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Enhancement of the field desorption mass spectra of phospholipids.

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ENHANCEMENT OF THE FIELD DESORPTION MASS SPECTRA OF PHOSPHOLIPIDS

by

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A Thesis
submitted to the Faculty of Graduate Studies
through the Department of Chemistry
in Partial Fulfillment of the requirements
for the Degree of
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1981

ABSTRACT

ENHANCEMENT OF THE FIELD DESORPTION MASS SPECTRA OF PHOSPHOLIPIDS

by

Susan Elizabeth Perkins

Criteria for judging the field desorption mass spectra of phospholipids are introduced. Methods of enhancing these spectra are compared including removal of sodium ions by both a modified Bligh-Dyer extraction and by high performance liquid chromatography, addition of *p*-toluene-sulfonic acid and addition of water.

ACKNOWLEDGEMENTS

I would first like to thank Dr. Wood for his resourcefulness and guidance. Also to be thanked are Dr. Thibert for assistance with clinical problems and the members of Dr. Wood's research group, Ron Collacott, Mei Au and Wing Sun for helpful discussions during my studies. Lastly, thanks are due to Alan Thibert for technical assistance.

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LIST OF ABBREVIATIONS

BAT	best anode temperature
CI	chemical ionization
EI	electron ionization
eV	electron volt
FD	field desorption
FDMS	field desorption mass spectrometry
FI	field ionization
HPLC	high performance liquid chromatography
LPA	lysophosphatidic acid
mA	milliampere
mol. wt.	molecular weight
m/z	mass to charge ratio
PA	phosphatidic acid
PC	phosphatidyl choline
PG	phosphatidyl glycerol
PI	phosphatidyl inositol
ppm	parts per million
PS	phosphatidyl serine
p-TSA	*p*-toluenesulfonic acid
rel. abund.	relative abundance
TIC	total ion count
TLC	thin-layer chromatography
µl	microliter
µm	micrometer

CHAPTER I

INTRODUCTION

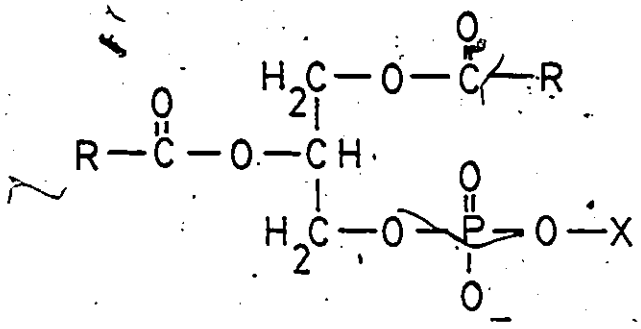
This thesis concerns the investigation of methods of improving the field desorption mass spectra (FDMS) of phospholipids.

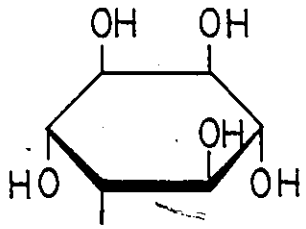
Lipids in general are a subject of great interest and importance in biological systems. They are valuable as sources and as storage and transport forms of metabolic energy as well as for protection and insulation not only of specific organs but also of whole organisms. Of all the lipids present in normal individuals, phospholipids are a major class, representing almost 50% of the total lipids of the blood plasma in man.[1]

Structurally, phospholipids are defined as being esters of fatty acids and alcohols (usually glycerol) with the additional identifying characteristic of a phosphate ester residue. The latter may be terminal, as in the case of phosphatidic acid and its analogues, or it may be esterified with another alcohol such as choline, ethanolamine, glycerol, inositol or serine (Figure 1).

The most abundant of the phospholipids in the plasma and various tissues in man are the lecithins, or phosphatidyl cholines (PC). They perform both metabolic and structural

Fig. 1: General Structure for Phospholipids



PC	$-CH_2CH_2\overset{+}{N}(CH_3)_3$
PA	$-H$
PG	$-CH_2\underset{OH}{CH}CH_2OH$
PE	$-CH_2CH_2\overset{+}{N}H_3$
PI	
PS	$-CH_2\underset{\overset{+}{N}H_3}{CH}COO^-$

roles, the latter in their capacity as a major lipid component of cell membranes along with glycolipids and cholesterol. In particular, dipalmitoyl lecithin is a powerful surfactant, important in the alveolar cells of the lungs. By preventing alveolar collapse on expiration, dipalmitoyl lecithin is critical in lung function. Since upon maturity of the fetal lung dipalmitoyl lecithin from the lung is found in the amniotic fluid, its presence there is the basis for tests of fetal lung maturity such as the lecithin/sphingomyelin ratio (L/S ratio),[2] total lecithin[3] and total choline-containing phospholipids[4] in amniotic fluid. More recently interest has centred on saturated PC[5,6] and the contents of lamellar bodies[7,8] which comprise the pulmonary portion of amniotic fluid.

Cephalins or phosphatidyl ethanolamines (PE), phosphatidyl inositols (PI), found mostly in the brain and phosphatidyl serines (PS) are also found in amniotic fluid. Recently there has been increased interest in phosphatidyl glycerol (PG) and its role in the surfactant properties of the alveoli.[8] Phosphatidic acids, on the other hand, are believed to be important mainly as intermediates in the synthesis of triglycerides and other phospholipids.

The analysis of phospholipid samples of both synthetic and biological origin presents certain problems with respect to storage and handling. Those lipids containing unsaturated fatty acids may undergo peroxidation at the

double bond when exposed to the oxygen of laboratory air or to peroxide impurities in organic solvents. For this reason it may be advisable to use freshly distilled solvents and in extreme cases to remove oxygen by nitrogen sparging. In addition, phospholipids should be stored in solution, preferably $CHCl_3/CH_3OH$ (1/1) to minimize contact with air.

Contact of phospholipids with acids or alkalis during analysis should be minimized. Bases cause hydrolysis at the fatty acid ester linkages yielding glycerol compounds and fatty acid soaps. At extremely high pH the phosphate-alcohol (x) linkage may even be destroyed. In contrast, unfavourably acidic conditions may encourage hydrolysis at the glycerol-phosphoric acid linkage producing a diacyl glycerol and a phosphoryl ester or phosphoric acid in the case of PA and its analogues. The latter class of compounds, and PG as well, present even further problems due to their hygroscopic nature.

In summary, phospholipids are best handled with minimal exposure to air, moisture and extremes of pH. In addition, storage at reduced temperatures of 0-15°C also reduces the chances of peroxidative or hydrolytic degradation.

Field desorption (FD) mass spectrometry[9] has been called "a black art . . . more mystical than scientific,"[10] however, it has been applied successfully to the elucidation of the structures of many hitherto troublesome, non-volatile molecules.[10-12] Among these compounds, which

for the most part are of a biological nature, are phospholipids.[13] Phospholipids are significantly polar, non-volatile and thermally labile, thus presenting unique problems when submitted for structural analysis by most mass spectrometric processes. Chemical ionization (CI) mass spectrometry of phospholipids[14] results in much fragmentation, although several structural features can be deduced from the spectra. Electron impact (EI) mass spectrometry yields useful information mainly on derivatized phospholipids.[15] However, the "soft ionization" technique of FDMS enables the identification of the $M^{+\cdot}$ or $[M+H]^+$ species while the low internal energy of the ions formed reduces the incidence of fragmentation.

The techniques of this fast expanding area of research are well-known and can be found in a number of excellent reviews.[10-12] Briefly, the procedure is as follows: the sample to be analyzed is placed on the anode, a tungsten wire 10 μm in diameter on which have been grown[16] 30 μm long carbon needles. Samples may be applied either by dipping into a sample solution or by direct delivery of the solution via a microliter syringe. When the solvent has evaporated, the anode is placed in the ion source where a vacuum is produced. Analysis is begun by slowly heating the anode in the presence of a large constant electric field.

It is postulated that at temperatures approaching the best anode temperature (BAT) the solid lattice of the sample

on the anode becomes semi-fluid, thus allowing molecules to migrate to the high field regions on the dendrite tips where the force is great enough to remove an electron by the well-known quantum mechanical tunnelling effect. In this way very little energy is transferred to the sample molecules and fragmentation is minimized. Phospholipids generally exhibit an $[M+H]^+$ peak rather than $M^{+\cdot}$, due to addition of a proton arising from another lipid molecule or from the solvent. This and other surface reactions such as methyl transfer processes occur on the anode.

All positive ions, however formed, desorb from the anode towards a negative counterelectrode forming the ion beam to be subsequently analyzed.

In comparison with other mass spectrometric processes, FD is considered superior for analyzing phospholipids, however there exist obstacles associated with both FD and the phospholipids themselves.

Inherent difficulties in FD that have been solved include unassignable ions produced from reactions taking place on the anode before desorption such as methyl transfer reactions[17,18] and cluster ion formation.[19] In addition, the very nature of the formation of ions in the absorbed layers on the anode predisposes the technique to be dependent on many parameters. These include anode preparation, field strength, temperature and thickness of the adsorbed sample layer.

The actual mechanisms of all of the above processes and their relative importance are the subject of a continuing controversy.[10,20-23]

Problems arising from the nature of the phospholipid samples or their preparation involve contamination by both organic and inorganic compounds. During laboratory synthesis or purification, impurities may be introduced. Because of the amphipathic nature of the phospholipid molecules these contaminants may be both non-polar and polar. Organic contaminants may include other lipids extracted during separation or purification procedures. Another interfering group of compounds are phthalate esters. These fat-soluble compounds are found in many commercial organic reagents as well as in plastic and other laboratory equipment.[24]

The main inorganic contamination is that of alkali metal ions, especially sodium. The latter is an insidious problem not only because of the ubiquitous nature of the ion in the laboratory environment but also because its effect on FD spectra is unpredictable. At its worst, sodium may cause low intensities, increased fragmentation and cluster ion formation, or possibly the absence of any spectrum at all. However, good spectra can also occasionally be obtained in the presence of moderate amounts of sodium. In fact, cationization with sodium was used successfully for molecular weight determination of certain polar organic molecules.[25,26]

Effects on FD mass spectra of various methods to remove sodium ions from phospholipid samples constitute the major portion of this thesis. Several procedures have been tried by previous investigators[13] with some degree of success. These include gel filtration, dialysis, TLC and complexing with ligands such as the crown ethers and cryptands. Often these techniques proved extremely time-consuming.

More recently with the advent of reverse-phase packings and their wide range of solvent applicability in the high performance liquid chromatography (HPLC) of phospholipids,[27-32] it was thought that it might also successfully separate them from their ionic contaminants.

The lipid purification method of Bligh and Dyer[33] has been used successfully in this regard. Thus its ability to remove sodium was investigated to determine if the resulting phospholipid specimens showed improved spectra after extraction.

The original Bligh-Dyer procedure involved initial extraction of lipids with a monophasic solution of composition chloroform/methanol/water (1/2/0·8). By the addition of one part chloroform and one part water, thus changing the ratio to (2/2/1·8), the lipids were effectively back-extracted into the lower chloroform layer while inorganic contaminants were removed in the upper methanol/water layer.

A modified Bligh-Dyer extraction has been proposed by Kates[34] in which the lipid is initially dissolved in chloroform/methanol (1/1) followed by the addition of the 0.9 parts water thus making the final ratio chloroform/methanol/water (2/2/1.8) as in the original method.

The present research compared the two methods for ability to remove sodium from a mildly contaminated sample of dipalmitoyl phosphatidyl choline and found the difference in sodium values for initial washes to be negligible.

Since the criterion of maximum sodium removal was satisfied it was decided to use Kates' modified version because it was simpler and less time-consuming. The latter reason is most important when extracting under acidic conditions as is the case for acidic phospholipids. Thus in the later part of this thesis, "Bligh-Dyer Extraction" in fact refers to the Kates' modified version.

Elimination of extraneous compounds is one method of improving mass spectra. Another approach may be to add a compound to the sample in order to enhance a merely mediocre spectrum. Studies are underway to improve spectra by the addition of acids as proton donors. Keough and DeStefano[35] had some success with this approach using *p*-toluenesulfonic acid (*p*-TSA) and zwitterions. The resulting spectra are characterized by a significant increase in intensity, a decrease in the BAT and thus a decrease in the number of fragment ions.

In similar experiments, Ligon[36] used polyphosphoric acid to enhance spectra in the field ionization (FI) mode.

The present paper details and compares some of the above methods of improving and enhancing the FD mass spectra of specific phospholipids, in particular those which have previously proved intractable to the technique.[37]

Phospholipids still present problems for mass spectrometrists who must deal with their thermal lability and sensitivity to the ever-present problem of sodium contamination. It has been necessary to find methods for improving spectra which are then first applied to commercial synthetic samples with a view to later adapting these procedures to the more complex area of FDMS of biological samples.

Hopefully, FDMS studies of phospholipids will add to the increasing quantity of data being collected on phospholipids in order to better understand their behaviour in the mass spectrometer. At the same time knowledge of both purification and enhancement techniques ensures that the best choice may be made when applying these techniques to phospholipids of differing structures and degrees of purity.

CHAPTER II

EXPERIMENTAL

A. Instrumental

All FD mass spectra were obtained from a Varian CH-5 DF mass spectrometer with an FD/FI/EI source, interfaced to an INCOS Model 2000 Data System and operated at a nominal resolution of 750. Calibration was accomplished using the EI spectrum of perfluorokerosene as the standard. Samples were dissolved in chloroform or chloroform/methanol mixtures depending on solubilities and applied to the anode by the dipping technique. When the solvent had evaporated the anode was introduced into the cool ion source through a vacuum lock. Vacuum was restored to 10^{-6} torr or greater and then an anode voltage of +3kV and a cathode voltage of -8kV (unless otherwise indicated) were applied. The anode was heated steadily at approximately 1·5mA per minute and the magnet was scanned from high to low mass in 12 second scans.

Total ion counts (TIC) were registered in arbitrary units where one unit is equivalent to approximately 1000 ions.

On the average, smooth sample desorption was obtained for 5 or 6 consecutive scans; however, results

tabulated could be for as many as 12 or as few as 2 useful scans.

On completion of a sample run, the anode was heated to a maximum of 50mA in order to remove all sample and inorganic contaminants before the next run.

Sodium levels were determined on an IL 251 aa/ae spectrophotometer at 589.2 nm in the flame emission mode with an air/acetylene flame. All Bligh-Dyer wash samples were aspirated at 1-2 ml per minute and read against a blank and standards in a matrix of methanol/water/chloroform (50/45/5). High performance liquid chromatography (HPLC) fractions were run against a blank and standards in a matrix of methanol/water/chloroform (84/10/6) at an aspiration rate of 1-2 ml per minute. These readings were compared to a pre-injection reading in order to detect a change in sodium levels.

HPLC separations were performed at room temperature without the use of the detector on a Spectra Physics SP-8000 liquid chromatograph equipped with an RP-8 column on 10μ Lichrosorb® and interfaced with a fraction collector, Model 328, from Instrumentation Specialties Company.

® T.M. of E. Merck, Darmstadt, W. Germany.

B. Chemicals

Palmitoyl LPA, oleoyl LPA, dipalmitoyl PA, dioleoyl PA, dipalmitoyl PG, dioleoyl PG, and dipalmitoyl PE were purchased from Serdary Research Laboratories, Incorporated, London, Ontario.

Dipalmitoyl PC was purchased from P-L Biochemicals Incorporated, Milwaukee, Wisconsin.

Methanol and chloroform used in HPLC experiments were HPLC grade purchased from Fisher Scientific Company and Caledon Laboratories Limited, Georgetown, Ontario, respectively.

All distilled water was passed through a Barnstead Ultrapure mixed bed column and then filtered with a 0.22 μm GS type filter from Millipore Corporation, Bedford, Massachusetts.

For other experiments, all solvents and other reagents were at least ACS grade.

Silica Gel H was purchased from Sigma Chemical Company.

C. Modified Bligh-Dyer Procedure

1. For Neutral Phospholipids

To 5 mg of phospholipid sample, in a 15 ml centrifuge tube, were added 2 ml chloroform and 2 ml methanol. The solution was swirled to dissolve all possible lipid, 1.8 ml deionized water were added, the tube was inverted 10 times and then centrifuged for 1 minute at 3500 rpm. The top methanol/water layer was removed and labelled

"wash #1." Two ml of methanol/water (10/9) were added and the inversion and centrifugation repeated for the second wash. The sample was washed up to 5 times. Upon removal of the final wash, the remaining chloroform layer containing the lipid was evaporated at 37°C under nitrogen. Benzene was added to remove any water present in the extract. All lipid samples were redissolved in chloroform or chloroform/methanol solutions to give a concentration of approximately 1 mg/0·1 ml and stored at -4°C before submission for FD mass spectrometric analysis.

2. For Acidic Phospholipids

The above procedure was carried out with the replacement of water by 0·1N hydrochloric acid. After all the washes were removed, the chloroform layer was neutralized with 0·2N methanolic ammonium hydroxide. The sample was evaporated, redissolved, stored and submitted in the same manner as neutral phospholipids.

D. High Performance Liquid Chromatography Procedures

All separations were performed on the RP-8 column using an isocratic solvent mixture of methanol/water/chloroform (84/10/6) at a flow rate of 2 ml per minute and a pressure of 400 psi. Prior to sample injection, the column was washed overnight with a total of 1800 ml filtered deionized water at 1·5 ml per minute. Ten µl of an equimolar solution of sodium iodide/dipalmitoyl PC in methanol were injected resulting in 0·14 nM of each on

on column. Fractions collected at 1 fraction per minute were first tested for sodium by flame emission spectrophotometry and then for phospholipids by TLC in order to find a reproducible lipid elution time. Fractions collected for FD mass spectrometric analysis were tested for sodium and then combined, dried down, redissolved, stored and submitted in the same manner as were Bligh-Dyer extracted samples.

E. Thin-Layer Chromatography

The TLC of phospholipids was performed on glass plates manually coated with 0·3 mm of silica gel H using a Desaga spreader and tray. Plates were run in a chloroform/methanol/water (65/25/4) solvent system and were sprayed for phosphate by the method of Hack and Ferrans.[38]

CHAPTER III

RESULTS AND DISCUSSION

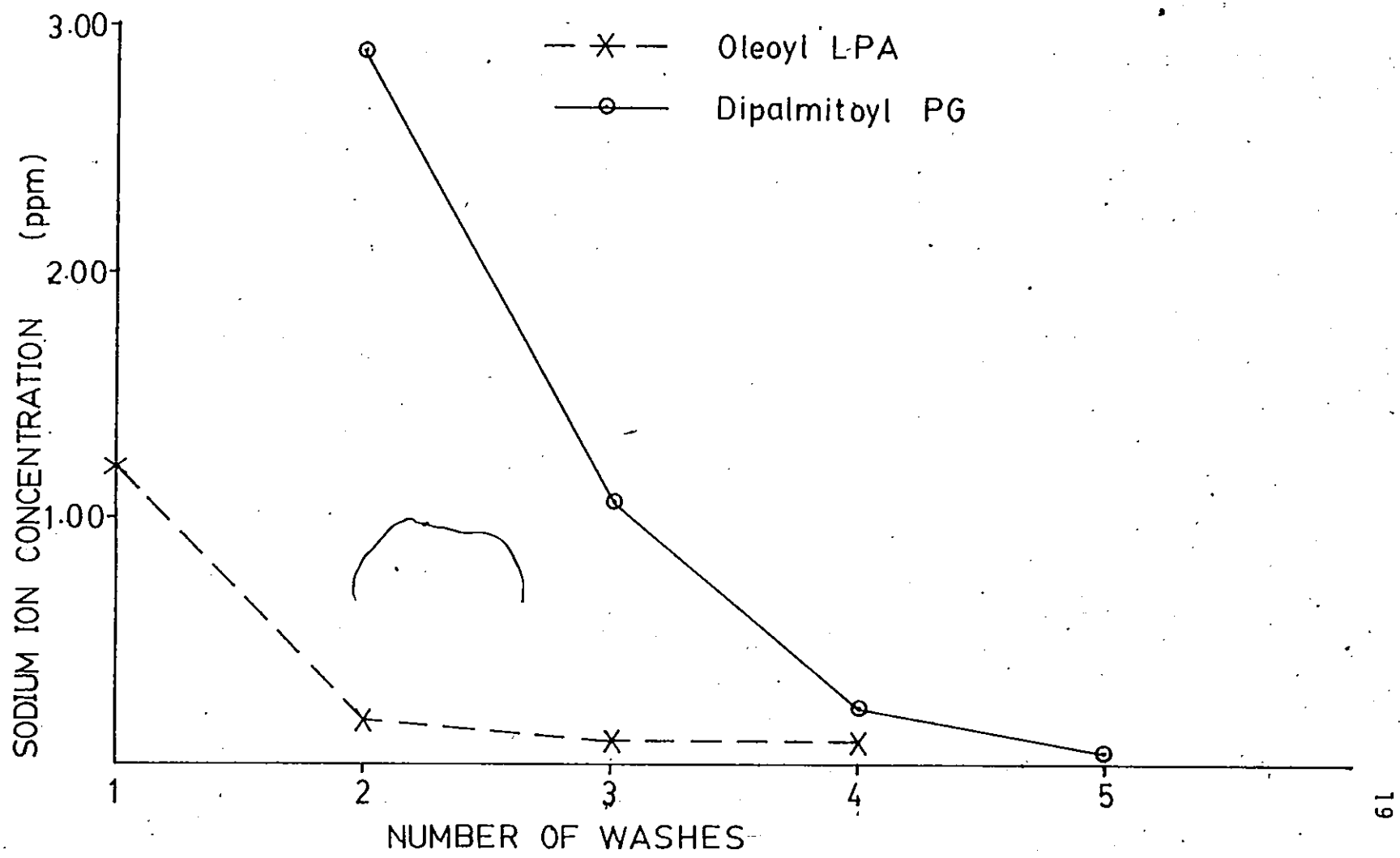
A. Sodium Determinations on Bligh-Dyer Washes

It is most important to begin by mentioning that the sodium contamination we are dealing with is in the ppm range, specifically between 0 and 5 ppm. All ppm are expressed as w/w, not w/v.

It should be realized that after extraction the composition of the washes was not methanol/water (10/9) but closer to methanol/water/chloroform (10/9/1) which is due to saturation of the methanol/H_2O phase with chloroform.[39] Thus sodium levels in the washes were determined by running samples against a blank and standards in a matrix of methanol/water/chloroform (10/9/1). This was necessary since the presence of chloroform has an apparent negative effect on the sodium response in flame emission.

The chloroform and methanol used in the extractions were found to contain sodium in concentrations of 0·01 and 0·20 ppm, respectively. The resulting sodium washes therefore have a minimum possible sodium level of 0·09 ppm. Extractions were repeated until sodium levels in the washes were constant. For moderately contaminated samples this required three washes and for highly contaminated samples, 5 washes (Figure 2).

Fig. 2: Sodium Concentration of Bligh-Dyer Washes


3.00
2.00
1.00
SODIUM ION CONCENTRATION (ppm)
Oleoyl L-PA
Dipalmitoyl PG
1
2
3
4
5
NUMBER OF WASHES

B. The Lipid Spectra

1. Introduction

Since the purpose of this thesis is to investigate methods of improving the FD mass spectra of phospholipids, it is necessary to establish criteria by which to judge them. However, because of the versatility of mass spectrometry in its many forms, many different kinds of information may be obtained from an analysis depending on which technique, whether FD, FI, EI, CI, is used and also on how it is run. Thus the label of "a good spectrum" may depend on the kind of information the investigator seeks. For instance, FD, because of the small amount of energy it transfers to sample molecules, minimizing fragmentation, is extremely useful in molecular weight determinations. On the other hand, fragmentation may be desired to elucidate structural features of the sample.

The qualities that were considered necessary in "a good phospholipid spectrum" in this research were the following: (1) a low emitter current, (2) a high total ion intensity per scan, (3) at least three consecutive scans of the desired quality, (4) a base peak of $[M+H]^+$, (5) high base peak abundance as a % of total ion intensity.

As mentioned in the introduction, sodium contamination of phospholipid samples produces deleterious but inconsistent results in their mass spectra. Any one or all of the above five criteria may not be fulfilled. In addition

one will also observe ions of the form $[X+Na]^+$ where X may be equal to the molecular weight or some characteristic fragment of that particular molecule. Thus, in the Bligh-Dyer results, a sixth criterion, the absence of $[X+Na]^+$ ions, was taken into account. The six criteria were used to compare spectra of samples before extraction, ("pre") and after extraction, ("post").

It is important to note that for every set of FD mass spectrometric results in this thesis possibly three or four have been discarded. That is, phospholipids are difficult to run well and the reader should not expect to be able to duplicate these results on the first try.

Table 1 lists the phospholipids that were studied.

2. Dipalmitoyl Phosphatidic Acid

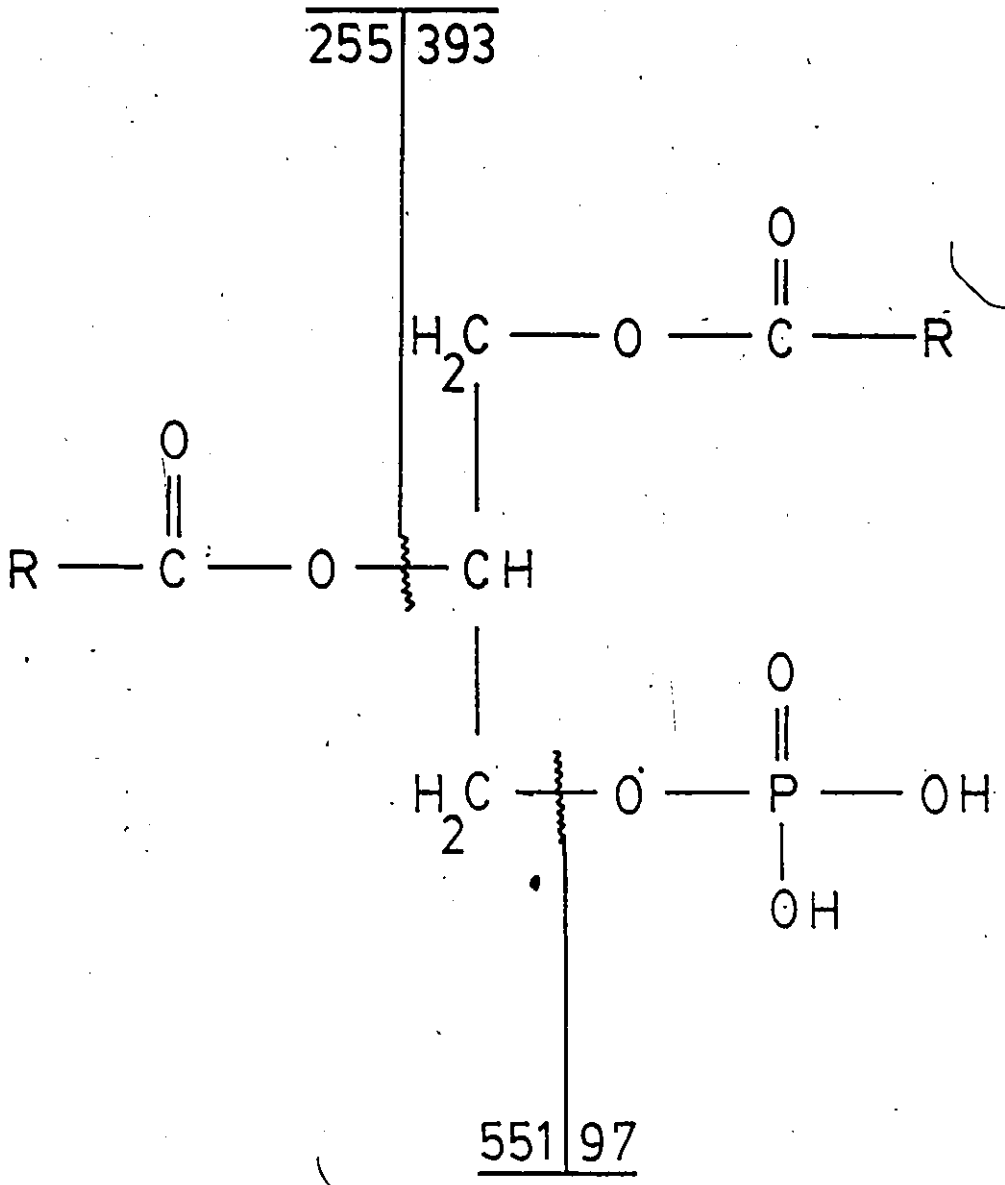
A phospholipid that has not behaved well during previous investigations[34] is dipalmitoyl PA (Figure 3). Highly contaminated with sodium initially, this sample did not yield a "pre" spectrum at all. After extraction however, a good spectrum was obtained (Table 2) with a high average intensity per scan and consistent desorption over eleven consecutive scans. The intensities of the isotope peaks related to the base peak, $[M+H]^+$, were close to the calculated values. Other recognizable fragments were also observed namely, $[M+H-RCOOH]^+$ at *m/z* 393, $[M-RCOO+2H]^+$ at *m/z* 395, $[H_3PO_4+H]^+$ at *m/z* 99 and the familiar ion at *m/z* 551 representing loss of the phosphate moiety, H_2PO_4.

Table 1

Phospholipids Studied

Name	Empirical Formula	Mol. Wt.	Theoretical Isotopic Abundances		
			M+1	M+2	M+3
dipalmitoyl PC	$C_{40}H_{80}O_8NP$	733.56	100.00	45.18	22.01
dipalmitoyl PA	$C_{35}H_{69}O_8P$	648.47	100.00	39.22	16.98
dioleoyl PA	$C_{39}H_{73}O_8P$	700.50	100.00	43.61	20.62
palmitoyl LPA	$C_{19}H_{39}O_7P$	410.24	100.00	21.42	5.99
oleoyl LPA	$C_{21}H_{41}O_7P$	436.26	100.00	23.62	6.98
dipalmitoyl PG	$C_{38}H_{75}O_{10}P$	722.52	100.00	42.64	20.18

Fig. 3: Structure and Fragmentation of Dipalmitoyl Phosphatidic Acid



$R = C_{15}H_{31}$

Table 2

FDMS of Dipalmitoyl Phosphatidic Acid

Post Bligh-Dyer Extraction

m/z	Assignment	Rel. Abund. (%)[a]
		Post 13.5-18.5 mA[b] TIC=3.7[c]
99	$[H_3PO_4+H]^+$	6
393	$[M+H-RCOOH]^+$	25
395		56
396		13
551	$[M-H_2PO_4]^+$	10
647	$[M-H]^+$	11
649	$[M+H]^+$	100
650		47
651		12
671	$[M+Na]^+$	1
683		8
684		7
789		7

[a]All ions greater than 5% rel. abund. are tabulated

[b]Anode heating current

[c]TIC = average total ion intensity per scan in arbitrary units

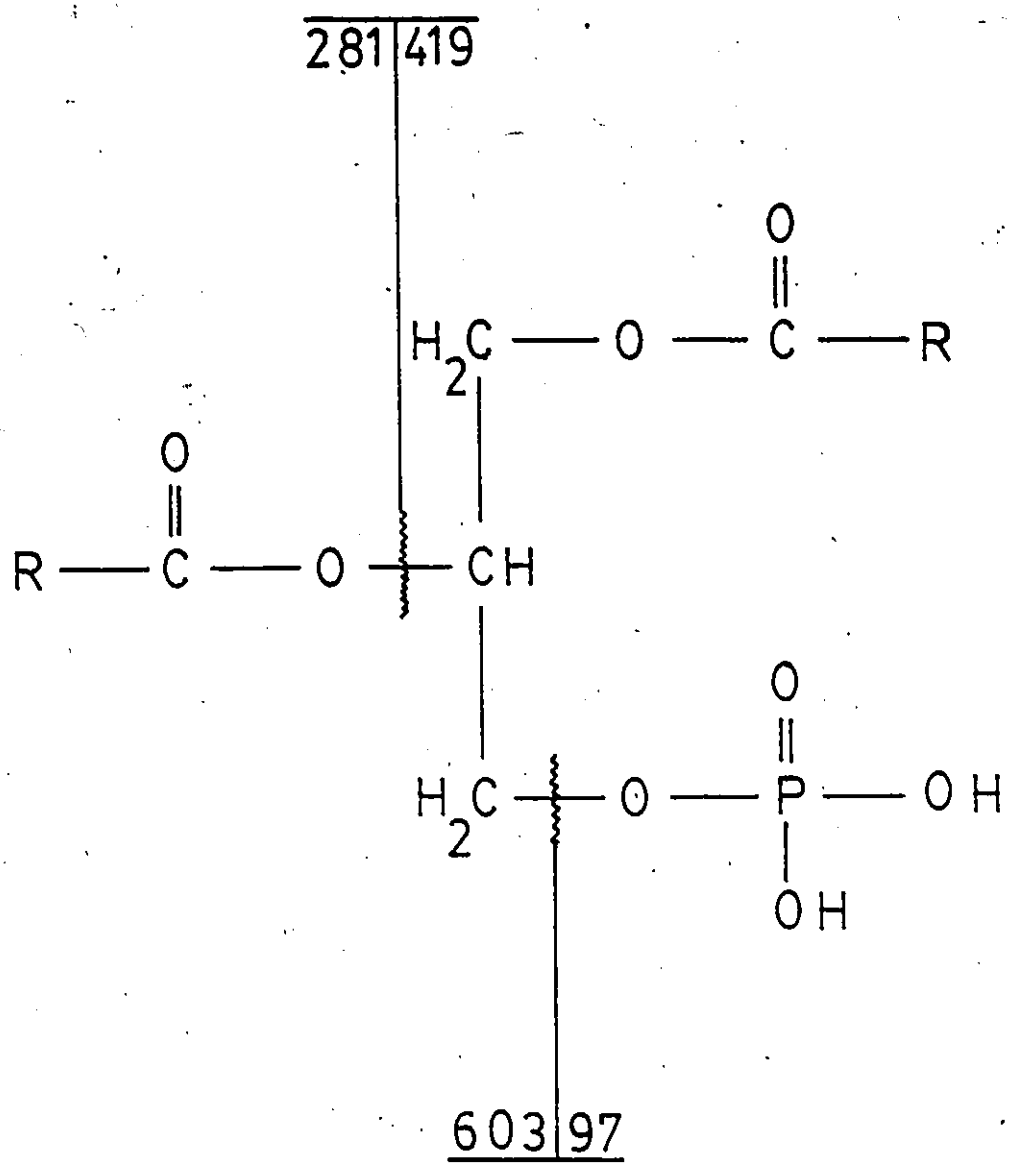
3. Dioleoyl Phosphatidic Acid

Dioleoyl PA, (Figure 4), an example of a moderately contaminated phospholipid gave $[M+H]^+$ as the base peak for both the "pre" and the "post" runs. However, the "post" spectrum did show significant improvements over the "pre" (Table 3). The anode currents at which the sample desorbed decreased and the number of useable scans increased from 4 to 11 as the result of the Bligh-Dyer clean-up even though the average total intensity per scan was down. The $[H_3PO_4 \cdot H_2O]^{+\cdot}$ peak at m/z 116, over 61% in the "pre" run, had completely disappeared after extraction. A smaller change was noted for $[M \cdot H_2O\text{-}RCOOH]^{+\cdot}$ at m/z 437. The expected $[M \cdot H_2O]^{+\cdot}$ was absent; nevertheless the greater average total intensity per scan of the "pre" sample leads to the speculation that water was absorbed prior to extraction. Some further results on this problem will be discussed later under "Additional Techniques."

Although a fragment of 5% intensity at m/z 419, representing a loss of oleic acid from M+H, had increased to 60% in the "post," overall the amount of fragmentation decreased because of the Bligh-Dyer clean-up. In addition, the base peak abundance as a percentage of the total intensity increased from 12% to 19% and the "post" isotope peaks compare slightly better with the theoretical values.

Perhaps the most striking evidence of the efficiency of extraction was the change in the relative abundance of the

Fig. 4: Structure and Fragmentation of Dioleoyl Phosphatidic Acid



$R = C_{17}H_{33}$

Table 3

FDMS of Dioleoyl Phosphatidic Acid

Pre and Post Bligh-Dyer Extraction

m/z	Assignment	Rel. Abund. (%)[2]	
		Pre 17.2-18.9 mA[b] TIC=5.8[c]	Post 14.1-18.5 mA[b] TIC=1.1[c]
116	$[H_3PO_4 \cdot H_2O]^{+\cdot}$	61	-
118		9	-
351		-	7
419	$[M+H-RCOOH]^+$	5	61
420		-	20
421		14	27
422		-	6
437	$[M \cdot H_2O-RCOOH]^{+\cdot}$	11	-
534		13	-
552		10	-
699	$[M-H]^+$	5	-
700	$M^{+\cdot}$	9	-
701	$[M+H]^+$	100	100
702		28	57
703		10	14
723	$[M+Na]^+$	13	1

[a] All ions greater than 5% rel. abund. are tabulated

[b] Anode heating current

[c] TIC = average total ion intensity per scan in arbitrary units

$[M+Na]^+$ ion. Prior to extraction it was greater than 12%, but after sodium removal it had dropped to less than 1%.

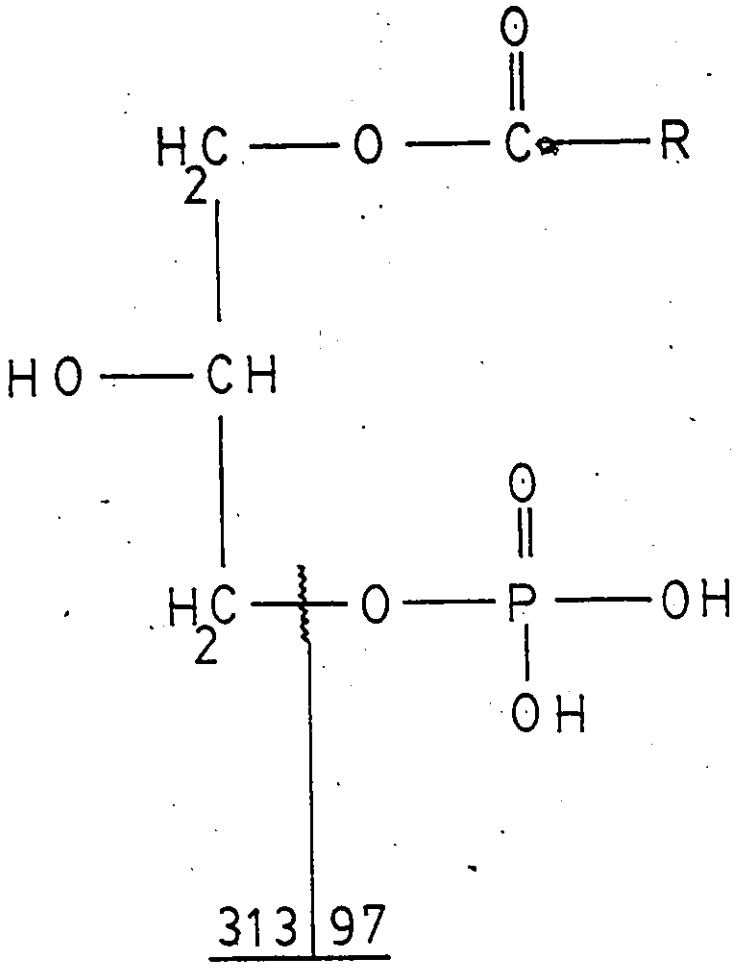
4. Palmitoyl Lysophosphatidic Acid

The case of palmitoyl LPA (Figure 5) presents some problems (Table 4). Although there was obvious improvement in the spectrum after removal of sodium, the base peak in both spectra was $[M-H_2O]^+$ at m/z 392. The relative abundance of $[M+H]^+$ increased with sodium removal, but only to 14%. There was a parallel increase in m/z 822 and m/z 823, either of which could be $[2M+H]^+$, mass assignment at that end of the spectrum being poor. Similarly an increase of $[M\cdot H_2O]^{+\cdot}$ at m/z 428 from 14% to 29% corresponded to the increase of m/z 840 which is probably $[2M\cdot H_2O]^{+\cdot}$. The $[M+Na]^+$ peak at m/z 433 dropped from 4% to an undetectable level, suggesting a successful removal of sodium overall.

5. Oleoyl Lysophosphatidic Acid

The second LPA, oleoyl LPA, (Figure 6), is interesting because in general the "pre" sample gave a better spectrum than the "post" (Table 5). Prior to extraction, emitter currents and fragmentation were lower, average total intensity per scan was higher and five times as many consecutive scans could be summed. The most significant observation, as we were to learn later, was the base peak of $[M\cdot H_2O]^{+\cdot}$ (see "Additional Techniques"). The protonated ions identified as $[H_3PO_2+H]^+$ and $[M+H]^+$ at m/z 99 and

Fig. 5: Structure and Fragmentation of Palmitoyl Lysophosphatidic Acid



$R = C_{15}H_{31}$

Table 4

FDMS of Palmitoyl Lysophosphatidic Acid

Pre and Post Bligh-Dyer Extraction

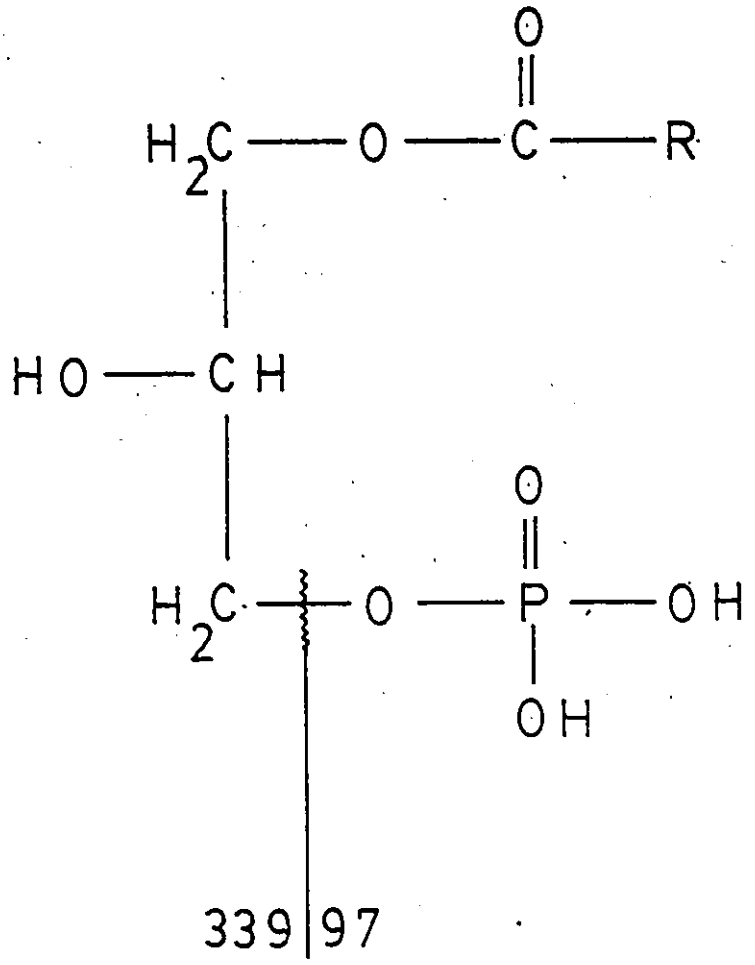
m/z	Assignment	Rel. Abund. (%)[a]	
		Pre 11.5-13.8 mA[b] TIC-0.9[c]	Post 8.9-11.3 mA[b] TIC=2.6[c]
392	$[M-H_2O]^{+\cdot}$	100	100
393		21	28
411	$[M+H]^+$	9	14
428	$[M\cdot H_2O]^{+\cdot}$	14	29
433	$[M+Na]^+$	4	-
821		14	-
822	$[2M+H]^+$?	15	34
823		-	14
840	$[2M\cdot H_2O]^{+\cdot}$?	-	10

[a]All ions greater than 5% rel. abund. are tabulated

[b]Anode heating current

[c]TIC = average total ion intensity per scan in arbitrary units

Fig. 6: Structure and Fragmentation of Oleoyl Lysophosphatidic Acid



$R = C_{17}H_{33}$

Table 5

FDMS of Oleoyl Lysophosphatidic Acid

Pre and Post Bligh-Dyer Extraction

m/z	Assignment	Rel. Abund. (%)[a]	
		Pre 6.8-9.6 mA[b] TIC=4.9[c]	Post 13.1-13.5 mA[b] TIC=2.0[c]
23	Na^+	-	5
39	K^+	-	3
99	$[H_3PO_4+H]^+$	-	6
116	$[H_3PO_4 \cdot H_2O]^{+\cdot}$	10	-
157		-	5
173		-	7
190		-	7
219		-	12
418	$[M-H_2O]^{+\cdot}$	13	7
436	$M^{+\cdot}$	-	8
437	$[M+H]^+$	17	100
438		-	15
439		-	1
454	$[M \cdot H_2O]^{+\cdot}$	100	52
455		16	-
459	$[M+Na]^+$	2	2
475	$[M+K]^+$	-	6
535	$[M+H_3PO_4+H]^+$	-	15
572		-	5
885	$[2M+H]^+$?	-	11
895	$[2M+Na]^+$?	11	-
915	$[2M+Na]^+$?	-	12

[a] All ions greater than 5% rel. abund. are tabulated

[b] Anode heating current

[c] TIC = average total ion intensity per scan in arbitrary units

m/z 437 respectively in the "post" sample, have their corresponding hydrated ions in the "pre" sample, $[H_3PO_4 \cdot H_2O]^{+\cdot}$ and $[M \cdot H_2O]^{+\cdot}$ at *m/z* 116 and *m/z* 454 respectively. Although oleoyl LPA was only moderately contaminated with sodium, needing only three washes before the levelling-off effect was observed (Figure 2), it is still surprising that the "post" was as contaminated as the "pre". In fact, potassium (*m/z* 39) was observed, as was $[M+K]^+$ at *m/z* 475 in the "post" only, suggesting that some contamination occurred during the Bligh-Dyer procedure. The fact that no *m/z* 23 was observed in the "pre" does not discount the presence of sodium since $[M+Na]^+$ at *m/z* 459 was present in the "pre" as well as an ion at *m/z* 895 which could quite likely be due to $[2M+Na]^+$ as could *m/z* 915 in the "post."

This sample is not a good demonstration of the efficiency of the Bligh-Dyer clean-up procedure, especially since the "pre" spectrum is further enhanced by the presence of water.

6. Dipalmitoyl Phosphatidyl Glycerol

In many ways the investigation of dipalmitoyl PG (Figure 7) produced one of the best examples of the efficiency of the Bligh-Dyer method. The initial sodium contamination of the PG was high, the first wash reading being greater than 3 ppm (Figure 2). Nevertheless, the "pre" sample, as well as the "post," yielded spectra when

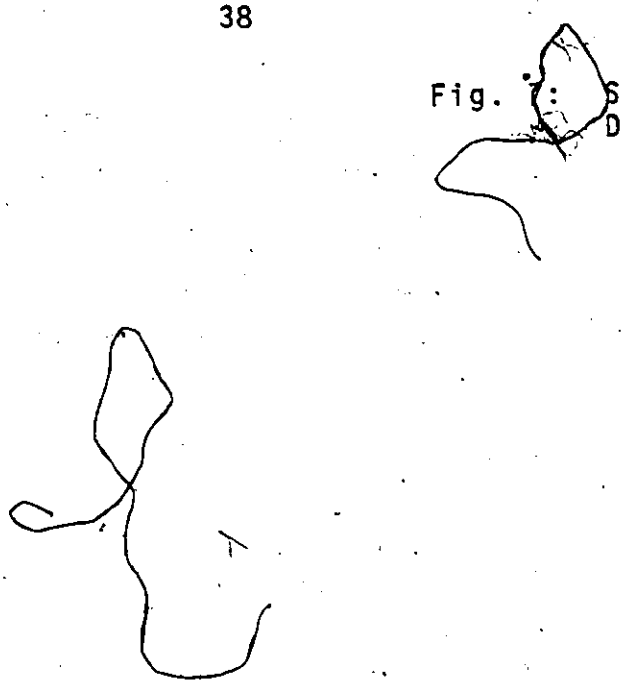
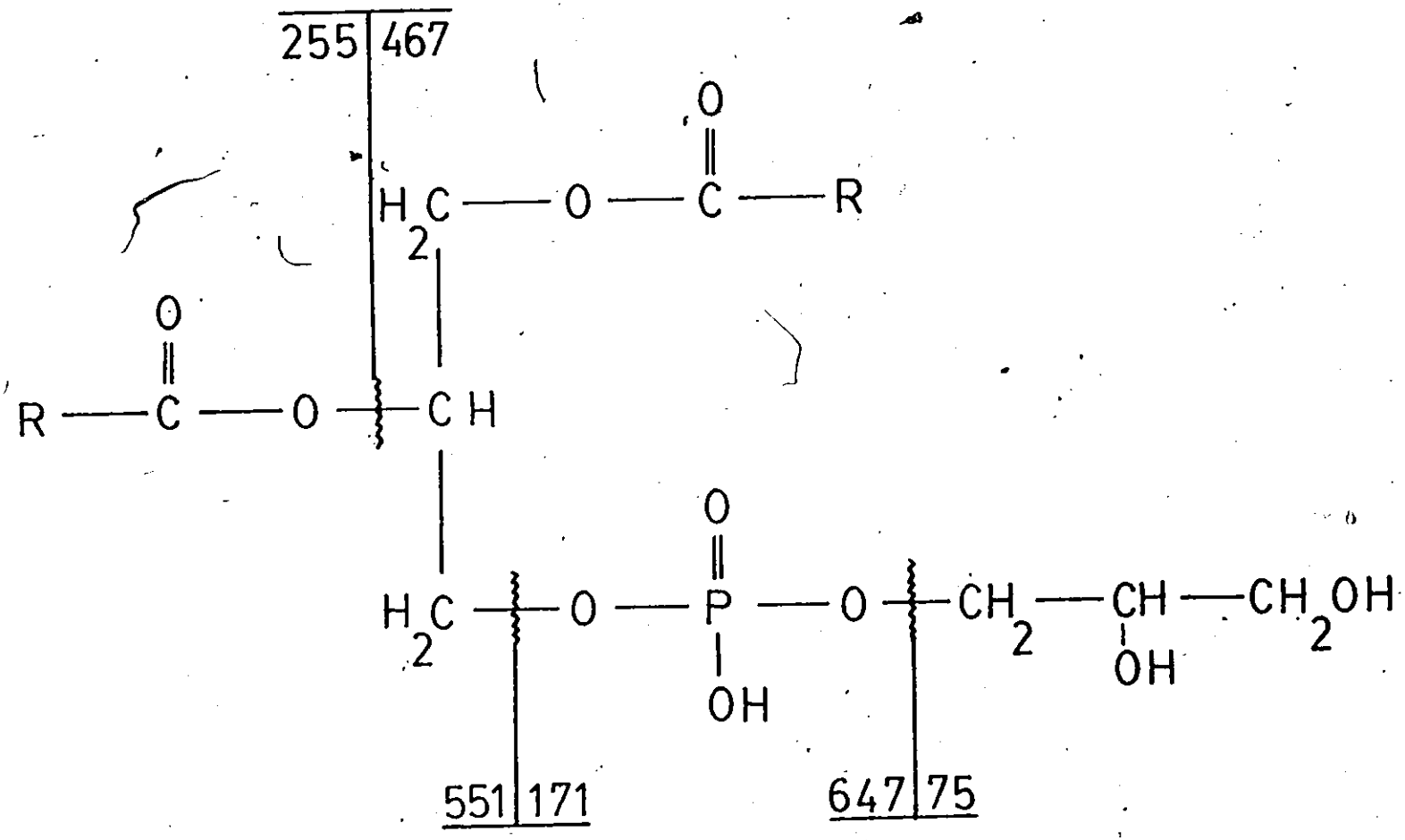


Fig. 7: Structure and Fragmentation of Dipalmitoyl Phosphatidyl Glycerol



$R = C_{15}H_{33}$

subjected to FD mass spectrometric analysis (Table 6). As expected, anode current values were down and the average total intensity per scan was up after extraction. In addition, the number of useful consecutive scans had increased. Most ions of the form $[X+Na]^+$ in the "pre" had corresponding $[X+H]^+$ values in the "post" sample, thus demonstrating the success of sodium removal. Peaks at *m/z* 745 and *m/z* 727 representing $[M+Na]^+$ and $[M-H_2O+Na]^+$ respectively, have parallels of $[M+H]^+$ at *m/z* 723 and $[M-H_2O+H]^+$ at *m/z* 705. The loss of water probably occurs across two glycerol carbons on the phosphoryl glycerol moiety in a process analogous to the loss of water from the glycerol backbone of LPAs. Ions at *m/z* 619 and *m/z* 597 are probably homologue contaminants from the commercial synthesis of the lipid since they are each 28 mass units higher than the base peaks in the respective samples. The cationized dipalmitoyl glycerols, which are the base peaks of the spectra, present the only unexpected results and probably derive from another contaminant, namely dipalmitoyl glycerol, introduced during lipid preparation. The first reason for this conclusion is that fragmentation between the phosphate and the glycerol backbone in most phospholipids usually occurs between the carbon and the oxygen, producing an *m/z* 551 peak in dipalmitoyl species. In the experience of this investigator, *m/z* 569 is rarely seen. Secondly, the percentage of *m/z* 569 ion did not

Table 6

FDMS of Dipalmitoyl Phosphatidyl Glycerol

Pre and Post Bligh-Dyer Extraction

m/z	Assignment	Rel. Abund. (%)[a]	
		Pre 17.4-18.9 mA[b] TIC=0.7[c]	Post 12.6-13.6 mA[b] TIC=3.4[c]
104		8	-
331	$[RCOOCH_2CHOHCH_2OH]$	-	8
569	[dipalmitoyl glycerol+H]$^+$	-	100
570		-	41
571		-	6
591	[dipalmitoyl glycerol +Na]$^+$	100	-
592		23	-
593		6	-
597	[569+28]	-	7
619	[591+28]	15	-
705	$[M-H_2O+H]^+$	-	5
723	$[M+H]^+$	-	5
727	$[M-H_2O+Na]^+$	11	-
740	$[M\cdot H_2O]^{+\cdot}$	-	-
745	$[M+Na]^+$	12	-
746		9	-

[a]All ions greater than 5% rel. abund. are tabulated

[b]Anode heating current

[c]TIC = average total ion intensity per scan in arbitrary units

increase with anode current, suggesting it did not originate from a decomposition of the molecule.

C. Additional Techniques for Phospholipid Spectra Enhancement

1. Adsorption of Water

(a) Introduction

During the course of this project it was observed that hygroscopic lipids allowed to remain on the bench in contact with a humid atmosphere exhibited large $[M \cdot H_2O]^{+\cdot}$ peaks that were often the base peak of the spectrum. This occurrence coincided with observable increases in total ion intensity, lowered emission current and sometimes less fragmentation. The hygroscopic lipids in this study were phosphatidic acids, lysophosphatidic acids and phosphatidyl glycerol.

The first results, some of which have already been noted in this thesis, were for samples that were allowed to adsorb water indiscriminantly from the atmosphere. The following experiments involved saturating a "post" Bligh-Dyer chloroform extract with one drop of deionized water. All samples were run both before and after H_2O adsorption, usually on the same day and on the same anode.

(b) Dioleoyl Phosphatidic Acid

In general, the spectrum of dioleoyl PA exhibited the expected changes due to the adsorption of water (Table 7). The emitter current decreased and the base peak changed

Table 7

FDMS of Dioleoyl Phosphatidic Acid

Before and After Water Adsorption

m/z	Assignment	Rel. Abund. (%)[a]	
		Before 14.1-18.5 mA[b] TIC=1.1[c]	After 10.5-12.4 mA[b] TIC=23.6[c]
351		7	-
418	$[M-RCOOH]^{+\cdot}$	-	20
419	$[M+H-RCOOH]^{+}$	61	6
420		20	-
421		27	-
422		6	-
436	$[M\cdot H_2O-RCOOH]^{+\cdot}$	-	21
437		-	5
438		-	6
454	$[M\cdot 2H_2O-RCOOH]^{+\cdot}$	-	13
468		-	7
682	$[M-H_2O]^{+\cdot}$	-	12
683		-	8
701	$[M+H]^{+}$	100	-
702		57	-
703		14	-
718	$[M\cdot H_2O]^{+\cdot}$	-	100
719		-	41
720		-	13
723	$[M+Na]^{+}$	1	1

[a] All ions greater than 5% rel. abund. are tabulated

[b] Anode heating current

[c] TIC = average total ion intensity per scan in arbitrary units

from $[M+H]^+$, the result of protonation and desorption, to $[M \cdot H_2O]^{+\cdot}$, the result of a one electron ionization. In addition, there were other notable changes in the fragment ions. Whereas m/z 419, representing $[M+H-RCOOH]^+$, was prominent in the spectrum without water, in the water-absorbed sample m/z 418, m/z 436 and m/z 454 were found, representing $[M-RCOOH]^{+\cdot}$, $[M \cdot H_2O-RCOOH]^{+\cdot}$ and $[M \cdot 2H_2O-RCOOH]^{+\cdot}$ respectively. Thus again we find a contrast between the desorption process of the initial sample and the ionization process of the H_2O-adsorbed sample.

(c) Dipalmitoyl Phosphatidic Acid

The other PA in this project was also investigated in a similar manner. The results are shown in Table 8. As expected, upon the addition of water the base peak changed from $[M+H]^+$ to $[M \cdot H_2O]^{+\cdot}$. In addition, the emitter current was lower and the total intensity increased. Some of the expected fragment ion changes were seen. The ion at m/z 393 representing $[M+H-RCOOH]^+$ was seen initially, whereas with water there appeared m/z 410 corresponding to $[M \cdot H_2O-RCOOH]^{+\cdot}$. However, the expected peaks at m/z 392 and m/z 428 corresponding to $[M-RCOOH]^{+\cdot}$ and $[M \cdot 2H_2O-RCOOH]^{+\cdot}$ were not found.

It is interesting to note that although the ion at m/z 410 was previously identified as $M^{+\cdot}$ for palmitoyl LPA and used to verify contamination of the commercial sample of dipalmitoyl PA by the corresponding LPA,[37] it now appears more likely that it represents the adsorption of one

Table 8

FDMS of Dipalmitoyl Phosphatidic Acid

Before and After Water Adsorption

m/z	Assignment	Rel. Abund. (%)[a]	
		Before 13.8-17.3 mA[b] TIC=1.3[c]	After 11.6-12.8 mA[b] TIC=2.1[c]
99	$[H_3PO_4+H]^+$	7	-
139		-	12
180		-	6
183		-	45
393	$[M+H-RCOOH]^+$	11	-
395		9	-
410	$[M\cdot H_2O-RCOOH]^{+\cdot}$	-	9
551	$[M-H_2PO_4]^+$	5	6
552		-	5
648		6	-
649	$[M+H]^+$	100	4
650		40	-
651		8	-
665		-	25
666	$[M\cdot H_2O]^{+\cdot}$	-	100
667		-	36
668		-	5
671		-	-

[a] All ions greater than 5% rel. abund. are tabulated

[b] Anode heating current

[c] TIC = average total ion intensity per scan in arbitrary units

molecule of water by the sample followed by a loss of the fatty acid.

An interesting result was recorded during one of the water-addition experiments with the lipid oleoyl LPA. During the analysis of the water-free sample, the instrument was focused on $[M \cdot H_2O]^{+\cdot}$ at m/z 454, not $[M+H]^+$ at m/z 437. As a result, the base peak of the spectrum was m/z 454. Now it is not surprising there is water in a sample that was thought to be water-free, especially since the sample is hygroscopic. What initially is surprising, however, is that $[M \cdot H_2O]^{+\cdot}$ is the base peak. However, this observation demonstrates a characteristic of FDMS that has been noted in this laboratory. The optimum focus may not be the same for all ions, especially if they are formed by different mechanisms. In this case it may be that focussing on the odd-electron species resulted in less than optimum focussing for the even-electron ion.

(d) Summary

Several conclusions may be drawn from the results of the water-adsorption experiments. The addition of water to the extracts of certain hygroscopic lipids results in improved spectra with base peaks of the form $[M \cdot H_2O]^{+\cdot}$. Other usual fragments may appear in a hydrated form.

The water molecules are most likely associated with the lipids through hydrogen-bonding. Thus the $[M \cdot H_2O]^{+\cdot}$ desorb at a low temperature. This is reasonable since increasing the emitter current supplies extra thermal

energy to the species which overcomes the hydrogen-bonding attraction. The site of complexation of the water molecule may be at the diol of the glycerol moiety of PG where hydrogen-bonding is believed to occur[37] and at the phosphate of the PAs and LPAs.

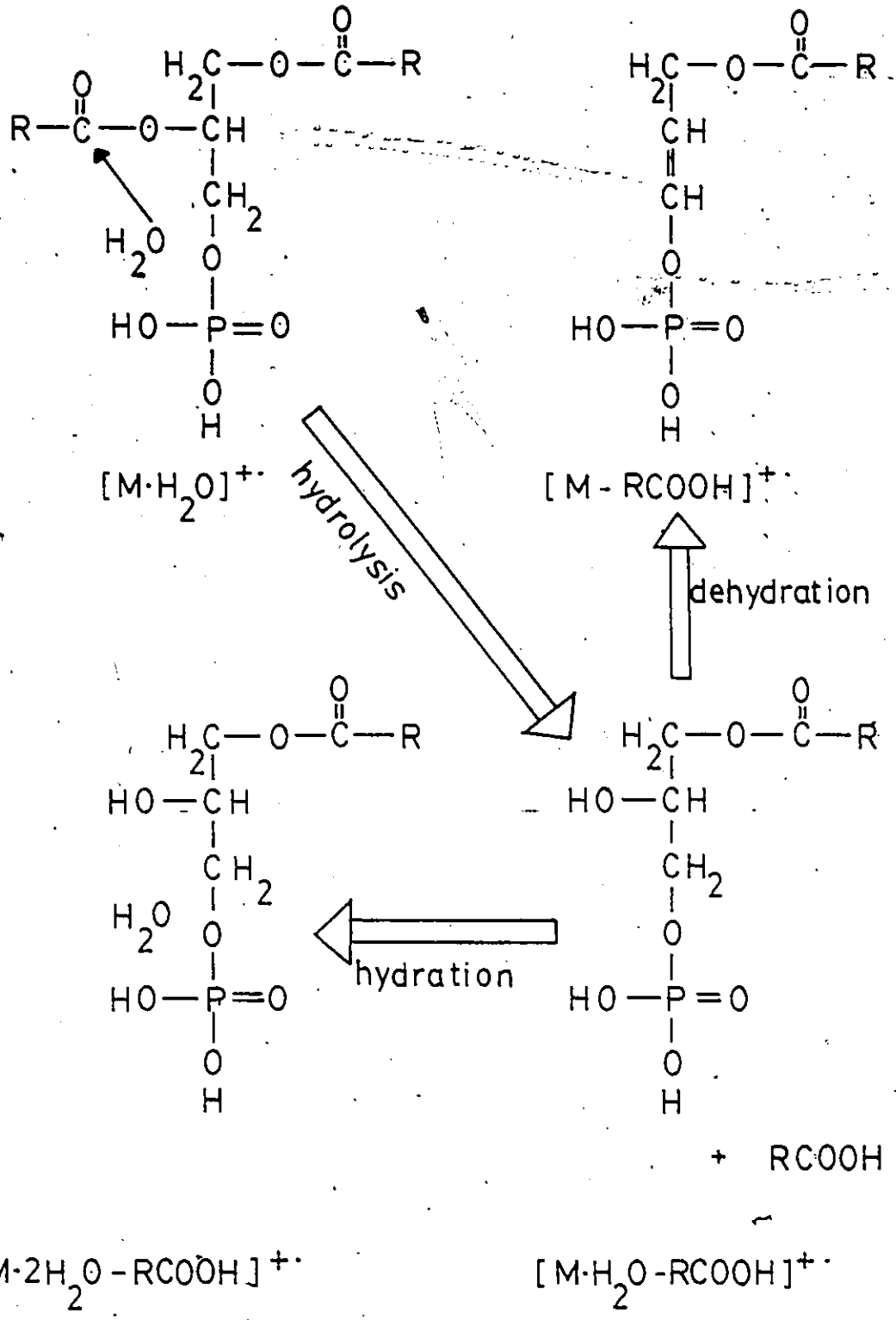
A water molecule in the vicinity of the phosphate group could induce hydrolysis of the ester linkage of PAs to produce the observed $[M\text{-}RCOOH]^{+\cdot}$, and subsequently $[M\cdot H_2O\text{-}RCOOH]^{+\cdot}$ and $[M\cdot 2H_2O\text{-}RCOOH]^{+\cdot}$ (Figure 10).

Improved characteristics of these spectra include lower anode currents, increased total intensity and less frequently, increased base peak abundance and decreased fragmentation.

2. *p*-Toluenesulfonic Acid

Purification and removal of extraneous compounds can only improve an FD mass spectrum to a certain extent. For further enhancement, other techniques must be used. In the FD mass spectra of lipids, the intact sample molecule generally appears as the protonated $[M+H]^+$ rather than the molecular ion $M^{+\cdot}$. Therefore, it seems likely that increasing the supply of protons to the sample might intensify the $[M+H]^+$ peak. This could be accomplished by dissolving the sample in a protic solvent or by mixing the sample with a proton donor. An example of the latter that has been used with some success[35] is *p*-toluenesulfonic acid (*p*-TSA). A good choice because it is a strong acid but non-volatile, it has been added to zwitterions prior to FD mass spectrometric analysis with the following

Fig. 8: Water-induced Hydrolysis of Phosphatidic Acids


[M·H2O]+·
hydrolysis
[M - RCOOH]+·
dehydration
hydration
+ RCOOH
[M·2H2O - RCOOH]+·
[M·H2O - RCOOH]+·

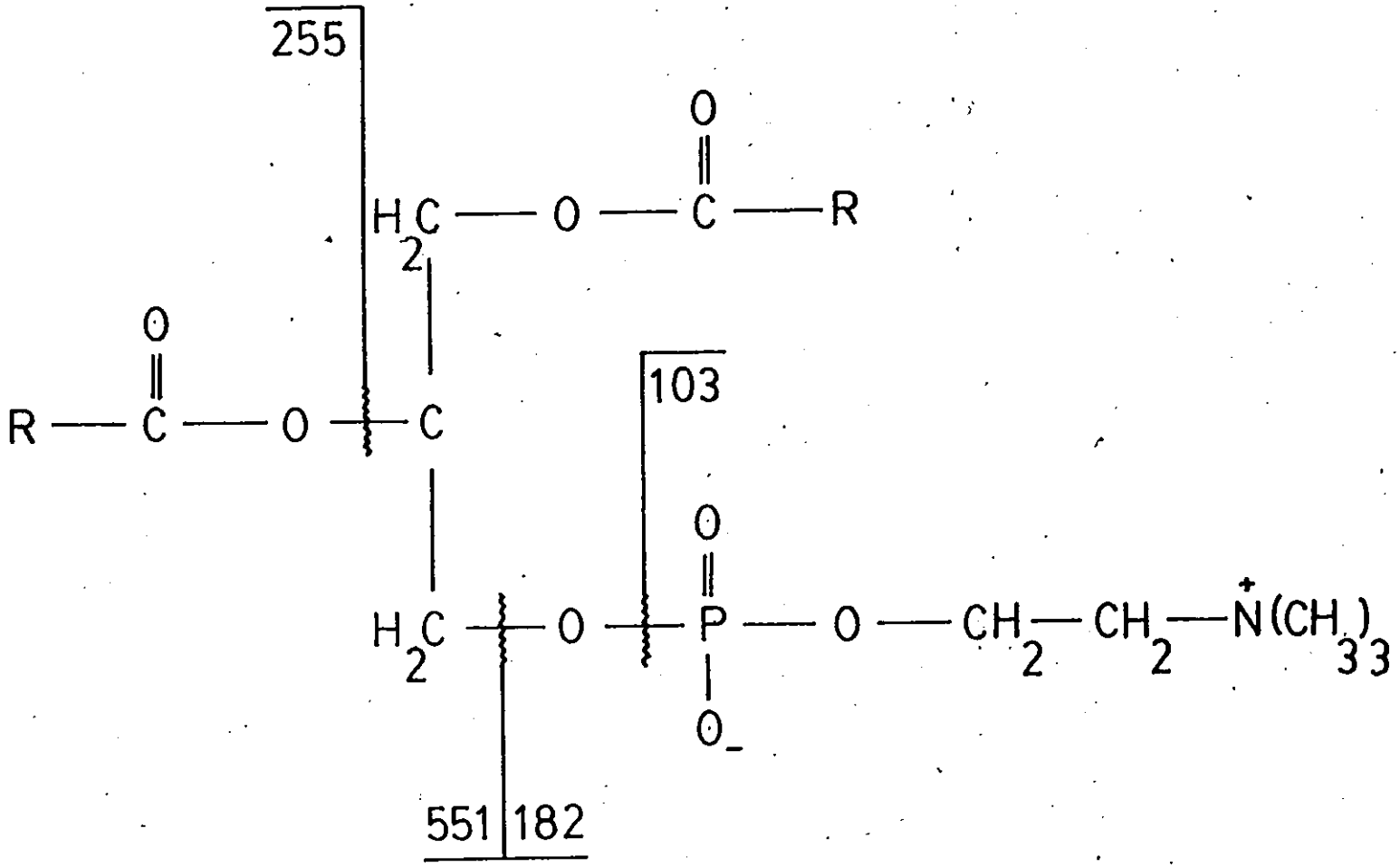
results: an increase in $[M+H]^+$, a decrease in the anode current and the elimination of fragmentation.

p-TSA was added to dipalmitoyl PC (Figure 9), in a 1/1 molar ratio immediately prior to sample loading in order to minimize the chance of acid hydrolysis of the lipid sample. Some degree of success was achieved (Table 9). The anode current for sample desorption was in the range of 7-11 mA, approximately 10 mA below the usual value. Fragmentation was virtually eliminated, the only ion with a relative abundance greater than 5% being $[M+H]^+$.

However, the sample behaviour was not consistent. That is, total intensities and the *m/z* value of the base peak changed from scan to scan. For example, total intensities varied between 1.4 and 5.3 and the base peak fluctuated between *m/z* 734 and *m/z* 735. Two scans with results consistent with calculated isotope ratios are shown in Table 9.

The problem with these results may be due to the fact that the *p*-TSA is too strong. It is thought[36] that the success of this technique is due to the fact that the large electric field increases the effective K_a of the acid. Thus weaker acids (perhaps polyacrylic acids) could be tried in future experiments.

Fig. 9: Structure and Fragmentation of Dipalmitoyl Phosphatidyl Choline



$R = C_{15}H_{31}$

Table 9

FDMS of Dipalmitoyl Phosphatidyl Choline

Plus *p*-Toluenesulfonic Acid

m/z	Assignment	Rel. Abund. (%)[a]	
		9.0 mA[b]	10.3 mA[b]
		TIC=7.0[c]	TIC=3.4[c]
733	$M^{+\cdot}$	6	-
734	$[M+H]^+$	100	100
735		37	58
736		11	11

[a]All ions greater than 5% rel. abund. are tabulated

[b]Anode heating current

[c]TIC = average total ion intensity of each scan in arbitrary units

3. High Performance Liquid Chromatography

As previously mentioned, HPLC has gained popularity in phospholipid analysis. Several investigators[24,28] have used it to separate and quantitate different classes of phospholipids using a variety of columns such as anion-exchange and silica gel and an assortment of detection techniques such as FID and uv. In similar studies, Jungalwala et al[30] used HPLC to separate and quantitate lecithins and sphingomyelins. Even species of PCs were separated according to fatty acid chain length on a partially aklylated Sephadex column.[28] On a preparative scale, Radin[31] used a silica gel column to purify crude egg lecithin extracts after an initial clean-up on alumina. One of the few applications of reverse-phase columns to phospholipid investigations was made by Porter et al[29] who used an RP-C-18 column again in order to separate lecithins by their "carbon number" or fatty acid chain length.

It seemed possible that a reverse-phase column that could retard different molecular species of PC could also separate a lecithin or other phospholipid from more polar contaminants by allowing them to elute before the lipid.

A moderately non-polar RP-8 column was chosen and various ternary solvent systems and compositions were tried in order to achieve separation of a 10 µl 1/1 molar mixture of NaI/dipalmitoyl PC in methanol. The best solvent

system was found to be methanol/water/chloroform (84/10/6) (Figure 10).

Experiments established that the bulk of the sodium ions eluted between 1 and 2 minutes and that the dipalmitoyl PC eluted between 8 and 24 minutes with a maximum between 10 and 12 minutes after injection . Attempts to narrow the lipid band also decreased the sodium-lipid separation thus lessening the applicability of the method to other more polar lipids. It was decided, therefore, to tolerate the wider lipid band.

Once reproducible elution times were established, lipid fractions between 9 and 18 minutes were collected, pooled, dried down and submitted for FD mass spectrometric analysis (Table 10). The peak at *m/z* 734 of 24% relative abundance representing $[M+H]^+$ suggests that the eluate contained considerable amounts of the intact phosopholipid. However, the average intensity per scan was low even though seven consecutive scans were acceptable enough for summation. There appears to be much fragmentation. Although the base peak was *m/z* 550, the 82% fragment at *m/z* 551 is too large to be an isotope of 550 and thus indicates a considerable amount of [M-phosphoryl choline]$^+$ was present. Other peaks characteristic of dipalmitoyl PC at *m/z* 104, 184, 86 and 129 represent choline, phosphoryl choline, $CH_2{=}CH\overset{+}{N}(CH_3)_3$ and $(CH_3)_3\overset{+}{N}CHCH_2OCH{=}CH_2$, respectively.

Fig. 10: HPLC Elution of Sodium Ion and Dipalmitoyl Phosphatidyl Choline

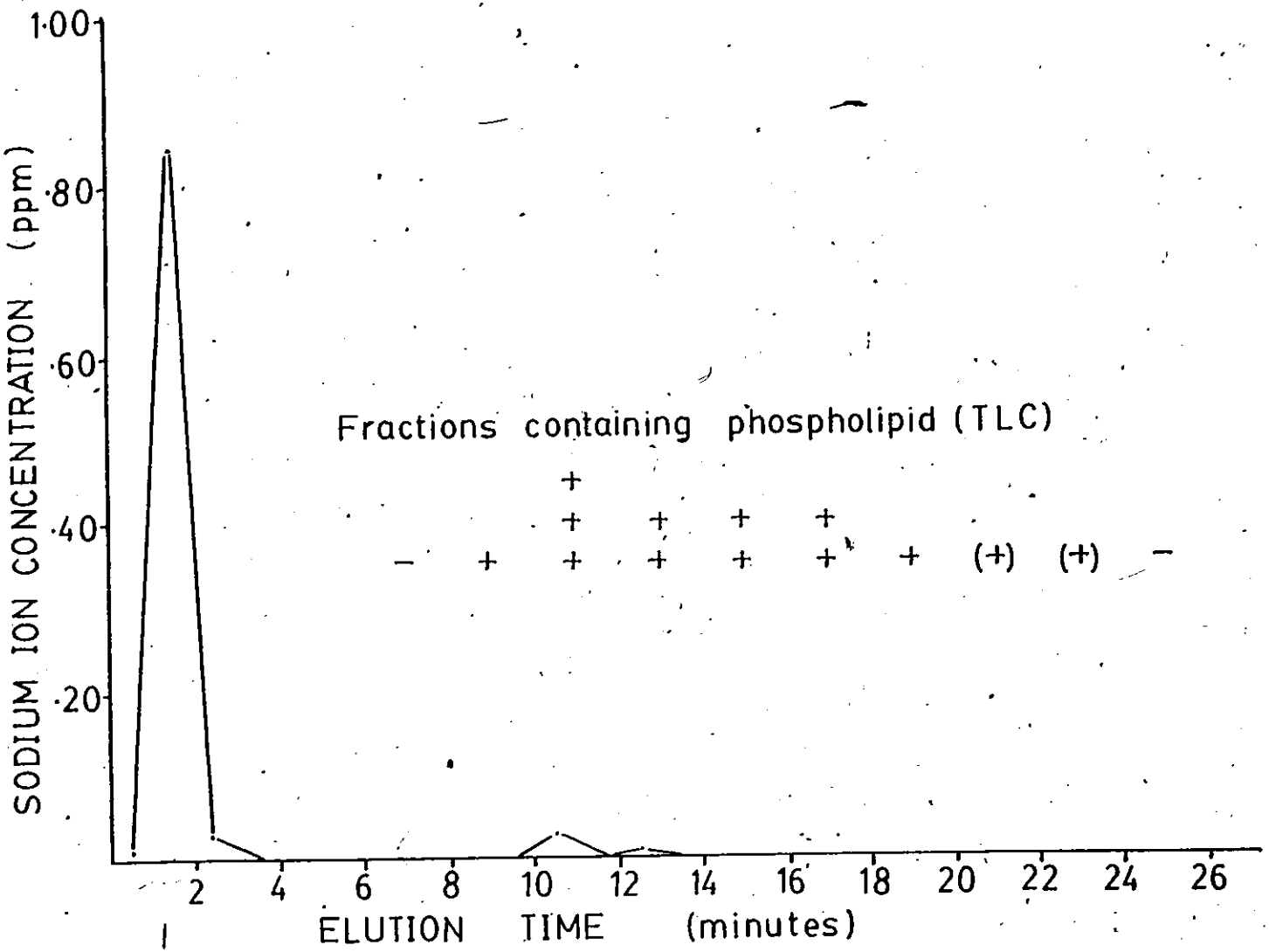

1·00
·80
·60
·40
·20
SODIUM ION CONCENTRATION (ppm)
Fractions containing phospholipid (TLC)
+
+ + + +
– + + + + + + (+) (+) –
2 4 6 8 10 12 14 16 18 20 22 24 26
ELUTION TIME (minutes)

Table 10

FDMS of HPLC-Eluted Dipalmitoyl Phosphatidyl Choline

m/z	Assignment	Rel. Abund. (%)[a] 18.0-20.1 mA[b] TIC=0.1[c]
86	$CH_2=CHN^+(CH_3)_3$	49
104	[choline]$^+$	52
129	$(CH_3)_3\overset{+}{N}CHCH_2OCH=CH_2$	27
184	[phosphoryl choline]$^+$	39
239		16
550		100
551	[M-phosphoryl choline]$^+$	82
552		19
734	[M+H]$^+$	24
735		55
736		15
756	[M+Na]$^+$	24
849	[M+$H_3PO_4 \cdot H_2O$]$^{+\cdot}$	13

[a] All ions greater than 5% rel. abund. are tabulated

[b] Anode heating current

[c] TIC = average total ion intensity per scan in arbitrary units

The $[M+Na]^+$ peak at *m/z* 756 suggests the presence of sodium even though most fractions collected showed no sodium. This could be explained by the fact that fractions with sodium levels below the flame emission detection limit, when combined and concentrated, resulted in a sample containing appreciable sodium.

CHAPTER IV

CONCLUSIONS

The results of this work have contributed to available data on the FD mass spectrometry of phospholipids, especially in the area of spectra enhancement. Several characteristics of the spectra have been confirmed; in addition, some new proposals have been introduced. The following is a list of the significant conclusions:

(1) The modified Bligh-Dyer extraction is a quick and efficient method of removing sodium from phospholipid samples thus providing samples of a purity that meets the requirements of FD mass spectrometry.

(2) The Bligh-Dyer method appears to be superior to HPLC separation on an RP-8 column for separating sodium from individual lipid samples.

(3) The removal of sodium may improve the phospholipid spectra in several ways by:
   (a) decreasing anode currents
   (b) increasing total intensity
   (c) decreasing fragmentation
   (d) increasing abundance of $[M+H]^+$

(4) The addition of water to hygroscopic phospholipids may improve mediocre spectra by (a) and (b) and sometimes (c) and (d).

(5) *p*-TSA addition has limited applicability to phospholipid FD mass spectrometric analysis.

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