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ABBREVIATIONS

ADP	adenosine diphosphate
ATP	adenosine triphosphate
t-BDMS	tert-butyldimethylsilyl
BHT	butylated hydroxy toluene
cAMP	cyclic adenosine monophosphate
CI/MS	chemical ionization mass spectrometry
CP	creatine phosphate
CPK	creatine phosphokinase
DEHP	di(2-ethylhexyl)phthalate
DHAP	dihydroxy acetone phosphate
DMA	dimethyl acetal
FAB/MS	fast-atom bombardment mass spectrometry
FAME	fatty acid methyl ester
GLC	gas-liquid chromatography
HPLC	high-performance (pressure) liquid chromatography
21:0 ME	methyl heneicosanoate
MAGD	monoalkyl glycerol diacetate
MEHP	mono (ethylhexyl)phthalate
NADPH	nicotinamide diphosphate
NL	neutral lipid
P	phosphate
PA	phosphatidic acid
Paf	platelet-activating factor
PC	phosphatidyl choline
PE	phosphatidyl ethanolamine

PGI_2	prostacyclin
PI	phosphatidyl inositol
PL	phospholipid
PRP	platelet-rich plasma
PS	phosphatidyl serine
SPH	sphingomyelin
TLC	thin-layer chromatography
TMS	trimethylsilyl

ABSTRACT

Platelets stored in PL 732 bags at 22°C for a minimum of three days lose their ability to respond to single stimuli. The aims of this study were to examine the effects of storage on platelet lipid composition, more specifically on the arachidonic acid and the alkylacyl class content of the major phospholipids.

A procedure for the determination of the proportions of diacyl, alkenylacyl and alkylacyl classes of glycerophospholipids was developed. The procedure involved: (1) methanolysis of the phospholipid followed by Bligh/Dyer extraction of fatty acid methyl esters (FAMEs) derived from acyl chain types, dimethylacetals (DMAs) derived from alkenyl ether chain types and lysoalkyl phosphatidic acids (lysoalkyl-PAs) derived from alkyl ether chain types; and (2) subsequent acetolysis to convert the lysoalkyl-PAs to monoalkyl glycerol diacetates (MAGDs). GLC analysis of the FAMEs and DMAs after methanolysis and MAGDs after acetolysis, with internal standard (21:0 FAME), was used for identification and quantification of each hydrocarbon chain types, allowing calculation of the proportions of the three molecular classes. The methanolysis/acetolysis procedure resulted in a mean phospholipid recovery of 96 $\pm$ 4%.

The results of application of this procedure to fresh resting platelets were in agreement with those obtained by

other procedures. Phosphatidylcholine (PC) contained 10% alkylacyl class and lesser amounts of alkenylacyl class. Molecular species analysis by fast-atom bombardment mass spectrometry (FAB/MS) indicated a high amount of 16:0-18:1 diacyl PC. Arachidonate was predominately associated with the diacyl PC. In addition, FAB/MS suggested the presence of Paf molecules in resting platelets, which might be an endogenous source of preformed Paf. Phosphatidylethanolamine (PE) analysis demonstrated a higher alkylacyl content (8.0%) than previously reported. The alkenylacyl content was 27% and the arachidonic acid content was 47%. FAB/MS studies indicated that the arachidonate was associated mainly with plasmalogen or alkenylacyl class of PE.

The effects of storage on platelet lipid components are listed below:

1) A significant decrease in the alkylacyl class (16 to 10 nmol per 10^9 cells) content of PC after three days of storage but the loss was not significant with respect to the availability of Paf precursor as only 2% would be sufficient to be activating;

2) No significant change in the total arachidonate concentration (245 nmol per 10^9 cells in fresh resting platelets);

3) No significant change in the cholesterol, total phospholipid or cholesterol to phospholipid molar ratio;

4) No significant changes in the phospholipid composition;

5) No significant change in the alkenylacyl or diacyl classes of total lipids, PC or PE;

6) No significant changes in the hydrocarbon chain composition of diacyl and alkenyl classes of total lipids, PC or PE.

These studies indicate that the lipid composition of stored platelets remains similar to that of fresh platelets: there was no significant loss of arachidonic acid or Paf precursors from endogenous phospholipids to account for the inability of stored platelet to respond to single stimuli. Taking into account other recent work [Imai, A., Takahasi, M. and Nozawa, Y. (1984). Cryobiology, 21, 51; Labow, R.S., Tocchi, M., Adams, G.A. and Rock, G. (1986). manuscript in preparation] and the present study, this inability of stored platelet to respond is suggested to be the result of the inhibition of the phospholipase A_2 activity by plasticizers from the PL 732 bags used for platelet storage.

A. INTRODUCTION

Platelets are the smallest cells in the circulating human blood. They number approximately 2×10^8 to 4×10^8 /ml (Weiss, 1975) and remain in the circulation for a lifespan of 7 to 10 days (Zucker, 1974). The platelet is derived from the largest cell in the bone marrow, the megakaryote, and is devoid of a nucleus, DNA, rough endoplasmic reticulum and ribosomes (Marcus, 1978).

The primary function of blood platelets is the prevention of hemorrhage by quick response to the exposure of subendothelium and collagen that results in the formation of a haemostatic plug at the site of injury. Besides collagen, other physiological agents such as adenosine diphosphate (ADP), thrombin, epinephrine and arachidonic acid are all able to elicit platelet response consisting of change of cellular shape, degranulation or secretion, and aggregation. Pairs of these agents act synergistically, that is, neither agent alone is stimulatory, but in combination, they produce full response. When platelets are isolated from whole blood, concentrated and stored as platelet concentrates for use in transfusion therapy, the platelet response to a single stimulus markedly decreases while its synergistic response is retained. Despite this decrease which can be noted after a minimum of three days of storage, the stored platelets remain therapeutically effective in thrombocytopenic patients.

Although the mechanisms that modulate the phenomenon of haemostasis are not yet completely elucidated, recent biochemical studies of the platelet membrane indicate that the composition of its phospholipid and their constituent molecular species are rapidly altered when platelets are activated by physiological agents such as thrombin (Skeaf and Holub, 1985).

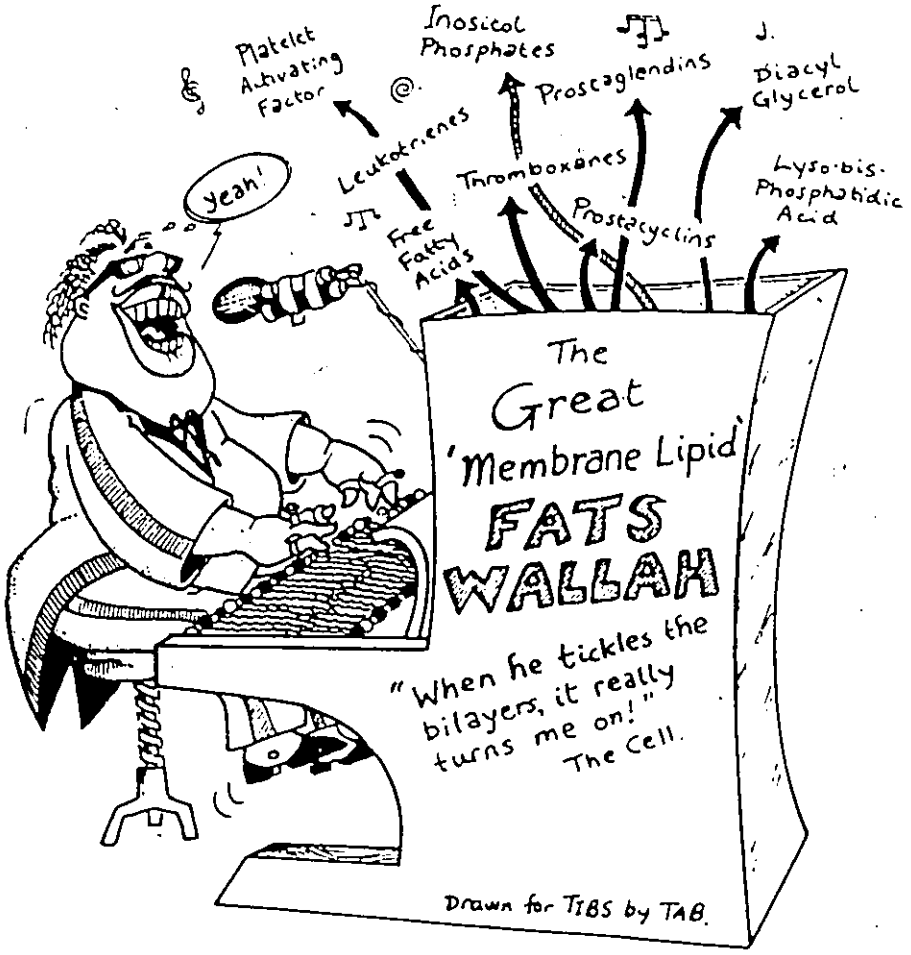
I. Pathways of Platelet Activation

Primary activators of platelets are collagen, ADP, epinephrine, arachidonate metabolites, thrombin and Paf which act through receptors on the platelet surface. The three most studied pathways are the release of ADP, the liberation and metabolism of arachidonic acid, and the synthesis of Paf. Each one of these pathways generates compounds which act as intercellular mediators to propagate the rapid activation of all platelets (Fig.1). These pathways are discussed further in detail below.

I.1 ADP

The mechanism of platelet activation by ADP has not yet been completely elucidated, however two hypotheses have been proposed. One hypothesis is the binding of ADP to a nucleotide diphosphokinase on the platelet surface. This would be consistent with the conversion of exogenous ADP to ATP during platelet aggregation (Mustard et al., 1975). The

Figure 1: Formation of Secondary Stimulants from Platelets (from Pagano and Sleight, 1985).


Platelet Activating Factor
Inositol Phosphates
Prostaglandins
Diacyl Glycerol
Leukotrienes
Thromboxanes
Lyso-bis-Phosphatidic Acid
Prostacyclins
Free Fatty Acids
Yeah!
The Great 'Membrane Lipid' FATS WALLAH
"When he tickles the bilayers, it really turns me on!" The Cell.
Drawn for TIBS by TAB.

shunting of the endogenous ATP to act as phosphate and energy donor to this process and away from normal phospholipid synthesis or cyclic adenosine monophosphate (cAMP) production could be the activation signal for the cellular response (Lam et al., 1982).

An alternative hypothesis is that a decrease in cAMP within the platelet results from the binding of ADP to a regulatory portion of the adenylate cyclase complex (Salzman, 1972; Gorman et al., 1977; MacFarlane et al., 1982). cAMP levels have been observed to drop slightly upon stimulation and large increases in cAMP inhibit platelet stimulation by even the most powerful stimulants. However, it is not clear how decreases in cAMP would initiate the intracellular events that occur before aggregation.

Exogenous ADP or ADP from platelets or from red blood cells can activate platelets. Removal of ADP by enzymes such as apyrase and creatine phosphate/creatine phosphokinase or by addition of exogenous ATP or other triphosphate nucleotides all inhibit specifically the ADP-induced platelet aggregation (Mustard et al., 1975; MacFarlane and Mills, 1975; Kinlough-Rathbone et al., 1977). These approachs have been used to determine the role of ADP released from platelet granules during platelet activation. A positive feedback amplification is possible: ADP released from platelets has been shown to be one of the amplification pathways whereby stimulants such as collagen and thrombin activate platelets

(Kinlough-Rathbone, 1977; Packham and Mustard, 1984).

I.2 Release and Metabolism of Arachidonic Acid

Arachidonic acid is released from membrane phospholipids by the direct action of phospholipase A_2 or by the indirect action of phosphatidylinositol-specific phospholipase C to form diglyceride and the subsequent actions of diglyceride lipase and monoglyceride lipase to liberate arachidonate (Fig. 2), (Bell et al., 1979; Prescott and Majerus, 1983). Arachidonate metabolism (Fig. 2) results in the formation of prostaglandins, thromboxanes and hydroxy fatty acids some of which constitute the second pathway for platelet activation (Lusher, 1978; Marcus, 1978; Moncada and Vane, 1979; Wautier and Caen, 1979 Mustard et al., 1980). Arachidonate metabolites, prostaglandins (PG)G_2 and H_2 and thromboxane A_2 (TXA_2), can all induce platelet aggregation and appear to work through the same receptor as trans-13-azaprostanoic acid (13-APA) which can block all three compounds. Prostaglandin D_2 inhibits platelet response through a receptor different from the PGI_2 and PGE_1 receptor (Fig. 3). A number of inhibitors for the arachidonate pathway at various levels have been reported. Mepacrine inhibits the arachidonic acid release from membrane phospholipids (Winocour et al., 1982; Chan et al., 1982). Non-steroidal; antiinflammatory drugs such as aspirin, sulfinpyrazone and indomethacin all inhibit the conversion of arachidonate to PGG_2 and PGH_2 by the enzyme

Figure 2: Arachidonic Acid Release and Metabolism (from Packham and Mustard, 1984).

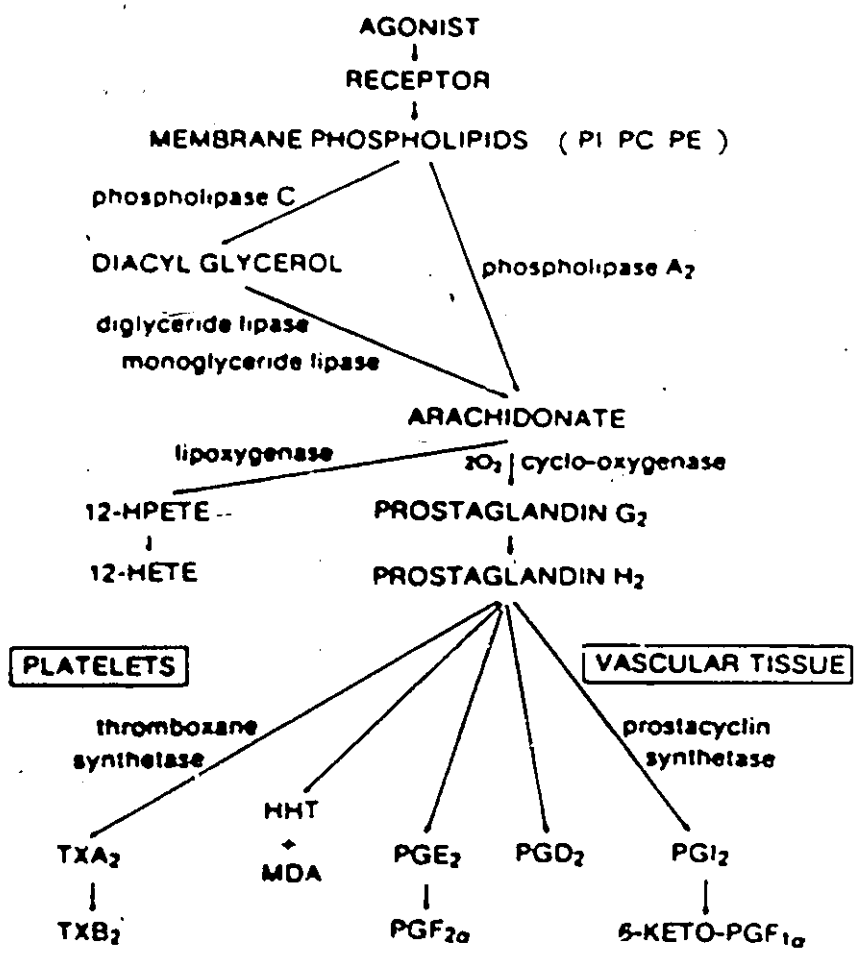
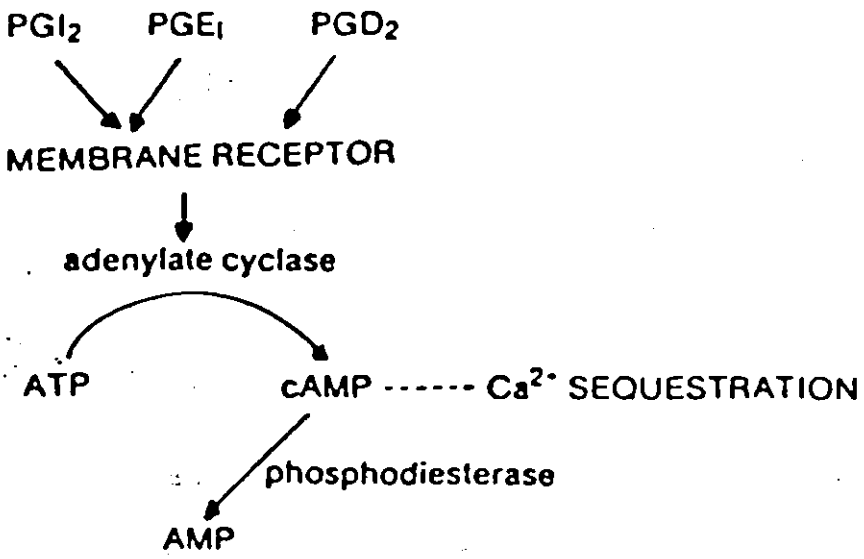

AGONIST
RECEPTOR
MEMBRANE PHOSPHOLIPIDS (PI PC PE)
phospholipase C
DIACYL GLYCEROL
phospholipase A2
diglyceride lipase
monoglyceride lipase
ARACHIDONATE
lipoxygenase
2O2 | cyclo-oxygenase
12-HPETE
12-HETE
PROSTAGLANDIN G2
PROSTAGLANDIN H2
PLATELETS
VASCULAR TISSUE
thromboxane synthetase
prostacyclin synthetase
HHT
MDA
TXA2
TXB2
PGE2
PGF2α
PGD2
PGI2
6-KETO-PGF1α

Figure 3: Role of Arachidonic Acid Metabolites in Platelet Activation (from Packham and Mustard, 1984)


PGI2
PGE1
PGD2
MEMBRANE RECEPTOR
adenylate cyclase
ATP
cAMP ------ Ca2+ SEQUESTRATION
phosphodiesterase
AMP

cyclo-oxygenase. Other compounds such as imidazole and 9,11-azoprosta- 5,13- dienoic acid, have been reported to inhibit thromboxane synthetase which converts PGH_2 into TXA_2 (Wautier and Caen, 1979).

I.3 Platelet-Activating Factor (Paf)

Platelet-activating factor (Paf) is a potent aggregator of platelets in the presence of aspirin (inhibits AA metabolism) and apyrase or CP/CPK (removal of ADP) indicating that there is a third pathway for platelet activation. The structure of Paf has been identified as 1-O-alkyl-2-acetyl sn-glycero-3-phosphocholine (Fig. 4), (Demopoulos et al., 1979; Benveniste et al., 1979; Blank et al., 1979; Hanahan et al., 1980). The structural characteristics of Paf which contribute to its potent biological activity have been examined by several laboratories. Among the structural features found to be functionally important were the hydrocarbon chain length and type of linkage found at the sn-1 and sn-2 positions, the nature of the head group and the stereochemistry of the glycerol moiety (Fig. 5), (Snyder, 1985). Paf molecules synthesized in vivo contain a heterogeneous mixture of chain lengths from C14 to C20 at the sn-1 position of the glycerol moiety. The most potent Paf molecule is 1-hexadecyl- 2-acetyl- sn-3-glycerophosphocholine which is active at a minimum concentration of 10^{-7} M for human platelets (Packham and Mustard, 1984).

Figure 4: Structural Formula for Paf

R_1= alkyl chain, n= 13 - 19

R_2= acetate group

R_3= phosphocholine head group

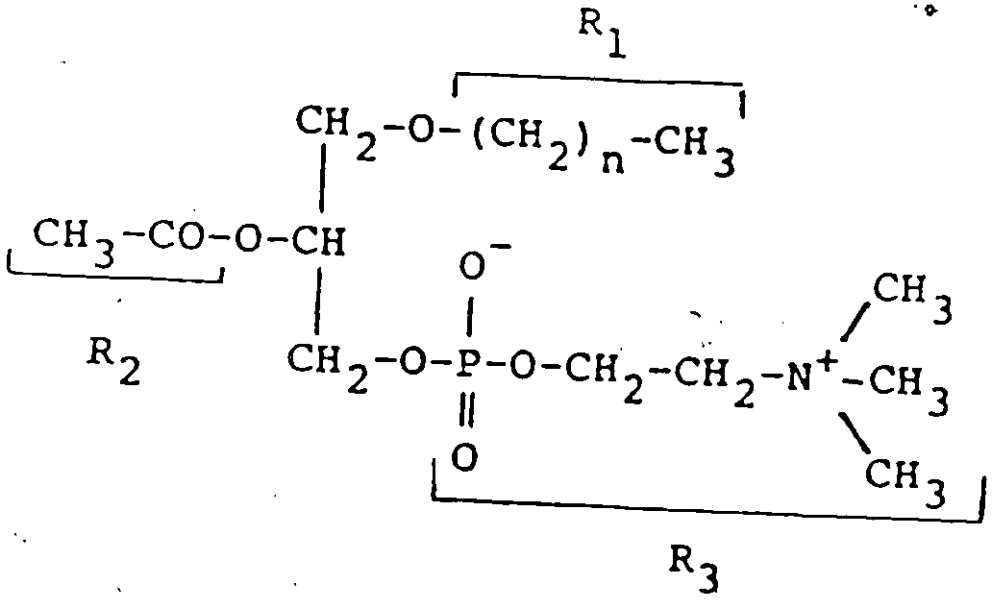
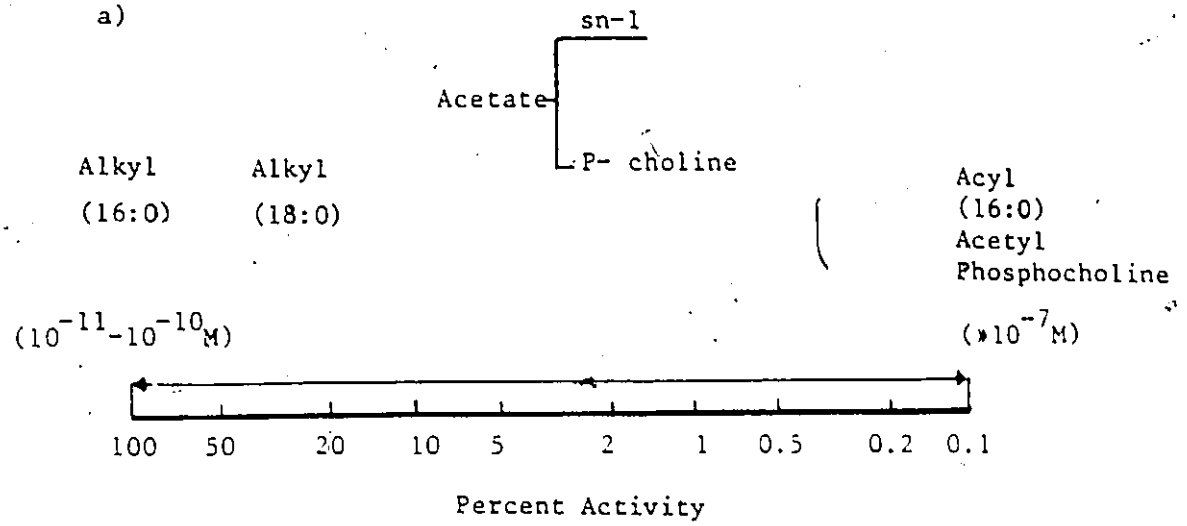
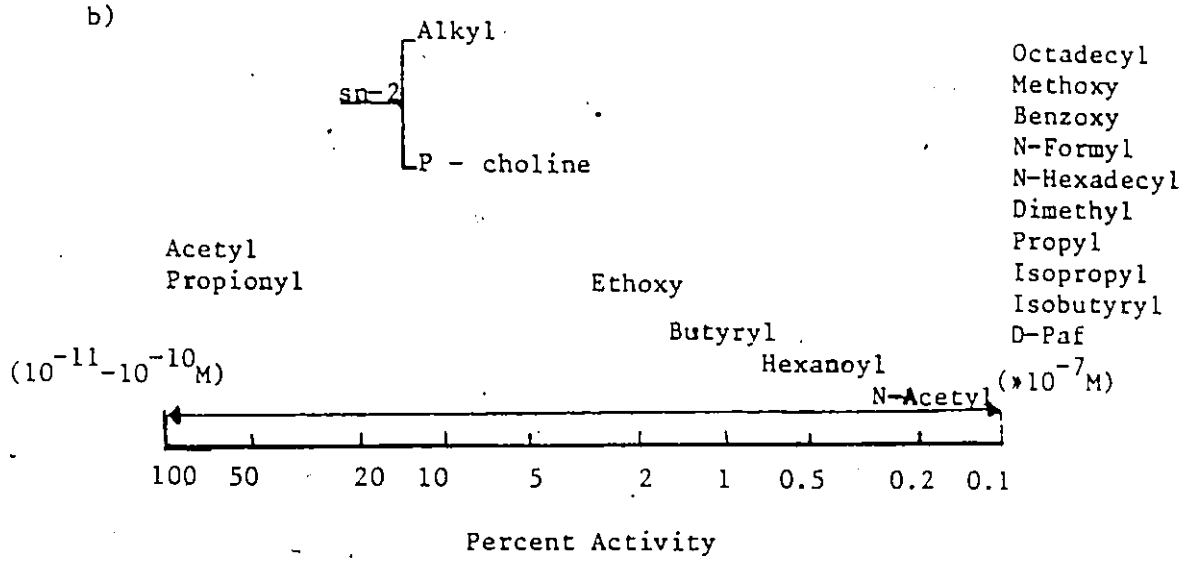

R1
CH2-O-(CH2)n-CH3
CH3-CO-O-CH
O-
CH3
R2
CH2-O-P-O-CH2-CH2-N+-CH3
O
CH3
R3

Figure 5: Effect of Various Chemical Substituents at a) sn-1
b) sn-2
c) sn-3
Positions of Paf Molecule on its Potentcy as Measured by Serotonin Release from Rabbit Platelets (from Snyder, 1985).

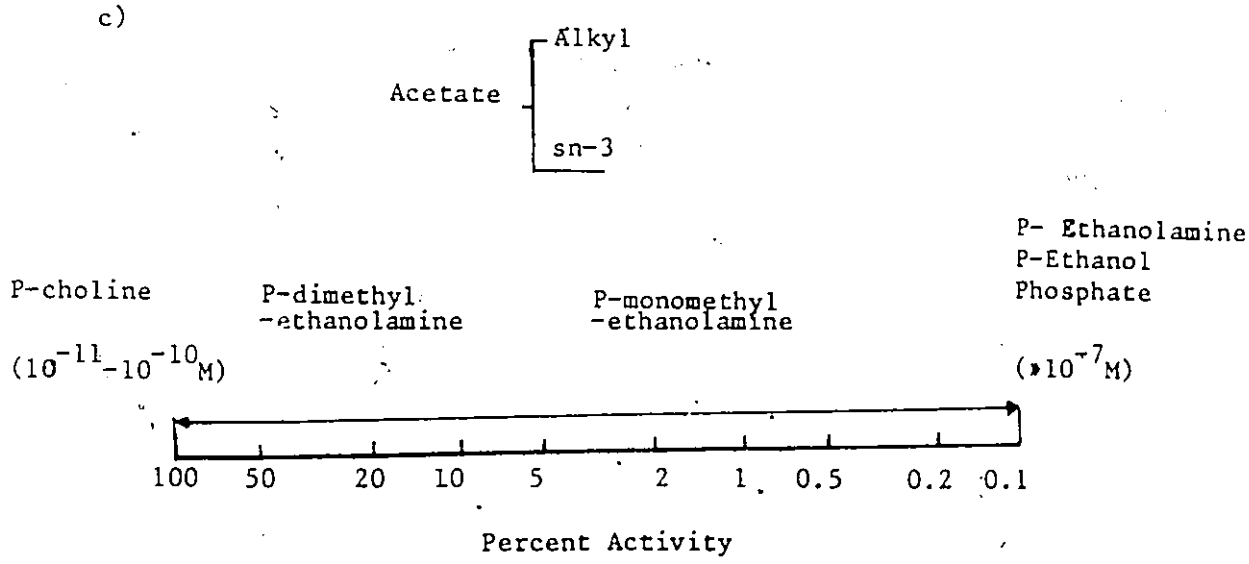
a)


sn-1
Acetate
P- choline
Alkyl
(16:0)
Alkyl
(18:0)
Acyl
(16:0)
Acetyl
Phosphocholine
(10^{-11}-10^{-10}M)
(»10^{-7}M)
100 50 20 10 5 2 1 0.5 0.2 0.1
Percent Activity

b)


Alkyl
sn-2
P - choline
Octadecyl
Methoxy
Benzoxy
N-Formyl
N-Hexadecyl
Dimethyl
Propyl
Isopropyl
Isobutyryl
D-Paf
Acetyl
Propionyl
Ethoxy
Butyryl
Hexanoyl
N-Acetyl
(10^{-11}-10^{-10}M)
(»10^{-7}M)
100 50 20 10 5 2 1 0.5 0.2 0.1
Percent Activity

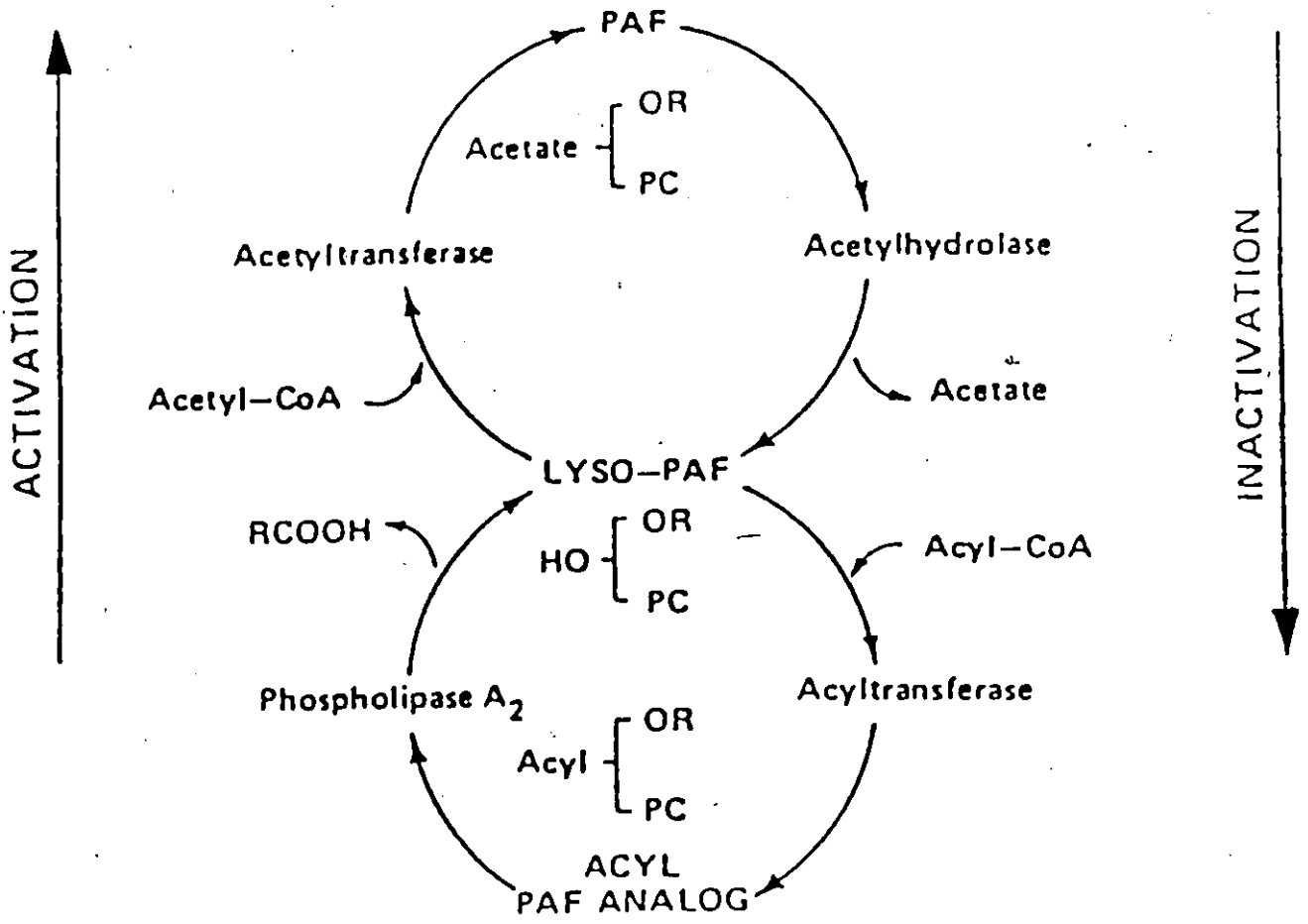
c)


Alkyl
Acetate
sn-3
P- Ethanolamine
P-Ethanol
Phosphate
P-choline
P-dimethyl
-ethanolamine
P-monomethyl
-ethanolamine
(10^{-11}-10^{-10}M)
(»10^{-7}M)
100 50 20 10 5 2 1 0.5 0.2 0.1
Percent Activity

Two pathways for the synthesis of Paf have been demonstrated. In the first pathway, 1-O-alkyl-2-acetyl-PC is synthesized from 1-O-alkyl-2-acetyl-glycerol by a CDP-choline: 1-O-alkyl-2-acetyl-glycerol choline transferase reaction (Renooij and Snyder, 1981). In the second pathway, the most studied of the two, Paf is synthesized from 1-O-alkyl-2-acyl-PC via a deacylation by a phospholipase A_2 reaction to a lyso-Paf and then acetylated by an acetyltransferase (Snyder, 1985).

Studies of the second pathway have led to the proposal that Paf regulation occurs via an activation -inactivation cycle illustrated in Fig. 6. The properties of the enzymes (acetyl CoA: alkyl-GPC acetyltransferase coupled with phospholipase A_2) involved in the conversion of alkylacyl PC to Paf, as well as the specific catabolic enzyme, acetylhydrolase have been characterized in human and rabbit platelets (Snyder et al., 1983; Benveniste and Vargaftig, 1983; Alam et al., 1983). Phospholipase A_2 inhibitors such as EDTA, bromophenacyl bromide, or mepacrine prevent the release of Paf (Mencia-Huerta et al., 1981) leading to the formation of 1-O-alkyl-GPC (lyso-Paf). That lyso-Paf was indeed the precursor of Paf was determined by experiments in which platelets were incubated with cold acetyl-CoA and tritiated lyso-Paf to yield radiolabelled Paf (Chap et al., 1981). This was strengthened by experiments showing incorporation of tritiated acetyl-CoA or tritiated lyso-Paf into the active

Figure 6: Activation and Inactivation Cycle for Paf Regulation


PAF
OR
Acetate
PC
Acetyltransferase
Acetylhydrolase
Acetyl–CoA
Acetate
LYSO–PAF
RCOOH
HO
OR
PC
Acyl–CoA
Phospholipase A2
Acyltransferase
Acyl
OR
PC
ACYL
PAF ANALOG
ACTIVATION
INACTIVATION

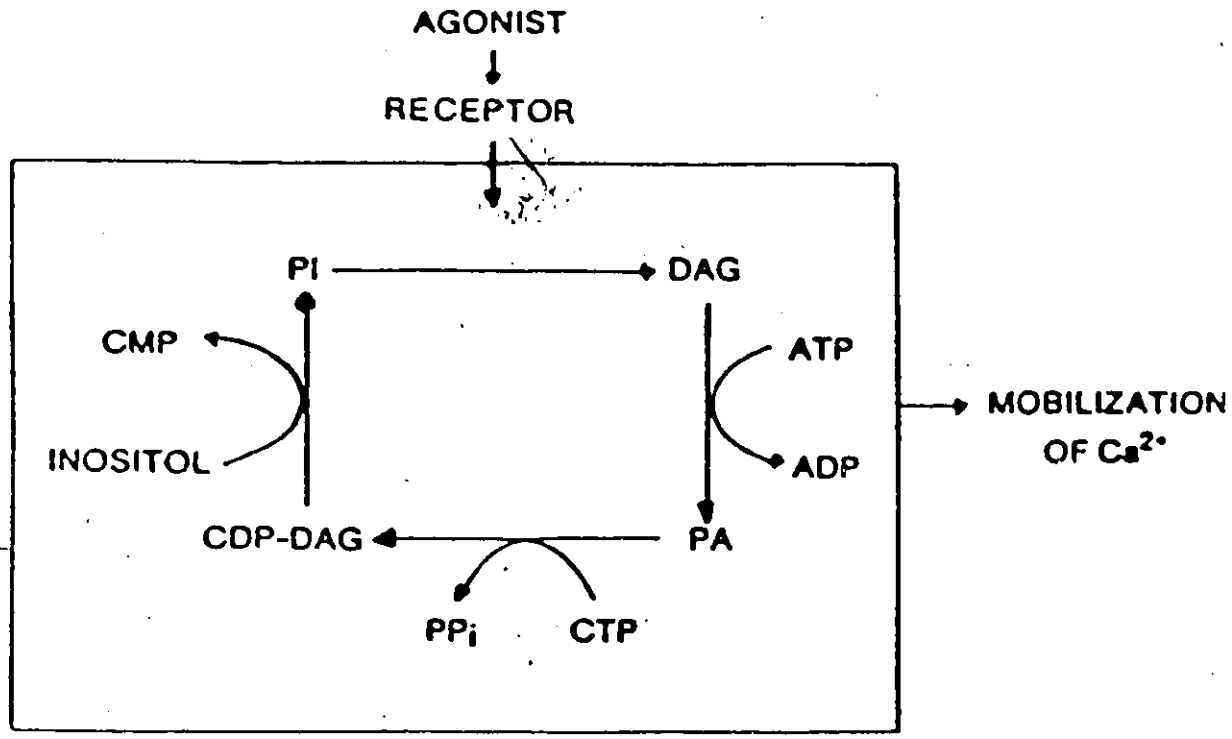
molecule (Ninio et al., 1982). Chap et al., (1981) also demonstrated incorporation of labelled acetate in the Paf released from platelets. The basal acetyltransferase activity is transiently increased upon thrombin activation reaching a maximum within 2 minutes. This process may then be regulatory in the formation of Paf in platelets (Coeffec et al., 1983).

I.4 Proposed Mechanism of Platelet Activation

Study of platelet activation suggest that all of the stimulating agents may act through a receptor and results in Ca^{2+}-mobilization (from 10^{-8} - 10^{-7} M to 10^{-6} - 10^{-5} M) which in turn causes certain biochemical changes (Lapetina, 1983; Packham and Mustard, 1984). One such biochemical change is the degradation of inositol phospholipids, especially phosphatidylinositol-4, 5-bisphosphate (PIP_2) (Fig. 7). These lipids are located at the cytoplasmic face of the platelet membrane and provide important cellular Ca^{2+}-binding sites (Dawson, 1965; Harrison and Long, 1968; Hendrickson and Reinertsen, 1969; Billah and Lapetina, 1982). The triggering of the PI cycle results in the subsequent formation of 1,2-diacylglycerol, inositol phosphatides and lysophosphatidic acid (LPA) (Lapetina, 1970; Lapetina et al., 1981; Lapetina, 1982; Billah and Lapetina, 1982). 1,2-diacylglycerol can activate protein kinase C and LPA might act as an intracelllular Ca^{2+}-ionophore (Gerrard et

Figure 7: Phosphoinositide Cycle

(from Packham and Mustard, 1984)


AGONIST
RECEPTOR
PI
DAG
CMP
ATP
INOSITOL
ADP
CDP-DAG
PA
PPi
CTP
MOBILIZATION
OF Ca2+

al., 1978) and thus cause further release of Ca^{2+} from the dense tubular system (Gerrard et al., 1978). This Ca^{2+} mobilization might then stimulate phospholipase A_2 which would act to liberate arachidonic acid from phospholipids such as PC, PE, PI and PA (Lapetina, 1970; Lapetina, 1982; Lapetina, 1983).

I.5 Synergisms

All of the aggregating agents (ADP, TXA_2, collagen, epinephrine, serotonin, thrombin, and many others) appear to act synergistically with each other so that concentrations that are themselves insufficient to cause aggregation can greatly amplify the response to a low concentration of another aggregating agent (Kinlough-Rathbone et al., 1977; Grant and Scrutton, 1980; Rao and White, 1981; Cameron and Ardlie, 1982; Rittenhouse and Allen, 1982; Di Minno et al., 1982, 1983; Vargaftig et al., 1983). It seems likely that, *in vivo*, platelets are rarely exposed to only one aggregating agent; the more-than-additive effects of several agents are undoubtedly important in haemostatic plug and thrombus formation. The existance of synergisms suggests a point of convergence of activation pathways at the level of an intracellular messenger. It is not clear how or why all these different stimulants result in the same responses.

I.6 Effect of Storage on Platelet Activation

Platelets are normally stored under blood banking conditions for three to five days (DiMinno et al., 1983). They retain unchanged serotonin uptake, but have a decreased hypotonic response and pH. The stored platelets exhibit change in cellular shape, loss of membrane glycoprotein and loss of glycogen, dense and alpha granules (Adams, 1982). In addition, there is a drop in the cell count after recovery with increase in the median cell volume. Cyclo-oxygenase (Roth et al., 1982), and phospholipase C remain unchanged while phospholipase A_2 activity is decreased (Labow et al., 1986a, manuscript in preparation).

These stored platelets do not respond to a single stimulus of epinephrine, ADP, collagen or arachidonic acid unless high concentrations are used; the synergistic reactions to pairs of these agents remain unchanged (Rock et al., 1985; Adams et al., 1986).

Furthermore platelet ability to correct prolonged bleeding time in thrombocytopenic patients is recovered within several hours upon transfusion (Slichter and Harker, 1976; Slichter, 1980). It is unknown how platelets are transformed or rejuvenated upon transfusion. Synergistic action of several stimuli might be the physiological pathway of activation. Thus stored platelets provide an interesting model to study the synergisms and the role of membrane phospholipids in platelet activation.

II. Platelet Membrane Composition

Marcus et al.(1969) have identified the major lipid classes in addition to the individual fatty acid components in isolated platelet plasma membranes. He reported that the ratio of lipid to protein was 0.58 by weight with phospholipids comprising 78% of the total lipid and cholesterol accounting for 90 % of the neutral lipid. The molar ratio of cholesterol to total phospholipid in these membranes was 0.53 which is in agreement with the value (0.50) obtained by Barber and Jamieson (1970) and Kaulen and Gross (1973).

II.1 Role of Cholesterol in Platelet Activation

Derksen and Cohen (1973) have shown that platelets are unable to synthesize cholesterol. They cannot form mevalonate nor can they incorporate mevalonate into cholesterol. The platelet cholesterol content reflects the initial composition of the megakaryocyte; although exchange of cholesterol with the plasma lipoproteins may result in modification of the endogenous platelet cholesterol content.

Previous studies suggest that changing the cholesterol content of the platelet membrane alters the platelet response to aggregating agents. Carvalho et al. (1973) studied platelet function in 17 patients with Type II hyperbetalipoproteinenemia, a condition in which there is an increased platelet cholesterol/phospholipid ratio (Bennett et

al., 1974). They found that platelets from these patients, as compared to normal subjects, had an enhanced response to epinephrine, collagen, and ADP. These experiments suggest a relationship between plasma content and platelet function, but did not establish the mechanism as normal platelets incubated with plasma from these patients did not have increased sensitivity. More direct evidence linking platelet cholesterol content to function was provided by Shattil and coworkers (1975). They prepared cholesterol/ phosphatidylcholine liposomes which were 'cholesterol normal' (cholesterol/ phosphatidylcholine mole ratio, 1:1), 'cholesterol rich' (cholesterol/ phosphatidylcholine ratio, 2:1) or 'cholesterol poor' (devoid of cholesterol). Incubation with cholesterol poor liposomes lowered platelet membrane cholesterol. Cholesterol normal liposomes did not change the platelet membrane cholesterol. The acquisition of cholesterol by platelets was associated with a 35-fold increase in sensitivity to epinephrine-induced aggregation. Reduction of platelet membrane cholesterol was associated with a 18-fold reduction in sensitivity to epinephrine. Of interest in this work is the concept that the decrease in sensitivity of platelets to stimulation after storage could be due to a decrease in cholesterol content.

II.2 Phospholipid Classes

Besides major classes, phospholipid components may be

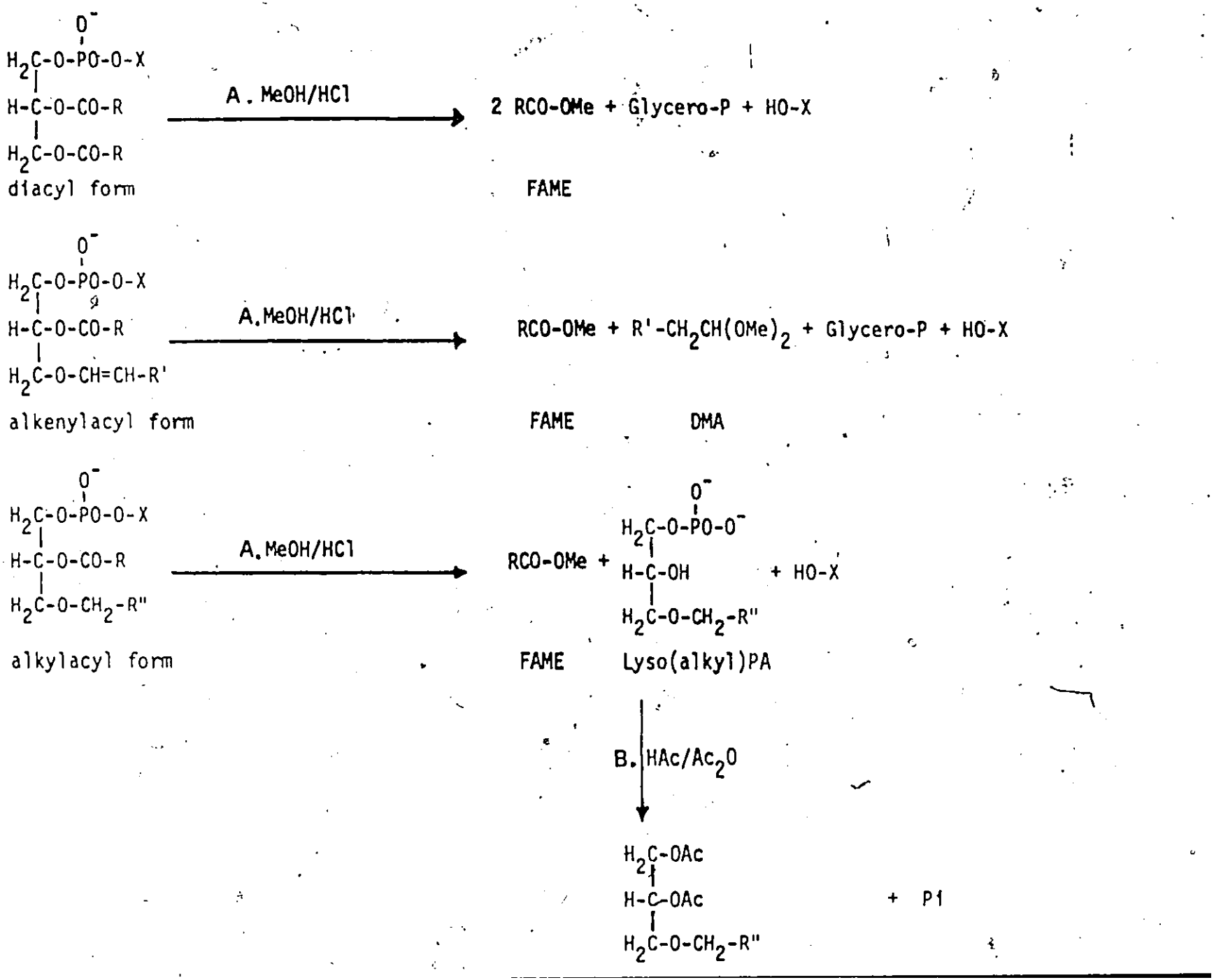
further grouped into three major subclasses depending on the nature of the linkage of the hydrocarbon chains to the glycerol backbone: those compounds having ester linkages at both sn-1 and sn-2 positions are "diacyl" phospholipids; those having a saturated ether linkage at the sn-1 position and an ester at sn-2 are "alkylacyl" phospholipids; and those having a vinyl ether linkage at the sn-1 and ester at sn-2 are "alk-1'-enylacyl" phospholipids (Fig. 8).

II.3 Properties of the Ether Linkage

Ether lipids are characterized by the presence of alkyl or (Z)-1'-alkenyl ether groups. The absence of the carbonyl dipoles in ether lipids makes them less polar than their ester analogues and consequently their abundance in biological membranes may contribute to: i) differences in dipolar membrane potential, compared to membranes containing mainly ester lipids; ii) to a reduction in hydrogen bonding between the phospholipid molecules and between potential hydrogen donors such as cholesterol, protein and water and iii) formation of closely packed hydrocarbon region and a less ordered polar head region of the membranes (Paltauf, 1983).

The alkyl ether bond resists chemical and enzymatic hydrolysis by phospholipases. The alk-1'-enyl ether bond however is acid labile, but stable towards alkali and phospholipase action. These chemical properties are useful to

Figure 8: Reaction Products of Diacyl, Alkenylacyl and Alkylacyl classes of Phospholipids
A) After Methanolysis
and B) After Acetolysis


A . MeOH/HCl
2 RCO-OMe + Glycero-P + HO-X
diacyl form
FAME
A. MeOH/HCl
RCO-OMe + R'-CH2CH(OMe)2 + Glycero-P + HO-X
alkenylacyl form
FAME
DMA
A. MeOH/HCl
RCO-OMe +
+ HO-X
alkylacyl form
FAME
Lyso(alkyl)PA
B. HAc/Ac2O
+ P1

separate diacyl forms from the ether lipids analytically. The double bond adjacent to the ether oxygen in alk-1'-enyl lipids is very susceptible to acid hydrolysis (Hanahan, 1972). Exposure of alk-1'-enyl glycerophospholipid to HCl produces fatty aldehydes or if the acid catalyzed reaction is carried out in methanol (methanolysis), dimethylacetals (DMAs) of the aldehydes and fatty acid methyl esters (FAMEs) of the acyl chains are formed while the saturated ether linkage remains intact to the mild acid hydrolysis resulting in the formation of 1-alkyl-2-lyso-PA (Fig. 8) (Gray, 1969). The 1-alkyl-2-lyso PA may be acetylated by acetolysis with acetic anhydride/acetic acid to form monoalkylglycerol diacetates (MAGDs) (Fig. 8). The DMAs, FAMEs and MAGDs can be separated according to chain length and number of double bonds using similar GLC conditions as for methyl ester analysis, and can be identified by their retention times relative to standards, or by using equivalent chain length (ECL) values.

A particularly useful reaction in establishing the nature of the ether bond involves the use of iodine and methanol (Siggia and Edsberg, 1948). This reaction forms the basis of a quantitative analytical procedure for alk-1'-enyl ethers (Gottfried and Rapport, 1962) in which uptake of iodine is measured colorimetrically:

$$R_1\text{-}CH_2\text{-}O\text{-}CH{=}CH\text{-}R_2 + I_2 \longrightarrow [R_1\text{-}CH_2\text{-}O\text{-}CH(I)\text{-}CH(I)\text{-}R_2]$$

$$[R_1\text{-}CH_2\text{-}O\text{-}CH(I)\text{-}CH(I)\text{-}R_2] \xrightarrow{CH_3OH} R_1\text{-}CH_2\text{-}O\text{-}CH(OCH_3)\text{-}CH(I)R_2 + HI$$

The reaction is fast and provides a facile approach to quantitative analysis of the vinyl ether bond in lipid mixtures.

The alk-1'-enyl group is relatively stable to alkali treatment. Pietruszko and Gray (1962) found that lysoplasmalogens and fatty acids were the sole lipid products when choline or ethanolamine phospholipid was hydrolysed in 0.5N NaOH (38°C). This technique was used to separate dimethylacetals from the fatty acid methanolysates after methanolysis of phospholipid and to see whether DMAs overlap or are hidden by FAMEs on GLC.

Hydrogenation of the double bonds in aliphatic groups including the one adjacent to the ether bond in plasmalogens, makes it possible to prepare stable derivatives of the alk-1'-enyl lipids. Hydrogenation can be carried out with Adams (PtO_2) or palladium (Pd/C) catalyst in 95% ethanol at room temperature and atmospheric pressure (Snyder and Blank, 1969). This reaction was useful in preparing a mixture of diacyl, alkenylacyl and alkylacyl PE from PE that contains mainly diacyl and alkenylacyl classes for preliminary assessment of the efficiency of the proposed method for analysis of these species (see Materials I.2).

II.4 Phospholipid Composition, Biosynthesis and Metabolism

Phospholipids in human platelets consist of about 40 mole% PC, 23-28 mole% PE, 9-11 mole% PS, 3-6 mole% PI and 18-20 mole% Sph (Cohen and Derksen, 1969; Owen et al., 1981;

Mahadevappa and Holub, 1982). PC and PE may be further subdivided into subclasses: PC was found to contain 80% diacyl, 10% alkylacyl and 9% alkenylacyl; PE contained 36% diacyl, 4% alkylacyl and 60% alkenlylacyl (Mueller et al., 1983).

Fatty acid analyses have revealed that the major fatty acid residues in all classes of choline and ethanolamine phospholipids in resting platelets are palmitic (16:0), stearic (18:0), oleic (18:1), linoleic (18:2) and arachidonic (20:4) acids. The alkylacyl and alkenylacyl PE species also contained significant amounts of 22:4, 22:5 and 22:6 acyl chains (Marcus et al., 1969; Mahadevappa and Holub, 1982; Mueller et al., 1983; Kramer and Deykin, 1983).

The fatty acid composition of each class of platelet phospholipid is unique but very little is known about the mechanisms which regulate the distribution of fatty acids among the phospholipid classes. Studies by several groups (Rittenhouse-Simmons and Deykin, 1981; Prescott and Majerus, 1983; Goracci et al., 1983) have shown that platelets can biosynthesize phospholipid by the de novo (Kennedy) pathway or by the remodelling of phospholipid via the deacylation -reacylation (Lands) pathway (Marcus, 1978; Rittenhouse-Simmons and Deykin, 1981). Precursors such as glycerol, phosphate and long chain fatty acids are incorporated into platelet phospholipid when supplied in the incubation medium (Marcus, 1978; Rittenhouse-Simmons, 1981;

Mahadevappa and Holub, 1983). The fatty acids can be incorporated either through de novo synthesis or through remodelling of the phospholipids.

There is now evidence for the de novo synthesis of the monoalkyl ether phospholipids in platelet membranes by a pathway involving alkyl-dihydroxyacetone phosphate (alkyl-DHAP) synthetase (Fig. 9). This enzyme, which has been solubilized and purified from platelets (Hajra et al., 1978; Brown and Snyder, 1982) carries out the exchange of the acyl group in the acyl DHAP with an alcohol group to form O-alkyl DHAP (Davis and Hajra, 1977; Hajra, 1983; Brown et al., 1985):

Acyl-DHAP + R-OH ----------> Alkyl-DHAP + RCOOH

Alkyl-DHAP synthetase has been shown to be quite specific for acyl-DHAP, so that neither acyl-DHA, acylglycerophosphate or DHAP could substitute for acyl-DHAP as substrate (Hajra et al., 1978; Hajra, 1983). However, the reaction itself was found to be non-specific with respect to various alcohols, so that any long chain primary alcohol above C10 or below C22, including polyunsaturated alcohols and diols may react with acyl-DHAP to form the ether bond (Hajra, 1983). Subsequent reduction of the O-alkyl-DHAP followed by acylation gives rise to the alkylacyl phosphatidic acid which can be converted to alkylacyl phospholipids (alkylacyl PC, alkylacyl PE, etc.) by the de novo (Kennedy) pathways (Hajra, 1983) (Fig. 9).

Figure 9: Pathways for the Biosynthesis of Ether Phospholipids
(from Snyder, 1985)

(1) acyl CoA: dihydroacetone-P acyltransferase

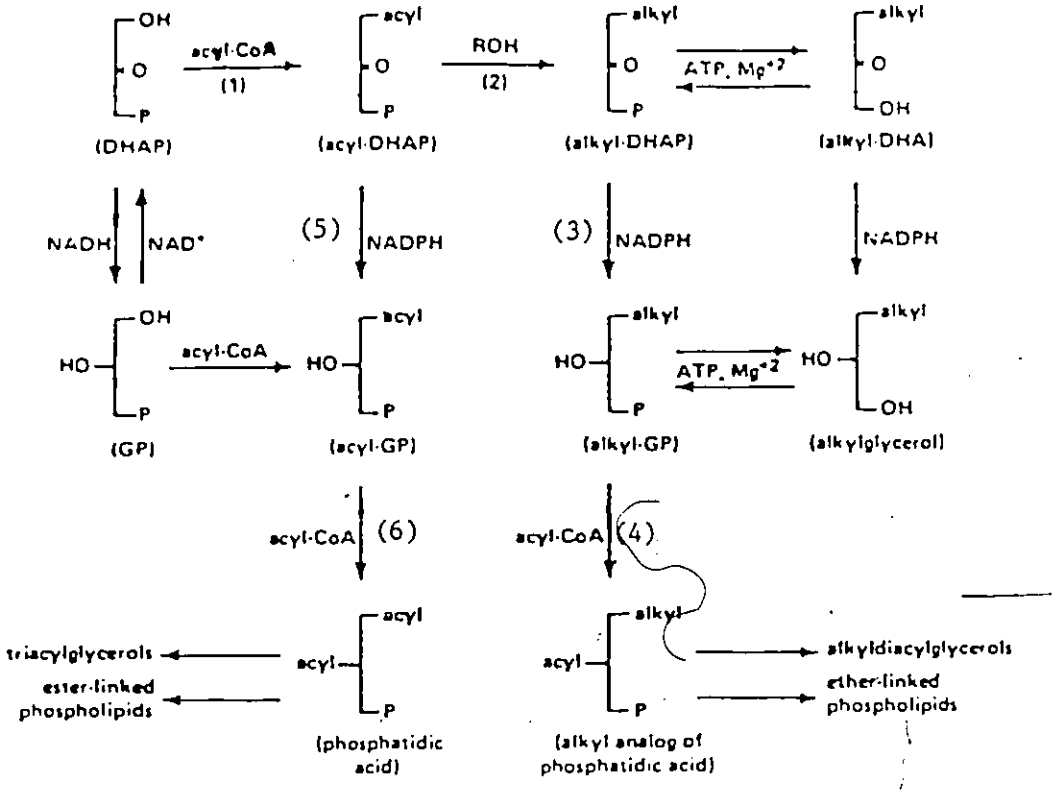
(2) alkyldihydroxyacetone-P synthase

(3) NADPH: alkyldihydroxyacetone-P oxidoreductase

(4) acyl-CoA: 1-alkyl-2-lyso-glycero-3-P acyltransferase

(5) NADPH: acyldihydroxyacetone-P oxidoreductase

(6) acyl CoA: 1-acyl-2-lyso-glycero-3-P acyltransferase


OH
O
P
(DHAP)
acyl-CoA
(1)
acyl
(acyl-DHAP)
ROH
(2)
alkyl
(alkyl-DHAP)
ATP, Mg+2
OH
(alkyl-DHA)
NADH
NAD+
(5)
NADPH
(3)
NADPH
NADPH
HO
(GP)
acyl-CoA
(acyl-GP)
(alkyl-GP)
(alkylglycerol)
acyl-CoA
(6)
acyl-CoA
(4)
triacylglycerols
ester-linked
phospholipids
(phosphatidic
acid)
(alkyl analog of
phosphatidic acid)
alkyldiacylglycerols
ether-linked
phospholipids

The formation of the alk-1'-enyl ether linkage in the alkenylacyl or plasmalogens occurs exclusively by the desaturation of the alkyl ether linkage in the alkylacyl PE by the microsomal bound cytochrome b_5 dependent desaturase system requiring O_2 and NADPH as cofactors (Paltauf, 1983):

$$\underset{\text{alkylacylPE}}{R\text{-}O\text{-}CH_2\text{-}CH_2\text{-}R} \xrightarrow[\text{alkylacylPE desaturase}]{O_2,\ NADPH} \underset{\text{alkenylacyl PE}}{R\text{-}O\text{-}CH{=}CH\text{-}R}$$

The alk-1'-enyl acyl PE formed may be converted to other alkenylacyl phospholipids such as PC, PI etc. and alkenyldiacyl glycerols by the same enzymatic reactions involved in the interconversion of the 1,2-diacyl analogues (Fig. 10) (Paltauf, 1983).

The O-alkyl and O-alk-1'-enyl bonds are resistant to the action of phospholipases (Fig. 11). In certain types of cells specific enzymes cleave the ether bond of 1-O-alkylglycerols by an oxidative mechanism (Tietz et al., 1964) to yield long chain aldehydes and glycerol. The same enzyme accepts 1-O-alkylglycerophosphatidylethanolamine (1-O-alkyl PE) as a substrate, but alkylglycerolipids containing acyl or free phosphate groups are not attacked (Snyder et al., 1973). The 1-O-alkenyl ether bond can be hydrolysed by membrane bound plasmalogenases (which have been identified in rat liver (Warner and Lands, 1961; Gunawan and Debuch, 1981) and rat brain (Ansell and Spanner, 1965). Products of the plasmalogenase-catalysed reaction are long chain aldehydes

Figure 10: Interconversion of Phospholipid Head Group

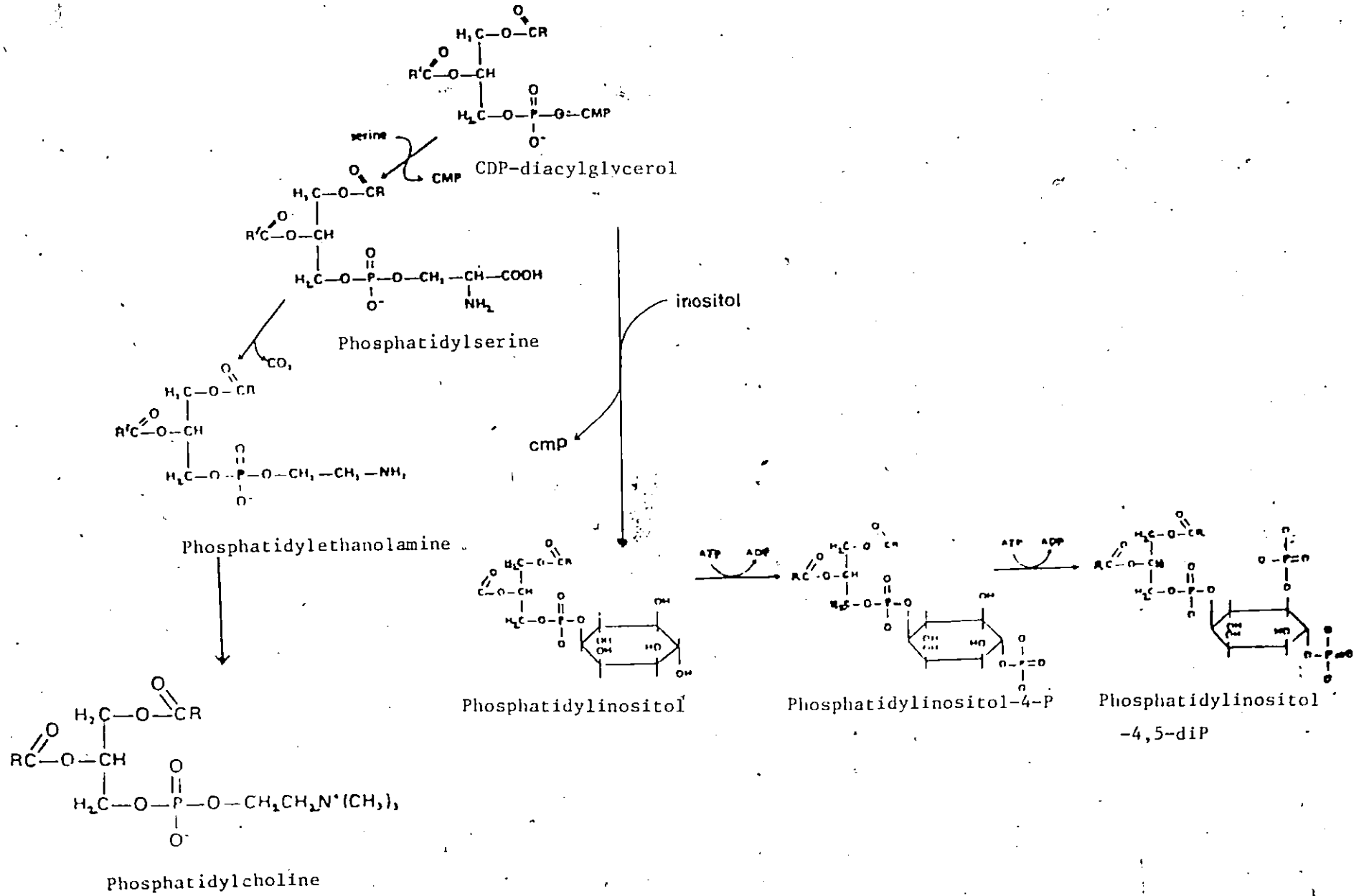

CDP-diacylglycerol
serine
CMP
Phosphatidylserine
CO2
Phosphatidylethanolamine
inositol
cmp
Phosphatidylinositol
ATP
ADP
Phosphatidylinositol-4-P
Phosphatidylinositol
-4,5-diP
Phosphatidylcholine

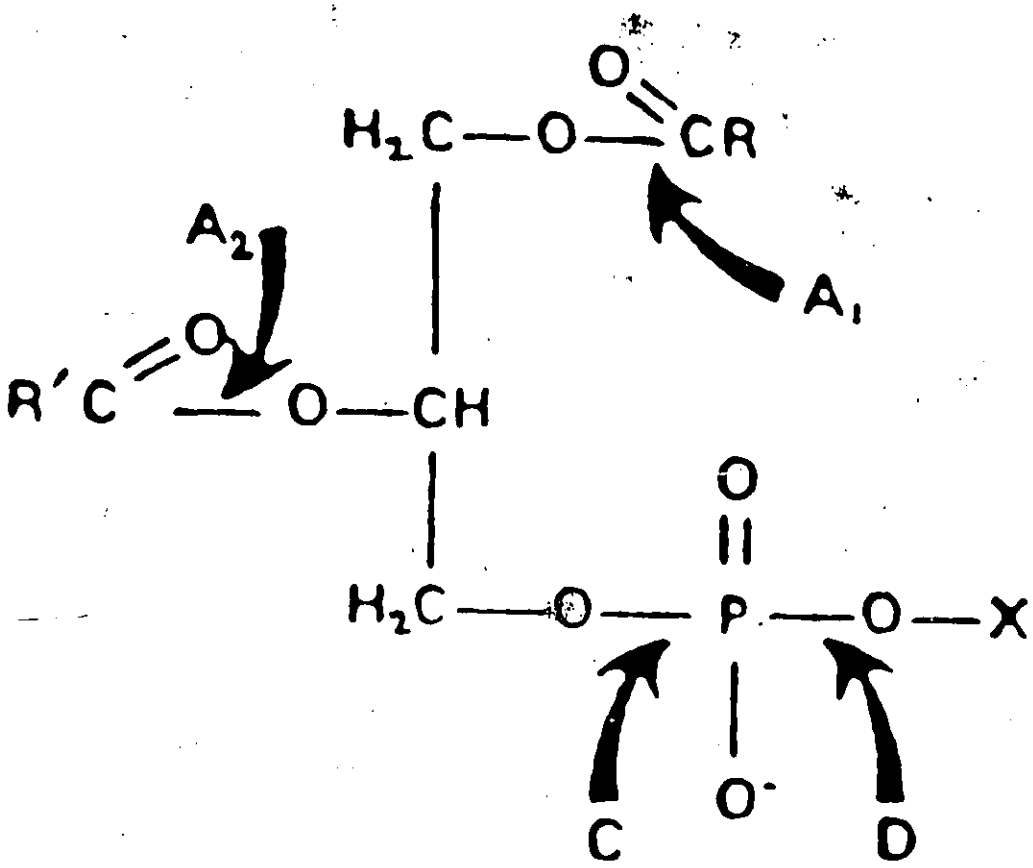
Figure 11: Enzymatic Hydrolysis of Diacyl Phospholipid

A_1 = phospholipase A_1

A_2 = phospholipase A_2

C = phospholipase C

D = phospholipase D


O
H2C—O—CR
A2
A1
O
R'C
O—CH
O
H2C—O—P—O—X
O-
C
D

and 2-acylglycerophospholipids. The enzymes necessary for degradaton of ether lipids have not yet been isolated from platelets. Acyl ester and phosphoester bonds of ether lipids are hydrolysed by the same enzymes phospholipases that hydrolyse the corresponding bonds in ester glycerolipids but at lower rates (Fig. 11) (Paltauf, 1983). However, little is known about the regulation of ether lipids such as Paf which has very potent biological activity. A better understanding of the regulation of ether lipid synthesis would be of great interest in connection with platelet activation.

III. Rationale and Aims of Present Work

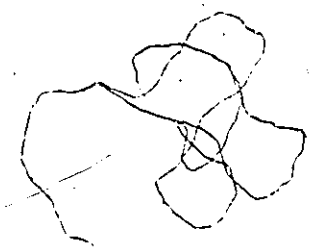
The roles of ether phospholipids in normal platelet function is not known. However, the presence of significant amounts of Paf precursor and the enzymes to metabolize and regulate Paf release in the platelet membrane indicates that Paf may indeed play a role in platelet maintenance of vascular integrity (Packham and Mustard, 1984; Snyder, 1985). The importance of arachidonate and metabolites on platelet function has long been recognised (Marcus, 1978; Rittenhouse-Simmons and Deykin, 1981). Present mechanistic concepts of haemostasis support the hypothesis that liberation of agents from platelets in response to exogenous stimuli amplify the initial stimulus and recruit more platelets to the site (Adams, 1985). The nature of the intercellular mediator is unknown, and has been speculated to

be thrombin, ADP, serotonin, thromboxane A_2, Paf and endoperoxides, as they all can be formed by the platelet in response to stimulation (Mustard et al., 1981). Then synergistic effect may indeed be the physiological mechanism for amplification of the haemostatic signal as calculations indicate that insufficient amounts of any one agent are produced at the site of vessel injury (Adams and Feuerstein, 1983).

The effect of storage has been shown to result in a decrease in platelet response to single stimulus with full response to pairs of stimuli. This effect may be the result of the loss of the platelet response to propagate the initial stimulus with endogenous agents. The immediate effect of storage would be expected to be reflected by changes in its membrane composition. Both arachidonate and Paf precursors are stored as membrane phospholipids. Therefore study of platelet phospholipid composition should indicate whether the storage effect on platelet function could be due to a loss in the precursor material for Paf or arachidonate metabolites on storage.

The aims of this thesis are: (1) to develop a method for the analysis of phospholipid molecular class composition and more specifically, the chain lengths and unsaturation of the fatty alcohol, fatty aldehyde and fatty acid components of the total lipids, and of the phospholipid classes, PC and PE; (2) to examine the effects of storage on platelet

phospholipid composition. The results obtained might indicate the potential role of diacyl, alkenylacyl and alkylacyl phospholipids in platelet function.



B. MATERIALS AND METHODS

I. MATERIALS

I.1 Platelets

Human blood collected from donors was obtained from the Canadian Red Cross, Ottawa Center, Blood Transfusion Service.

I.2 Chemicals

All solvents were glass-distilled before use. All other chemicals were of "Reagent Grade" unless otherwise specified. Fatty acid methyl ester standards were from NuChek (Elysian, MN). Aldehyde dimethyl acetal standards were synthesized by methanolysis of sodium bisulfite-adducts of palmitaldehyde, stearaldehyde and oleylaldehyde (K+K Lab Inc., Plainview, NY) (Kates, 1972). Monoalkyl glycerol diacetate standards were synthesized by acetylation of chimyl (16:0), batyl (18:0) and selachyl (18:1) alcohols purchased from Western Chemical Industries Ltd., (Vanocouver, BC), (Renkonen, 1965). The synthesized standards were checked for purity and characterized by analytical TLC, GLC and mass spectrometry.

Paf (1-O-hexadecyl-2-acetyl-3-glycerophosphocholine) and plasmalogen PE (bovine brain extract) were purchased from Sigma Chemical Co. (St. Louis, Mo.). The PE was partially hydrogenated with PtO_2 as catalyst (Kates, 1972) to form a mixture of alkylacyl, alkenylacyl and diacyl classes.

II. METHODS

II.1 Preparation of Platelets

Fresh human platelets were prepared using conventional methods (Mustard et al., 1972). In brief, human venous blood was collected from donors into citrate-phosphate-dextrose-adenine (CPDA-1) anticoagulant and separated into platelet-rich plasma (PRP) and packed red cells by centrifugation at 150xg for 10 minutes. The PRP was transferred to PL 732 blood bag and recentrifuged at 3000xg for 7 minutes to concentrate the platelets.

For the study of the effects of storage, platelet concentrates from 8 volunteers were pooled, aliquoted into separate PL732 bags and stored as concentrates at 10^9 platelets per ml in 60 ml of plasma. The concentrates were stored at 22°C with tumbler agitation for up to five days. Subsamples were withdrawn using aseptic procedure from platelet concentrates on the day of collection (Day 0 or fresh), and after three and five days of storage. The sample was acidified using 0.1 N sodium citrate to pH 6.3 and washed according to the procedures of Mustard et al.(1972). In the final wash, the platelets were washed with Tyrode's ion-free buffer. The platelet concentration was determined by electronic particle counting (Coulter Inc.).

II.2 Platelet Function Testing

The platelets were diluted to 3 X 10^8 cells/ ml with autologous plasma and stimulated with epinephrine (Eastman-Kodak, Toronto, Ont.), 5 X 10^{-5} M; adenosine diphosphate (Sigma Chemical Co.), 1 X 10^{-5} M; collagen (Sigma), 5 X 10^{-6} g/ml; ionophore A23187 (Sigma), 1 X 10^{-5} M; arachidonic acid (Biodata Corp., Hatboro, Penn.), 0.5 mg/ml; or pairs of these stimulants at the concentrations above. Aggregations proceeded for 4 minutes and were performed in a Payton aggregometer; the extent of aggregation was measured using turbidmetric methods (Born, 1962). To graphically illustrate the results, a synograph was constructed by plotting the percentage of aggregation to the various stimuli on each spoke of a 16 spoke polar graph. Aggregation was defined as the maximum change in transmittance after stimulation.

II.3 Lipid Extraction

Total cellular lipids were extracted by the method of Bligh-Dyer (1959) immediately after cell isolation. The total amount of lipid extracted was calculated from the dry weight of an aliquot. It is expressed in terms of milligrams of total lipid extracted per total number of platelets (mg/10^9 cells).

Total cholesterol was determined by the colorimetric method of Zlatkis et al. (1963) and expressed as percentage

of dry weight of the total lipid and as a molar ratio to the total phospholipid determined by the method of Bartlett (1959) except that samples were digested with perchloric acid (Kates, 1972).

II.4 Thin-layer Chromatography

i. Analytical TLC

Analytical TLC was performed on 20 X 20 cm^2 plates precoated with 0.25 mm silica gel H (Whatman k5 plates, Whatman Chemical Separation Inc., Clifton, NJ). Samples were spotted under N_2 on plates previously washed twice with $CHCl_3$-CH_3OH, 1:1. Phospholipid standards were obtained from Sigma Chemical Co. (St. Louis, Mo.). The solvent systems used for chromatography were as follows:

A. petroleum ether/ethyl ether/acetic acid (70/30/10 v/v/v) for separation of neutral lipids;

B. chloroform/methanol/ammonium hydroxide/water (70/30/4/1 v/v/v/v) with double development for separation of platelet phospholipids;

C. chloroform/methanol/acetic acid/water (50:28:10:5 v/v/v/v) for separation of phospholipids;

D. chloroform/ methanol/ water (65:35:5 v/v/v) for separation of phospholipids.

Following development of the chromatograph, spots were located or characterized by spraying or exposing the TLC

plate with the following general reagents (Kates, 1972):

a. iodine vapour;

b. 10% H_2SO_4 in ethanol followed by charring of the plate;

c. 'NRC' spray (1% Cerium sulfate hydrate and 1.5 wt% molybdic acid in 15% aqueous sulfuric acid) and charring;

and specific stains:

d. Ninhydrin (2.5 g% ninhydrin in 10% lutidine-acetone solution) for amino groups (Marinetti, 1964);

e. Molybdate reagent (Dittmer and Lester, 1964) for phosphate groups;

f. 2,4-dinitrophenyl hydrazine (saturated solution of 2,4-dinitrophenyl hydrazine in 10% conc.H_2SO_4 in ethanol) for carbonyl compounds (Marinetti, 1964).

ii. Preparative TLC

Preparative TLC was performed on 20 X 20 cm^2 plates precoated with 0.75 mm silica gel H (Whatman pK5) plates with double development in the solvent system of $CHCl_3$-CH_3OH-NH_4OH-H_2O (70:30:4:1). The separated phospholipids were visualised by exposure to iodine vapour. The bands were marked, scraped into tubes and the phospholipids were extracted from the silica gel with $CHCl_3$-CH_3OH (1:1). Lipid phosphate recoveries from extraction of known amounts of phospholipid from preparative TLC plates were 90 $\pm$ 10 %.

II.5 General Analytical Procedures

i. Phosphate Determination

The phospholipid phosphorus was determined by the modified method of Bartlett (Kates, 1972). The total phosphorus was expressed as a percentage of the lipid dry weight (mgP/ mg lipid X 100).

ii. Vinyl Ether Assay

Vinyl ether content was assayed by the method of Bottfried and Rapport (1962, 1963) modified as follows: an aliquot of chloroform solution containing 0.05 to 0.10 µmole of vinyl ether lipid was transferred to a 15 ml glass stoppered tube. The solvent was evaporated under a stream of nitrogen and the residue suspended in 0.5 ml of methanol; then, 0.5 ml of iodine solution (0.6×10^{-3} N) was added and the tube was shaken for 30 minutes at room temperature. The mixture was then diluted with 4.0 ml of 95% ethanol and the absorbance read at 355 nm against a blank containing 0.5 ml of 3% KI instead of the iodine solution. A control tube containing the iodine solution without the lipid sample was also measured. The average extinction coefficient of the iodine solution was determined by measuring absorbance of varying amounts of the iodine solution in the above system without the lipid sample (molar extinction coefficient, ca. 27,500).

Calculation:

$$\frac{\mu\text{moles vinyl ether groups}}{g} = \frac{(A_{\text{control}} - A_{\text{sample}}) \times 5}{e \times 10^{-3}}$$

where e is the molar extinction coefficient of I_{λ}.
The content of vinyl ether was then expressed as a percentage of the phospholipid used in the assay.

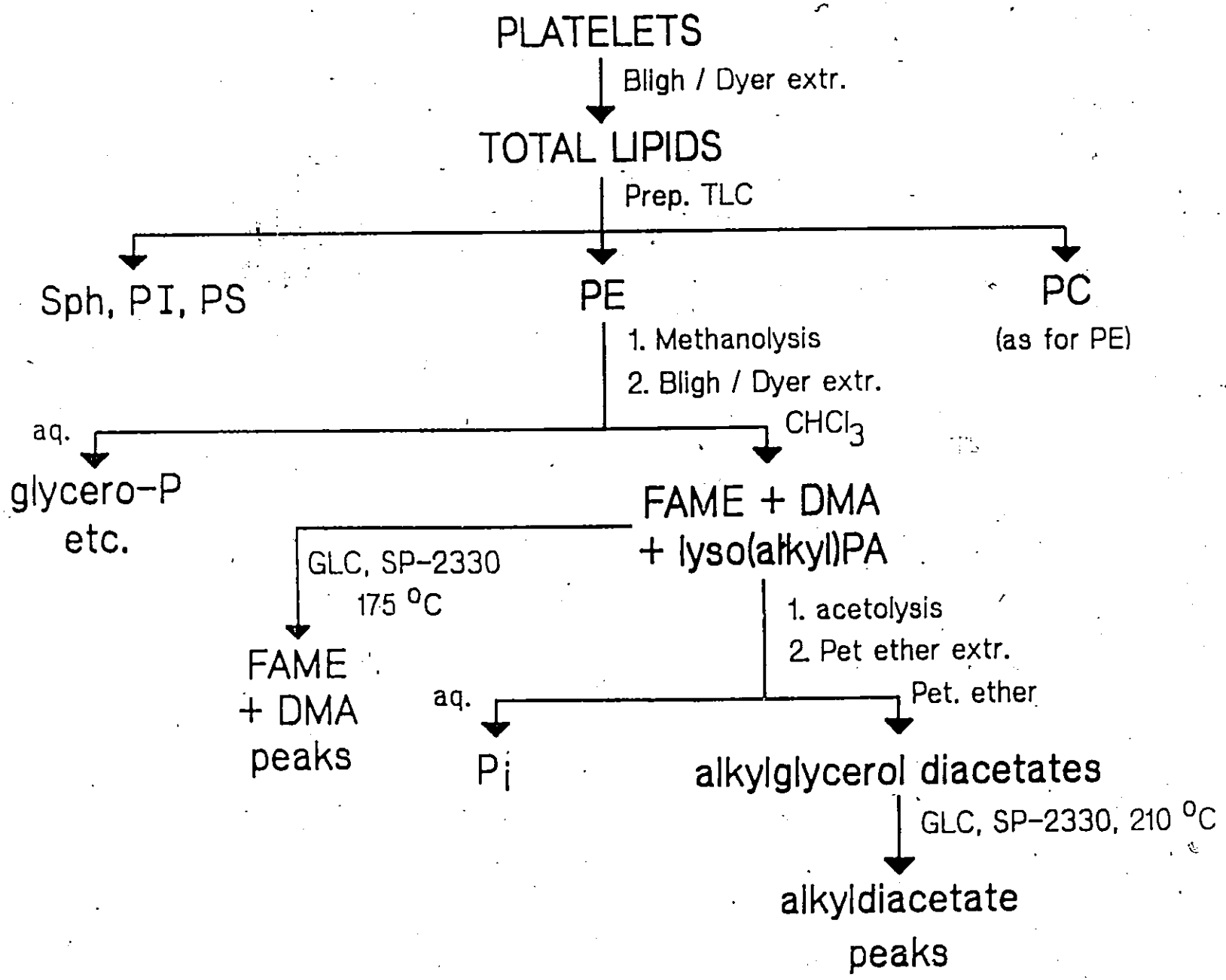
II.6 Procedures for Platelet Phospholipid Analysis

The phospholipid components of human platelets were separated by preparative TLC of 10-15 mg of total lipid extract (Bligh-Dyer) of whole cell lysate as described above (Fig. 12).

i. Methanolysis Procedure (modified method of Kates, 1972)

An aliquot of chloroform solution (1-2 μmol of phospholipid) of total lipid or PE or PC fractions was pipetted into a 20-ml screw-capped test tube and a known quantity (0.4 x moles of phospholipid used) of internal standard, methyl heneicosanoate (21:0ME) was added. The solvent was evaporated under a stream of nitrogen; 4.5 ml of 2.5% methanolic-HCl (made by bubbling 2.5 g of $HCl_{(g)}$ in 100 ml of methanol) was added; the tube was stoppered and heated for 1-2 hours at 70-75°C in a temperature controlled block heater (Blok Heater, Canlab). Then, 4.0 ml of water and 4.5 ml of chloroform were added to make a two phase Bligh-Dyer

Figure 12: Analytical Scheme for Study of Platelet Phospholipids


PLATELETS
Bligh / Dyer extr.
TOTAL LIPIDS
Prep. TLC
Sph, PI, PS
PE
PC
(as for PE)
1. Methanolysis
2. Bligh / Dyer extr.
aq.
$CHCl_3$
glycero-P
etc.
FAME + DMA
+ lyso(alkyl)PA
GLC, SP-2330
175 °C
FAME
+ DMA
peaks
1. acetolysis
2. Pet ether extr.
aq.
Pet. ether
Pi
alkylglycerol diacetates
GLC, SP-2330, 210 °C
alkyldiacetate
peaks

system. The lower chloroform phase was removed and the upper phase was washed with chloroform three more times. The chloroform phases were pooled and concentrated under a stream of nitrogen. The residual lipids (FAMEs, DMAs, lysoalkyl-PA) were made up to a known volume in chloroform and appropriate aliquots were taken for GLC analysis and acetolysis.

ii. Analysis of Fatty Acids and Aldehydes by Gas-liquid Chromatography

Fatty acid methyl esters (FAMEs) and dimethyl acetals (DMAs) prepared as described above, were analysed by gas liquid chromatography on a Pye Unicam (Cambridge, Eng.) gas chromatograph, on a 2 m x 4 mm column of 10% SP 2330 on 100/120 chromosorb W AW (Supelco, Belleforte, PE) at 195°C. Inlet carrier gas (nitrogen) pressure was 1.2 Kg/cm^2 and the gases fed to the flame ionization detector were hydrogen and compressed air (Union Carbide, Ottawa). Peak areas were measured by the retention time x peak height method of Carroll (1961) suitably corrected for attenuation of the peaks.

iii. Acetolysis (Renkonen, 1965)

An aliquot of the chloroform phase of the methanolysate described in B.II.6.i containing 1-2 mg of lipid were added to a screw-capped test tube and the solvent evaporated under nitrogen; 1 ml of acetic acid- acetic anhydride (3:2) was

added, and the mixture was refluxed at 150°C for 48 hours in the capped tube. To the cooled tube, 4.5 ml of methanol and 0.5 ml of water (9:1) were added and the MAGDs formed from lysoalkyl-PAs, along with the remaining FAMEs and DMAs extracted with petroleum ether.

iv. Analysis of MAGDs by Gas-liquid Chromatography

The MAGDs were analysed on the same column as for FAMEs and DMAs (see above) but at a temperature of 210°C and carrier gas pressure at 1.5 Kg/cm². MAGD peaks were identified using standard monoalkyl glycerol diacetates and quantified using the 21:0 ME as internal standard.

v. Molecular Class Calculation

Molecular class composition was determined by quantification of the FAMEs and DMAs obtained by methanolysis and the MAGDs obtained by acetolysis, using GLC with 21:0 ME as internal standard. Each mole of methyl ester was taken to represent 1 mole of 1-O-alkyl-2-acyl-, 1 mole of 1-O-alkenyl-2-acyl, or 0.5 mole of 1,2-diacyl phospholipid.

$$\text{moles \% diacyl PL} = \frac{\sum\text{moles FAME} - \sum\text{moles DMA} - \sum\text{moles MAGD}}{2 \times \text{moles PL used}} \times 100$$

$$\text{moles \% alkenylacyl PL} = [\sum\text{moles DMA} / \text{moles PL}] \times 100$$

$$\text{moles \% alkylacyl PL} = [\sum\text{moles MAGD} / \text{moles PL}] \times 100$$

where PL is phospholipid abbreviated.

vi. Statistical Analysis

All of the platelet samples reported in this study were from one platelet pool. The "n" used in statistical analysis refers to the number of repeated analysis of each sample. The results were expressed as the mean ± standard deviation for n>3 and for n=2, the average of the difference between the means is used as the deviation.

Statistical evaluation of the final results were performed using a paired Student's t-test represented by the following equation (Steel and Torrie, 1980):

$$T = \frac{M_1 - M_2}{\sqrt{(SE_1)^2 + (SE_2)^2}}, \quad \text{where } SE = \frac{SD}{\sqrt{n}}$$

M_1 and M_2 are means; n = number of samples; SD = standard deviation.

vii. Mass Spectrometry

a. Fast-atom Bombardment Mass Specrometry (FAB/MS)

A FAB source fitted to a VG 7070E mass spectrometer (VG Analytical, Manchester, Eng.) was operated at an accelerating potential of 6 kV and a potential of 9 kV at 1.5 mA was applied to the xenon gun used to form the fast atom beam.

0.5 umol of PC and PE fractions prepared by preparative TLC (see section II.4.ii) were treated by mild acid Bligh-Dyer partition (Kates, 1972) to remove salts. The samples were introduced at room temperature on a stainless

steel probe tip of approximately 2 mm diameter. The sample was dissolved directly in about 1 ul of glycerol on the probe tip. The probe was then inserted into the source. Dithioerythritol and dithiothreitol (1:1) (Magic Bullet) were used to increase the solubility of the phospholipid if glycerol alone was not sufficient.

The spectra were acquired by a DEC PDP 8 data system (Digital Equipment Co.) using dipalmitoyl PC and dipalmitoyl PE (Sigma Chemical Co., St. Louis, Mo.) to standardize the mass calibration.

b. Chemical Ionization Mass Spectrometry (CI/MS)

Chemical ionization mass spectrometry was used to corroborate GLC identification of MAGDs. Ethyl ether was the source of ions. The same instrumentation as for FAB analysis was used. Batyl diacetate and chimyl diacetate were used as standards for mass calibration.

C. RESULTS

I. Development of Analytical Procedures

Platelet phospholipids consist of complex mixtures of molecular species of diacyl, alkenylacyl and alkylacyl classes. Although analysis of intact glycerophospholipid species would be desirable, no chromatographic system is known that would resolve the molecular classes of an intact phospholipid. Conventional methods of analysis involve modification of the phospholipid, by enzymatic hydrolysis (phospholipase C) followed by chemical derivatization (acetate, t-BDMS, TMS) of the isolated diradyl glycerol products and quantification of the derivative by GLC, GC/MS or HPLC (Renkonen, 1965, 1966; Wood and Snyder, 1969; Mueller et al., 1983; Myher and Kuksis, 1983; El Tamer et al., 1984) This approach is limited by the lack of broad substrate specificity of the phospholipase C and the necessity of isolating the diradylglycerol products and their derivatives. Alternatively, acetolysis has been used for the direct conversion of phospholipids into diglyceride acetates (Renkonen, 1965) but it has become apparent that plasmalogens cannot withstand the severe conditions of this procedure, and even in diacyl phospholipids, some changes in the positional distribution of the fatty acids takes place (Kuksis, 1972) as well as selective degradation of polyunsaturated fatty acids (Renkonen, 1966; Nutter and Privett, 1966; Privett and

Nutter, 1967).

We have simplified the conventional procedure by replacing the phospholipase C hydrolysis by chemical methanolysis followed by acetolysis (Fig. 8). Methanolysis products of the acid sensitive acyl ester and vinyl ether linkages produces fatty acid methyl esters and dimethylacetals, respectively, which can be extracted by petroleum ether and quantified by GLC (Fig. 13a).

The acid-resistant saturated ether linkages in alkylacyl phospholipids are converted by methanolysis to lysoalkyl-PA's which are extracted into chloroform (Bligh-Dyer) and derivatized by acetolysis to monoalkyl glycerol diacetates (MAGDs) (Fig. 8) and quantified by GLC (Fig. 13b).

I.1 Standardization of Analytical Procedures

This procedure was checked for: i) efficiency of the methanolysis step for formation of FAMEs and DMAs; ii) efficiency of extraction of the lyso-PAs from the methanolysate by chloroform (Bligh-Dyer) and iii) optimal conditions for the acetolysis procedure.

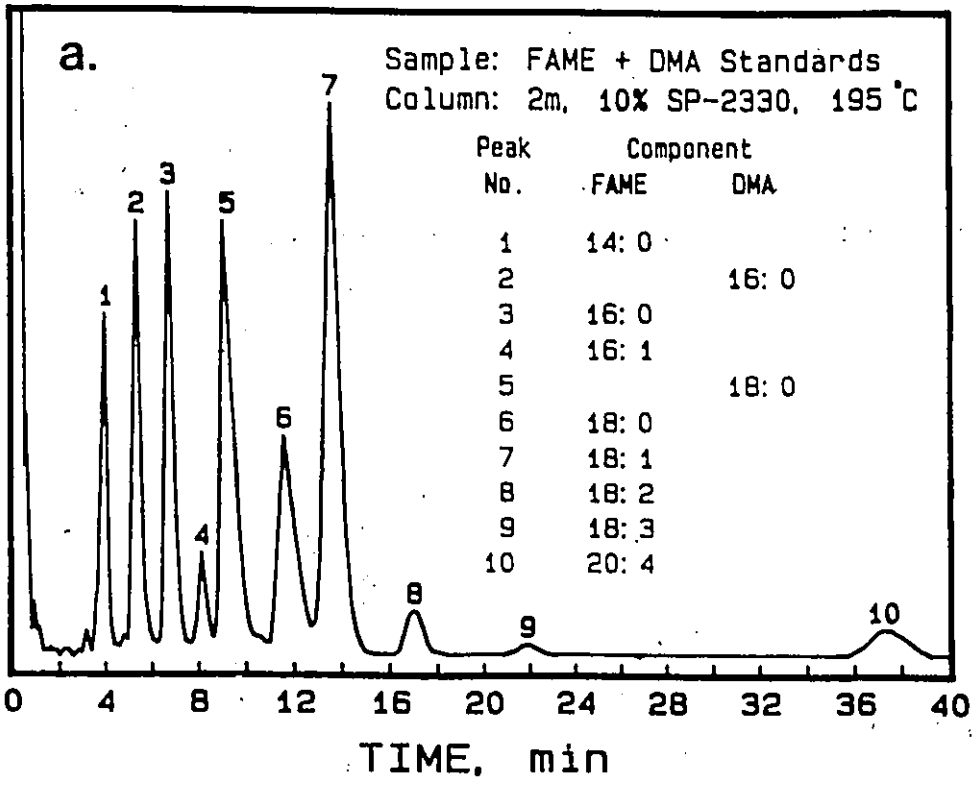
i) Efficiency of Methanolysis Procedure for FAME and DMA Formation

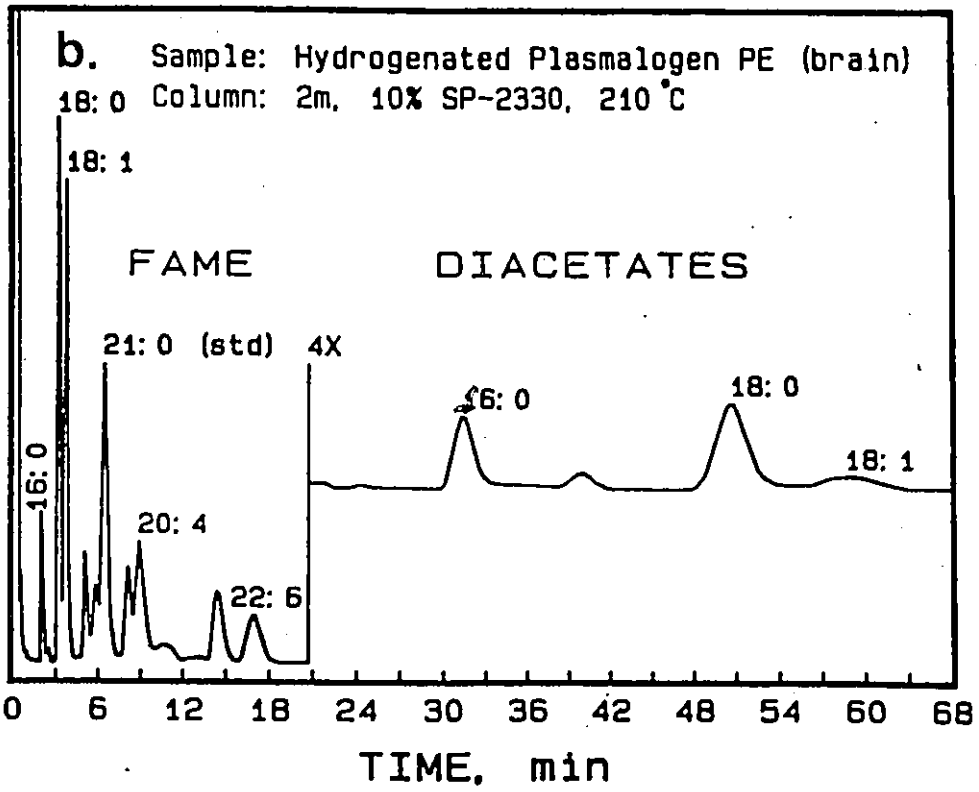
The efficiency of the petroleum ether extraction of the DMAs and FAMEs and their quantification after GLC analysis were determined, by carrying out the methanolysis on known

Figure 13: Gas-Liquid Chromatographic Separation of:

A. FAMEs and DMAs after Methanolysis

B. MAGDs after Methanolysis and Acetolysis


a.
Sample: FAME + DMA Standards
Column: 2m, 10% SP-2330, 195 °C
Peak No. | Component FAME | DMA
1 | 14: 0 |
2 | | 16: 0
3 | 16: 0 |
4 | 16: 1 |
5 | | 18: 0
6 | 18: 0 |
7 | 18: 1 |
8 | 18: 2 |
9 | 18: 3 |
10 | 20: 4
0 4 8 12 16 20 24 28 32 36 40
TIME, min


b.
Sample: Hydrogenated Plasmalogen PE (brain)
Column: 2m, 10% SP-2330, 210 °C
18: 0
18: 1
FAME
DIACETATES
21: 0 (std)
4X
16: 0
16: 0
18: 0
18: 1
20: 4
22: 6
0 6 12 18 24 30 36 42 48 54 60 68
TIME, min

amount of mixtures of authentic standard FAMEs (14:0, 16:0, 16:1, 18:0, 18:1) and DMAs (16:0, 18:0), followed by repeated (3X) extraction of the methanolysate (after addition 10% of water) with petroleum ether and analysis by GLC after addition of a known amount of internal standard (21:0 ME). The results showed very good quantification of the FAMEs and DMAs tested as well as good overall recoveries from the methanolysis step (95.3 ± 9.8 %), (Table 1).

ii) Efficiency of Extraction of Lyso-PAs after Methanolysis

When a known amount (27.8 µgP) of standard commercial platelet-activating factor (1-O-hexadecyl-2-acetyl-phosphatidylcholine) was subjected to methanolysis and extraction, the petroleum ether phase was found to contain lipid-P (0.30 µgP) which was characterized by TLC as lysoalkyl-PA. When the methanolysate was then subjected to Bligh-Dyer partition, a further amount of lysoalkyl-PA (31.7 µgP) was extracted. These results showed that low recoveries of lysoalkyl-PA are obtained in the chloroform extract (Bligh-Dyer) of the methanolysate following the petroleum ether extraction (Table 2).

In order to improve recovery of the lysoalkyl-PA and also retain the FAMEs and DMAs, it was found necessary to omit the petroleum ether extraction and to subject the methanolysate directly to Bligh-Dyer partition ($CHCl_3$-MeOH-H_2O, 1:1:0.9). Using this modification, authentic Paf was subjected to

TABLE 1: Analysis and Recovery of *Standard Aldehyde Dimethyl Acetals and Fatty Acids Methyl Esters After Methanolysis

	Known Composition (n = 2)			Estimated (n = 3)		
Chain Type	µmoles	moles %	µmoles**	moles %	area %†	µmoles recovery %
FAME						
14:0	4.6 ± 0.2	13.3 ± 0.6	4.2 ± 0.3	12.7 ± 1.3	10.3 ± 1.2	91.3 ± 10.9
16:0	8.3 ± 0.4	24.1 ± 1.2	8.1 ± 0.2	24.4 ± 0.4	23.4 ± 0.4	97.5 ± 7.2
16:1	2.6 ± 0.1	7.4 ± 0.4	2.5 ± 0.1	7.5 ± 0.3	7.2 ± 0.3	99.6 ± 7.7
18:0	4.3 ± 0.2	12.4± 0.6	4.3 ± 0.1	13.0 ± 0.2	13.7 ± 0.2	100.9 ± 7.0
18:1	14.7 ± 0.7	42.7± 2.0	14.1 ± 0.3	42.5 ± 0.9	44.7 ± 1.0	95.9 ± 6.8
DMA						
16:0	9.4 ± 0.5	57.3± 3.0	8.4 ± 1.0	56.3 ± 6.1	54.0 ± 6.6	89.4 ± 15.9
18:0	7.0 ± 0.4	42.7± 2.4	6.5 ± 0.5	43.6± 3.1	45.7 ± 3.9	92.9 ± 12.9

*NIH standards FAMEs of known composition (Nuchek, Elysian, MN). Synthetic DMAs prepared by Methanolysis as described and aliquot used to determine dry weight and a known volume is taken for analysis.

** $\mu mol = \frac{\text{peak area (component)}}{\text{peak area (int. std.)}} \times \frac{\text{mg of int. std.}}{\text{mol. wt. of component}}$

†Calculated from areas of peaks (Carroll, 1961).

The results are the means ± standard deviation of n determinations.

TABLE 2: Efficiency of Extraction of Lysoalkyl-PA After Methanolysis and After Acetolyis Using Authentic PAF

Phase	P-recovered* μgP	% P
1) pet. ether extract of methanolysate	27.8 ± 0.1	47.2 ± 0.2
2 methanol-water phase after Bligh-Dyer extraction of methanolysate	0.3 ± 0.2	0.6 ± 0.3
3) acetolysis reaction mixture after pet. ether extraction	31.7 ± 2.0	53.8 ± 3.4
Total recovery	59.8 ± 2.3	101.6 ± 3.9

*Total μgP in Paf used was 58.9 ± 2.0. P-determination was by the modified method of Bartlett (Kates, 1972) and performed in duplicate for each sample.

$$\% \text{ P recovered} = \frac{\mu \text{ gP recovered}}{\mu\text{gp in PAF}} \times 100$$

All data are corrected for appropriate blanks.

The results are the mean ± deviation for duplicate sample analysis.

methanolysis and Bligh-Dyer extraction; all of the lipid-P appeared in the chloroform phase (as lysoalkyl-PA, 96.6± 0.6%) and negligible amounts were retained in the methanolysate (2.5± 0.1%) after Bligh-Dyer extraction (Table 3). As a further check, recovery of lysoalkyl-PA (and subsequently MADGs) from hydrogenated plasmalogen PE (see Materials and Methods) was estimated and found to be 94.8 ± 4.9 %. (Table 3).

iii) Optimal Conditions for Acetolysis Procedure

In the procedure developed here, we use the same acetolysis conditions of Renkonen (1965) (Ac_2O-AcH, 2:3, 150°C, 48 hours) to make monoalkyl glycerol diacetates from lyso alkyl PA (see B.II.6.iii). These conditions were checked for complete acetolysis of lysoalkyl-PA using two standards: commercial Paf and partially hydrogenated plasmalogen PE. The reaction was followed by quantification of the MAGDs recovered by GLC (with 21:0 ME as internal standard) for different reaction times (Fig. 14). Acetolysis was complete after 48 hours at 150°C.

GLC analysis of the Paf alkylglycerol diacetates recovered after acetolysis and identified by comparison with standard synthetic diacetates indicated that Paf contained only a single alkyl chain, hexadecyl (Table 4) and was 99.5% pure, in agreement with the manufacturer's product analysis.

CI/MS analysis of Paf acetylosis product (Appendix, CI/MS

Table 3: Recovery of Lysoalkyl-PA after Methanolysis and Acetolysis of Authentic Paf and Plasmalogen PE

	Paf		Plasmalogen PE	
Sample	µgP recovered*	%	µgP recovered*	%
µgP in sample	68.3 ± 3.4	100 ± 5.0	72.0 ± 3.6	100 ± 5.0
aqueous phase of methanolysate**	1.7 ± 0.2	2.5 ± 0.3	58.3 ± 2.7	81.0 ± 3.8
aqueous phase of acetolysate+	66.0 ± 0.4	96.6 ± 0.6	10.1 ± 0.1	13.9 ± 0.1
total recovery	67.7 ± 0.6	99.1 ± 0.9	68.4 ± 2.8	94.9 ± 4.9

* See Methods Section II.6; P-determination by the modified method of Bartlett (Kates, 1972).

** After Bligh/ Dyer extraction and removal of chloroform.

+ After pet. ether extraction.

Results are the mean ± deviation for duplicate sample analysis.

Figure 14: Rate of Acetolysis Reaction after Methanolysis for a) Paf and b) Partially Hydrogenated Plasmalogen PE

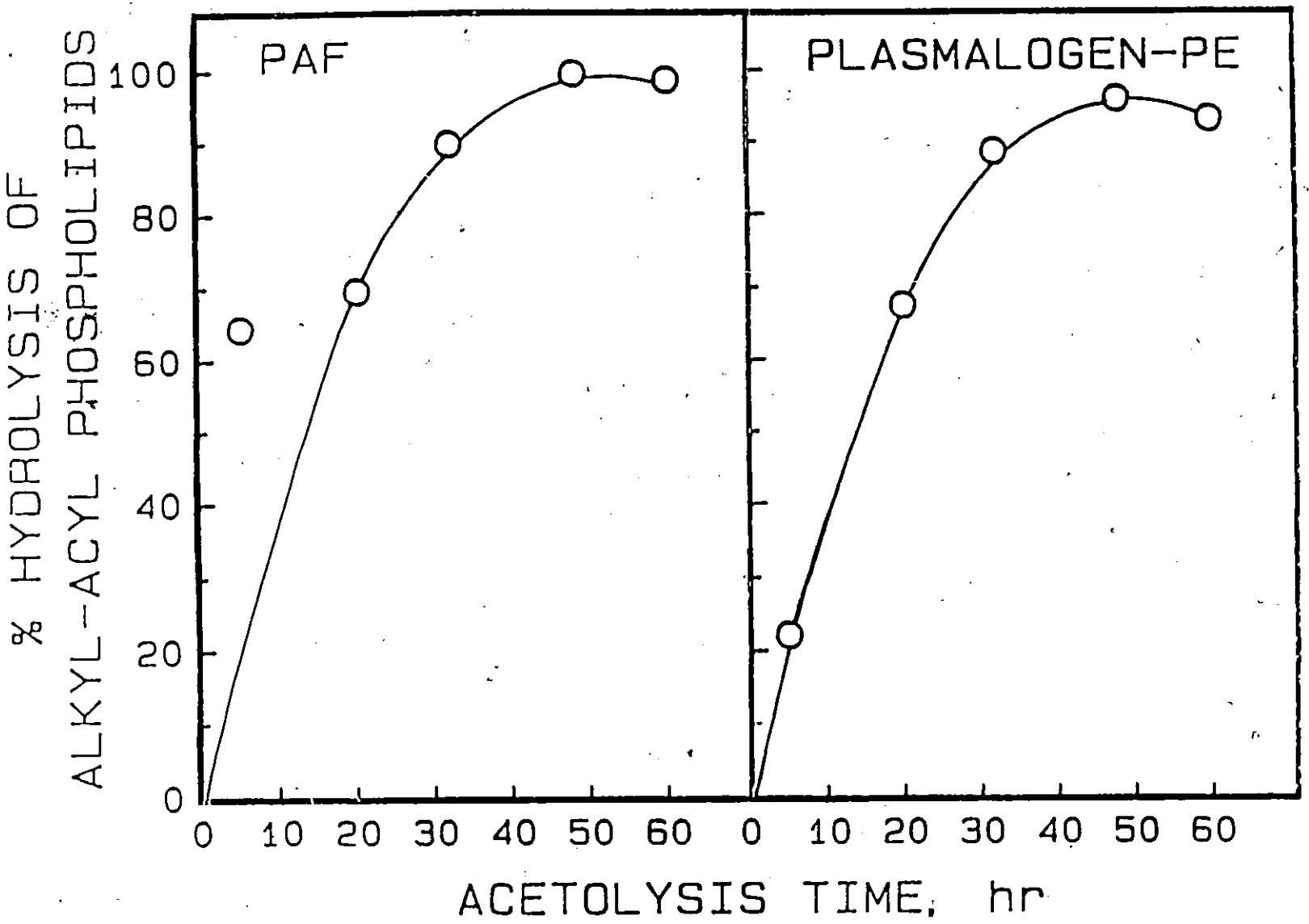

PAF
PLASMALOGEN-PE
% HYDROLYSIS OF ALKYL-ACYL PHOSPHOLIPIDS
0
20
40
60
80
100
0 10 20 30 40 50 60
0 10 20 30 40 50 60
ACETOLYSIS TIME, hr

TABLE 4: Recovery of Monoalkylglycerol Diacetates From Synthetic PAF and and Partially Hydrogenated Plasmalogen-PE*

	PAF	Plasmalogen-PE	
Chain	moles %	nmole/nmole PE X 100	moles %
16:0	99.5 ± 0.1	4.7 ± 0.9	31.8 ± 6.1
18:0	n.d.**	8.6 ± 1.7	58.1 ± 11.5
18:1	n.d.**	1.5 ± 0.3	10.1 ± 2.0
Total recovery	99.5 ± 0.1	14.8 ± 2.9	100.0 ± 19.6

*After methanolysis [0.6M methanol-HCl, 70-80°C, 1-2 hours], acetolysis [HAc:Ac_2O (3:2), 150°C, 48 hours], and analysis of FAMEs, DMAs + MADGs by GLC with 21:0 ME internal standard.

**n.d., not detected.

The results are the mean ± deviation of duplicate sample analysis.

-2) showed a parent ion peak [MH^+] with m/z 401 [$C_{25}H_{44}O_5$ + H^+], corresponding to hexadecyl glycerol diacetate, (Appendix, CI/MS-1). and a base peak at m/z 341 [$C_{21}H_{40}O_3^+$] corresponding to [MH^+ - acetic acid]. Other ion peaks were observed as follows: m/z 297 [$C_{19}H_{37}O_2^+$] due to a loss of acetic acid from m/z 341; and 280 [$C_{19}H_{36}O^+$] due to the loss of two molecules of acetic acid from [MH^+]; m/z 159 [$C_7H_{11}O_4^+$] corresponds to the glycerol backbone with its two acetyl groups intact. This pattern of fragmentation agrees with that reported by Jost (1974) and Rock and Snyder (1975) for chimyl diacetate.

Analysis of the partially hydrogenated PE indicated its saturated ether components were hexadecyl, octadecyl and octadecenyl and the total alkylacyl composition was 15% (Table 5). CI/MS analysis was more complicated as FAMEs and DMAs peaks were also present; however, the molecular ions for 16:0, 18:1 and 18:1 diacetates [MH^+] at 401, 429 and 427, repectively, were observed (Appendix, CI/MS-3).

Molecular class composition of plasmalogen PE was determined by quantification of the FAMEs and DMAs after methanolysis and MAGDs after acetolysis by GLC using an internal standard, 21:0ME. Each mole of methyl ester was taken to represent 1 mole of 1-O-alkyl-2-acyl-, 1 mole of 1-O-alk-1'-enyl-2-acyl-, or 0.5 mole of 1,2 -diacyl of phospholipid. The plasmalogen PE contained 68% diacyl, 7% alk-1'-enylacyl and 15% alkylacyl with about 90% recovery of

TABLE 5: Analysis of Molecular Classes of Partially Hydrogenated Plasmalogen-PE*

Molecular class	moles %**
Diacyl	67.7 ± 5.1
Alkenylacyl	6.7 ± 3.5
Alkylacyl	14.8 ± 2.9
Total recovery	89.2 ±11.5

*After methanolysis and 48 hour acetolysis at 150°C (see Table 4).

**Calculated as follows:

$$\text{moles \% Diacyl} = \frac{\Sigma \text{ moles FAME} - \Sigma \text{ moles DMA} - \Sigma \text{ moles MAGD}}{2 \times \text{moles PE}} \times 100$$

$$\text{moles \% Alkenylacyl} = [\Sigma \text{ moles DMA} / \text{moles PE}] \times 100$$

$$\text{moles \% Alkylacyl} = [\Sigma \text{ moles MAGDs} / \text{moles PE}] \times 100.$$

moles PL is calculated from determination of lipid-P.

The values are the mean ± deviation for duplicate analysis.

the total phosphate which is quite acceptable (Table 5). On the basis of these results, the following procedure was proposed for analysis of the platelet phospholipid molecular class composition.

I.2 Outline of Final Analytical Procedure

As summarised in Figure 12, the final analytical procedure involves preliminary separation of total lipids (obtained by Bligh-Dyer extraction of platelets) into the major phospolipid components (Sph, PI, PS, PE and PC) by preparative TLC in solvent system chloroform: methanol: ammonium hydroxide: water (70:30:4:1). Total lipids or each isolated component (PC and PE) are subjected to methanolysis followed by Bligh-Dyer extraction and subsequent acetolysis and petroleum ether extraction as detailed in the Methods section.

II. Application to Fresh Resting Platelets

The method summarized above (see Fig. 12) was applied to the analysis of lipids of fresh resting platelets and the results compared to previously reported analyses. The major phospholipid components identified by analytical TLC using different solvent systems, various spray reagents and comparison with phospholipid standards were: Sph, PI, PS, PC and PE (Fig. 15 , Table 6).

Figure 15: Analytical TLC of Platelet Phospholipid Fractions

solvent system: $CHCl_3$:CH_3OH:NH_4OH:H_2O (70/30/4/1) with one development.

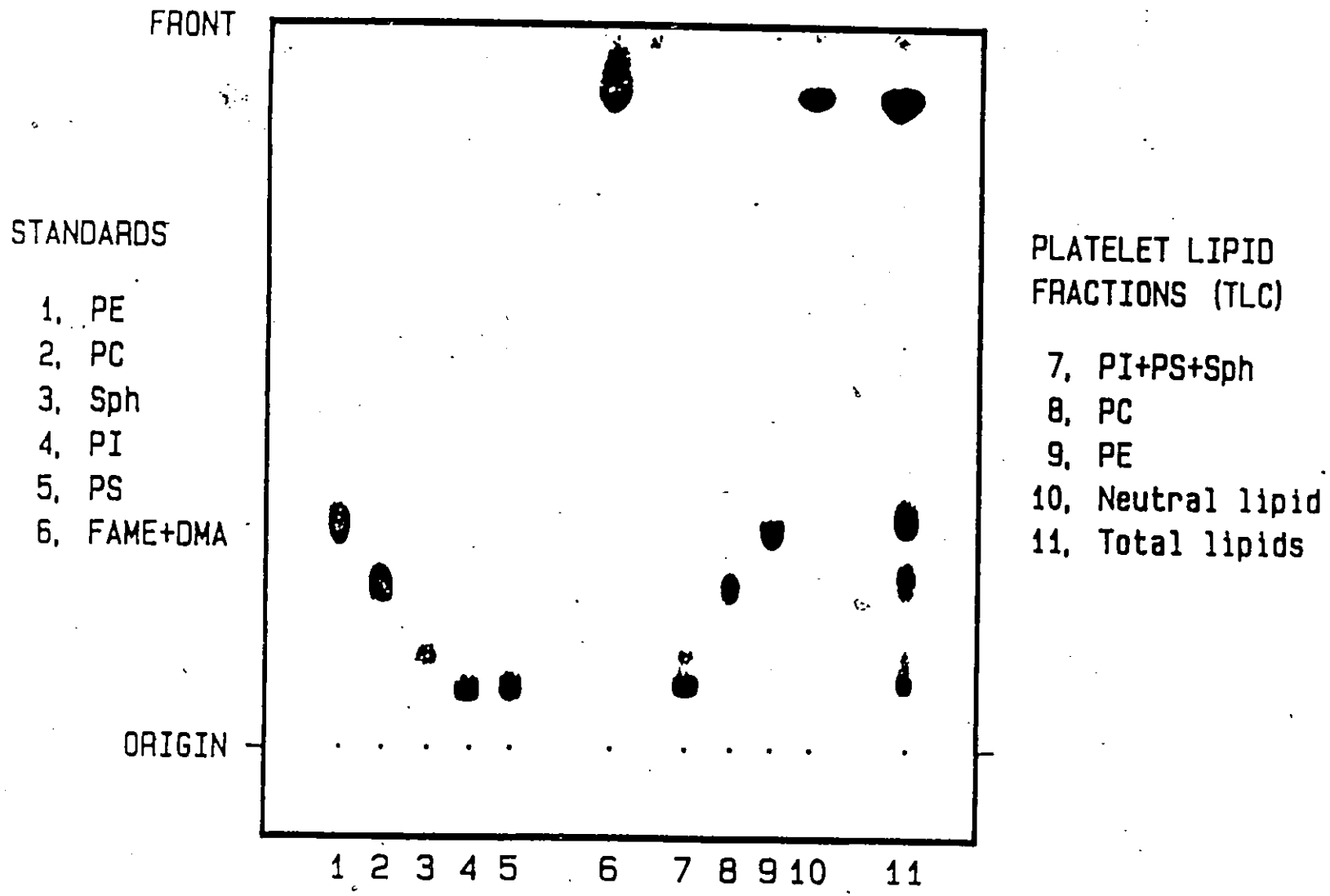

FRONT
STANDARDS
1, PE
2, PC
3, Sph
4, PI
5, PS
6, FAME+DMA
ORIGIN
PLATELET LIPID FRACTIONS (TLC)
7, PI+PS+Sph
8, PC
9, PE
10, Neutral lipid
11, Total lipids
1 2 3 4 5 6 7 8 9 10 11

TABLE 6: Characterization of Phospholipid Components of Platelets by Analytical TLC and Staining Behaviour

Phospholipid	Iodine stain	Sulfuric Acid and charred	Ninhydrin stain	Phosphate stain	Carbonyl stain	Solvent B	Solvent C
origin	+	+	-	-	-	-	-
Sph	+	+	-	+	-	0.35	0.30
PI/PS	+	+	+	+	-	0.22	0.55
LPE	+	(+)	+	+	-	0.38	0.43
LPC	+	(+)	-	+	-	0.19	0.16
PC	+	+	-	+	(+)	0.52	0.46
PE	+	+	+	+	+	0.63	0.81
Solvents front (NL)	+	+	-	-	(+)	1.00	1.00

+ = positive reaction

- = no result or absence of reaction

(+) = faint positive reaction

Solvent B = $CHCl_3$:MeOH:Amm:H_2O (70:30:4:1) double development

Solvent C = $CHCl_3$:MeOH:AA:H_2O (50:28:10:5)

Trace amounts of lyso compounds were observed after preparative plate elution of phospholipid from the silica gel (Table 6). The acid solvent system (chloroform:methanol: acetic acid:water) resolved the phospholipids, with PS and PI running high and well separated from the other lipids; however, the yields of PC and PE plasmalogen after preparative TLC and elution were low. The basic solvent system (chloroform/ methanol/ ammonium hydroxide/ water, 70:30:4:1) with double development separated the PC and PE from the other major phospholipids (Fig. 15) and resulted in better plasmalogen recoveries as indicated by analytical TLC of the PC and PE separated components. The basic solvent was then used routinely to avoid breakdown of plasmalogen forms. The staining characteristics and TLC mobilities of the different phospholipid components are summarised in Table 6.

The phospholipid compositon of fresh platelets was based on phosphorus determination of components separated by preparative TLC in the basic solvent system (Table 7). The major phospholipid classes were PC (38%), PE (32%) and sphingomyelin, PI and PS (30% combined). These findings are in close agreement with previous reports on phospholipid composition of human platelets (Cohen and Derksen, 1969; Broekman et al., 1980; Mahadevappa and Holub, 1982; Mueller et al., 1983).

The molecular class composition of PC and PE (see Table 7) was determined by the method outlined in Figure 12, as

TABLE 7: Phospholipid Composition of Fresh Resting Platelets

component	% Total PL present study	Mueller et al. 1983 (n = 3)	Mahadevappa and Holub, 1982 (n = 4)
SPH + PI + PS	29.3 ± 1.3[a]	32.9 ± 4.1	32.4 ± 1.4
PC	38.2 ± 2.7[a]	38.0 ± 1.6	38.3 ± 0.9
diacyl	85.8 ± 2.0[b]	81.8 ± 2.6	-
alkylacyl	10.4 ± 1.0[b]	9.7 ± 0.3	-
alkenylacyl	1.4 ± 0.4[b] (1.0.7)[c]	8.8 ± 2.4	-
PE	32.2 ± 1.3[a]	25.3 ± 1.8	29.4 ± 1.4
diacyl	59.7 ± 2.0[b]	36.1 ± 0.3	-
alkylacyl	8.0 ± 0.4[b]	3.5 ± 0.1	-
alkenylacyl	27.4 ± 1.5[b] (24.5±3.2)[c]	60.0	-

[a]Phospholipids were separated by TLC and quantified by P-analysis as described in Materials and Methods. Results are the mean ± deviation for duplicate analysis.

[b]Quantified by GLC method as described and expressed as % of individual PL component PC or PE respectively. The values represent mean ± standard deviation for triplicate analysis.

[c]Alkenylacyl content as determined by I_2 addition method described in Material and Methods. The values are the mean ± deviation for duplicate analysis.

All samples were from one pool of platelets.

detailed above for the standard plasmalogen PE. The PC fraction was found to contain 86% diacyl, 10% alkylacyl and 1.4% alkenylacyl classes. PE contained a larger amount (27%) alkenylacyl, and lesser amount of alkylacyl (8.0%) and mainly diacyl (60%) classes. These molecular class compositions generally agree with other studies, although Mueller et al.(1983) report higher amounts of plasmalogen in PE (60%). However, the plasmalogen content in PE has been reported to range from 30-60% (Horrocks, 1972; Horrocks and Sharma, 1982).

The fatty acid, alkyl and alkenyl chain distribution of platelet PC and PE are shown in Tables 8 and 9, respectively. The major fatty acid residues in both PE and PC were 16:0, 18:0, 18:1, 18:2 and 20:4 in agreement with previous reports (Horrocks, 1972; Horrocks and Sharma, 1982; Mahadevappa and Holub, 1982; Mueller et al., 1983; Skeaf and Holub, 1985).

The alkyl ether chains of PC consisted mainly of 16:0, 18:0 and 18:1 with smaller amounts of 14:0 and 20:0 (Table 8). This again agrees with Mueller et al. (1983) who also found that 16:0, 18:0 and 18:1 were the major alkyl chains in platelet PC. In the PE fractions, the alkyl ether chains range from 14:0 to 18:2 (Table 9). The major chains were 14:0, 14:1 and 18:2 with very low amounts of 16:0 and 18:0. There were no literature values for comparison.

Analysis of the alkenyl chain distribution of the PC

TABLE 8: Fatty Acid, Alkenyl and Alkyl Ether Composition of Phosphatidylcholine in Fresh Human Platelets

Chain	Present Study	Mueller et al., 1983	Mahadevappa and Holub, 1982
		mol %	
		Fatty Acid Methyl Esters	
14:0	1.1 ± 0.1	-	-
16:0	27.1 ± 2.4	30.2 ± 0.8	30.5 ± 0.6
16:1	1.9 ± 0.1	1.3 ± 0.2	-
18:0	14.9 ± 1.9	13.0 ± 0.6	13.4 ± 0.5
18:1	24.9 ± 1.4	23.8 ± 0.7	24.1 ± 0.9
18:2	9.5 ± 1.6	9.0 ± 0.3	11.2 ± 0.9
20:0	1.1 ± 0.4	0.8 ± 0.2	1.3 ± 0.3
20:1	1.6 ± 0.4	0.9 ± 0.1	1.4 ± 0.1
20:3	2.4 ± 0.6	1.7 ± 0.1	2.0 ± 0.2
20:4	18.5 ± 1.4	16.0 ± 0.7	14.1 ± 0.5
other			1.7 ± 0.3
		ALKYL-ETHER (AS MAGDs)	
14:0	18.3 ± 1.0	-	
16:0	29.6 ± 0.5	43.6	
18:0	27.5 ± 3.0	36.6	n.d.
18:1	21.3 ± 0.2	15.6	
20:0	2.3 ± 2.3	4.1	
		ALKENYL-ETHER (AS DMAs)	
16:0	82.4 ± 4.0	34.0	
18:0	17.4 ± 2.0	52.0	n.d.
18:1	-	9.2	
18:2	-	4.8	

n.d., not determined.

The results are the mean ± standard deviation for triplicate analysis. All samples were from one pool of platelets.

TABLE 9: Fatty Acid, Alkenyl and Alkyl Ether Chain Composition of Phosphatidyl Ethanolamine in Fresh Human Platelets

Chain	Present study	Mueller et al. 1983	Mahadevappa and Holub, 1982
		mol %	
		Fatty Acid Methyl Esters	
14:0	0.4 ± 0.1	0.2 ± 0.2	-
16:0	7.0 ± 0.7	3.9 ± 0.9	8.5 ± 0.7
16:1	0.7 ± 0.4	0.6 ± 0.2	-
18:0	17.0 ± 1.2	13.8 ± 2.3	20.5 ± 0.7
18:1	9.0 ± 0.9	7.5 ± 0.6	11.3 ± 0.7
18:2	5.7 ± 0.5	2.4 ± 0.4	3.3 ± 0.2
20:0	0.6 ± 0.1	0.37 ± 0.2	0.8 ± 0.3
20:1	0.5 ± 0.1	0.44 ± 0.2	-
20:3	0.8 ± 0.1	0.78 ± 0.2	-
20:4	47.0 ± 3.4	52.8 ± 5.0	43.6 ± 1.6
22:4	4.8 ± 2.0	8.4 ± 0.8	6.7 ± 0.2
22:5	4.7 ± 0.5	5.2 ± 0.9	2.7 ± 0.1
22:6	2.1 ± 0.9	2.6 ± 0.5	2.5 ± 0.5
other		1.0	-
		ALKYL ETHER (AS MAGDs)	
14:0	26.7 ± 2.4	n.d.	n.d.
14:1	29.0 ± 3.6		
16:0	6.6 ± 1.0		
18:0	12.7 ± 2.0		
18:2	25.0 ± 2.5		
		ALKENYL ETHERS (AS DMAs)	
16:0	25.0 ± 0.9	27.6	32.1 ± 4.9
18:0	56.0 ± 1.6	40.5	54.9 ± 9.9
18:1	17.4 ± 3.1	30.2	13.0 ± 2.5
18:2	-	1.7	-

n.d., not determined.

The results are the mean ± standard deviation for triplicate analysis.

All samples were from one pool of platelets.

indicated that there were only two major chains, 16:0 and 18:0 (Table 8). Mueller et al. (1983) have reported 18:0 (52%) and 16:0 (34%) as major chains with 18:1 and 18:2 as minor components. PE alkenyl chain composition was in agreement with published results: 18:0 (56%), 16:0 (27%) and 18:1 (17%) (Table 9). GLC analysis of the DMAs extracted after alkaline hydrolysis of FAME and DMA mixtures indicated that these indeed were the major aldehyde chains in PC and PE. There was no overlap in retention times of FAMEs with DMAs.

Although fatty acyl residues show little variability from study to study, there seems to be more variability in the ether chain composition (Tables 8 and 9). The causes of variability in the ether chain composition are unknown.

III. Effect of Storage on Platelets

Platelets are routinely stored as platelet-rich plasma in PL 732 bags at 22°C for up to five days during which they retain their therapeutic effectiveness. There are however changes in platelet response to certain single stimuli with no alteration to the platelet synergistic pairs of stimuli. In this section, the effect of storage on platelet function and on the composition of specific components such as cholesterol and phospholipids will be presented.

III.1 Platelet Function

Platelet function was measured by its ability to aggregate in response to certain specific stimuli and the results are presented in the form of a synograph (Fig. 14). The percentage aggregation is expressed as the radial distance from the center along the spoke corresponding to the stimulus under study (Fig. 16). The synograph, prepared for freshly collected platelets and after 72 and 120 hours of storage, indicates that platelet response to all single stimulus tested decreased considerably with the exception of calcium ionophore. The synergistic effect of these activators however was not affected (Rock et al., 1985; Adams et al., 1986).

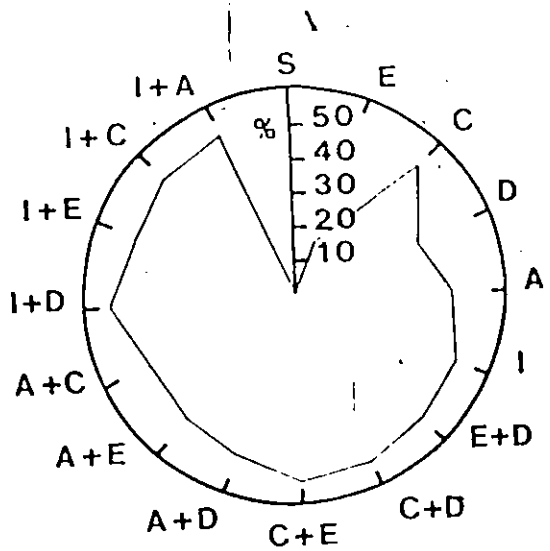
III.2 Total Lipid Composition

Total lipid extracted by the Bligh-Dyer procedure remained constant (0.44 mg lipid dry weight/10^9 cells). Preliminary studies by analytical TLC (figure 17) indicated that there were no qualitative changes in the proportions of lipid components observed with storage time.

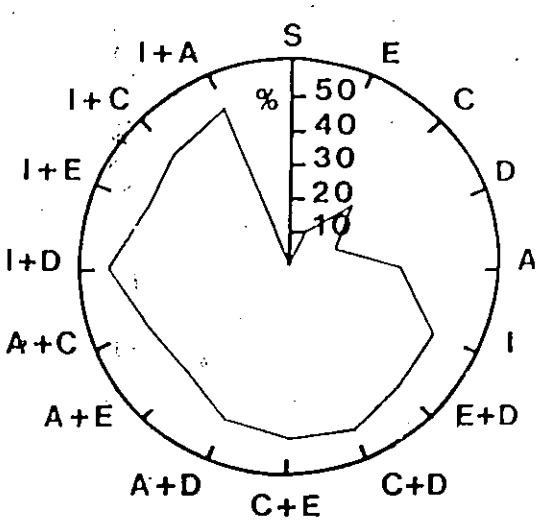
Study of the cholesterol content (Table 10) indicated that there were no significant changes in cholesterol content or cholesterol: phospholipid mole ratio in the total lipids during storage. Total phospholipid content also shows no significant change on storage. The cholesterol to

Figure 16: Synographs of the Effect of Storage on Platelet Aggregation by Various Stimulants

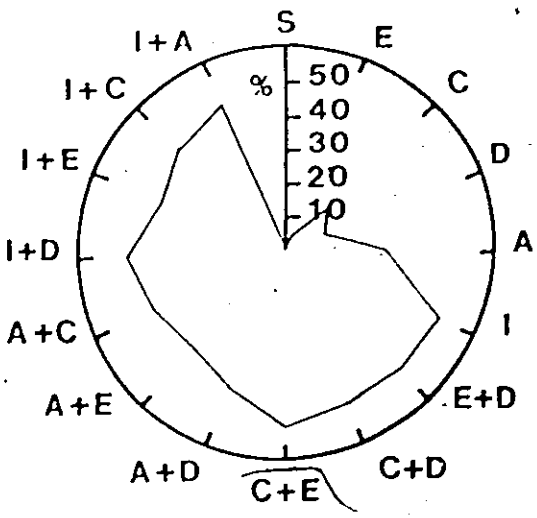
The percentage aggregation is expressed as the radial distance from the center along the spoke corresponding to the stimulus under study: E, epinephrine; C, collagen; D, ADP; A, arachidonic acid; I, ionophore or pairs of these stimulants at the stated concectrations (see methods). Platelets were fresh or stored at 22°C for 72 and 120 hours in Fenwal PL 732 blood bags at a concentration of 10^8 cells/ ml.



Fresh



72 Hours



120 Hours

Figure 17: Analytical TLC of the Effect of Storage and BHT on Platelet Phospholipids

1. PE standard
2. PC standard
3. SPH standard
4. PI/PS standards
5. Fresh Platelets (Day 0)
6. Day 3 of Storage
7. Day 3 of Storage with BHT
8. Day 5 of Storage
9. Day 5 of Storage with BHT

Solvent System $CHCl_3$:CH_3OH:NH_4OH:H_2O (70/30/4/1) with double development

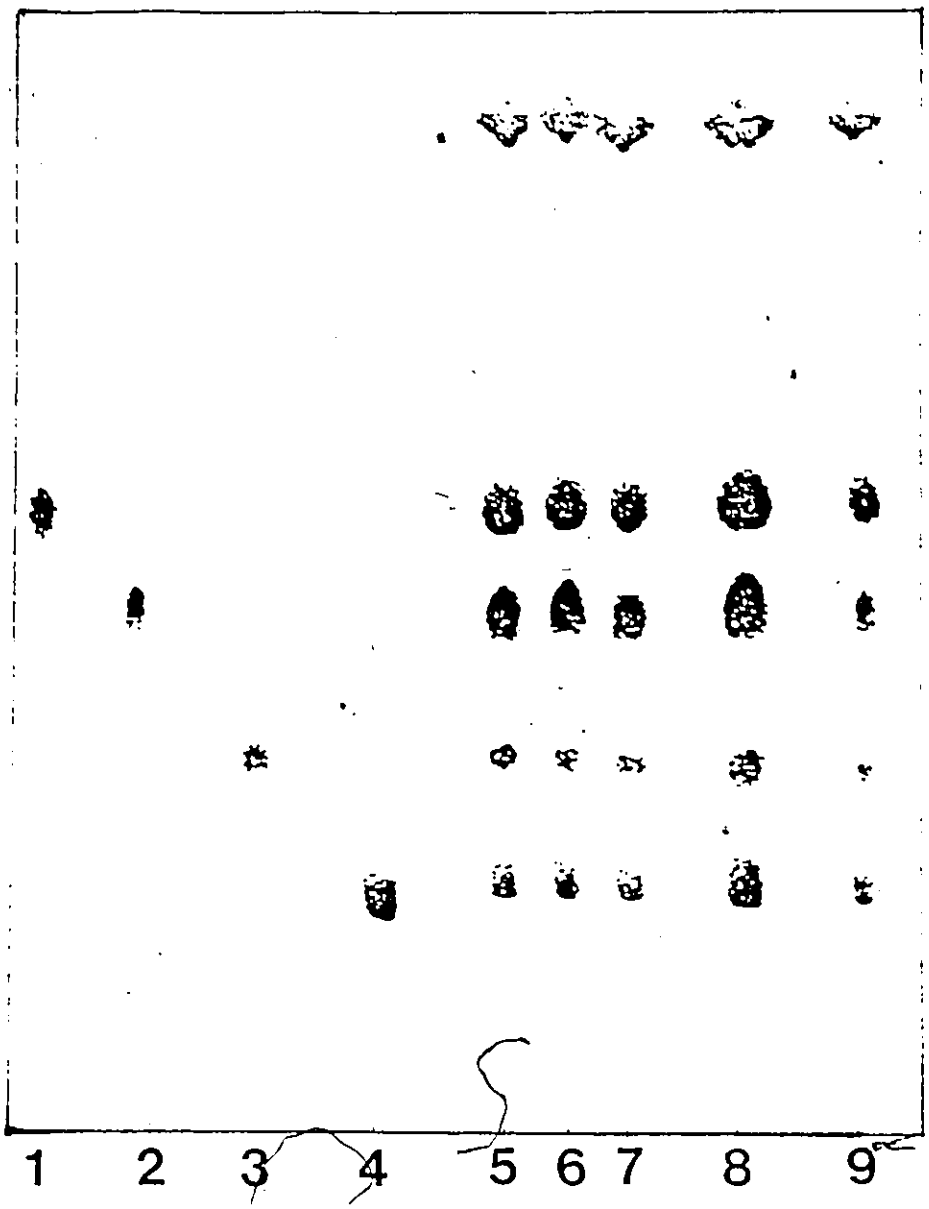

1
2
3
4
5
6
7
8
9

Table 10: Effect of Storage on Platelet Lipid Components

Storage time, days	Weight % of Total Lipid		mole ratio of cholesterol: phospholipid **
	Phospholipid*	Cholesterol	
0	67.5 ± 2.9	13.2 ± 0.6	0.40 ± 0.02
3	63.3 ± 2.3	10.8 ± 0.7	0.34 ± 0.03
5	62.5 ± 1.9	10.9 ± 0.4	0.35 ± 0.02

Values given are mean ± deviation for duplicate samples.

* weight % phospholipid was calculated assuming an average molecular weight of phospholipid, 800.

** mole ratio of cholesterol to phospholipid is calculated as follows:

$$\text{mole ratio} = \frac{\text{wt. \% cholesterol}}{\text{wt. \% phospholipid}} \times \frac{800}{386}$$

(800 = average PL molecular weight; 386 = molecular weight of cholesterol).

All samples were from one pool of platelets.

phospholipid mole ratio (0.34-0.40) was lower than the value (0.5) previously reported (Table 10).

III.3 Phospholipid Composition

The major phospholipid components of fresh platelets were PC (38.2%), PE (32.2%) and Sph/PI/PS (29.6% combined) (Table 11). There were no significant changes observed as a result of storage, the proportions of the phospholipids remaining constant within experimental error.

The molecular class compositions determined as described above are summarized in Table 12. Overall, the total lipids of fresh platelets contained mainly 70% diacyl, 9.5% alkenylacyl and 8.3% alkylacyl phospholipid class composition and no significant changes were observed on storage.

For the individual PC component, the alkylacyl content significantly decreased after 3 days of storage from 10.4% to 6.7%, then remaining constant upto five days. In contrast, no significant changes were noted for the diacyl or the alkenylacyl classes of PC (Table 12).

Furthermore, this trend was also observed in the isolated PE fraction: no changes in either the diacyl or the alkenylacyl composition were seen; again a decrease in the alkylacyl composition was noted. In this case, the decrease in three days was more dramatic (from 8% to 3 %) representing a 62% decrease and persisted for up to five days of storage

TABLE 11: Effect of Storage on Platelet Phospholipid Composition

Storage time	Sph/PI/PS*		PC*		PE*	
days	nmol/100mg lipid	moles %	nmol/100mg lipid	moles %	nmol/100mg lipid	moles %
0	22.0 ± 1.0	29.6 ± 1.3	28.4 ± 2.0	38.2 ± 2.7	23.9 ± 1.0	32.2 ± 1.3
3	23.2 ± 1.0	29.0 ± 1.3	32.2 ± 1.0	40.3 ± 1.3	24.6 ± 1.0	30.7 ± 1.2
5	18.6 ± 1.0	26.9 ± 1.4	27.3 ± 1.0	39.4 ± 1.4	23.3 ± 1.0	33.7 ± 1.4

*Based on P-determination by the modified method of Bartlett, (Kates,. 1972). The results are the mean ± deviation for triplicate analysis. All samples were from one pool of platelets.

Table 12: Effect of Storage on the Molecular Classes of Platelet Phospholipids

Storage time, days	moles %*			Total Recovery
	Diacyl	Alkenylacyl	Alkylacyl	
		Total Lipids		
0	69.6 ± 2.3 (571)+	9.5 ± 1.2 (89)	9.5 ± 1.2 (78)	87.4 ± 4.7
3	78.3 ± 5.1 (546)	10.1 ± 1.2 (88)	6.6 ± 1.5 (52)	95.0 ± 7.8
5	73.8 ± 6.8 (569)	10.7 ± 1.0 (73)	8.3 ± 0.9 (61)	92.8 ± 8.7
		PC		
0	85.8 ± 2.0	1.4 ± 0.4	10.4 ± 1.0	97.6 ± 3.4
3	86.2 ± 4.1	1.8 ± 0.4	6.7 ± 1.1**	94.7 ± 5.6
5	82.0 ± 4.0	2.5 ± 0.5	7.0 ± 1.4**	91.5 ± 5.9
		PE		
0	59.7 ± 2.0	27.4 ± 1.5	8.0 ± 1.4	98.2 ± 4.9
3	59.5 ± 5.0	30.5 ± 3.0	3.0 ± 1.0**	93.0 ± 9.0
5	56.6 ± 5.0	25.2 ± 1.4	3.6 ± 1.0**	85.4 ± 7.4

* mol % data calculated in terms of moles phospholipid phosphorus as in Table 5.

** significantly different from day 0 values at $p < 0.05$.

+ data in parentheses are values in nmol/mg total lipid.

Results are the mean ± standard deviation for triplicate analysis. All samples were from one platelet pool.

(Table 12).

Fast-atom bombardment mass spectrometry (FAB/MS) was used to study the molecular species of isolated PC and PE. PC contained major molecular species with protonated parent ions [MH+] having m/z 782, 760 and 758 (Table 13) corresponding to diacyl molecular species with palmitate at the sn-1 carbon, and arachidonate, oleate or linoleate, respectively, at the sn-2 carbon. Other parent ions easily identified included species with m/z 810 (18:0-20:4), 788 (18:0-18:1) and 786 (18:0-18:2) (Appendix, FAB/MS-4; Table 13).

In addition, the FAB/ MS of the PC component (Table 13) showed protonated molecular ion [MH+] peaks for Paf at m/z 524, 522, 552 and 550 corresponding to ether linked hexadecyl, hexadecenyl, octadecyl and octadecenyl groups at the sn-1-glycerol position and acetate at sn-2-glycerol position. The fragmentation pattern of these compounds were compared with authentic Paf (hexadecyl) standard. An abundant molecular ion appeared at m/z 524 (Appendix, FAB/MS-3). This protonated molecular ion appears to lose hydrogen (m/z 522), the elements of ketene (m/z 482) and acetate with proton transfer (m/z 466). The fragmentation pattern observed indicated that preformed Paf molecules are present in the platelet lipids (Appendix, FAB/MS-4). The fragmentation pattern seen for standard Paf is consistant with other reports (Clay et al., 1984).

FAB/MS of platelet PE (Table 14, Appendix, FAB/MS-5)

Table 13: Analysis of Fresh Platelet Phosphatidylcholine Molecular Species by FAB/MS

Molecular Class	[MH+]	Assignment (sn-1/sn-2)	% Area Modified*	%**
Diacyl				
	734	16:0/16:0	7.3	5.7
	756	16:0/18:3	5.1	4.0
	758	16:0/18:2	23.5	18.2
	760	16:0/18:1	60.9	47.2
	762	16:0/18:0	7.5	5.8
	782	16:0/20:4	17.5	13.6
	784	18:0/18:3; 16:0/20:3	7.4	5.7
	786	18:0/18:2	10.6	8.2
	788	18:0/18:1	20.0	3.8
	808	18:1/20:4	4.9	3.8
	810	18:0/20:4	9.6	7.4
	812	18:0/20:3	1.5	1.2
Alkenylacyl (P) or Alkylacyl (A)				
	746	16:0/18:0 (A)	1.9	1.5
	772	18:0/18:0 (P); 18:0/18:1(A) 18:0/18:1 (A)	1.6	1.2
	774	18:0/18:0 (A)	2.3	1.8
	796	18:0/20:3 (A)	1.5	1.2
Paf Analogues				
	522	16:1/acetate	55.7	54.7
	524	16:0/acetate	32.7	31.9
	550	18:1/acetate	12.0	11.8
	552	18:0/acetate	1.6	1.6

* Data from mass spectrometer print-out (appendix, FAB/MS-4) given as % of peak with maximum intensity.

** Data expressed as % of the total peak intensities ([MH+] 734-812).

Results are based on a single analysis.

Table 14: Analysis of Fresh Platelet Phosphatidylethanolamine Molecular Species by FAB/MS

Molecular Class	[MH+]	Assignment (sn-1/sn-2)		χ* 1	χ* 2
Diacyl					
	718	16:1/18:0;	16:0/18:1	-	1.2
	722	18:1/18:2		-	1.2
	740	16:0/20:4		-	3.9
	742	16:0/20:3		-	1.5
	744	16:0/20:2		2.6	5.3
	748	16:0/20:0;	18:0/18:0	-	1.1
	766	18:1/20:4;	16:1/22:4	10.0	2.3
	768	18:0/20:4		28.4	18.9
	769			4.2	-
	770	18:0/20:3		-	1.4
	794	18:1/22:6		-	4.7
	795			-	1.4
	796	18:0/22:6;	18:1/22:5	-	1.5
	810	18:0/22:5		-	1.1
Alkenylacyl (P) or Alkylacyl (A)					
	724	16:0/20:4(P)		8.9	8.4
	725			2.8	2.7
	750	18:1/20:4(P)		8.7	-
	751			10.0	20.9
	752	18:0/20:4(P);	18:1/20:4(A)	11.6	-
	753			-	2.6
	754	18:0/20:3(P);	18:0/20:4(A)	2.7	2.4
	776	18:0/22:6(P);	18:1/22:5(P)	-	2.2
	778	18:0/22:5(P);	18:1/22:4(P)	3.5	5.1
		18:0/22:6(A);	18:1/22:5(A)		
	779			-	1.3
	780	18:0/22:4(P)		3.2	5.2
	782	18:0/22:3(P);	18:0/22:4(A)	-	1.4

* Data expressed as % of total peak intensities ([MH+] 718- 810) (Appendix, FAB/MS-5); shown are two analyses of the same sample.

demonstrated an abundance of alkenylacyl or plasmalogen class including major protonated parent ions at m/z 724, 750 and 752 corresponding to arachidonate at the sn-2 carbon and 16:0, 18:1 and 18:0 alkenyl chains, respectively, at the sn-1 carbon. Longer polyunsaturated fatty acid chains were also observed with the alkenyl chains; for example, m/z 778, and 780 represented parent ions with 22:4 at the sn-2 position and 18:0 or 18:1 alkenyl chains, respectively, at the sn-1 carbon.

Predominant diacyl molecular species of the PE (Table 14) consisted of arachidonate at the sn-2 carbon and palmitate (m/z 740), stearate (m/z 768) or oleate (m/z 766) at the sn-1 carbon.

Previous studies by Gross (1984, 1985) indicated that diacyl, but not alkenylacyl undergoes a facile loss of ethanolamine phosphate after ionization and desorption from the glycerol matrix. Therefore, quantificaton of the molecular species based on the relative intensities of the parent ions would be incorrect as the relative intensities would not accurately reflect their relative abundance due to differences in the rate of unimolecular decomposition of ionized plasmalogen and diacyl phospholipids. In addition, although the major [MH$^+$] ion peaks present in choline or ethanolamine phospholipids in a given sample were reproducibly identified, the relative intensities of peaks on replicate spectra of the same sample could differ by about

15%. The presence of sodiated parent ions could also lead to substantial errors in quantifying the relative abundance of phospholipid molecular species. Therefore, results of fast-atom bombardment mass spectrometry of complex mixtures should be considered only as semi-quantitative. GLC data, being more reproducible was used for quantitative analysis of molecular species of platelet PC and PE (see Tables 15-17)

GLC analysis of platelet PC and PE FAMEs, DMAs and MAGDs corroborated the molecular species identified by FAB/MS. The major fatty acids in PC from fresh platelets were palmitate (27.1%), stearate (15%), oleate (25%) and arachidonate (19%). This profile showed no significant change with storage time except for a minor decrease in arachidonic acid (Table 15). The predominant vinyl ether linked chains were palmityl and stearyl aldehydes in the mole ratio 4.7:1. The proportion of 16:0 to 18:0 aldehyde chains decreased with storage time and remained at about 1:1 after 5 days of storage. The alkyl ether linked groups were composed mainly of 16:0, 18:0 and 18:1. The proportions of these chains did not vary significantly with storage time (Table 15).

The results of GLC analysis of platelet PE FAMEs, DMAs and MAGDs are summarised in Table 16. Arachidonate and stearate are the major fatty acid chains; as with the PC component there were no significant changes observed on storage. The alkenyl chains were primarily of 18:0, with smaller amounts of 16:0 and 18:1. The proportions of these

TABLE 15: Effect of Storage on the Fatty Acid (FAME), Alkenyl (DMA) and Alkyl Ether (MAGD) Components of PC

Chain Type	Day 0 (Fresh)	Day 3	Day 5
		moles %	
FAME			
14:0	1.0 ± 0.7	0.8 ± 0.2	1.0 ± 0.3
16:0	27.1 ± 2.4	25.0 ± 0.0	25.8 ± 0.5
16:1	1.9 ± 0.1	1.4 ± 0.7	1.8 ± 0.5
18:0	14.9 ± 1.9	14.4 ± 0.9	14.7 ± 0.2
18:1	24.9 ± 1.4	27.2 ± 0.9	26.5 ± 0.3
18:2	9.5 ± 1.6	10.4 ± 0.1	9.9 ± 0.5
20:0	1.0 ± 0.4	0.9 ± 0.1	1.2 ± 0.5
20:1	1.6 ± 0.4	1.6 ± 0.4	1.5 ± 0.6
20:3	2.4 ± 0.6	2.7 ± 0.6	1.9 ± 0.2
20:4	18.5 ± 1.4	16.2 ± 0.3	16.0 ± 0.3
DMA			
16:0	82.4 ± 4.0	42.7 ± 1.1	48.4 ± 1.2
18:0	17.6 ± 2.0	57.4 ± 1.1	51.6 ± 1.2
MAGD			
14:0	18.3 ± 1.0	7.7 ± 0.8	17.0 ± 3.9
16:0	29.6 ± 0.5	33.3 ± 0.5	30.6 ± 2.2
18:0	27.5 ± 3.0	27.8 ± 0.9	28.3 ± 0.5
18:1	21.3 ± 0.2	23.5 ± 1.4	20.8 ± 1.1
18:2	-	7.7 ± 1.9	4.9 ± 1.6
20:0	2.3 ± 2.3	-	-

The results are the mean ± standard deviation for triplicate analysis. All samples were from one pool of platelets.

TABLE 16: Effect of Storage on the Fatty Acid (FAME), Alkenyl (DMA) and Alkyl Ether (MAGD) Components of PE

Chain Type	Day 0 (Fresh)	Day 3	Day 5
		moles %	
FAME			
14:0	0.4 ± 0.1	0.6 ± 0.1	0.6 ± 0.02
16:0	7.0 ± 0.7	5.7 ± 0.4	5.8 ± 0.8
16:1	0.7 ± 0.4	0.7 ± 0.1	0.6 ± 0.2
18:0	17.0 ± 1.2	16.5 ± 0.4	18.2 ± 2.3
18:1	9.0 ± 0.9	7.5 ± 0.2	6.6 ± 0.03
18:2	5.7 ± 0.5	4.0 ± 0.0	2.9 ± 0.3
20:0	0.6 ± 0.1	0.6 ± 0.1	0.8 ± 0.3
20:1	0.5 ± 0.1	0.5 ± 0.1	0.9 ± 0.3
20:3	0.8 ± 0.1	0.9 ± 0.0	1.6 ± 0.2
20:4	47.0 ± 3.4	46.9 ± 1.2	42.9 ± 2.5
22:2	-	0.7 ± 0.0	1.1 ± 0.1
22:4	4.8 ± 2.0	6.9 ± 0.2	5.9 ± 0.6
22:5	4.7 ± 0.5	4.5 ± 0.1	-
22:6	2.1 ± 0.9	2.8 ± 0.3	2.4 ± 0.2
DMA			
16:0	27.0 ± 0.9	28.0 ± 0.5	32.0 ± 4.2
18:0	56.0 ± 1.6	53.4 ± 2.9	50.4 ± 2.3
18:1	17.4 ± 3.1	18.6 ± 2.9	17.5 ± 1.6
MAGD			
14:0	26.7 ± 2.4	17.9 ± 0.7	9.8 ± 0.5
14:1	29.0 ± 3.6	31.6 ± 2.8	21.2 ± 6.9
16:0	6.6 ± 1.0	8.3 ± 0.1	30.5 ± 0.3
16:1	-	7.3 ± 0.5	-
18:0	12.7 ± 2.0	10.0 ± 2.1	38.7 ± 7.0
18:2	25.0 ± 2.5	24.5 ± 1.6	-

The results are the mean ± standard deviation for triplicate analysis. All samples were from one pool of platelets.

TABLE 17: Effect of Storage on Fatty Acid (FAME) Alkenyl (DMA) and Alkyl Ether (MAGD) Component of Total Lipids

Chain Type	Day 0 (Fresh)	Day 3	Day 5
		mole %	
FAME			
14:0	tr	tr	tr
16:0	15.2 ± 1.2	14.2 ± 1.3	13.9 ± 0.1
16:1	tr	tr	tr
18:0	19.0 ± 0.6	18.7 ± 1.6	18.7 ± 0.4
18:1	18:0 ± 0.2	17.7 ± 1.1	17.2 ± 0.3
18:2	6.0 ± 0.1	6.4 ± 0.9	6.6 ± 0.1
20:0	1.2 ± 0.1	0.9 ± 0.2	0.9 ± 0.1
20:1	1.0 ± 0.1	0.9 ± 0.2	0.7 ± 0.1
20:3	1.4 ± 0.1	1.1 ± 0.3	1.4 ± 0.1
20:4	29.1 ± 1.2	29.1 ± 1.5	30.3 ± 1.2
22:4	3.1 ± 0.4	2.5 ± 0.9	2.9 ± 0.3
22:5	2.2 ± 0.4	1.2 ± 1.1	1.4 ± 0.8
22:6	1.6 ± 0.3	1.6 ± 0.6	1.9 ± 0.2
DMA			
16:0	31.2 ± 2.8	29.3 ± 0.4	30.3 ± 0.4
18:0	55.1 ± 2.4	53.9 ± 3.2	54.1 ± 1.6
18:1	13.8 ± 0.9	16.8 ± 2.8	15.5 ± 1.2
MAGD			
14:0	35.8 ± 3.0	29.9 ± 1.9	32.0 ± 1.8
14:1	21.8 ± 2.0	12.1 ± 0.6	11.2 ± 2.2
16:0	11.5 ± 2.0	18.9 ± 2.6	17.0 ± 0.4
18:0	15.4 ± 2.0	16.9 ± 3.4	19.4 ± 2.5
18:1	10.7 ± 1.7	11.9 ± 2.1	14.5 ± 1.9
18:2	3.6 ± 0.6	6.6 ± 1.7	8.3 ± 1.4
20:0	4.9 ± 0.9	3.2 ± 0.1	-

The results are the mean ± standard deviation for triplicate analysis. All samples were from one pool of platelets.

chains did not show any alteration with storage time. The alkyl linked chains demonstrated a wider range of chain lengths and a varying profile.

The fresh platelet PE contained mainly 14:0, 14:1 and 18:1 and 18:2 alkyl groups. After five days of storage, there were higher proportions of 16:0 and 18:0 alkyl chains, but the overall proportion of alkylacyl class had decreased (Table 12).

Analysis of total lipids also showed no significant changes in its fatty acid, alkenyl or alkyl chain components with storage time (Table 17), indicating that none of the ether phosholipid components have undergone changes during storage.

D. DISCUSSION

I. Errors and Limitations of Procedure

The method developed in this study was effective for the analysis of platelet samples with an overall reproducibility of $\pm$ 11 % for molecular class composition of triplicate samples. This error can be accounted for in the GLC analysis where the error can vary from $\pm$ 2 to $\pm$ 20 % depending on the chain type and its proportion (Table 12). The following errors and consequent assumptions are involved in the GLC analysis.

I.1 Peak Area Errors

The method of Carroll (1961) which was used to measure the GLC peak areas of FAMEs, DMAs and MAGDs is based on the assumption that peaks obtained closely approximate a Gaussian distribution curve. Carroll's method is rapid and convenient, allowing for analysis of large numbers of samples within a reasonable acceptable range of error. The probable error inherent in Carroll's method is $\pm$ 1.5 to $\pm$ 2.0% (Carroll, 1961); in addition, errors of $\pm$ 0.5 to 1.0% may be introduced through errors of measurement of peak height, and retention time giving an overall error range of $\pm$ 2.0 to $\pm$ 3.0% (absolute). Consequently, only changes in proportions of major components of FAMEs, DMAs and MAGDs that are $\geq$ 4% were considered significant in the present studies. Absolute

values and changes reported for minor components (<4%) cannot be regarded as significant, and therefore will not be considered in the discussion.

I.2 Error in Internal Standard

The internal standard 21:0 ME was chosen because its retention time does not overlap with major FAME, DMA or MAGD peaks. In addition, its retention time falls in the middle of the chart; therefore, the internal standard is easily identified in the higher temperature analyses as well as the low temperature analysis.

However, several assumptions were made in calculating the mol% of the peaks. It was assumed that there is a direct correlation of the weight of the internal standard FAME to the area of its peak and that this correlation applies equally to saturated and unsaturated FAMEs, DMAs and MAGDs. The response of the flame-ionization detector is proportional to the mass or weight of the component and may differ for each of these chain types. Calibration with known amounts of each chain type should be carried out and correction factors calculated as follows:

$$\frac{\text{Area of chain type peak}}{\text{Area of internal standard}} \times \frac{\text{mg chain type}}{\text{mg internal standard}}$$

For example, calibration with known amounts of DMA gave a factor of 0.91 $\pm$ 0.10. Thus, use of 21:0 ME as internal standard resulted in the following errors for analysis of

saturated FAMEs 2%, unsaturated FAMEs 5%, DMAs 10%, and MAGDs 12%.

Summation of all these errors gave an overall error 11%, 14% and 16% for each molecular class of diacyl, alkenylacyl and alkylacyl respectively with a range of ± 2.0 to ± 20 % (average ± 11 %) observed (Table 12). However for the total recoveries, the average error was ± 5 %, agreeing well with that of the major molecular class of each phospholipid, namely, the diacyl class.

I.3 DMA Formation

DMAs are easy to prepare in quantitative yield from aldehydes (Gray, 1969) and are well resolved from FAMEs under the GLC conditions already described. DMAs may decompose to form alk-1-enyl methyl esters if aluminium columns or stationary phase containing acid are used in the gas chromatograph (Mahadevan, 1971). However if glass columns are used with packing materials that contain catalyst-free materials, then artifact formation is negligible and acetal derivatives are recommended for analysis of aldehydes (Gray, 1976). To check for any breakdown of DMAs during GLC, the amount of alkenyl ether lipid was also quantified by iodine assay. DMA quantification by GLC was in agreement (≤ 2.9%) with the colorimetric assay of vinyl ether determination using the iodine addition method (see B.II.5.ii), (Table 7).

II. Application of Results of Present Study of Whole Platelet Lipids to Plasma Membrane Lipids

Studies by Skeaf and Holub (1985) and Broekman et al., (1980) indicate that the phospholipid changes observed during activation are detectable in phospholipids of the whole cell lysate. In their studies the fatty acid and alkenyl composition of whole cell lysate PC and PE and isolated membrane PC and PE were shown to be identical. Therefore, results of the studies of the changes in phospholipid composition in whole cell lysates would reflect changes in the plasma membrane phospholipid composition.

III. EFFECTS OF STORAGE ON PLATELETS

III.1 Platelet Function

Storage of human platelet concentrates at 22°C with agitation in PL 732 bags resulted in the virtual disappearance of platelet response to single stimuli, including epinephrine, collagen, ADP, and arachidonic acid, with the exception of calcium ionophore (Fig. 14). Calcium ionophore may indeed be the common intracellular trigger for platelet activation by various agents (Gerrard et al., 1981; Rittenhouse, 1982).

The synergistic response by stored platelets using pairs of activating agents studied, produce normal platelet aggregation and release responses (DiMinno et al., 1983). The

stored platelets when transfused into thrombocytopenic patients function normally in vivo (Adams et al., 1986).

III.2 Lipid Composition

No change in cholesterol concentration or its mole ratio to phospholipid was observed on storage of platelets. In addition, there was no change in proportions of platelet lipid components and no breakdown of phospholipid components into lyso compounds or oxidation of polyunsaturated or aldehyde linkages (Tables 10 - 17). These results all support the conclusion that the platelet lipid composition does not undergo changes on storage up to five days. This conclusion may also be applicable to the plasma membrane lipids (see preceding section D. II.)

III.3 Preformed Paf

FAB/MS studies of isolated PC indicate the presence of Paf molecules with alkyl chains of 16:0, 16:1, 18:0 and 18:1 (Table 13). The presence of these intact molecules of Paf in fresh resting platelets suggest the existence of a pool of preformed Paf in the platelet. Others (Benveniste et al., 1977; Camussi et al., 1983) have reported that Paf is not a preformed mediator as mechanical disruption of cells did not induce Paf release. Other than through the FAB/MS analysis, our method of study did not distinguish Paf molecules from other PC analogues. New methods involving mass spectrometry

are being developed to quantify Paf in phospholipid mixtures (Clay et al., 1984; Varenne et al., 1985).

III.4 Quantification of Paf Precursor Present in Platelets

Decreases in the proportion of the alkylacyl class were observed in the total lipids and isolated PC and PE, but the proportions of alkyl and acyl chains were maintained. Quantification of the alkylacyl class, specifically in PC, would indicate the amount of precursor available for the synthesis of Paf in the platelet and this can be taken as an indication of the "potential" for Paf, estimated as 4.8 μmoles/L of blood (Table 18). Concentrations of 10^{-7} M - 10^{-8} M of Paf are activating for human platelets; thus only 0.2 to 2 % of the Paf precursor available as alkylacyl PC need be converted to Paf to be activating (Chignard et al, 1983; Tence et al., 1985). Hence, there is more than sufficient precursor available with storage, in spite of ~ 40% loss during storage. Therefore, the decreased platelet response is not a result of a loss of Paf precursor material during storage but may be due to changes in enzymes or other factors involved in Paf formation and release.

Other sources for Paf precursors exist in the platelet. Alkylacyl PE can be converted to alkylacyl PC via methyltransferases in the platelet membrane (Hirata et al., 1978). The amount of alkylacyl class of PE as previously seen for the alkylacyl PC also shows a significant decrease from

Table 18: Calculations of Paf and Arachidonate Potential of Fresh and Stored Platelets

Storage Time, days	Paf: nmol alkylacylPC per 10^9 cells[a]	Paf: μM[b]	Total Arachidonate: nmol per 10^9 cells[c]	Total Arachidonate: μM[d]
0	15.9 ± 3.1	4.8	245 ± 35	73
3	10.1 ± 2.1	3.0	262 ± 40	79
5	10.2 ± 2.3	3.1	266 ± 37	80

Results are mean ± standard deviation based on triplicate analyses of each sample, all originating from one pool of platelets.

[a] nmol alkylacyl PC = 0.44 mg lipid x $\frac{\text{wt. \%PC X \% alkylacyl}}{742}$ per 10^9 cells.

(742 = average molecular weight of alkylacylPC as calculated from GLC data). Refer to Tables 11 and 12.

[b] μM alkylacyl PC = 0.3 x nmol alkylacyl PC per 10^9 cells. (1 ml of blood = 3×10^8 cells).

[c] nmol arachidonate = 0.44 mg lipid x wt. % FA x $\frac{\text{\% arachidonate}}{304}$ per 10^9 cells.

(304 = molecular weight of arachidonate). Refer to Tables 12 and 17.

[d] μM arachidonate = 0.3 x nmol arachidonate per 10^9 cells.

8% to 3% after 3 days of storage (a 63% decrease). Although most of the previous studies on Paf regulation have focussed on the deacylation and acetylation cycle, our present studies indicate that alkylacyl PE may also play a major role as a Paf precursor, and this possibility warrants futher study.

III.5 Effect of Storage on the Arachidonic Acid Content

No significant changes in the arachidonate levels were observed with storage for up to five days in the total lipids, PE or PC (Tables 15 - 17) indicating that the arachidonate "potential" (245 nmol per 10^9 cells or 73 µmol per L of blood) of the cell had not changed (Table 18). The range of arachidonate concentration that induces platelet aggregation is 100 - 300 uM; PGG_2 and PGH_2 arachidonic acid metabolites are potent at concentrations of 200 nM (Adams, 1985). Therefore, only 0.3 % of the total arachidonate need be metabolized for platelet activation (Table 18). The results of our study indicates that there is sufficient arachidonate present to support synergism or propagation of a primary signal which would require sub-maximal concentrations; however, not enough arachidonate is present for arachidonate to be the primary stimulant of platelet aggregation. As previously concluded for Paf, a deficiency of arachidonate is not the apparent cause of the lack of platelet response to single stimuli.

IV. Model for Changes in Platelet Activation on Storage

It has been established that stimulation of intact human platelets induces significant alterations in their phospholipid composition (Broekman et al., 1980; Holub, 1984; Skeaf and Holub, 1985). Most notable among the changes in stimulated platelets are the quantitative losses of PC via phospholipase A_2 and PI via phospholipase A_2 and phospholipase C activity in addition to the resynthesis of PIP_2 (Broekman et al., 1980; Prescott and Majerus, 1981; McKean et al., 1981; Broekman et al., 1981; Berridge, 1984; Holub, 1984; Skeaf and Holub, 1985). The active hydrolysis of these phospholipids provides for the release of arachidonic acid, which is converted via the cyclooxygenase complex to prostaglandins (Hamberg et al., 1975; Chau and Tai, 1982; Billah et al., 1981; Prescott and Majerus, 1983); and for the release of Paf via the deacylation-reacylation cycle from the alkylacyl PC analogues (Chap et al., 1983; Chignard et al., 1983).

On storage, the platelet's ability to synthesize the secondary stimuli in response to single stimuli is greatly diminished; this inability would then affect the amplification of the first signal and therefore, an abolition of platelet response.

Studies of platelet phospholipases A_2 and C show a decrease in the activity of phospholipase A_2, while

phospholipase C activity is unaffected on storage of platelets (Labow et al., 1986a). This decrease has been shown to be the result of inhibition by plasticizers, di(2-ethylhexyl)phthlate (DEHP) and its metabolite mono (ethylhexyl)phthalate (MEHP) which are contaminants of the PL 732 bags used in storage. The inhibition is reversible with the effect reduced after platelet washing procedures using buffer containing albumin. The amount of plasticizer leaching into the plasma increases with storage time (Labow et al., 1986b). Inhibition of phospholipase A_2 would block the release of arachidonic acid and prevent formation of Paf. The lack of formation of these intercellular messengers (Paf and arachidonate) would then explain the impairment of the platelet to respond to single stimulus after 3 and 5 days of storage.

In this connection, Imai et al., (1985) have studied platelets stored as PRP in glass tubes (no plasticizers), and found no change in phospholipase C activity but an increase in phospholipase A_2 activity. As a result, the levels of lyso PC and lyso PE components increased after 12 and 48 hours of storage. In addition, response of stored platelet activation to thrombin stimulus, as measured by serotonin release was slower than that of fresh platelets. These results indicate that although there is reversible inhibition of phospholipase A_2 when platelets are stored in plastic bags, the plasticizer might prevent the membrane phospholipid components from

degradation resulting from an increased phospholipase A_2 activity during storage.

Therefore, the defect in the stored platelet response to single stimuli appears to be due to plasticizer inhibition of phospholipase A_2 activity which in turn decreases the platelet's ability to release Paf and arachidonic acid from platelet phospholipids to amplify the primary signal.

It has been demonstrated that DEHP binding to the platelet membrane is reversible and also that MEHP and DEHP have a very high affinity binding for albumin (Labow et al., 1986b). Washing procedures with albumin should be effective in removing plasticizer from the platelet membrane. However, it remains to be tested whether simple washing of the platelets after storage would remove the plasticizer from platelets and thus alleviate the phospholipase A_2 inhibition and whether indeed this inhibition is the only action of plasticizer on platelets. It should be noted that platelets are hemostatically dysfunctional for the first few hours after transfusion. It is possible that this is the time required to wash out the DEHP and allow enzyme activation.

V. Future Aspects

The present study has demonstrated that there were no significanat changes in the platelet lipid composition during storage that could account for the change in platelet inability to respond to single stimulus. From the findings of

Imai et al.,(1984), and Labow et al.,(1986a; 1986b), we have speculated that the decreased ability is due to the plasticizer inhibition of phospholipase A_2 activity blocking arachidonic acid and Paf release from the platelet phospholipids. But how much of the physiological response is Phospholipase A_2 dependent? Can the inhibition of the plasticizer be reversed by washing the platelets with albumin? Does the plasticizer have other effects; for example, modulation of other enzyme activites? These are a few interesting questions that need further study.

There are other questions that arise from the results of the present study. Significant decreases in the alkylacyl PC and PE content is observed. What are the regulatory features of alkylacyl phospholipid synthesis from diacyl phospholipid. Is alkyl-DHAP synthase affected by storage or by plasticizer to explain this decrease in alkylacyl content?

In addition, the abundance of molecular ion peaks for Paf suggest a pool of Paf in the resting platelet. This should be verified by separating the Paf molecules from other PC analogues of fresh resting platelets. Thus, there are many questions still to be studied in the field of platelet phospholipids, metabolism and the effects of storage.

CONCLUSIONS

I. Advantages of Present Analytical Procedure of Methanolysis/Acetolysis:

1) Avoids difficulties due to lack of broad substrate specificity of the enzyme leading to incomplete hydrolysis of the substrate.

2) The present procedure is conveniently carried out by sequential methanolysis and acetolysis reactions which give quantitative yields of the corresponding products.

3) Eliminates need for TLC separation and isolation of the reaction products.

4) Analysis of products by GLC with a single internal standard gives data on the type and chain length of hydrocarbon groups that can be used to calculate the molecular class composition.

5) The present procedure is well suited to replicate and multi-sample analysis within a reasonable time period (2-3 days).

6) Products are recovered in an overall yield of 96 $\pm$ 4%.

II. Application of Procedure to Fresh Resting Human Platelets:

1) PC contained mainly diacyl (86%) with lesser amounts

of alkylacyl (10%) and alkenylacyl (1.4%) classes.

2) PE contained mainly diacyl (60%) with 27% alkenylacyl and 8% alkylacyl class.

III. Effect of Storage on Platelet Lipid Composition

1) No significant changes in cholesterol, phospholipid or related diacyl and alkenylacyl classes or in their proportions of chain types were noted.

2) Decrease in the alkylacyl class of PC (36 %) and PE (63 %) after 3 days of storage was observed; however, calculations indicate that in spite of this loss there is more than sufficient amount of Paf precursor available.

In summary, the present study indicates that the inability of the stored platelet to respond to single stimuli does not result from loss of lipid components or precursors required for the generation of secondary stimulants, specifically Paf and arachidonate metabolites.

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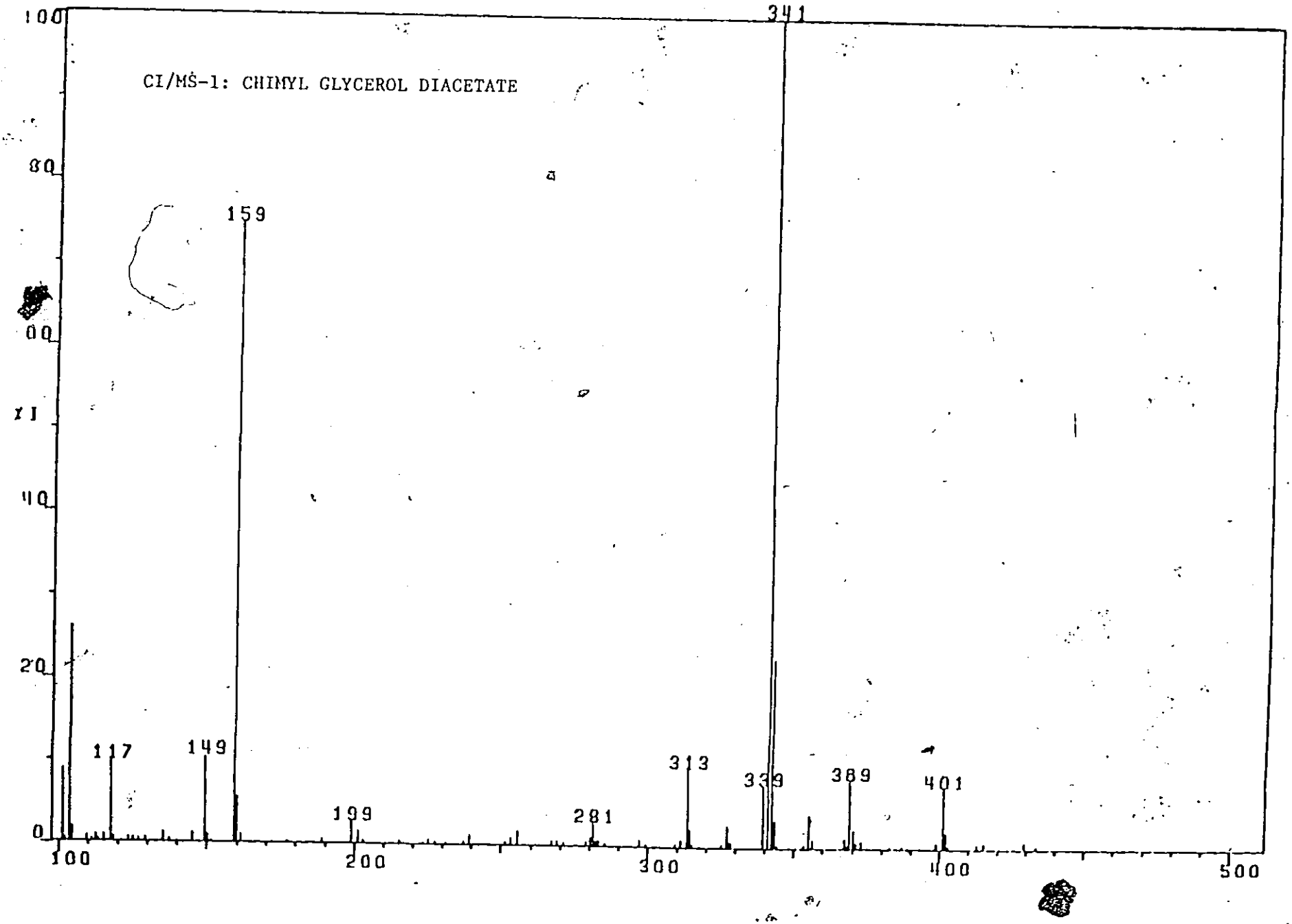
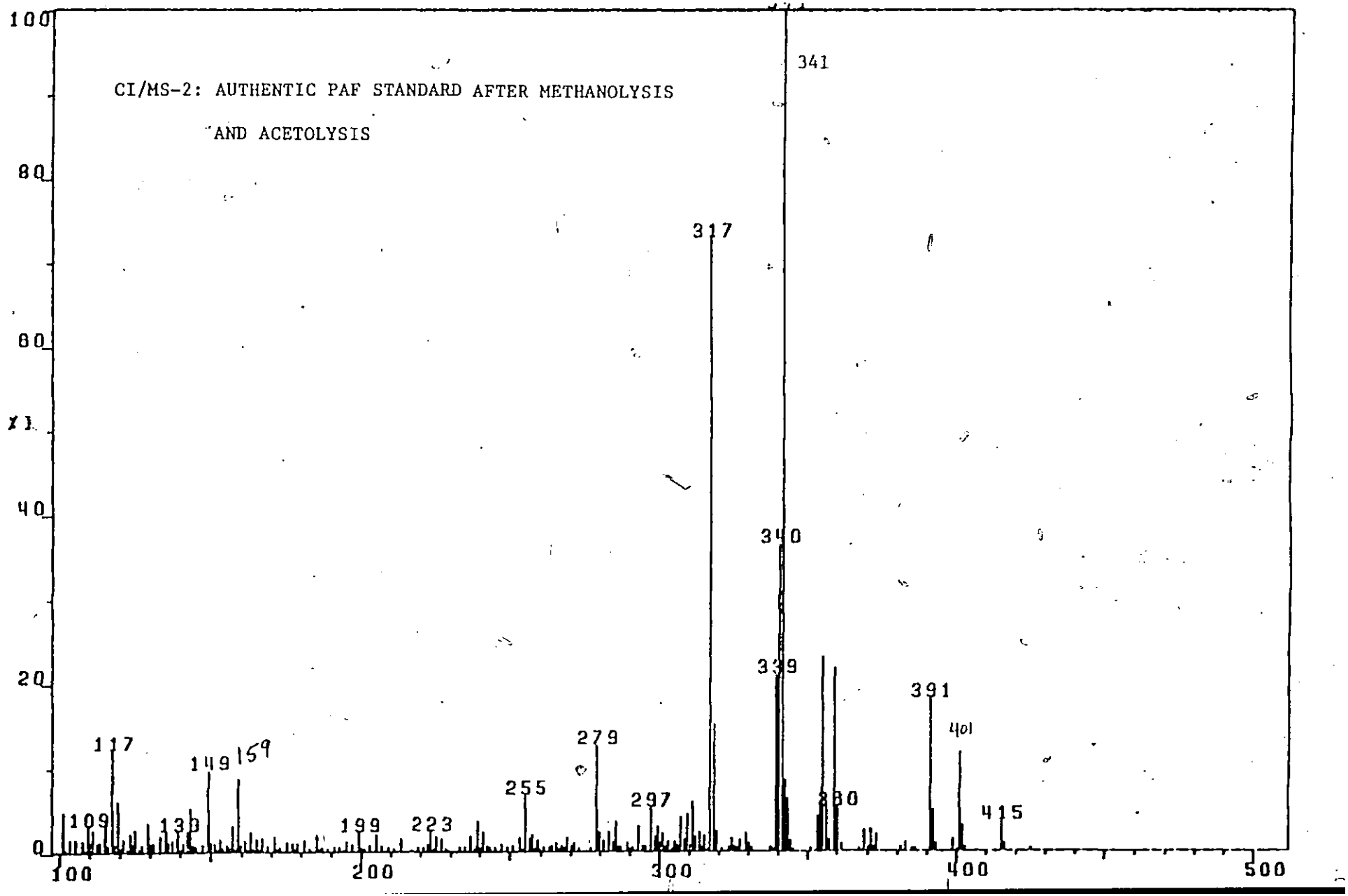
APPENDIX


CI/MS-1: CHIMYL GLYCEROL DIACETATE
100
80
60
40
20
0
159
341
117
149
199
281
313
339
389
401
100
200
300
400
500

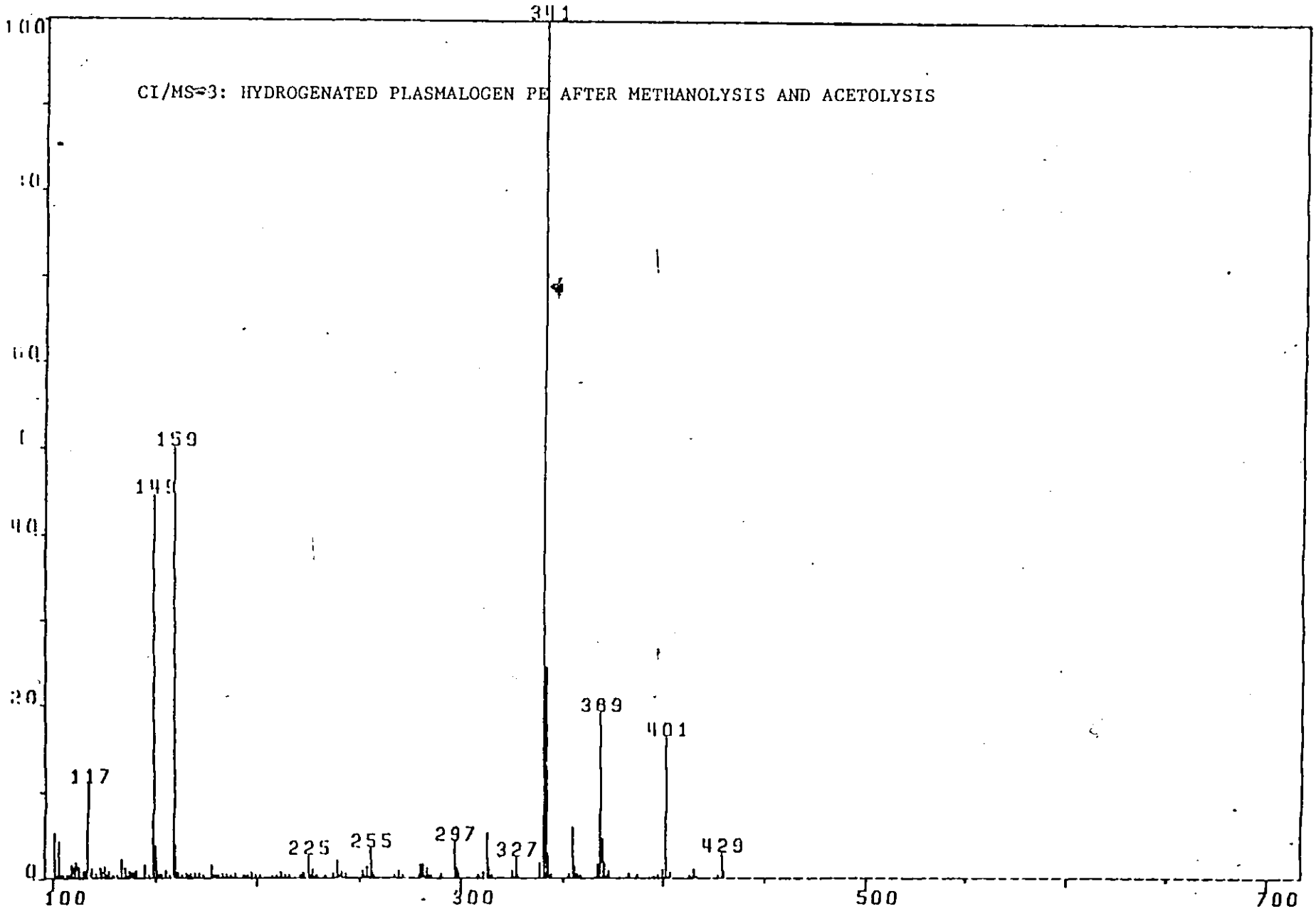
CI/MS-1: CHIMYL GLYCEROL DIACETATE

PEAK NO.	MASS	%AR. MOD.	PEAK NO.	MASS	%AR. MOD.
1	100.9	8.7	110	314.0	2.0
2	102.0	0.4	111	315.0	0.3
3	103.1	26.0	112	325.0	0.4
4	104.1	1.7	113	327.0	2.6
6	109.0	0.6	114	328.0	0.6
7	111.0	0.4	117	339.0	7.3
8	112.0	0.8	119	340.9	100.0
9	113.0	0.4	120	341.9	22.9
10	115.0	0.8	121	343.0	3.2
11	117.0	9.6	122	344.0	0.4
12	118.0	0.7	123	353.0	0.4
15	123.0	0.4	124	355.0	3.9
16	125.0	0.5	125	356.0	0.9
17	126.9	0.3	128	367.0	1.1
18	128.9	0.4	129	368.0	0.3
21	135.0	1.2	130	368.9	8.0
22	137.0	0.4	131	370.0	2.1
26	145.0	1.2	132	371.0	0.6
28	149.0	10.1	133	372.9	0.8
29	150.0	0.9	137	382.9	0.2
30	151.0	0.2	138	386.9	0.2
35	158.9	74.5	139	398.9	0.6
36	159.9	5.5	141	400.9	7.3
37	160.9	1.0	142	401.9	2.0
45	177.0	0.2	143	402.9	0.4
51	188.9	0.4	145	412.9	0.4
53	198.9	2.4	146	414.9	0.7
55	200.9	1.5	147	428.9	0.7
56	202.9	0.2	149	432.9	0.3
61	215.0	0.3			
64	223.0	0.2			
65	225.1	0.5			
67	227.0	0.3			
69	230.9	0.2			
71	237.0	0.3			
73	239.0	1.1			
77	251.0	0.3			
79	253.0	0.8			
81	255.1	1.6			
86	267.0	0.4			
87	269.0	0.5			
88	271.0	0.3			
90	279.0	0.5			
91	280.0	1.0			
92	281.0	2.5			
93	282.0	0.6			
94	283.0	0.7			
96	285.0	0.3			
101	297.0	0.7			
103	299.0	0.3			
106	309.0	0.3			
107	310.9	0.8			
109	313.0	9.3			


CI/MS-2: AUTHENTIC PAF STANDARD AFTER METHANOLYSIS
AND ACETOLYSIS
100
80
60
40
20
0
X1
100
200
300
400
500
341
317
340
339
391
401
279
255
297
380
415
117
149
159
109
139
199
223

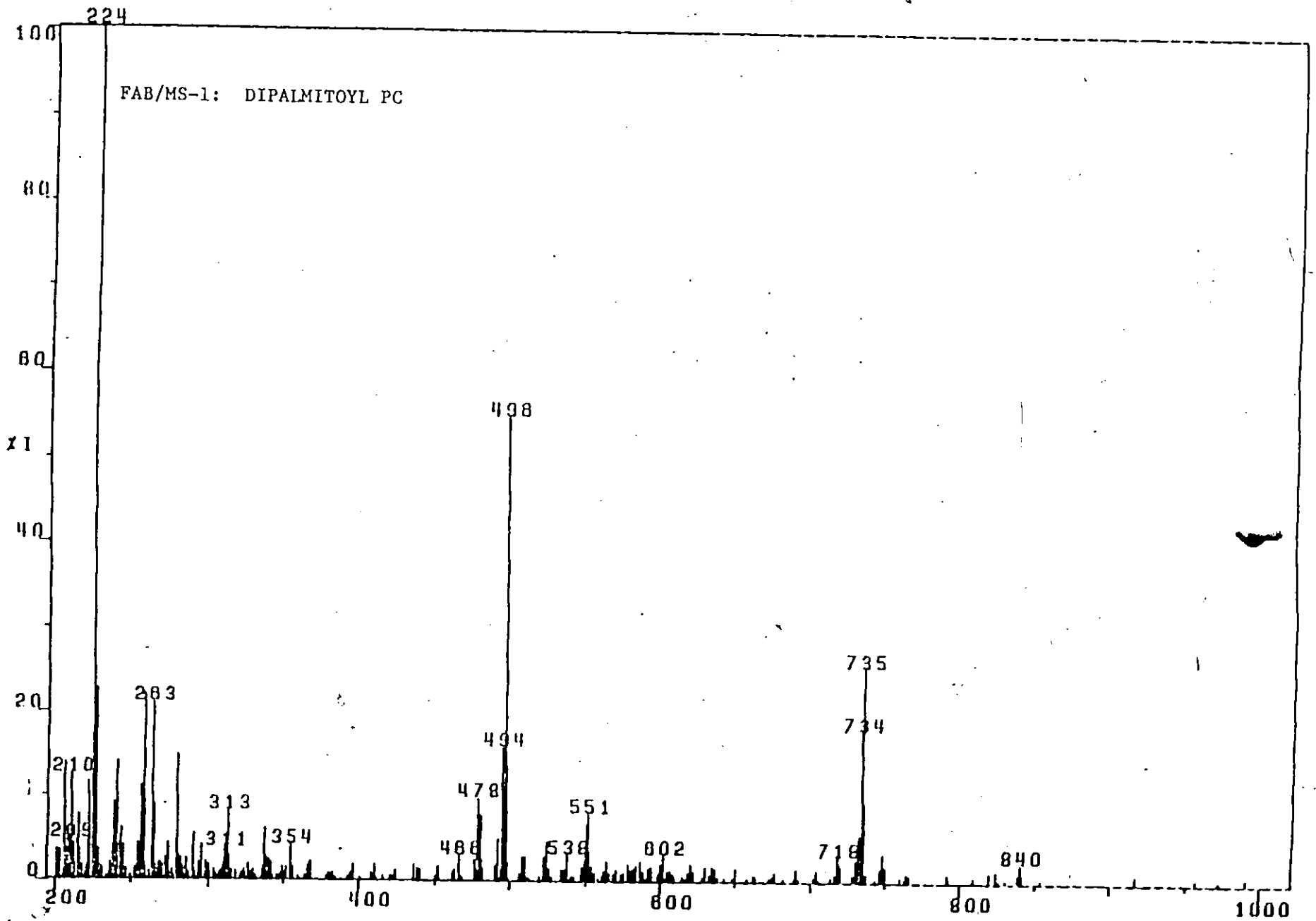
CI/MS-2: AUTHENTIC PAF STANDARD AFTER METHANOLYSIS AND ACETOLYSIS

PEAK NO.	MASS	%AR. MOD.	PEAK NO.	MASS	%AR. MOD.	PEAK NO.	MASS	%AR. MOD.	PEAK NO.	MASS	%AR. MOD.
58	163.0	2.3	129	247.0	0.8	188	312.0	1.7	249	415.0	3.4
59	164.0	0.4	131	248.9	0.5	189	313.0	2.3	250	416.0	1.0
60	165.0	1.4	132	250.9	0.7	190	314.0	0.6	252	424.9	0.3
61	166.0	0.3	133	253.0	1.6	191	314.9	1.9			
62	167.0	1.7	134	254.0	0.4	192	316.0	0.4			
64	169.0	0.6	135	255.0	6.5	193	317.0	72.9			
65	171.0	1.8	136	256.0	1.7	194	318.0	14.9			
66	172.0	0.4	137	257.0	1.9	195	319.0	2.4			
67	173.0	0.5	138	258.0	0.4	197	320.9	0.2			
68	175.0	1.1	139	258.9	1.3	199	323.0	0.4			
69	177.0	1.0	140	259.9	0.2	200	324.2	1.4			
70	178.0	0.2	141	260.9	0.5	201	325.0	0.6			
71	179.0	1.0	142	263.0	0.4	202	326.0	0.5			
73	180.9	1.4	143	264.0	0.6	203	326.9	1.3			
75	183.0	0.4	144	265.0	0.9	205	328.9	2.0			
76	185.0	1.9	145	266.0	0.3	206	329.9	0.9			
77	186.0	0.2	146	267.0	0.6	207	330.9	0.4			
78	186.9	0.5	147	267.9	0.5	208	337.0	0.4			
79	188.9	0.4	148	269.0	1.6	209	338.0	0.2			
80	190.9	0.5	149	270.0	0.6	210	339.0	20.8			
81	192.9	0.4	150	271.0	1.0	211	340.0	36.3			
82	194.0	0.2	152	273.0	0.4	212	341.1	100.0			
83	195.0	1.1	154	277.0	0.2	213	341.9	8.4			
85	196.9	0.7	155	278.9	12.5	214	343.0	6.2			
87	199.0	2.2	156	279.9	2.3	215	344.0	1.2			
88	199.9	0.4	157	281.0	1.2	216	344.9	0.3			
89	200.9	0.3	159	283.0	2.3	217	350.9	0.2			
91	203.0	0.7	160	284.0	1.1	218	353.0	4.1			
93	205.0	2.0	161	285.0	3.6	219	354.0	5.8			
95	207.0	0.7	162	286.1	0.6	220	355.0	22.9			
97	208.9	0.5	163	287.0	0.5	221	356.0	5.4			
98	210.9	0.4	165	289.0	1.0	222	357.0	1.3			
100	213.0	1.5	166	290.0	0.4	224	358.9	21.7			
103	216.9	0.2	167	291.0	0.4	225	359.9	5.0			
104	218.9	0.5	168	292.0	0.2	226	360.9	0.8			
106	220.9	0.5	169	292.9	2.9	227	367.0	0.4			
107	222.0	0.8	170	293.9	0.6	228	369.0	2.4			
108	222.9	2.2	171	295.0	0.7	229	370.0	0.4			
109	223.9	0.3	173	297.0	4.9	230	370.9	2.6			
110	225.0	1.8	174	298.0	1.8	231	371.9	0.7			
112	227.0	1.5	175	299.0	2.9	232	372.9	2.0			
113	227.9	0.3	176	300.0	1.2	234	380.9	0.5			
114	228.9	0.2	177	301.0	2.2	235	383.0	0.9			
116	232.9	0.4	178	301.9	0.6	236	384.9	0.3			
118	234.9	0.5	179	303.0	1.2	237	385.9	0.4			
120	236.9	1.7	180	304.0	0.4	239	390.9	17.9			
121	237.9	0.4	181	305.0	1.1	240	391.9	4.8			
122	238.9	3.6	182	306.0	0.8	241	392.9	1.0			
123	239.9	0.5	183	307.0	4.0	244	398.9	1.4			
124	240.9	2.3	184	308.0	1.5	245	399.9	0.2			
125	242.0	0.6	185	309.0	4.4	246	400.9	11.6			
126	243.0	0.5	186	310.0	1.0	247	401.9	3.1			
127	245.0	0.5	187	311.0	5.7	248	402.9	0.5			


CI/MS=3: HYDROGENATED PLASMALOGEN PE AFTER METHANOLYSIS AND ACETOLYSIS
100
80
60
40
20
0
311
159
149
117
225
255
297
327
369
401
429
100
300
500
700

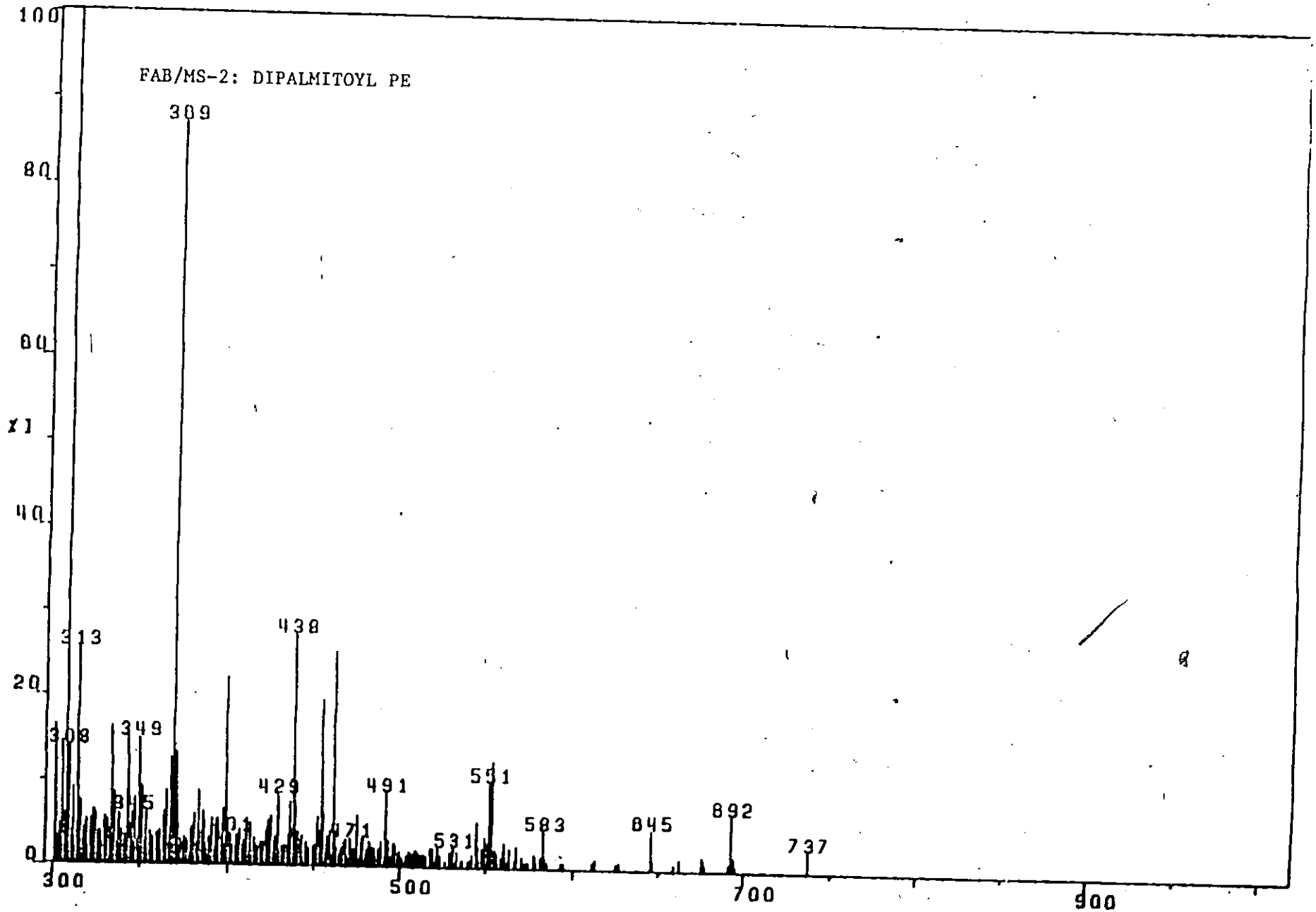
CI/MS-3: HYDROGENATED PLASMALOGEN PE AFTER METHANOLYSIS AND ACETOLYSIS

PEAK NO.	MASS	%AR. MOD.	PEAK NO.	MASS	%AR. MOD.	PEAK NO.	MASS	%AR. MOD.
1	100.9	5.1	61	179.0	0.4	123	308.0	0.4
2	102.0	0.3	62	180.0	0.3	124	309.0	0.3
3	103.0	4.2	63	191.0	0.3	125	311.0	0.6
4	104.1	0.3	64	183.1	0.5	127	312.9	5.2
5	105.0	0.3	65	185.0	0.5	128	314.0	1.2
6	107.0	0.3	66	187.0	0.3	129	315.0	0.3
7	108.0	0.3	67	188.9	0.5	130	325.0	0.9
8	109.0	1.5	68	193.1	0.3	131	326.0	0.2
9	110.0	1.2	69	194.1	0.2	132	326.9	2.2
10	111.0	1.7	70	195.1	0.3	133	327.9	0.5
11	112.0	1.3	72	197.0	0.6	135	338.8	1.8
12	113.0	0.8	73	199.0	0.4	136	340.9	100.0
14	115.0	0.8	74	201.0	0.3	137	342.0	24.6
15	116.0	0.8	75	207.0	0.2	138	343.0	2.9
16	117.0	10.7	76	209.0	0.4	139	344.0	0.5
17	118.0	0.7	77	211.0	0.7	140	352.9	0.4
18	119.0	1.1	78	213.0	0.4	141	355.0	5.9
19	121.0	0.4	79	215.0	0.3	142	356.0	1.3
21	123.0	1.1	80	221.0	0.3	143	357.0	0.4
22	124.1	0.8	81	222.1	0.6	144	359.0	0.4
23	125.1	1.3	82	223.1	0.6	145	367.0	1.6
24	126.1	0.4	84	225.1	2.4	146	367.9	0.5
25	127.1	0.8	85	226.1	0.4	147	368.9	19.1
26	129.0	0.4	86	227.0	1.0	148	369.9	4.6
27	130.9	0.4	87	228.0	0.2	149	370.9	1.8
28	133.0	2.1	88	229.0	0.3	150	372.0	0.3
30	135.0	1.1	89	233.0	0.2	151	372.9	0.8
31	136.0	0.2	91	237.0	0.4	153	383.0	0.6
32	137.0	0.9	93	239.0	2.0	154	386.9	0.4
33	138.0	0.6	94	240.0	0.4	155	395.0	0.2
34	139.0	0.7	95	241.0	0.6	156	396.9	0.4
35	140.0	0.3	96	243.0	0.5	157	398.9	0.9
36	141.0	0.8	100	251.0	0.7	158	401.1	16.1
38	145.0	1.5	101	252.0	0.4	159	402.9	0.7
39	147.0	0.3	102	253.0	1.4	160	412.9	0.3
40	149.0	44.3	103	254.1	0.3	161	415.0	0.9
41	150.0	3.8	104	255.1	3.2	162	415.9	0.2
42	151.0	0.9	105	256.1	0.7	163	427.0	0.3
43	152.0	0.5	106	257.0	0.3	164	428.9	2.7
44	153.0	0.6	108	267.0	0.4	165	429.9	0.8
45	155.0	0.8	109	269.0	0.8			
46	157.0	0.2	111	271.0	0.4			
47	158.9	49.8	112	279.0	0.3			
48	159.9	3.8	113	280.0	1.5			
49	160.9	0.6	114	281.0	1.7			
50	163.0	0.3	115	282.0	0.4			
51	165.1	0.5	116	283.0	1.2			
52	166.1	0.4	117	284.0	0.3			
53	167.1	0.4	118	285.0	0.4			
55	169.0	0.5	119	290.2	0.5			
56	171.0	0.4	120	297.0	4.1			
57	173.0	0.3	121	298.0	1.1			
59	177.0	1.4	122	299.0	0.6			


FAB/MS-1: DIPALMITOYL PC
100
80
60
40
20
0
X1
224
263
210
313
311
354
498
494
478
486
551
538
802
735
734
718
840
200
400
600
800
1000

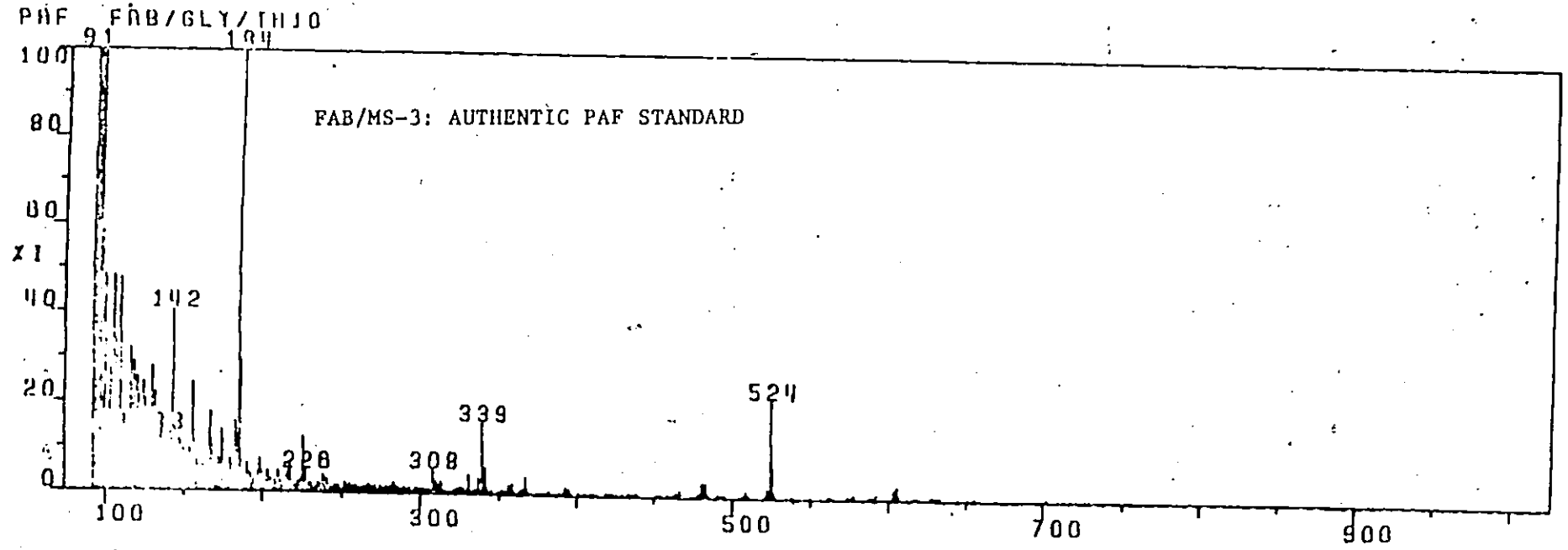
FAB/MS-1: DIPALMITOYL PC

PEAK NO.	MASS	%AR. MOD.	PEAK NO.	MASS	%AR. MOD.	PEAK NO.	MASS	%AR. MOD.	PEAK NO.	MASS	%AR. MOD.	PEAK NO.	MASS	%AR. MOD.	PEAK NO.	MASS	%AR. MOD.	PEAK NO.	MASS	%AR MOD
1	201.0	3.4	54	259.0	1.8	107	322.0	0.8	160	424.0	1.2	213	551.1	7.7	266	648.0	0 4			
2	202.0	1.7	55	260.0	0.8	108	324.0	1.1	161	436.0	1.8	214	552.1	3.6	267	650.1	1.5			
3	203.0	3.4	56	261.0	1.0	109	325.0	0.7	162	438.0	1.4	215	553.0	1.4	268	658.0	0.4			
4	204.1	0.5	57	263.0	20.8	110	326.0	1.7	163	440.0	1.4	216	554.0	1.8	269	662.0	0 6			
5	205.1	13.7	58	264.0	8.9	111	327.1	0.9	164	450.0	0.6	217	556.0	0.9	270	663.0	0.4			
6	206.1	3.9	59	265.0	1.6	112	328.0	0.8	165	451.0	0.4	218	558.0	0.4	271	672.0	0 4			
7	207.0	5.6	60	266.1	1.6	113	328.9	0.4	166	452.1	1.6	219	561.1	0.6	272	674.1	0.4			
8	208.0	6.3	61	267.1	1.2	114	329.9	1.2	167	453.0	0.4	220	562.1	1.1	273	676.1	0.9			
9	209.0	4.6	62	268.0	1.8	115	332.0	0.4	168	462.1	1.0	221	563.1	0.4	274	681.9	0.4			
10	210.0	12.3	63	269.0	2.0	116	335.0	0.4	169	463.1	0.4	222	564.1	2.3	275	689.1	0.4			
11	211.0	2.7	64	270.0	1.8	117	336.0	1.4	170	464.1	1.3	223	565.1	0.9	276	690.1	1.4			
12	212.0	4.2	65	271.0	0.4	118	337.0	5.9	171	466.1	2.8	224	566.0	0.9	277	692.1	0.4			
13	213.1	2.2	66	272.0	2.6	119	338.0	2.7	172	467.0	0.4	225	568.0	0.9	278	702.1	0.5			
14	214.0	7.6	67	274.0	4.1	120	339.0	0.5	173	468.1	0.5	226	570.1	1.4	279	704.1	1.4			
15	215.0	3.6	68	275.0	0.9	121	340.0	2.4	174	476.1	2.5	227	574.1	0.7	280	706.1	0.4			
16	216.0	1.5	69	277.1	1.2	122	341.0	1.5	175	477.0	1.8	228	576.0	0.9	281	714.1	0.4			
17	217.0	1.8	70	278.0	0.6	123	342.1	2.1	176	478.1	9.5	229	578.0	2.0	282	716.2	0.8			
18	218.0	1.0	71	279.1	14.7	124	343.0	0.5	177	479.0	2.4	230	579.0	1.0	283	717.1	0.4			
19	219.0	1.2	72	280.0	3.0	125	345.0	0.5	178	480.1	7.8	231	580.0	1.2	284	718.2	2.8			
20	220.0	1.1	73	281.0	2.4	126	345.9	0.5	179	481.0	2.5	232	581.0	0.5	285	719.2	2.0			
21	221.0	1.5	74	282.0	2.6	127	347.0	0.4	180	482.1	1.5	233	581.9	1.4	286	720.2	1.8			
22	222.0	11.5	75	283.1	1.6	128	348.0	0.6	181	490.0	1.6	234	584.0	1.8	287	728.1	0.8			
23	223.0	5.6	76	284.0	1.5	129	348.9	1.4	182	492.1	4.8	235	586.0	2.3	288	730.2	2.5			
24	224.0	100.0	77	285.1	0.4	130	350.9	0.8	183	493.0	1.4	236	586.9	0.4	289	731.1	1.4			
25	225.1	9.4	78	286.0	2.5	131	352.0	1.5	184	494.0	15.6	237	588.0	1.2	290	732.2	5.5			
26	226.0	22.7	79	287.1	0.9	132	354.0	3.8	185	495.0	4.4	238	590.1	0.4	291	733.7	17.8			
27	227.0	2.8	80	288.0	0.9	133	355.0	1.6	186	496.0	54.8	239	592.1	1.4	292	734.5	25.2			
28	228.0	3.6	81	289.9	5.3	134	356.0	1.4	187	497.1	13.6	240	593.0	0.5	293	735.2	9.3			
29	229.0	1.5	82	291.0	1.0	135	359.9	0.4	188	498.0	2.3	241	594.1	1.6	294	737.1	1.8			
30	230.0	1.6	83	293.0	0.4	136	364.1	0.4	189	499.0	0.4	242	598.0	0.9	295	744.0	0.4			
31	231.0	1.2	84	294.0	2.0	137	365.0	0.4	190	506.0	0.9	243	600.0	1.9	296	746.0	1.6			
32	233.0	0.5	85	295.1	1.4	138	366.0	1.6	191	508.0	2.8	244	601.0	0.4	297	747.0	1.2			
33	235.0	0.7	86	296.1	4.1	139	367.0	2.0	192	510.1	2.8	245	602.0	3.0	298	748.1	3.7			
34	236.1	2.0	87	297.1	1.8	140	368.0	2.0	193	511.0	0.4	246	603.1	0.5	299	749.1	1.0			
35	237.1	1.6	88	298.0	2.1	141	378.0	0.4	194	512.1	0.4	247	604.1	1.1	300	750.0	1.7			
36	238.0	9.0	89	299.0	1.1	142	380.0	0.9	195	520.0	0.6	248	606.1	1.2	301	760.0	0.5			
37	239.1	5.3	90	300.0	1.7	143	381.0	0.4	196	522.1	2.7	249	607.1	0.6	302	763.0	0.4			
38	240.0	13.9	91	301.0	1.0	144	382.0	0.9	197	523.1	0.5	250	608.1	0.8	303	764.0	0.9			
39	241.0	4.1	92	304.0	1.1	145	384.0	0.4	198	524.1	4.5	251	609.1	0.5	304	766.0	0.8			
40	242.0	6.0	93	305.0	0.7	146	392.0	0.4	199	525.1	1.3	252	614.1	0.5	305	791.3	0.9			
41	243.1	2.6	94	306.0	0.5	147	393.0	0.4	200	526.1	1.5	253	616.1	0.4	306	792.1	0.5			
42	244.1	4.1	95	307.0	0.9	148	394.1	0.9	201	528.1	0.5	254	617.9	0.9	307	808.2	0.5			
43	245.0	0.4	96	308.0	0.9	149	395.1	0.5	202	534.1	1.3	255	620.1	1.9	308	819.1	1.2			
44	246.0	1.3	97	309.0	1.1	150	396.0	1.8	203	535.2	0.5	256	621.0	0.4	309	824.1	1.2			
45	249.0	0.4	98	310.0	2.0	151	400.8	0.4	204	536.1	1.3	257	622.0	1.1	310	826.0	0 4			
46	250.0	0.5	99	311.1	3.5	152	408.0	0.8	205	537.1	1.1	258	628.1	0.5	311	830.0	0 4			
47	252.0	1.3	100	312.0	3.9	153	408.9	0.5	206	538.1	2.9	259	630.1	1.6	312	838.1	1.0			
48	253.1	1.7	101	313.1	7.8	154	409.9	1.7	207	539.1	0.4	260	632.1	0.9	313	839.0	0.8			
49	254.0	4.2	102	314.0	2.7	155	410.9	0.4	208	540.0	0.5	261	634.2	1.7	314	840.0	2.2			
50	255.1	2.6	103	315.0	1.0	156	416.0	0.5	209	542.0	0.5	262	635.1	0.5	315	841.2	1 0			
51	256.0	10.9	104	316.0	1.2	157	420.1	0.4	210	548.0	1.7	263	636.2	1.4	316	854.1	0.4			
52	257.0	2.3	105	318.0	1.0	158	422.0	0.5	211	549.1	2.3	264	638.1	0.4	317	917.1	0 9			
53	258.0	21.9	106	321.0	0.4	159	423.0	0.4	212	550.0	6.5	265	642.0	0.4	318	957.9	0.5			

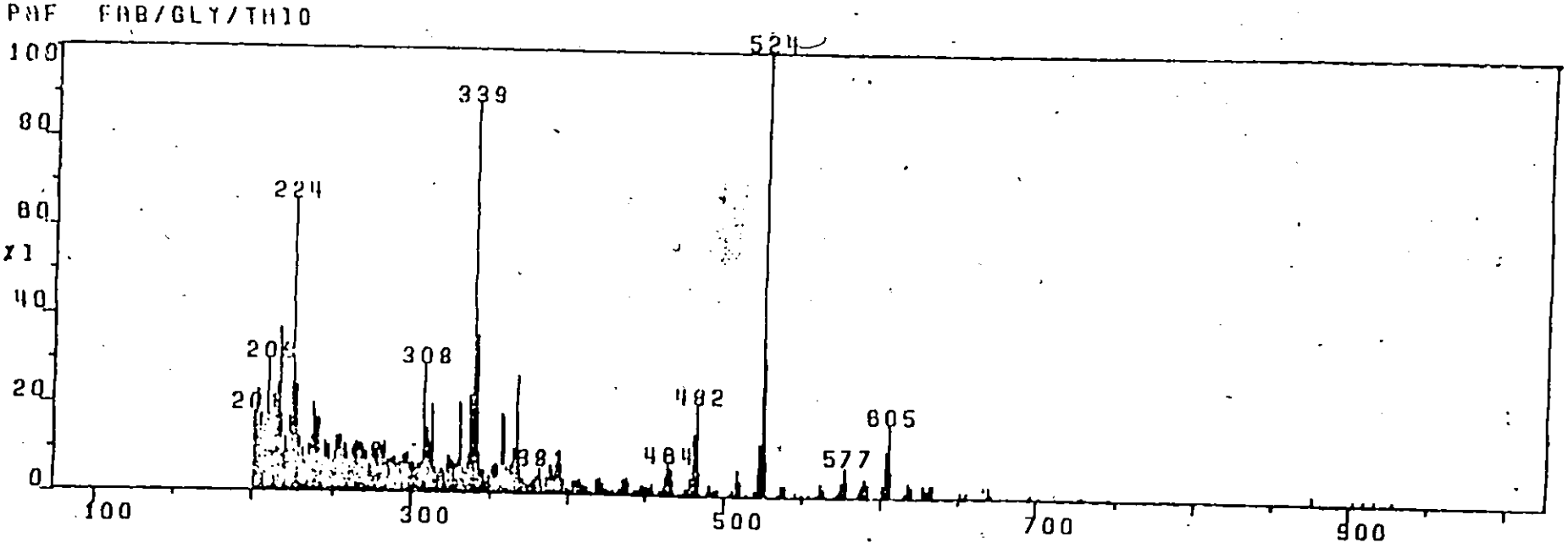

FAB/MS-2: DIPALMITOYL PE
100
80
60
40
20
0
X1
309
313
438
308
349
429
491
531
583
845
892
737
300
500
700
900

FAB/MS-2: DIPALMITOYL PE

PEAK NO.	MASS	%AR MOD.	PEAK NO	MASS	%AR MOD	PEAK NO	MASS	%AR MOD	PEAK NO	MASS	%AR MOD	PEAK NO	MASS	%AR MOD	PEAK NO	MASS	%AR MOD
1	301.0	16 3	54	354 0	2 9	107	407 0	4 1	160	463.0	2 3	213	521 0	2 3	266	627 1	0 7
2	302.0	3.4	55	355.0	3 9	108	408.0	2.5	161	464 0	1 4	214	523 1	1 5	267	645 1	1 6
3	303.0	4 9	56	356.0	2 8	109	409.9	2.9	162	465.0	1.7	215	526 0	0 6	268	646 1	1 2
4	304.0	3.5	57	357.0	3 3	110	410.0	2.7	163	466.1	2.0	216	528 1	1 0	269	659 0	0 6
5	304.9	14.4	58	358.0	1.2	111	411.0	3.8	164	467.0	2.2	217	530 1	0 7	270	661 9	1 4
6	306.0	6.2	59	359.0	3 0	112	412.0	1.6	165	468.0	3.0	218	531 1	2 3	271	675 0	1 5
7	306.9	100.0	60	360.0	2.8	113	413.0	5.0	166	469.0	1.3	219	532 1	0 7	272	676 1	1 3
8	308.0	13.7	61	360.9	4.1	114	414.0	2.3	167	470.0	0.7	220	533 1	1 0	273	677 1	0 6
9	308.9	13.0	62	361.9	1.6	115	415.0	3.3	168	470.9	3.3	221	535 1	0 6	274	690 1	0 7
10	310.0	4.0	63	363.0	6 2	116	416.0	2.6	169	472.0	2.1	222	536 2	0 7	275	691 1	0 6
11	311.0	9.1	64	364.0	2 0	117	417.0	2.1	170	473.0	2.2	223	539 1	0 7	276	692 3	0 4
12	312.1	3.2	65	364.9	0.0	118	418.1	2.2	171	474.0	2.0	224	540 1	0 7	277	693 1	1 6
13	313.1	25.3	66	366.0	3.0	119	419.0	2.0	172	475.0	6 0	225	541 1	0 6	278	694 0	0 7
14	314.1	6.3	67	367.0	12 6	120	420 1	1.0	173	475.9	1 4	226	542 1	1 5	279	737 1	2 4
15	315.0	7.6	68	368.0	3 5	121	421.0	2.0	174	477.0	2.9	227	544 1	5 4			
16	316.0	3 0	69	369.0	06.9	122	422.1	4.2	175	478.0	3.5	228	545 1	2 6			
17	317.0	4.4	70	370.0	13.2	123	423.1	5.3	176	479 0	1.5	229	546 1	0 9			
18	318.0	2.6	71	371 0	5 5	124	424.1	3.3	177	480.1	2.1	230	547 1	2 2			
19	319.0	5.3	72	372.0	2.2	125	425.1	5.0	178	481.0	1.3	231	548 1	0 6			
20	320 0	2.5	73	373.0	3.1	126	426.1	1.8	179	482.1	2 9	232	549 1	3 5			
21	321.0	5.4	74	374.0	2.7	127	427.0	3 4	180	483.1	2 3	233	550 2	2 9			
22	322.0	3.3	75	375.0	3.3	128	428.1	2.5	181	484 0	2.2	234	551 2	9 9			
23	323.0	6.5	76	376.0	0.6	129	428.9	0.2	182	485.0	2.2	235	552 2	4 0			
24	324.0	6.2	77	377.0	2.9	130	430.0	2.1	183	486.1	1.6	236	553 0	12 4			
25	325.0	3.0	78	378.0	1.0	131	431.0	2.3	184	487.0	2 2	237	554 0	2 2			
26	326.0	4 1	79	379.0	4 4	132	432.0	1 2	185	489 0	2.0	238	555.0	2 1			
27	327.0	3.0	80	380.0	3.3	133	433.0	2.5	186	490.1	0.6	239	556 1	1 6			
28	328.0	2.6	81	381.0	5.9	134	434.1	2.4	187	491 0	0.5	240	557 0	0 7			
29	329.0	5.7	82	382.0	2.7	135	435.0	3.0	188	492 0	2 9	241	558 0	1 3			
30	330.0	2.6	83	383.0	0.7	136	436.1	7.6	189	493 0	3 0	242	559 1	0 6			
31	331.0	5.3	84	384.0	3.9	137	437.0	4 4	190	494.0	1.5	243	560 0	2 9			
32	332.0	4.4	85	385.0	6.3	138	438.1	27.1	191	496.1	2 7	244	561 0	1 4			
33	333.0	16.1	86	386.1	5.3	139	439.0	7.6	192	497 0	2.5	245	562 0	0 0			
34	334.0	3.5	87	387.0	2.0	140	440.0	2.0	193	498 1	1 6	246	563 0	2 3			
35	335.0	0 5	88	388 1	2 0	141	441.0	4.0	194	499 0	1.7	247	566 0	0 0			
36	336.0	2.0	89	389.0	3 3	142	442.0	3.0	195	501.0	1 2	248	567 0	2 6			
37	337.0	6.0	90	390.0	2 0	143	443 0	3.6	196	502 0	0 6	249	570 1	1 3			
38	338.0	2.6	91	391.0	5.1	144	443.9	0.6	197	503 0	1 4	250	572.0	0 6			
39	339.0	4 0	92	392.1	2.4	145	445.0	2.7	198	503.9	1 3	251	573 1	0 6			
40	340.0	2.6	93	393.0	5.2	146	447.0	2.1	199	505.0	1.0	252	573 9	0 6			
41	341 0	3.6	94	394.0	2 9	147	449.0	2.2	200	505.9	1.6	253	577.1	1 6			
42	342 0	2.5	95	395.0	4.0	148	450.0	2.5	201	507.0	1 5	254	578.0	1 3			
43	343.0	15.1	96	396 0	3.7	149	451.0	1.0	202	508 0	0.6	255	579 0	0 6			
44	344 0	4.7	97	396.9	6.6	150	452 1	5.0	203	509.0	1 9	256	581 0	1 4			
45	345.0	6 1	98	398.0	2.6	151	453.0	4.2	204	510 0	1.0	257	583 0	4 3			
46	346.0	2.6	99	398.9	22.0	152	454.1	19.3	205	512.0	1 5	258	584 0	1 3			
47	347.0	6.6	100	399.9	5 0	153	455.0	6 3	206	513.0	1 5	259	585 0	0 0			
48	348.0	3.1	101	400 9	3 7	154	456.1	1.6	207	514.1	0.9	260	593 0	0 6			
49	348.9	14.7	102	401.9	1 4	155	457.0	3.5	208	515 0	1 3	261	595 1	0 6			
50	350.0	4 7	103	403.0	2 4	156	459.0	4.2	209	517.1	2 3	262	611 0	0 0			
51	350 9	9 2	104	404.0	2 3	157	460 0	1.5	210	518 1	1 3	263	612 0	0 7			
52	352 0	2.9	105	405.0	3.6	158	461 0	25 2	211	519 0	2 3	264	613 0	1 2			
53	353 0	6 5	106	406.0	2 4	159	462 0	4 2	212	520.1	1 3	265	625 0	0 6			

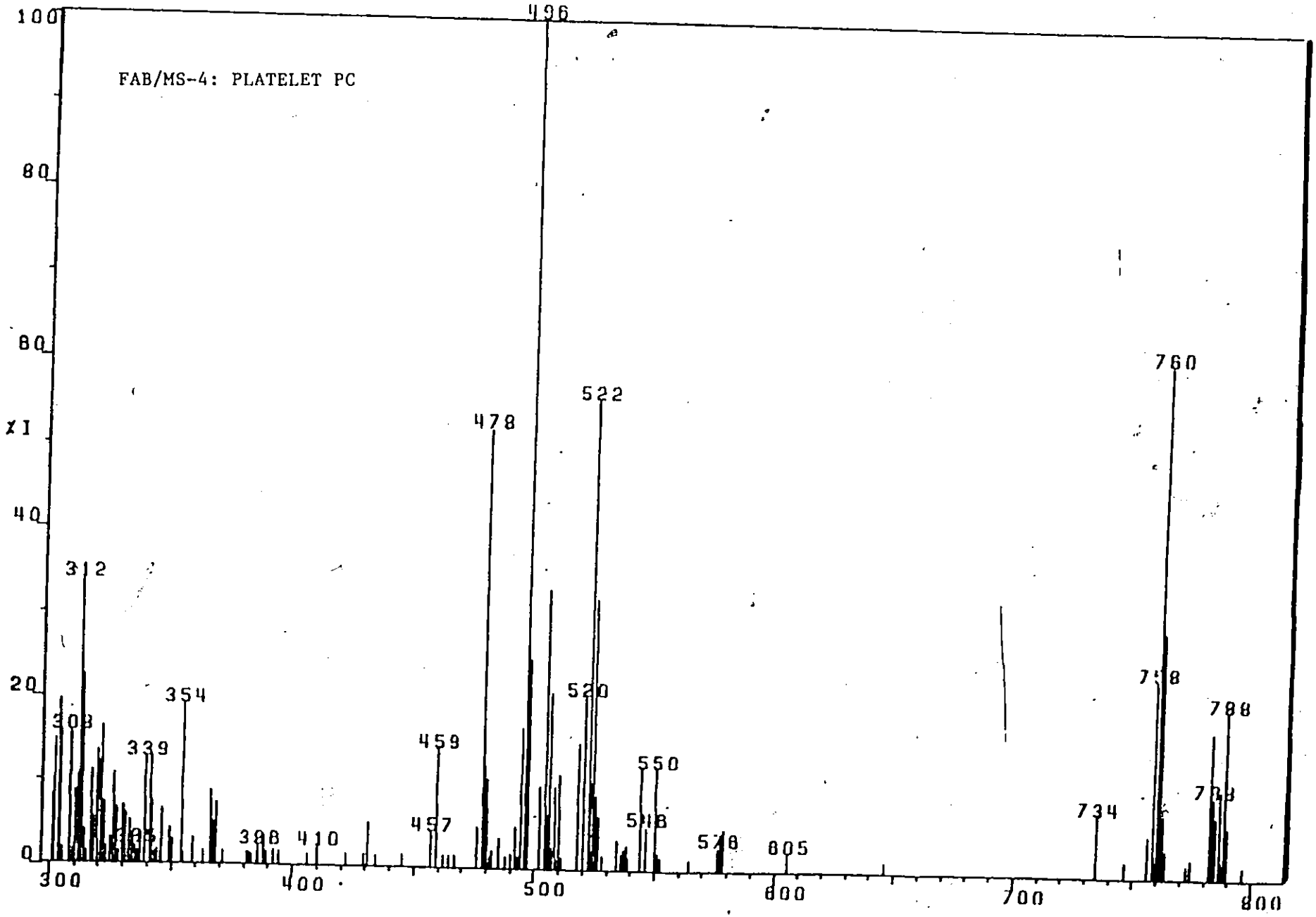


FAB/MS-3: AUTHENTIC PAF STANDARD



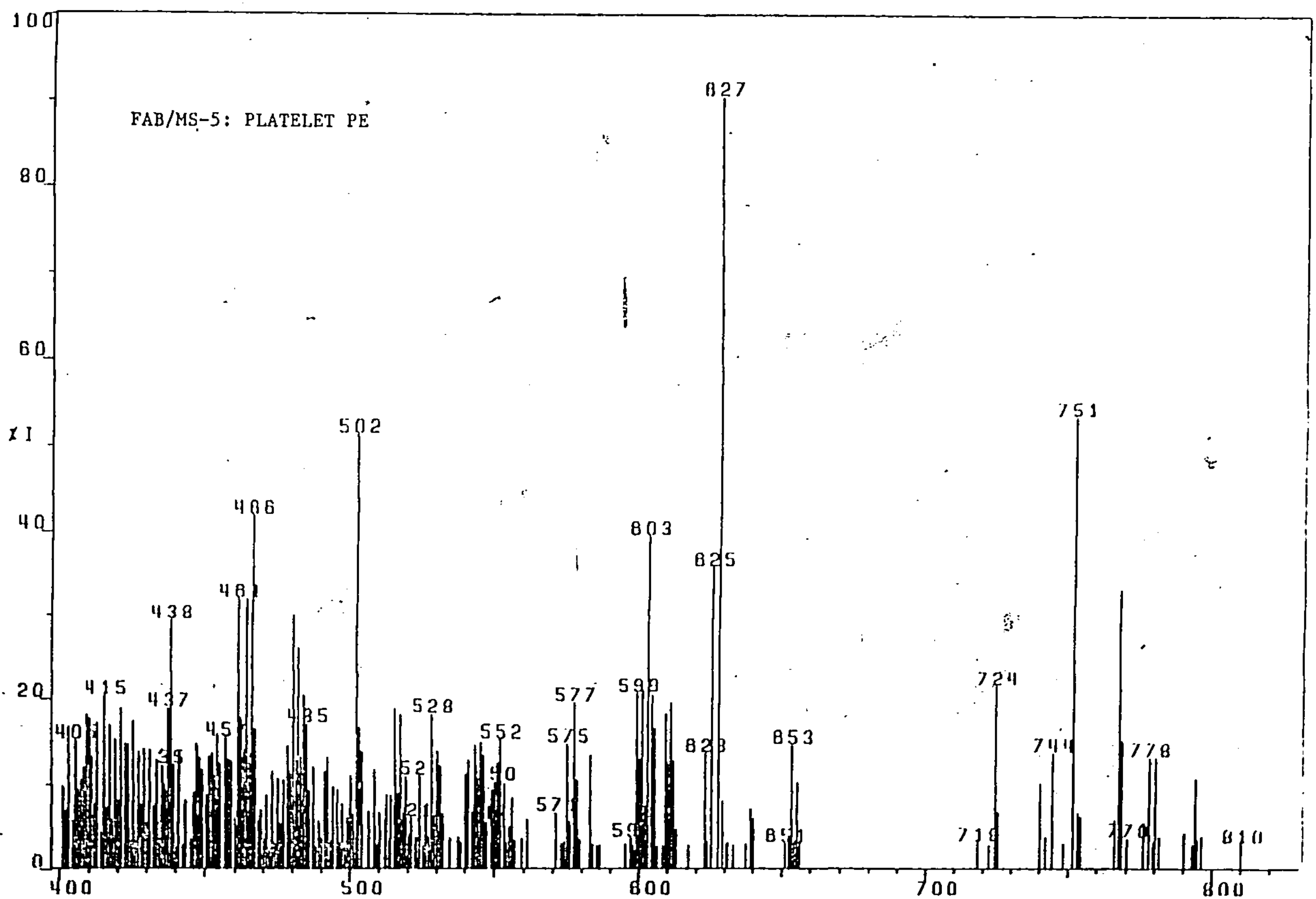
FAB/MS-3: AUTHENTIC PAF STANDARD

PEAK NO.	MASS	%HT. MOD.	PEAK NO.	MASS	%HT. MOD.
347	438.0	0.8	448	586.9	0.5
348	438.9	0.6	449	588.1	0.6
349	439.9	0.4	450	589.1	0.7
356	451.1	0.5	452	591.1	1.2
357	452.1	0.5	454	601.1	0.5
358	453.0	0.5	456	603.1	2.1
359	454.0	0.5	457	605.0	2.8
360	455.0	0.4	458	606.1	1.2
362	458.0	0.6	461	615.0	0.4
363	459.0	0.5	462	617.0	0.7
364	461.0	0.5	463	619.0	0.5
365	462.0	0.6	469	627.1	0.7
366	463.0	0.8	470	627.9	0.5
367	464.0	1.1	471	629.0	0.6
368	465.1	0.8	473	631.1	0.6
869	466.1	1.3	474	632.1	0.4
370	467.0	0.7	475	633.1	0.8
375	474.0	0.4	477	644.0	0.4
377	477.1	0.5	478	651.1	0.5
378	478.0	1.0	480	655.0	0.7
379	479.0	0.6	482	668.9	0.4
380	480.0	3.3	485	701.9	0.6
381	481.0	0.8	486	727.9	0.5
382	482.1	3.4	487	729.9	0.5
383	483.0	1.1			
384	484.1	0.6			
389	490.1	0.5			
390	491.0	0.7			
391	492.1	0.4			
393	494.1	0.6			
395	496.0	0.4			
399	505.2	0.5			
400	506.0	0.6			
401	508.0	1.6			
402	509.1	0.5			
403	510.0	0.8			
408	521.1	0.6			
409	522.1	2.3			
410	523.1	0.8			
411	524.0	21.9			
412	524.9	7.2			
413	526.0	1.5			
414	527.1	0.6			
419	536.0	0.4			
420	537.0	0.5			
421	538.0	0.5			
429	550.1	0.5			
434	561.0	0.7			
435	563.1	0.5			
440	574.1	0.6			
441	575.1	0.7			
442	577.1	1.3			
443	578.1	0.8			


FAB/MS-4: PLATELET PC
100
80
60
40
20
0
%I
300
400
500
600
700
800
496
478
522
760
312
354
339
459
520
550
518
457
410
398
578
805
734
788

FAB/MS-4: PLATELET PC

PEAK NO.	MASS	%AR. MOD.
1	301.0	8.12
2	301.9	14.64
3	303.2	19.42
4	304.0	15.80
5	305.0	1.88
6	308.0	15.51
7	309.0	1.74
8	310.0	8.55
9	311.1	10.72
10	312.0	33.62
11	313.2	22.46
12	314.0	4.06
13	315.1	1.59
14	317.0	11.01
15	318.0	5.51
16	319.0	13.33
17	320.0	12.17
18	321.0	16.23
19	322.0	7.25
20	323.1	2.17
21	324.0	1.59
22	325.0	3.04
23	326.0	10.58
24	327.0	6.67
25	328.0	1.59
26	329.9	6.81
27	330.9	5.94
28	332.2	3.33
29	333.0	5.22
30	333.9	3.62
31	335.0	2.03
32	336.0	1.59
33	337.1	3.77
34	339.1	12.46
35	341.1	12.90
36	341.9	7.39
37	343.0	1.45
38	344.0	1.74
39	345.0	1.45
40	345.9	6.38
41	349.0	4.20
42	350.0	2.90
43	354.0	18.84
44	355.0	1.30
45	358.9	3.04
46	363.0	1.59
47	366.0	8.70
48	367.1	5.22
49	368.0	7.25
50	371.0	1.59
51	381.1	1.45
52	382.0	1.45
54	385.0	1.45
55	385.9	2.17
56	388.2	3.62
57	389.1	1.59
58	392.0	1.74
59	394.0	1.59
60	406.1	1.30
61	410.1	2.03
62	422.1	1.45
63	429.0	1.45
64	431.1	5.22
65	434.1	1.30
66	445.1	1.45
67	457.1	4.06
68	459.1	14.06
69	462.1	1.45
70	464.2	1.45
71	467.2	1.45
72	476.2	4.78
73	478.1	52.03
74	479.1	12.32
75	480.1	10.72
76	481.1	1.45
77	482.1	2.03
78	485.1	3.48
79	488.1	1.45
80	490.0	1.74
81	492.1	4.93
82	493.0	1.59
83	494.1	16.96
84	496.1	100.00
85	497.1	25.22
86	502.1	9.86
87	504.1	33.33
88	505.1	6.67
89	506.1	21.16
90	507.1	2.75
91	508.1	9.86
92	509.0	1.45
93	510.1	11.30
94	511.0	1.45
95	518.1	15.22
96	520.0	20.72
97	522.1	55.65
98	523.1	10.43
99	524.1	32.46
100	525.1	8.84
101	526.0	6.38
102	528.0	1.74
103	534.0	3.62
104	536.1	1.88
105	537.0	2.46
107	538.9	1.59
108	544.0	12.03
109	546.0	5.22
110	550.1	12.03
111	551.1	2.17
112	552.0	1.59
113	564.0	1.30
114	576.0	2.75
115	577.2	3.91
116	578.1	5.07
117	605.2	2.03
118	646.1	1.30
119	734.3	7.25
120	746.2	1.88
121	756.2	5.07
122	758.2	23.48
123	759.1	2.75
124	760.1	60.87
125	761.2	29.28
126	762.2	7.54
127	763.1	3.33
128	772.4	1.59
129	774.1	2.32
130	782.1	17.54
131	783.0	9.71
132	784.1	7.39
133	786.2	10.58
134	786.9	2.32
135	788.1	20.00
136	789.1	6.09
137	796.0	1.45
138	808.0	4.78
139	810.1	9.57
140	812.0	1.45


FAB/MS-5: PLATELET PE
%I
100
80
60
40
20
0
400
500
600
700
800
827
751
502
406
803
825
438
724
415
437
577
599
528
552
575
853
778

FAB/MS-5: PLATELET PE

PEAK NO.	MASS	%AR. MOD.	PEAK NO.	MASS	%AR. MOD.	PEAK NO.	MASS	%AR. MOD.
266	468.0	1.14	319	522.1	0.73	372	604.3	4.09
267	469.0	1.39	320	523.0	0.75	373	605.1	3.31
268	470.1	0.71	321	523.9	2.17	374	606.1	0.56
269	471.0	1.75	322	525.1	0.54	375	608.0	0.56
270	472.0	0.54	323	526.0	1.44	376	609.0	3.65
271	473.1	2.29	324	527.1	0.78	377	610.0	2.51
272	474.0	0.66	325	528.0	3.60	378	611.0	3.89
273	475.0	2.12	326	529.1	1.31	379	612.0	2.56
274	476.0	1.10	327	529.9	2.77	380	612.9	0.92
275	477.0	2.09	328	531.0	2.41	381	616.9	0.54
276	478.0	2.90	329	532.0	1.34	382	623.0	2.70
277	479.0	2.31	330	534.0	0.71	383	624.0	0.63
278	480.0	5.96	331	536.9	0.75	384	625.0	7.13
279	481.0	2.68	332	537.9	0.63	385	627.3	18.06
280	482.0	5.18	333	539.9	2.21	386	629.0	1.61
281	483.0	2.65	334	541.0	2.56	387	630.2	0.58
282	484.0	4.06	335	542.0	1.31	388	631.1	0.61
283	485.0	3.38	336	543.1	2.90	389	633.0	0.54
284	486.0	1.87	337	544.0	2.12	390	637.1	0.58
285	487.1	2.39	338	545.1	2.97	391	639.1	1.41
286	488.1	0.54	339	546.1	2.68	392	640.1	1.19
287	489.1	1.14	340	547.1	1.14	393	650.9	0.58
288	490.0	0.85	341	549.0	1.87	394	652.0	0.71
289	491.0	2.29	342	550.0	2.02	395	653.1	2.90
290	492.1	2.60	343	550.9	2.48	396	654.0	0.68
291	493.1	0.71	344	551.8	3.02	397	655.1	2.04
292	494.1	1.95	345	553.0	2.00	398	656.0	0.61
293	495.1	1.67	346	553.9	0.68	399	718.0	0.61
294	496.0	0.63	347	555.1	1.00	400	722.0	0.56
295	497.1	1.53	348	556.1	1.68	401	724.2	4.28
296	497.9	0.71	349	557.1	0.68	402	725.0	1.36
297	499.1	1.24	350	559.1	0.71	403	739.9	2.00
298	500.1	2.19	351	561.1	1.17	404	741.8	0.75
299	501.1	1.34	352	571.1	1.31	405	744.1	2.70
300	501.9	10.22	353	573.1	0.61	406	748.0	0.58
301	502.4	3.19	354	574.1	0.63	407	751.3	10.61
302	503.0	3.33	355	575.0	2.92	408	752.9	1.34
303	504.0	2.77	356	576.1	1.12	409	754.0	1.22
304	506.1	1.36	357	577.1	3.92	410	766.0	1.17
305	508.0	2.31	358	578.0	2.09	411	767.5	6.57
306	508.9	0.58	359	579.1	0.71	412	768.2	3.04
307	510.0	1.31	360	583.1	2.68	413	770.0	0.71
308	512.0	1.17	361	584.1	0.58	414	775.9	1.10
309	512.6	1.73	362	585.1	0.54	415	778.0	2.60
310	513.1	0.73	363	586.1	0.54	416	779.2	0.66
311	514.0	1.75	364	595.1	0.58	417	780.0	2.63
312	515.1	3.75	365	597.2	0.71	418	781.6	0.73
313	516.0	1.80	366	598.0	0.58	419	790.0	0.83
314	517.1	3.60	367	599.1	4.11	420	793.0	0.58
315	517.9	1.36	368	600.1	2.60	421	794.1	2.12
316	519.0	2.12	369	601.0	4.23	422	795.0	0.71
317	**519.9**	**0.83**	**370**	**602.0**	**1.56**	**423**	**796.2**	**0.78**
318	**521.0**	**1.19**	**371**	**603.1**	**7.59**	**424**	**810.0**	**0.58**