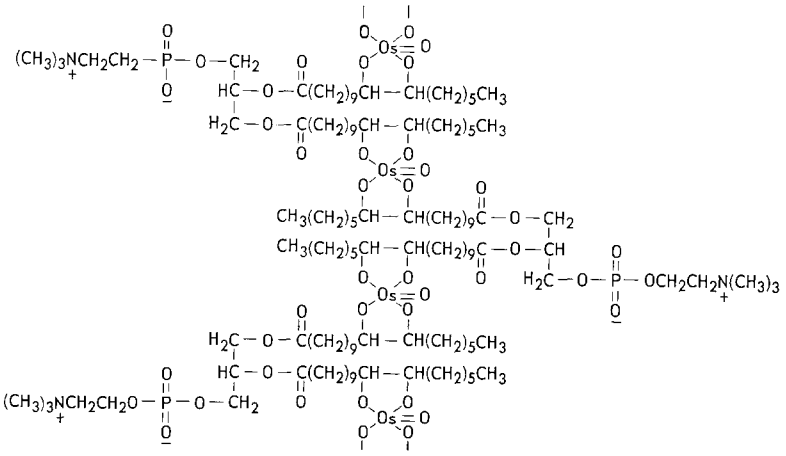


FIGURE 11 One possible structure for the methanol-insoluble osmium derivative of di-11-octadecenoyl phosphatidylcholine.

groups. The reactions proposed in Fig. 8 provide a mechanism whereby osmium could be released while the fatty acyl chains are still linked as diesters of osmic acid. Under the reaction conditions described in the present paper, however, little if any osmium appears to be bound to anionic groups. The solubility of most of the products, the specific radioactivity data, the chromatographic properties, and the phosphorus analyses all indicate that the osmium derivatives contain only the one molecule of esterified osmium for every two molecules of fatty acid. Whether a reaction between osmium and the polar groups of lipids occurs during biological fixations remains for future research to determine.

Received for publication 6 January 1967.

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