DEDICATED TO THE GLORY OF GOD

AND TO MY DEAR WIFE, JANET AND TWO

CHILDREN, CLAUDIA AND DAVID

NMR LIPID PROFILING AND LIPID METABOLISM IN PLATELETS

BY

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A thesis submitted in partial fulfilment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

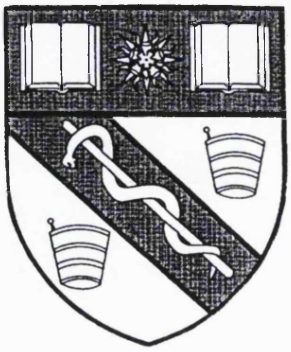
(PHARMACEUTICAL CHEMISTRY & BIOCHEMISTRY)

FACULTY OF MEDICINE

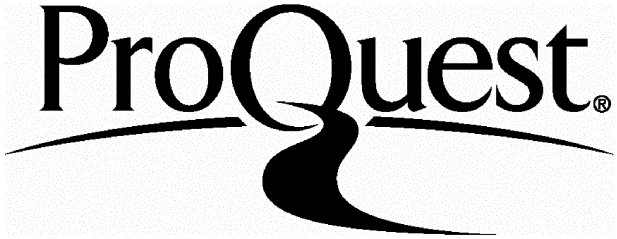
at the

SCHOOL OF PHARMACY

(UNIVERSITY OF LONDON)



January, 1995.



ACKNOWLEDGEMENTS

It is my great pleasure to extend my profound gratitude and heartfelt thanks to Professor Willie Gibbons for his tremendous support and assistance during the course of this work. He has been a source of constant inspiration and motivation for the past three years and the considerable effort he made to secure me a springboard for the future will ever stay in my heart. Willie, may the Lord richly bless you!

Many thanks are also conveyed to Professor Florence, the Dean, without whose timely intervention I would not have been able to continue this work.

I also wish to thank Gill (the secretary), Colin, Jason (multi-media unit) and Annie (photo unit) for their immense assistance in diverse ways.

Dr Marie Anderson deserves a special note of thanks for her assistance and support during my early days in the School of Pharmacy.

I also want to thank Dr Anna Nicolau, Dr Klara Valko and all my colleagues including Vadim, Tamara, Reimel, Gao Bin, Mireh, Qi Fei, Yang, Susan and Luma; their presence and assistance sometimes made the load less tiresome.

David, Roy, Lionnel and Wilfe have in various ways contributed to make this work possible. To you all I say thank you.

Much appreciation is also due to Dr Victor Preedy, Professor John Martin and Dr Angie Brown for their assistance during my short but fruitful work at King's College hospital.

I also thank the staff at the Hospital Unit of the North London Blood Transfusion Unit for the adequate supplies of blood for my work.

Not also forgetting my numerous friends at Hughes Parry Hall who made my stay there very enjoyable. Thanks a lot.

Finally, but not the least, I want to thank my wife and children for standing behind me during a difficult but fulfilling time of my life and also my parents.

God bless you all!!

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ABBREVIATION AND SYMBOL

ADP	adenosine 5'-disphophate
AMP	adenosine 3',5'-cyclic monophosphate
Arach	arachidonic acid
ARG	arginine
ASPG	asparagine
ASPT	aspartate
ATP	adenosine 5'-triphosphate
BSA	bovine serum albumin
BLK	blank
CAD	cadaverine
CA	L-cysteic acid
CSA	L-cysteine sulphinate
CNS	central nervous system
Chaps	(3-[(3-cholamidopropyl) dimethyl-ammonio] 1-propanesulphonate
CHOL.	cholesterol
Con-A	concanalin A
COSY	2-D correlated spectroscopy
CON	control
DAG	diacylglycerol
DEAE	diethylaminoethyl
Doco	docosahexanoic acid
FFA	free fatty acid
FID	free induction decay
fplc	fast protein liquid chromatography
GABA	glutamate amino butyric acid
GLY	glycine
5-HT	5-hydroxyptamine

HCA	L-homocysteic acid
HCSA	L-homocysteic sulphinate
HETE	hydroxyeicosatetraenoic acid
HIS	histamine
HPETE	hydroperoxyeicosatetraenoic acid
IP_3	inositol 1,4,5,-triphosphate
LPC	lysophosphatidylcholine
MET	methionine
Mr	molecular weight
NMDA	N-methyl-D-aspartate
NO	nitric oxide
NMR	nuclear magnetic resonance
NOR	norvaline
OPA	O-phthaldehyde
PAF	platelet-activating factor
PAGE	polyacrylamide gel electrophoresis
PC	phosphatidylcholine
PDE	phosphatidyldimethylethanolamine
PE	phosphatidylethanolamine
PDGF	platelet-derived growth factor
PGG_2	prostaglandin G_2
PGH_2	prostaglandin H_2
PGH_2	prostaglandin E_2
PGI_2	prostacylin
PIP_2	phosphatidylinositol-4,5-bisphosphate
PLA	plasmalogen
PK	protein kinase
PRP	platelet-rich-plasma
PS	phosphatidylserine
PUFA	polyunsaturated fatty acid

PUT	putrescine
SAA	sulphur amino acids
SAH	S-adenosyl-L-homocysteine
SAM	S-adenosyl-L-methionine
SDS	sodium dodecyl sulphate
SF	solvent front
SPD	spermidine
SM	sphingomyelin
SPM	spermine
SPH	sphingolipids
TAU	taurine
TBC	thromboglobulin
TXA_2	thromboxane A_2
TYR	tyrosine
VAL	valine
CM	centimetre
^{14}C	carbon-14
°C	degree centigrade
c.p.m.	counts per minute
d.p.m.	disintegrations per minute
hr	hour(s)
^{3}H	tritium
KDa	kilodalton(s)
M	molar
uci	microcurie(s)
ug	microgram(s)
uM	micromolar
mg	milligram
min	minutes
mol	mole(s)

ng	nanogram
pI	isoelectric point
^{32}p	phosphorus - 32
ppm	parts per million
rpm	revolutions per minute
S	seconds
V/V	volume per volume

ABSTRACT

Lipid profiles of human blood platelets were obtained employing high-resolution nuclear-magnetic resonance (nmr) in the one-dimensional and two-dimensional modes. The relative amounts of major lipids and their partial assignments were obtained from the spectra of the non-hydrolysed total lipid mixture. The results obtained agreed with similar data obtained by purely chromatographic means, but the nmr method proved more rapid, comprehensive and just as accurate. To extend the information obtained by the nmr approach, the latter was combined with hplc fractionation. The subsequent nmr analysis of the fractionated lipid extracts permitted analysis of the diacylglycerolcholines and ethanolamines, the ether ethanolamines and cholines and the corresponding ceramide (sphingoid) phospholipids. It was also possible to analyse for the C-1 unsaturated and saturated lipids attached to the alkyl-acyl ether lipids.

Using the same nmr approach as a novel diagnostic investigative tool, the platelet lipid profile of three cardiac patients were analysed. The results depicted differences in their lipid profiles compared to normal individuals and suggested that this methodology could be used to study lipid metabolic fluxes in pathological conditions involving platelets.

The phospholipid transmethylation pathway was also investigated in platelet plasma membranes, purified by a combination of electrophoretic and chromatographic procedures. The major lipid methylating enzyme(s), according to this work, had a molecular weight of 65 $\pm$ 2KDa and existed in both acidic and basic forms. These data are consistent with the hypothesis that several SAM-dependent isozymes exist that are responsible for the methylation process. These isozymes may exhibit tissue and substrate specificities.

CHAPTER 1

INTRODUCTION

Chapter 1

1.1 Ultra Structure and Morphology of Platelets

Platelets are cytoplasmic enucleate fragments of the megakaryocytes found in bone marrow. They are the smallest elements in peripheral blood, measuring 2 to 3μ in diameter (Fig.1). They are disc shaped, and there are 200,000 to 400,000 of them in every cubic millimetre of blood. Platelets remain in the circulation for an average of 7 days, and are removed from it by the spleen and lungs.

Platelets are formed in the bone marrow and are derived from the megakaryocytes, probably by fragmentation of the cytoplasm, both by the pinching off of parts of megakaryocyte pseudopodia and by mechanical fragmentation of larger portions of megakaryocyte cytoplasm, during passage through the microcirculation of the lung (Pennington, 1981).

The discoid shape of resting platelets is maintained by a ring of microtubules running around the edge of the disc immediately below the plasma membrane (Fig.1). Chilling platelets or treating them with drugs such as colchicine disrupts this microtubule ring and results in the loss of their discoid shape (White and Krivit, 1967; White, 1968). This also occurs when platelets are activated by most stimulatory agonists.

Platelets are surrounded by an 80Å thick cell membrane with an external fuzzy coat averaging 500Å in thickness (Fig.1). The plasma membrane of platelets is rich in glycoproteins, some of which are thought to be components of the receptors

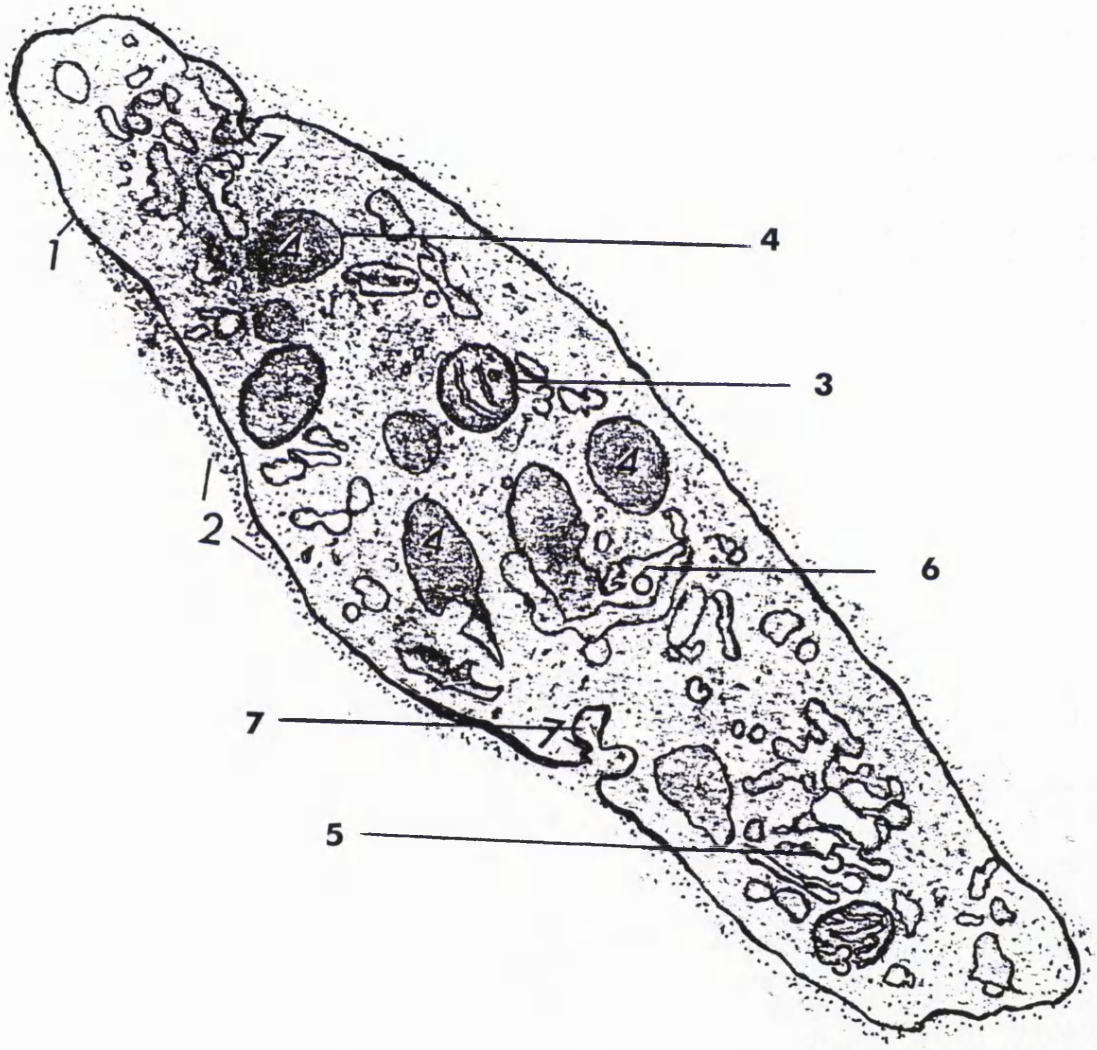


Fig.1 Blood Platelet (EM x 47,000)

1. surface membrane 2. fuzzy coat 3. mitochondria
4. alpha granules 5. golgi complex 6. smooth tubules
7. smooth tubules connected to surface membrane

present at the platelet surface, although the precise function of most of these glycoproteins is unknown. This plasma membrane is invaginated to form the surface-connected canalicular system, which greatly increases the surface area of the platelet and which is in close proxim-ity to a network of dense tubules derived from the smooth endoplasmic reticulum of the megakaryocytes. This association of surface invagination with the dense tubular system is similar to the association in skeletal muscle between transverse tubules and the sarcoplasmic reticulum (White, 1972). Functional similarities also may exist because dense tubular systems, like the sarcoplasmic reticulum, are sites of storage of Ca^{2+} (Skaer et al, 1974) which may be released as occurs too during platelet activation. Platelets also contain actin and myosin and can be regarded as contractile cells.

Platelets contain few mitochondria, with sparse cristae (Fig.1), but contain many glycogen granules; metabolic energy is mainly derived from glycolysis rather than oxidative phosphorylation (Holmsem, 1977). There are three types of storage granules (Table 1), the contents of which can be released on platelet activation. The *dense granules* contain high concentrations of biogenic amines (mainly 5-HT in human platelets), adenine nucleotides (ADP and ATP), and Ca^{2+}, the release of which can affect vascular tone as well as the thrombus-forming ability of other platelets. The second type of releasable granule is the heterogeneous population of α-*granules* (0.2μ in diameter), which mainly

Table 1 Types of platelet granules and their most important contents

TYPE	CONTENTS
Dense granules	5-hydroxytryptamine (serotonin) ADP, ATP, Calcium*
Alpha granules	Proteins not present in plasma: platelet factor 4 (PF-4), beta-thromboglobulin (β-TBC), platelet-derived growth factor (PDGF)
Lysosomes	Hydrolytic enzymes

*Magnesium in some non-human species

contain proteins that can influence blood vessel function and the clotting cascade, such as platelet factor 4, β-thromboglobulin, and platelet-derived growth factor. These granules also contain fibrinogen, which is important for platelet aggregation, as well as being the substrate for thrombin which converts it to fibrin, the physical basis of the mesh of a blood clot. Platelets also contain *lysosomal granules*, the contents of which also can be released but only after the platelets are stimulated with powerful aggregating agents, such as thrombin or high concentrations of collagen. In addition to the contents of these three types of granules, activated platelets can release pharmacologically active substances that are newly synthesised rather than stored, such as prostaglandins, TXA_2 and PAF, which affect vascular tone and permeability and activate other platelets.

Platelets contain 86% to 88% water. Nineteen per cent of their dry weight is lipid (phospholipids, triglycerides, cholesterol, cholesterol esters), and 57% is protein. Some of the inorganic constituents present are sodium, potassium, manganese, iron, copper, magnesium and calcium. They contain enzymes, eg. glucuronidase, catalase, amylase, phosphomonoesterase, lecthinase, histaminase, trypsin, acid phosphatase, lactic and glutamic dehydrogenases, and others. They also contain agglutinogens, apparently comparable to those of the red cells, and they may agglutinate or clump for that reason.

1.2 Platelet Responses

In stirred suspension, platelets challenged with activating agents normally change shape, aggregate, and release their contents in that order and with increasing concentrations of agonists (Zucker and Nachmias, 1985). Secretion may be triggered by products of released arachidonic acid, but these compounds are not required with strong stimuli such as thrombin. With such a stimulus, secretion is initiated in 1 to 2 seconds and is essentially complete in a few minutes (Holmsem and Weiss, 1979).

Platelet responses can be observed either in anti-coagulated platelet-rich plasma, or in buffer after carefully washing the platelets free of plasma by centrifugation and/or gel filtration.

A shape change is observed, as a small decrease in light scattering or transmission, as the platelets lose their discoid shape, become spherical and extrude pseudopodia. This change in shape is associated with constriction of the microtubule ring. It has been shown that shape change is not an essential pre-requisite for aggregation (Mustard and Packham, 1970) indicating that the development of platelet stickiness is not simply a result of the change in shape of platelets exposing new adhesive sites.

Aggregation of platelets is accompanied by a rapid increase in light transmission and is dependent on extracellular Ca^{2t} and fibrinogen, which forms the bridges between platelets during rapid stirring. Weak aggregating agents or low

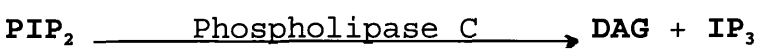
concentrations of stronger ones cause reversible ("primary") aggregation, whereas stronger stimuli cause an irreversible ("secondary") aggregation, which is associated with prostaglandin synthesis and release of granule contents. Aspirin inhibits both of these secondary events, suggesting that the release reaction caused by many agonists is mediated by arachidonic acid metabolism (Mustard et al, 1975) (Fig.2). This aggregation-induced synthesis of TXA_2 is enhanced by reduced extracellular Ca^{2t} levels such as are found in citrate-containing plasma (Packham et al, 1989). The substrates released from the dense granules, in particular ADP, also play an important role in enhancing secondary aggregation induced by other agonists. Thus there are at least two inter-related forms of positive feedback control, release of ADP and synthesis of prostanoids, and for strong stimuli such as collagen and thrombin there may be a third factor, the synthesis and release of PAF (Chignard et al, 1979, 1980).

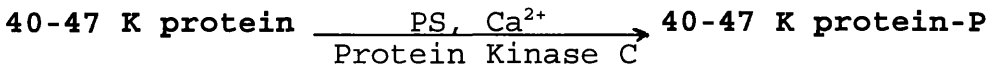
1.3 Stimulus-Response Coupling

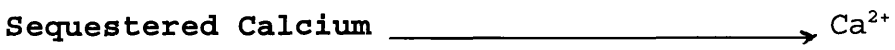
Platelet function is controlled by two types of agonists: stimulatory and inhibitory, which may be non specific or non competitive. The stimulatory agonists in general act via the phospholipase pathway to generate the second messengers Ca^{2+} and DAG, whereas inhibitory agonists act via stimulation of adenylate cyclase to generate the second messenger cyclic AMP. These effects are both thought to be coupled to receptor occupation via guanine nucleotide-binding proteins (G-proteins) and are probably mediated by protein phosphorylation (Feinstein, 1989; Siess, 1989). The responses appear to be sequential (Fig.2).

1.3.1 Role of cytoplasmic calcium

The role of Ca^{2+} in platelet activation was suggested by the finding that the Ca^{2+} ionophore A23187 causes platelet shape change, aggregation, and secretion, responses essentially the same as those produced by thrombin (Massini and Luscher, 1974; White et al, 1974). This supports the view that, as in other cells, ionized calcium is an important intracellular second messenger. Platelet responses to A23187 and thrombin are well maintained in the absence of external calcium except for aggregation. This indicates that an increase in cytoplasmic Ca^{2+} is from intraplatelet sources. Platelets contain a high concentration of Ca^{2+}.







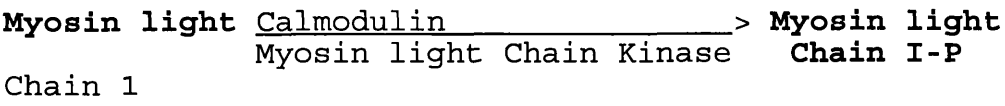


Fig.2 **Proposed sequence of reactions that follow the association of an agonist with its receptor on platelet membranes.**

About 60% is in the dense granules from where it can be secreted into the medium although it does not reach the cytoplasm (Holmsem and Karpatkin, 1983). The calcium concentration is very low in the cytoplasm (100nM or less) (Rink et al, 1982). Some calcium may be associated with the plasma membrane and the remainder of the functional platelet calcium is probably located in the dense tubular system.

1.3.2 Role of sodium and pH

Na^+/H^+ exchange may play a role in the activation of phospholipase A_2 and the formation of TXA_2, and Ca^{2+} mobilisation is enhanced by this pathway, at least for weak stimulants such as ADP, adrenaline, and low concentrations of thrombin, because inhibitors of the exchange block arachidonic acid release (Sweatt et al, 1985). Alternatively, Na^+/H^+ exchange may enhance Ca^{2+} mobilisation independently of phospholipase A_2 activation (Siffert et al, 1990). However, although ADP does stimulate Na^+/H^+ exchange, overall acidification of the cytoplasm is observed and amiloride and its analogues do not inhibit ADP-induced aggregation at concentrations that block Na^+/H^+ exchange, suggesting that this exchange is not required for aggregation (Funder et al, 1988). Also, the Ca^{2+} mobilisation caused by thrombin peaked before any alkalinisation occurred (Sage et al, 1990). It has been suggested that extracellular Na^+ itself rather than Na^+/H^+

exchange may be required for full aggregation (Krishnamurthi et al, 1990).

1.3.3 Changes in inositol phosphatides

Alterations in phosphatidylinositides occur during stimulation of many types of cells including platelets. Decreases in the concentration of phosphatidylinositol-4, 5-bisphosphate (PIP_2) occurs less than 5-10 seconds after horse or human platelets have been stimulated with thrombin (Broekman, 1984). With rabbit platelets, ADP has been observed to decrease PIP_2 concentration and increase its specific activity (Vickers et al, 1982a); thrombin decreases both parameters (Vickers et al, 1984). It has been suggested that agonists cause PIP_2 to decrease because the ATP necessary to maintain its normal level is also decreased (Vickers et al, 1984). This view is supported by the observation that PGE, or PGI_2, cause shape change in rabbit platelets, perhaps by diverting ATP for use in forming cyclic AMP (Vickers et al, 1982b). Others, however, have suggested that the decrease in PIP_2 after platelet stimulation results from phosphoesteratic activity of a phosphatidylinositide-specific phospholipase C (Vickers et al, 1982a; Rittenhouse-Simmons, 1979), leading to the formation of DAG and inositol-1,4,5-triphosphate (IP_3) (Fig.2).

The DAG formed from PIP_2 is rapidly phosphorylated to phosphatidic acid (PA) (Fisher et al, 1984). DAG also

acts as a cofactor in the activation of protein kinase C (Sano et al, 1983). DAG lowers the concentration of Ca^{2+} required by the enzyme, presumably to the level in platelet cytoplasm. The activated enzyme phosphorylates a protein with a molecular weight of between 40-47 KDa. Phosphorylation is associated with secretion (Nishizuka, 1984) but may also occur with weak stimuli that do not cause secretion.

1.3.4 Role of arachidonic acid and responses to collagen

Arachidonic acid is found in the 2-position of platelet phospholipids, especially the phosphatidylinositides and phosphatidylethanolamine (PE) (Marcus et al, 1962; Broekman et al, 1976). The free arachidonic acid necessary for T_xA_2 synthesis is cleaved from PI, PE and phosphatidylcholine (PC) by phospholipase A_2 (Imai et al, 1982), which is activated by calcium (Rittenhouse, 1982). There is some evidence that it is also cleaved from DAG by a diglyceride lipase (Bell et al, 1979). Whereas thrombin, PAF-acether and ADP exert their major effects directly, the effects of moderate amounts of collagen depend to a considerable extent on arachidonate metabolism (Fig.3). In a stirred system such as an aggregometer, inhibition of T_xA_2 formation with NSAI prevents PA formation (a reflection of PIP_2 hydrolysis), as well as responses such as shape change and

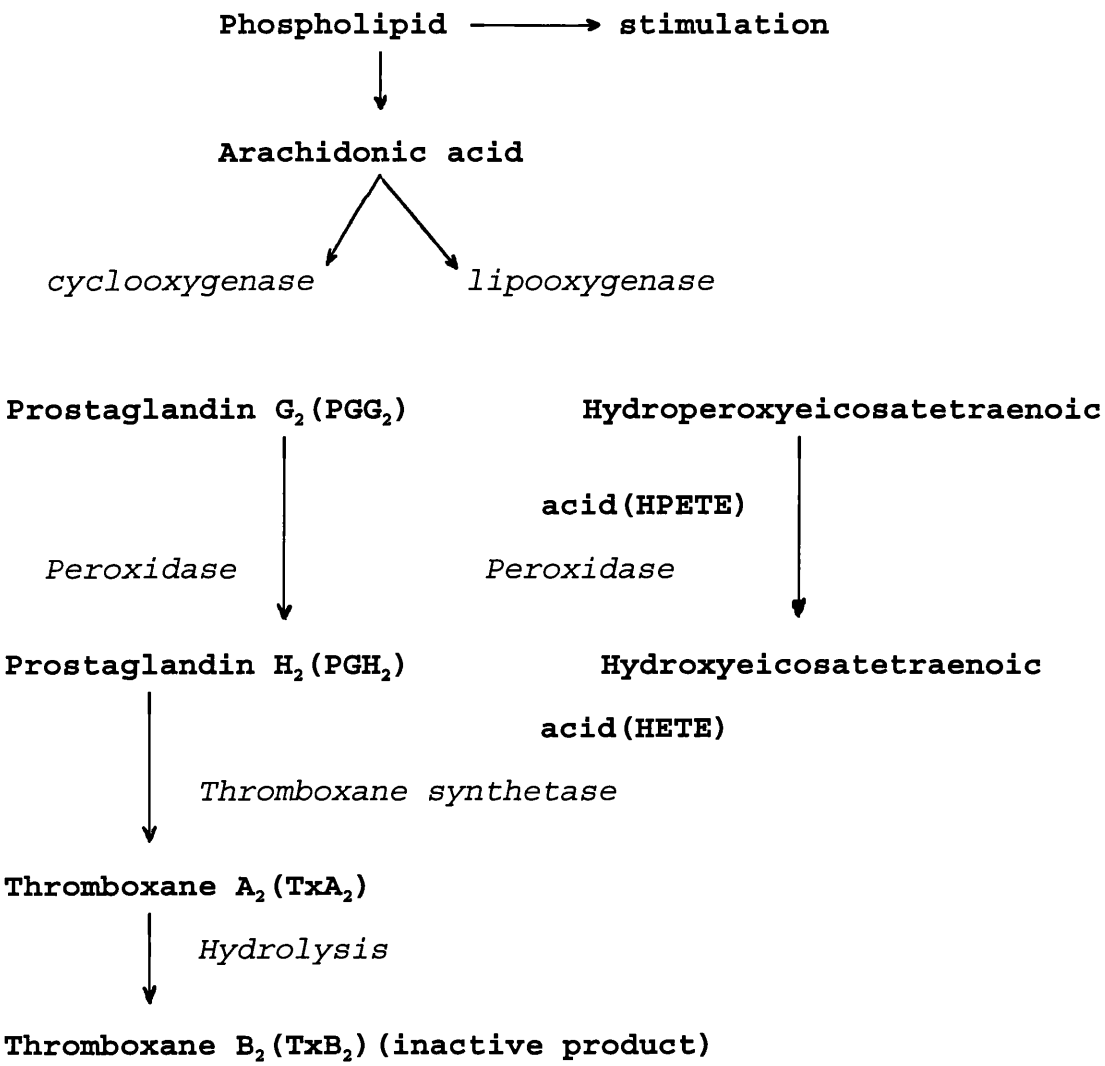


Fig.3 Arachidonic acid metabolism in platelets

secretion. Thus, PIP_2 hydrolysis depends upon formation of endoperoxides and T_xA_2 (Siess et al, 1983). The direct responses of the platelets that adhere to collagen must be different, since degranulation (ie. secretion) is not blocked by NSAIDS and is thus not dependent upon T_xA_2 formation (Charo et al, 1977).

1.3.5 Effects of aggregation

A number of effects reportedly occur only when platelets are aggregated, but not when they are activated without coming into contact with one another. Among these aggregation-dependent effects are: secretion induced by ADP and epinephrine (Zuker and Peterson, 1968; Charo et al, 1977); increase in platelet cyclic GMP (Davies et al, 1976); activation of the calcium-dependent protease (Fox et al, 1983); rapid dephosphorylation of actin binding protein (Carroll et al, 1982); and, possibly, inclusion of GPIIb and GPIIIa in the cytoskeleton. Close platelet contact can also stimulate platelet metabolism (Karpatkin et al, 1970).

1.4 Pharmacological Receptors on Platelets

1.4.1 Receptors for stimulatory agonists

A. Adenosine 5' Diphosphate

Although the effects of ADP have long been known, more is still being discovered on the nature of the ADP receptor,

its coupling mechanisms and its physiological importance (Cusack and Hollrani, 1991). ADP in vitro causes shape change, aggregation and release of granule contents in the presence of reduced Ca^{2+} levels. It is also known to inhibit adenylate cyclase (Halsalm, 1973), and controversy exists as to whether aggregation and the inhibition of adenylate cyclase are mediated by one or two types of ADP receptor (MacFarlane, 1987).

There is a high degree of structural specificity of ADP associated with platelet activation. For example, any modifications to the purine ring of ADP at the N^1, C^8 or C^6 results in loss of activity (Stone et al, 1976). Modifications also to the ribose sugar, such as removal or inversion of configuration of a hydroxyl group or periodate cleavage of the ring, result in weakly active compounds (Pearce et al, 1978). The ADP receptor is stereospecific; the unnatural L-enantiomers of ADP, 2-chloro-ADP and 2-azido-ADP (which contain L-ribose instead of the normal D-ribose), are inactive (Cusack et al, 1979). Overall, the platelet ADP receptor does not appear to be identical with any other receptor for adenine nucleotides.

B. Catecholamines (α-Receptor)

Aggregation to adrenaline is species dependent and does not occur in most non-primate mammals, although in some species adrenaline has a "proaggregatory" effect, potentiating responses to other agonists, when even its direct effect is too weak to be detected (Kerry and Scrutton, 1985). The

observed response to adrenaline seems to depend on the number of stimulatory α-adrenoceptors present, as well as the ratio of these receptors to the inhibitory β-adrenoceptors also present on platelets (Kerry et al, 1984). α-Adrenoceptors have been divided into two types, α_1 and α_2, on the basis of selective agonists and antagonists. α_2-receptors can mediate post-junctional effects as well as pre-synaptic inhibition of transmitter release. The platelet α-receptor has been characterised pharmacologically as an α_2-receptor (located outside the synaptic cleft) and therefore responds preferentially to blood-borne catecholamines.

C. 5-Hydroxytryptamine

The platelet 5-HT_2 receptor is coupled to phospholipase C, and receptor activation results in rapid phosphoinositide breakdown, increases in intracellular Ca^{2+} levels, and appropriate protein phosphorylation (De Chaffoy de Courcelles et al, 1984). Desensitization of 5-HT receptors on human platelets, which then fail to aggregate following successive doses of 5-HT, has been shown to correlate with decreased mobilization of intracellular Ca^{2+}, apparently due to a negative feedback mechanism involving protein kinase C, because stimulators of this enzyme (such as phorbol 12-myristyl 13 acetate) inhibit 5-HT-induced phosphatidyl-inositol (PI) turnover and Ca^{2+} mobilization (Erne and Pletscher, 1985a). Uptake of 5-HT into platelets has been

shown to be inhibited by propranol, but in a non-stereospecific manner (Rudnick et al, 1981).

D. Vasopressin

Vasopressin is capable of inducing irreversible aggregation and the release of dense granule contents. There are mainly two types of vasopressin receptors, V_1 (with a vasoconstrictor effect) and V_2 (with an antidiuretic effect) (Haslam and Rosson, 1972). Vasopressin V_1 receptors have recently been subdivided using new synthetic analogues and platelet V_1 receptor appears to be of the V_{1A} subtype, similar to those on the liver and vasculature rather than the V_{1B} subtype as found in the anterior pituitary (Launay et al, 1987). The V_1 receptors are responsible for aggregation and for weak inhibition of adenylate cyclase in platelet membrane preparations.

E. Prostaglandins and Thromboxane

The study of the receptors mediating aggregation induced by the endoperoxides and by TXA_2 is complicated by their instability. PGH_2 has a half life of approximately 5 minutes and is rapidly converted, mainly by thromboxane synthetase, to TXA_2 (which breaks down to inactive TXB_2 with a half life of <1 minute), but it can also be converted into inhibitory PGD_2 as well as the essentially inactive PGE_2 and $PGF_{2\alpha}$. TXA_2 causes shape change, aggregation and release of granule contents and is believed to act via membrane receptors coupled by a G protein to phospholipase

C (MacIntyre et al, 1987). Various studies have revealed the existence of a single TXA_2 receptor coupled to two effector systems (Takahara et al, 1990; Hirata et al, 1991).

F. Platelet-activating Factor (PAF)

PAF has the structure 1-0-alkyl-2 acetyl-sn-glycerol-3-phosphorylcholine, where the alkyl group on the C_1 position is usually hexadecyl or octadecyl (Benveniste et al, 1979). PAF is a potent aggregating agent, it is released from platelets following stimulation by A23187, thrombin or collagen, but not by ADP, arachidonic acid or PAF itself (Chignard et al, 1980).

PAF release has been suggested to be the "third pathway", in addition to ADP release and arachidonic acid metabolism, by which thrombin and collagen activate platelets (Chignard et al, 1979). As well as its potential role in platelet function, PAF has also been implicated in numerous physiological and pathological processes including pregnancy, asthma, inflammation, transplant rejection and gastrointestinal ulceration and arrhythmias (Braquet et al, 1987, Flores and sheridan, 1990). Although the phospholipid nature of PAF raises the possibility that its actions are due to some non specific perturbation of membranes, the clearly defined structure-activity relationships, the existence of competitive antagonists, and the ability to detect saturatabale binding sites shows that the actions of PAF are receptor mediated. Earlier attempts to solubilise and isolate the PAF receptors were complicated by several

problems associated with the phospholipid nature of PAF, which made it difficult to detect PAF binding to isolate receptors (Valone, 1988).

G. Thrombin

Thrombin is a clotting factor (factor IIa) that has an important role in blood coagulation, in cleaving fibrinogen (factor 1) to yield fibrin which then forms the fibrillar basis of the clot, and in activating factors V, VIII and XIII. Although an enzyme, it is not clear what role this enzymic activity plays in aggregation. In favour of a receptor-mediated process are the findings that thrombin acts via a G protein to activate phospholipase C, resulting in increases in DAG and Ca^{2+} levels. In addition, the kinetics of activation by thrombin are consistent with a receptor-mediated effect, because the extent of the response is dependent on the thrombin concentration. If an enzymic reaction were involved, it would be expected that only the rate of response would vary and that even low doses would eventually cause a full response (Detwiler and McGowan, 1985).

H. Collagen

Platelets adhere to the connective tissue protein collagen and this adhesion results in platelet activation, arachidonic acid metabolism, release of dense granule contents and aggregation; the latter process is largely dependent on the released ADP and PGH_2/TXA_2. Just as

platelet-platelet interaction (via fibrinogen) causes activation and release, so does platelet-collagen interaction. Thus, the collagen "receptor" on platelets is clearly more than just a nonspecific adhesive site (Valone, 1988).

A summary of platelet receptors and their coupling systems is shown on Table 2. Fig.4 also illustrates the interaction of pharmacological receptors on platelets and related receptors on blood vessels and some of their released mediators.

1.5 Physiology and Pathology of Platelets

1.5.1 The role of platelets in blood coagulation

The major physiological function of platelets is in haemostasis. At sites of vascular injury, platelets adhere to exposed subendothelial collagen and release the contents of their dense granules and α-granules, thereby recruiting more circulatory platelets which then clump together to form a haemostatic plug. The substances released include ADP, a powerful stimulant for platelet aggregation, and serotonin, a powerful vasoconstrictor that narrows the blood vessel wall locally and thus helps to restrict the bleeding area.

Simultaneously factor V and other clotting factors released from the α-granules aid in the local conversion of plasma

Table 2 Platelet receptors (Hourani and Cusack, 1991)

AGONIST	ANTAGONIST	RECEPTOR TYPE	COUPLING SYSTEMS	STRUCTURAL INFORMATION
ADP	ATP	P2T (unique)	?Ca2+ ↓ Cyclic AMP	100-KDa protein
Adrenaline (α-receptor)	Yohimbine	alpha2A	↓ Cyclic AMP	64-KDa protein
5-HT	Ketanserine	5-HT2	PL-C	232-KDa complex
Vasopressin	dVDAVP	V1A	PL-C ?↓ Cyclic AMP	125-KDa protein
TXA2/PGH2	13-A PA, SQ 29, 548, S-145	TP (TXA2/ PGH2)α	PL-C ↓ Cyclic AMP	37-KDa protein
PAF	BN 52021 Kadsurenone		PL-C ↓ Cyclic AMP	180KDa?, 160KDa? 220KDa?, 52KDa?
Thrombin	SC 42619		PL-C ↓ Cyclic AMP	GP16 (180KDa, 15KDa sub units)
Collagen			? Adhesion induced release	GP1a? GP1V? Platelet factor XIII?
Adenosine	Theophylline	A2	↑ Cyclic AMP	
$PG1_2/PGE_1$		1P	↑ Cyclic AMP	180KDa (85KDa + 95KDa sub units)
PGD_2	AH 6809 N-0164	DP	↑ Cyclic AMP	
Adrenaline (β-receptor)	Butoxamine propranolol	β2	? Cyclic AMP	

(Ref: Hourani and Cusack, 1991)

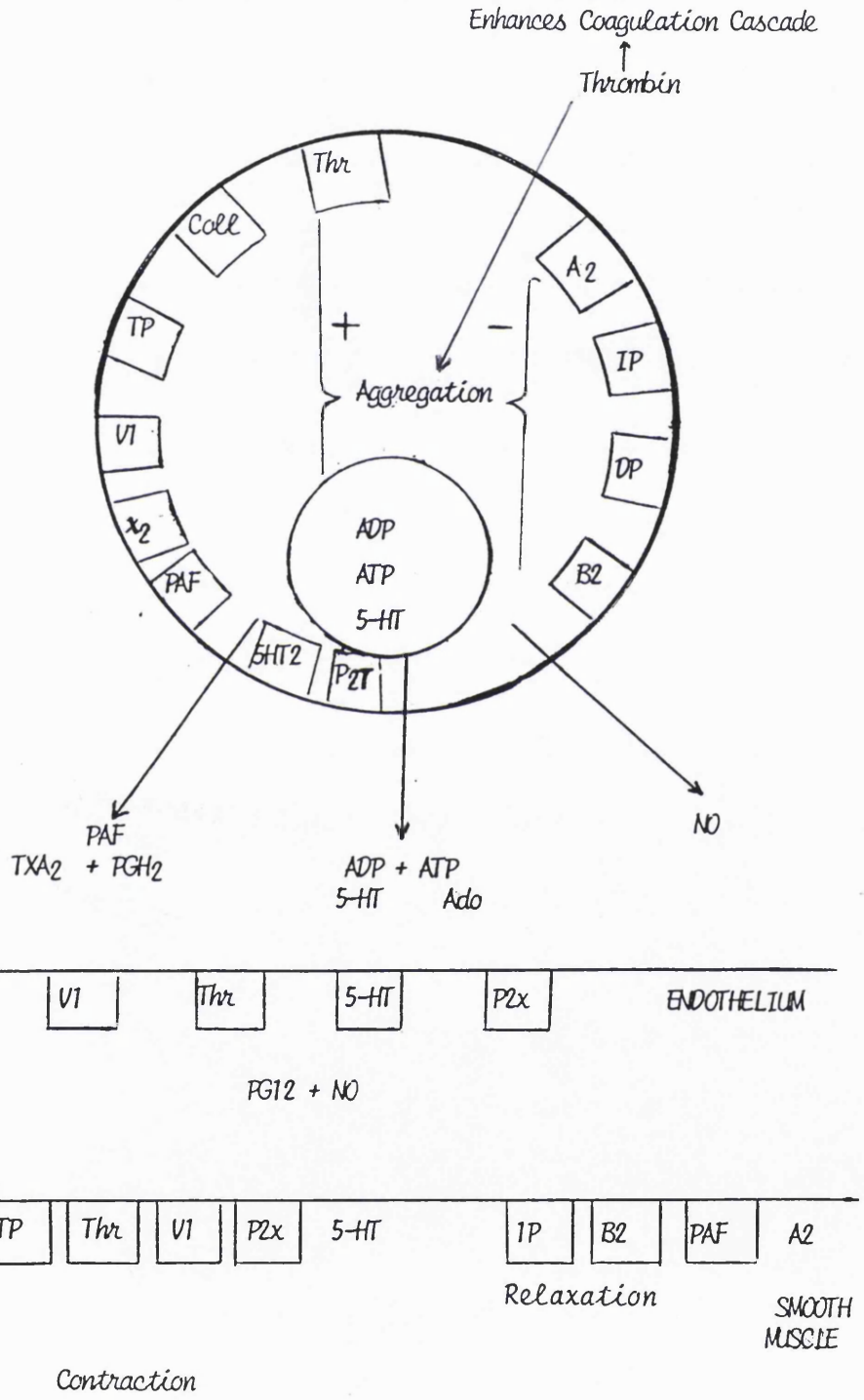


FIG.4 Schematic diagram illustrating interactions of pharmacological receptors on blood platelets and related receptors on blood vessels and some of the mediators released by platelets and endothelial cells. In the presence of intact endothelium, these mediators cause vasodilation and inhibition of aggregation via release of PGI2 and nitric oxide (NO), whereas in the absence of an endothelial layer, the overall response is vasoconstriction and enhancement of aggregation. Thr, thrombin; Coll, collagen; Ado, adenosine; PL-C, phospholipase C.

prothrombin into thrombin. Thrombin in turn stimulates further release of platelet granule material, thus providing strong local amplification of the clotting process. Thrombin also converts plasma and platelet fibrinogen into fibrin, which enmeshes and reinforces the platelet plug and entraps erythrocytes and other formed elements of the blood. Retraction of the platelet-fibrin clot then occurs as a result of the presence of the contractile protein *actomyosin* (thrombosthenin) in platelets, and a firm mass is formed. Thus smaller, severed blood vessels are closed. Larger wounds may require the application of external pressure.

Still another substance present in platelet granules is involved in the clotting process. Arachidonic acid is converted to an endoperoxide and thromboxane A_2, a metabolite of the prostaglandins (Fig.2). Thromboxane A_2 stimulates the secretion of ADP from the dense granules, further increasing platelet aggregations and enhancing the formation of the platelet plug. Studies have shown that aspirin impairs thromboxane A_2 formation by inhibiting the enzyme fatty acid cyclooxygenase, involved in the biosynthesis of thromboxane A_2. Another metabolite of the endoperoxides, prostacyclin, is an inhibitor of platelet aggregation, thus providing a countercontrol mechanism. Prostacyclin is synthesized in endothelial cells of blood vessels. A summary of the biochemical reactions that culminate in the formation of a fibrin clot during blood coagulation is shown in Fig.5.

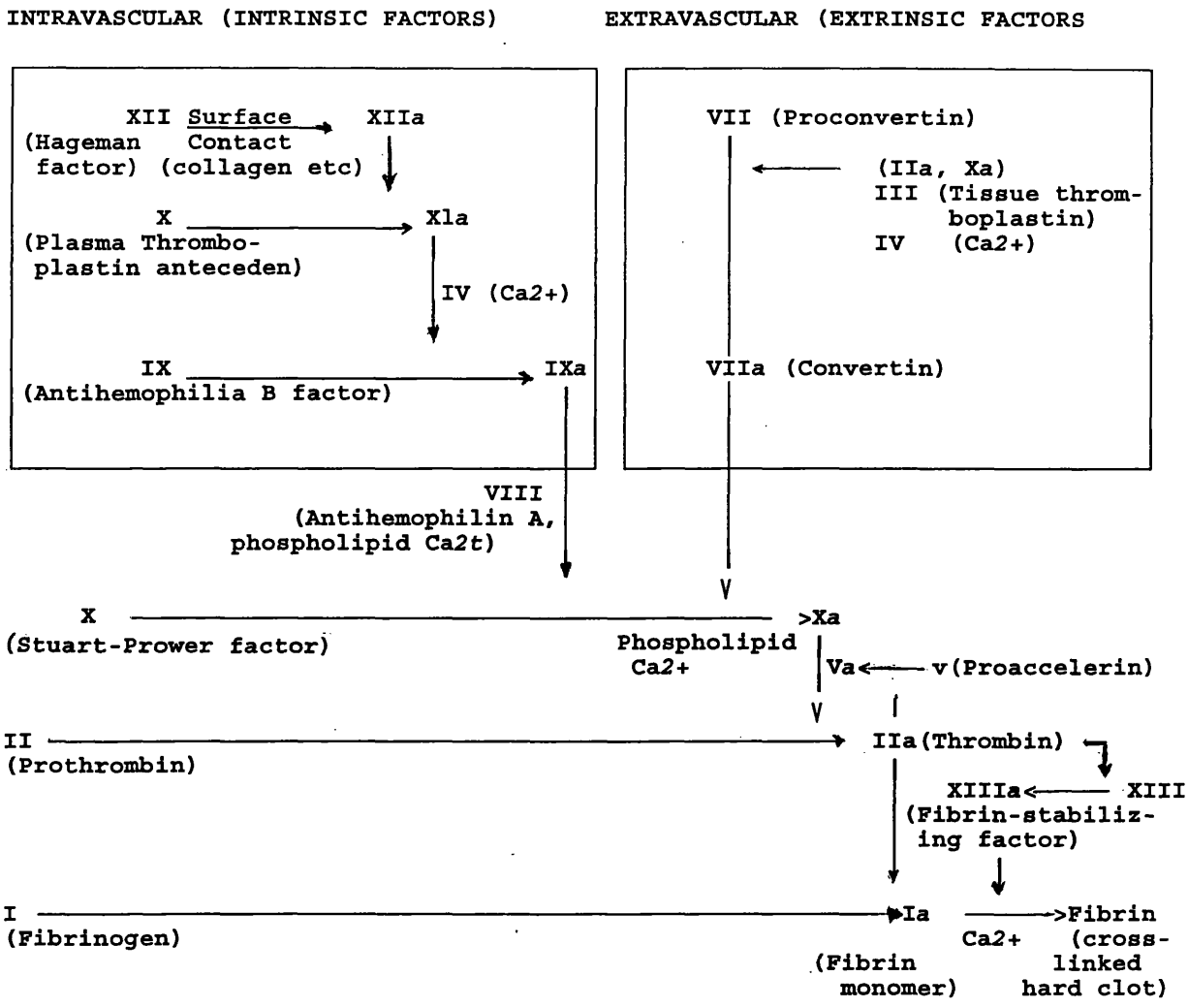


Fig.5 Schematic representation of principal reactions involved in the coagulation of blood. Roman numerals indicate International Commission on Designations of Clotting Factors. An italic 'a' indicates activated factor.

Table 3 Some abnormalities in human platelet function (Weiss H J; 1975)

Disease	Biochemical defect in platelet functions
Thrombocytopenia (congenital or acquired)	Deficient platelet production in bone marrow (due to leukemia, aplastic anemia, ionizing radiation, toxic agents, certain drugs)
Idiopathic thrombocytopenic purpura	Unknown (petechial hemorrhages under skin)
Storage pool disease	Impaired aggregation due to absence of dense granules
Thrombasthenia	Impaired aggregation probably due to deficiency of fibrinogen-binding surface glycoprotein
Congenital Afibrinogenemia	Congenital lack of fibrinogen
von Willebrand's disease	Lack of factor required for platelets to adhere to Collagen (also lack of anti-hemophilia A factor
Benard-Soulier (giant platelet) syndrome	Probable deficiency of surface glycoprotein that binds von Willebrand factor
Aspirin toxicity	Inhibition of fatty acid cyclooxygenase and thromboxane A_2 formation

Table 4 Some abnormalities of the blood clotting factors (Weiss, 1975)

Factor	Abnormality
I	Afibrinogenemia, congenital deficiency, hypofibrinogenemia, low plasma levels, dysfibrinogenemia, abnormal fibrinogen molecule
II	Hypoprothrombinemia, deficient or abnormal prothrombin molecule, vitamin K deficiency, liver disorders
III	Tissue factor deficiency (?), impaired thrombin formation, impaired clotting
IV	Hypocalcemia
V	Congenital parahemophilia, abnormal molecule or deficiency
VII	Congenital absence or abnormal molecule, vitamin K deficiency
VIII	Hemophilia A (Classic), deficient , inhibited, or abnormal molecule, sex-linked inheritance, low plasma level in von Willebrand's disease
IX	Hemophilia B, deficient or abnormal molecule, vitamin K deficiency
X	Stuart-Prower factor deficiency, deficiency or abnormal molecule, vitamin K deficiency
XI	Hemophilia C, factor absent or not released from platelet
XII	Hageman deficiency, factor absent, or defective molecule
XIII	Congenital deficiency, impaired fibrin polymerization, antithrombin III, thrombophilia, low plasma level, thrombosing tendency, reduced titer in some females using oral contraception

The vital role of platelets in the clotting of blood is further emphasized by the marked effects of abnormalities in platelet function on coagulation, especially on bleeding time (the latter is the time required for cessation of bleeding from a small cut in the skin of the forearm). Several of the major abnormalities of human platelets, together with the principal functions impaired, are summarized in Table 3. Dysfunction of the clotting process may occur also, secondary to various pathological conditions, such as liver disorders.
A number of abnormalities of blood coagulation due to dysfunction of the major clotting factors are also summarized in Table 4.

1.5.2 The role of platelets in atherogenesis/atherosclerosis

Platelets are thought to play a role in atherosclerosis both by forming mural thrombi, which become covered with endothelium and are incorporated into atherosclerotic plaques, and by secreting mitogenic factors such as platelet-derived growth factor (PDGF), which stimulate the proliferation of cells in the vessel wall; both processes lead to a narrowing of the blood vessel lumen (Sussman, 1985). According to the response-to-injury hypothesis, arteriosclerosis (thickening and loss of elasticity of the arterial cell wall), and eventually atherosclerosis (involvement of lipid in arterial wall thickening), are

caused by the platelet-vessel wall interaction that occurs following arterial injury and endothelial loss.

Although the exact role of platelets in atherogenesis is still not very clear, their importance in thrombosis is undoubted; they form part of and may initiate the occluding thrombus. The prophylatic use of antiplatelet drugs in an attempt to reduce the occurrence of thrombosis has had limited success, although recent results with aspirin have been more encouraging (Fuster et al, 1989, Juul-Moller et al, 1992).).

In their recent review, Flores and Sheridan (1994) concluded that, although clinical and experimental evidence suggests that platelet activation occurs during ischaemia which contributes to tissue necrosis and arrhythmogenesis, greater understanding of the cellular mechanisms of platelet activation and knowledge of specific receptor mediated effect should allow better control of platelet activation and aggregation during myocardial ischaemia.

1.6 Lipid Metabolism in Platelets

Platelets are capable of synthesing complex lipids from acetyl CoA, glycerol, and from preformed fatty acids (Fig.6). In addition alterations in lipid metabolism and in the orientation of lipids in membranes are integral to normal function.

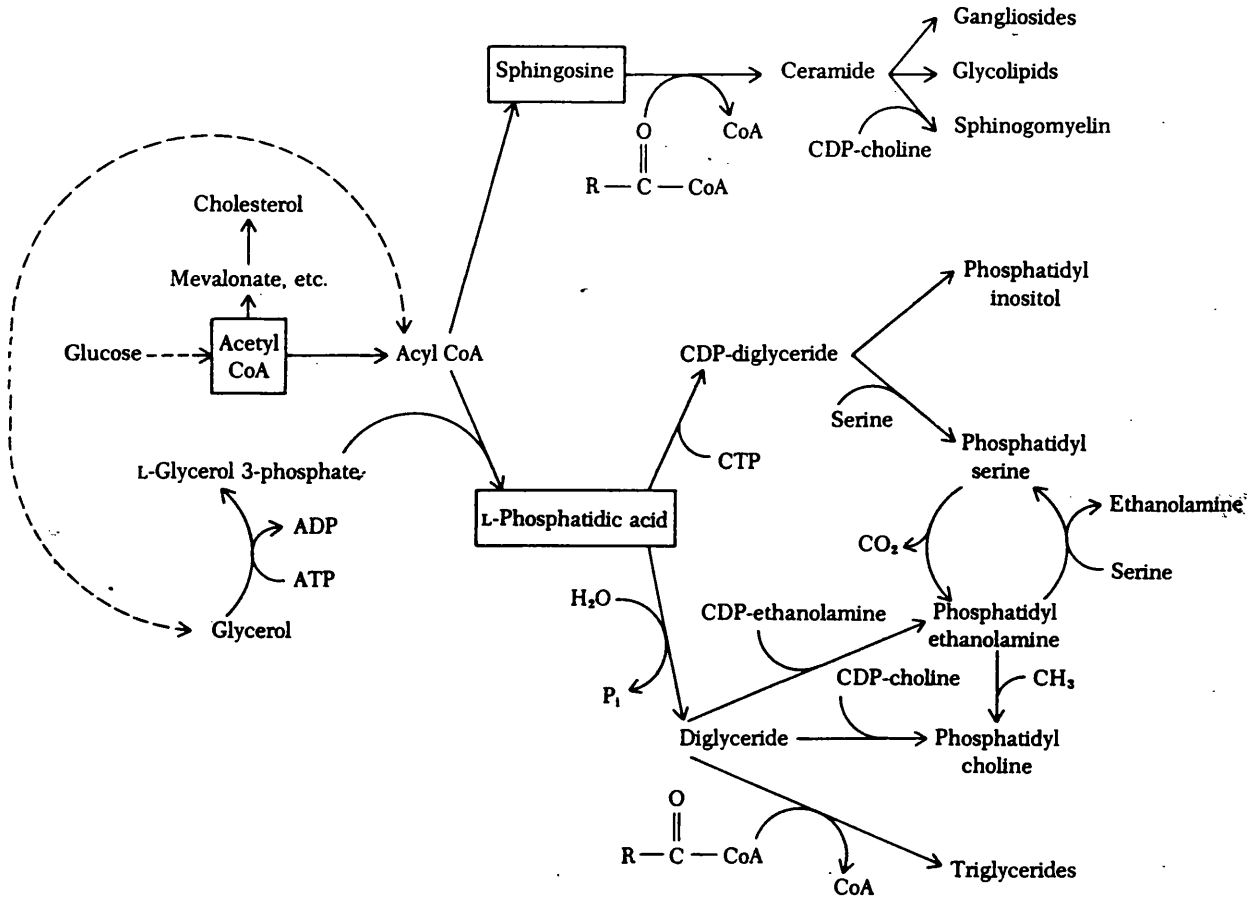


Fig.6 Summary of biosynthetic pathway for various lipids

1.6.1 Synthetic pathways for fatty acids, cholesterol and glycosphingolipids

Marcus et al (1969), showed that neither platelet membranes nor granules were characterized by a specific lipid or fatty acid. As in other tissues, they showed that the predominant fatty acids in the platelet phospholipids were palmitic, stearic, oleic and arachidonic acids. Both phosphatidylethanolamine and phophatidylinositol in platelet membrane and granules were enriched in arachidonic acid, which comprised 40% of the total fatty acids present in the phosphatidylinositides. Marks and his associates (1960) first showed that platelets can incorporate labelled acetate into fatty acids. This was later confirmed by Hennes et al (1966) who showed that both the *de novo* and chain elongation pathways of fatty synthesis exist in platelets, and by Majerus and his associates (1969), who demonstrated acetyl-CoA carboxylase activity in platelet extracts. Deykin and Dresser (1968) reported that fatty acids formed by *de novo* synthesis exchange with plasma free fatty acids, but those formed primarily by chain elongation did not. They also showed that in platelets, the synthesis of ceramides were very active. The platelet uptake of free fatty acids from plasma albumin has been studied by Spector et al (1970), Hoak et al (1972), and Cohen et al (1970). Fatty acids are taken up by platelets first as fatty acids bound to the membrane. The initial binding of the fatty acids is not energy-dependent, but dependent on continued

incubation. Free fatty acids are then transferred by an energy-dependent mechanism to internal sites within the platelet, where they may be either oxidized or incorporated into complex lipids. Thrombin, at high concentrations has been shown to alter platelet synthesis of fatty acids from acetate and the incorporation of fatty acids into complex lipids (Deykin, 1973). Although the initial binding of fatty acids to the platelet membrane is not impeded, the incorporation of saturated acids (palmitic, stearic) into both phospholipids and glycerides is depressed. By contrast, incorporation of oleic into phospholipids (but not into glycerides) is enhanced by thrombin, possibly due to the acylation of newly exposed monacyl receptors.

Platelets cannot synthesize cholesterol since they lack the enzyme system responsible for the formation of mevalonic acid from acetate and for the incorporation of mevalonate into cholesterol (Derksen and Cohen, 1973). Therefore, the cholesterol content of the platelet reflects the initial composition of the megakaryocyte, although exchange of cholesterol with plasma lipoproteins may result in remolding of the endogeneous cholesterol content of platelets.

In their study, Deykin and Desser (1968) reported that over 40% of the acetate incorporated into complex lipids was present in long-chain saturated fatty acids in ceramides, the precursors of glycosphingolipids. Subsequently, Krivit and Hammerstrom (1972) examined the composition of free ceramides in platelets. They found that although the

platelet ceramides resembled plasma ceramides in their composition, C_{24} acids predominated in platelet ceramides. Analyses of the gangliosides by Snyder et al (1972) and by Marcus et al (1972) both showed that hematoside (glucose: galactose: sialic acid ratio 1:1:1) is the predominant platelet ganglioside, comprising over 92% of the total gangliosides. Although no specific function for the platelet gangliosides has yet been established, Marcus et al (1975) have speculated that they may be involved in the initial binding of serotonin by platelets.

1.6.2 Uptake, release and metabolism of arachidonic acid by platelets

Arachidonate is metabolized to a variety of active mediators (Fig.2) that function in inflammation, coagulation, wound healing, and immunological reactions. The capacity to produce these substances and the control of their production is determined by cellular mechanisms for uptake and release of arachidonate from phospholipids. The initial enzymes (cyclooxygenase and lipoxygenase) that convert arachidonate to mediators are constitutively active within cells; therefore, the production of products is controlled by the availability of arachidonate.

In unstimulated platelets, free arachidonate is present n only trace quantities compared to other long-chain fatty acids. Low levels of free arachidonate prevent its metabolism by the unstimulated cells. These levels are maintained

when arachidonate bound to albumin is added to platelets; arachidonate is esterified into phospholipids without the production of oxygenated metabolites. Majerus et al (1983) speculated that arachidonyl CoA synthetase provides the mechanism by which platelets are able to esterify arachidonate rapidly and specifically into phospholipids via the formation of arachidonyl CoA esters.

Attempts to define mechanisms for arachidonate release from stimulated cells have been difficult because only a small fraction of the arachidonate is released when cells are stimulated. In the case of platelets, approximately 10% of the total is released, and in most other cells the proportion is less (Majerus et al, 1983). The original proposed mechanism for arachidonate release was via the action of a phospholipase A_2. According to this hypothesis, when platelets are stimulated by thrombin, part of the stimulus-secretion coupling mechanism involves activation of a phospholipase A_2 which cleaves arachidonate from several different phospholipids. Bell et al (1979) also proposed an alternative pathway for arachidonate release from PI involving a phospholipase C and diacylglycerol lipase (Fig.7).

The released arachidonic acid is then converted by cyclooxygenase, into prostaglandin A_2 (PGG_2) (Fig.2), a cyclic endoperoxide that has a half-life of less than 5 min. PGG_2 is a powerful initiator of platelet aggregation, but it is further transformed to a more potent mediator, thromboxane A_2, an unstable oxane which persists less than

40 seconds in plasma before decomposing to a stable, inert compound, thromboxane B_2. PGG_2 may further be metabolised to the classical stable prostaglandins PGE_2 and $PGF_{2\alpha}$. However, it has been established (Hamberg et al, 1974b) that in response to thrombin at least 100 times more PGG_2 is formed than PGE_2 and $PGF_{2\alpha}$, and since PGG_2 and its derivative thromboxane A_2 are far more powerful than PGE_2 and $PGF_{2\alpha}$, thromboxane A_2 is considered to be far more important. In addition to transformation by cyclooxygenase, arachidonic acid is also metabolized by platelet lipoxygenase, into 12, L-hydroxy-5,8,10,14 eicosatetraenoic acid or HETE (Fig.2). This compound has no apparent effect on platelet function, but it has been shown to be a chemo-attractant for human polymorphonuclear lenkocytes *in vitro* (Turner et al, 1975). Hamberg and co-workers (1974b) have reported that aspirin and indomethacin inhibit platelet cyclooxygenase, but HETE formation is actually enhanced in aspirin-treated platelets.

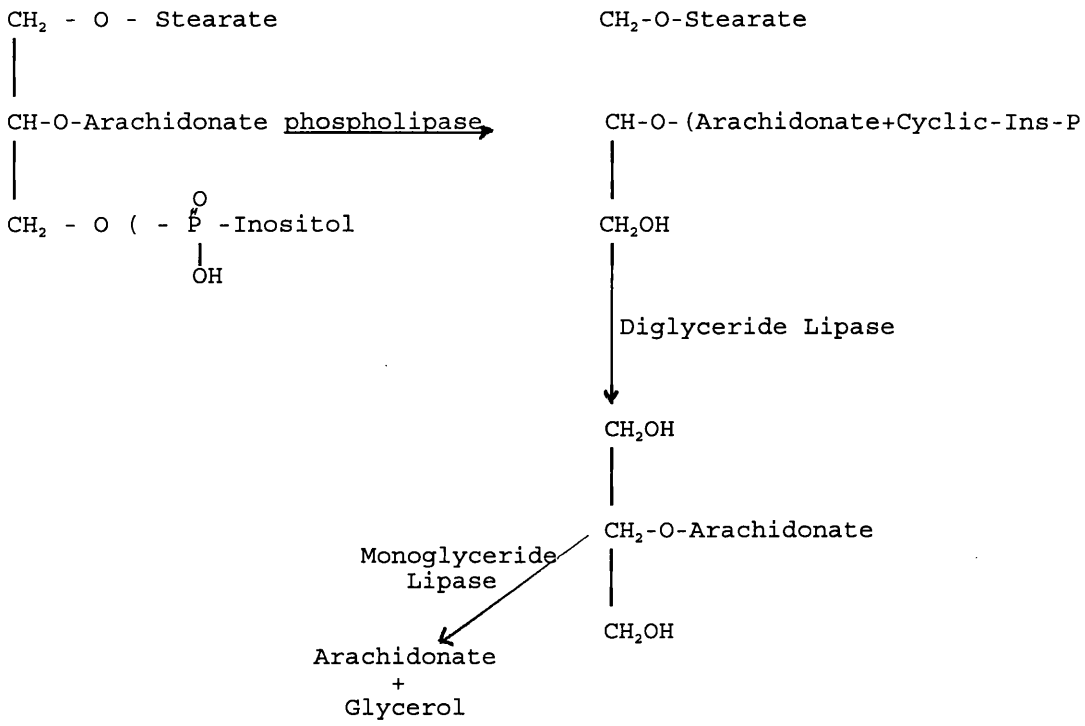


Fig.7 **Pathway for arachidonate release from phosphatidyeinositol (PI). A PI-specific phospholipase C-Catalyzed reaction yields a 1,2 diacylglycerol. The sn-1 position fatty acid (usually stearate) is subsequently released; and then arachidonate.**

1.6.3 De novo phospholipid synthesis and the effect of agonists on the pattern of synthesis

Platelets had been shown to be capable of incorporating both orthophosphate and glycerol into phospholipids (Lewis et al, 1969). In resting platelets, the predominant lipids to be labelled were phosphatidylcholine, phosphatidylinositol and phosphatidic acid, but phosphatidylserine and phosphatidylethanolamine were labelled as well. When platelets were treated with thrombin, total incorporation of glycerol into platelet phospholipids decreased, with a marked decrease in the labelling of phosphatidylcholine. In contrast, they observed that labelling of phosphatidylserine was sharply, but transiently, increased above basal levels. Deykin (1973), also observed that thrombin and poly-l-lysine both inhibited the incorporation of glycerol into phosphatidylserine, suggesting that the impaired phospholipid synthesis was not a specific thrombin-mediated event but rather reflected the consequences of aggregation itself. In a subsequent study Deykin and Synder (1973) examined the effect of aggregation by epinephrine on glycerol incorporation in platelets in platelet-rich plasma. In contrast to the observations made with thrombin-treated washed platelets, they observed that, epinephrine produced a marked, sustained increase in phosphatidylinositol synthesis. The effect was not immediate but occurred only after the release reaction had been completed. Aspirin inhibited but did not completely

suppress the stimulation of phosphatidylinositol synthesis; however, the stimulation of phosphatidylinositol did not occur in platelets from patients with storage pool disease, in which the release reaction was absent (Hutton and Deykin, 1973). Bevers et al (1982) in their study, reported that activation of platelets simultaneously by collagen and thrombin results in an increased susceptibility of phospholipids towards exogeneously added phospholipase A_2 in such a way that a substantial amount of the negatively-charged phosphatidyl-serine becomes exposed at the membrane outer surface. It has been suggested that this property of activated platelets is of significant importance for their role in the hemostatic process, since negatively-charged phospholipids markedly enhance the conversion of factor X to factor X_a by a complex of factor IX_a, factor $VIII_a$ and Ca^{2+}, as well as the conversion of prothrombin to thrombin by a complex of factor X_a, factor V_a and Ca^{2+} (Fig.5) (Papahadjopoulos et al, 1964, Nesheim et al, 1979). Zwaal et al (1989) have also shown that undoing normal assymmetry of membrane phospholipids in activated blood platelets is presumably mediated by increased transbilayer movement of phospholipids and seems to be strictly correlated with alterations in cytoskeletal organization.

1.7 Phospholipid methylation and cellular significance

1.7.1 SAM-dependent phospholipid methylation

A significant metabolic role of SAM involves methylation of phosphatidylethanolamine (PE) to phosphatidylcholine (PC) in hepatic microsomes. This important reaction, first identified by Bremer and Greenberg (1961), involves a 3-step sequential methylation of the primary $-NH_2$ group of ethanolamine to the quaterary ammonium moiety of choline (Fig.8a). The pathway has been identified in several cell types, including hepatocytes, neuronal and glial cells, erythrocytes, enterocytes (Brassitus et al, 1985; Hirata and Axelrod, 1978; Kelly et al, 1984; Lakher and Wurtman, 1978). The PC formed by this route is rich in polyunsaturated fatty acids (Akeson, 1983; Audubert et al, 1989; Lakher and Wurtman, 1987), presumably because the PE molecules rich in these types of fatty acids are preferred substrates for the enzyme(s) involved. The major route for phosphatidylcholine synthesis in most cells is via the CDP-choline pathway (Fig.8b). However, the methylation pathway is responsible for 15-20% of PC synthesis in the liver (Sundler et al, 1975). What function this PE-derived PC has in liver physiology is still not certain. PE methylation activity is very low in extrahepatic tissues, and it is generally assumed that this pathway does not contribute significantly to PC synthesis (Ridway and Vance, 1992).

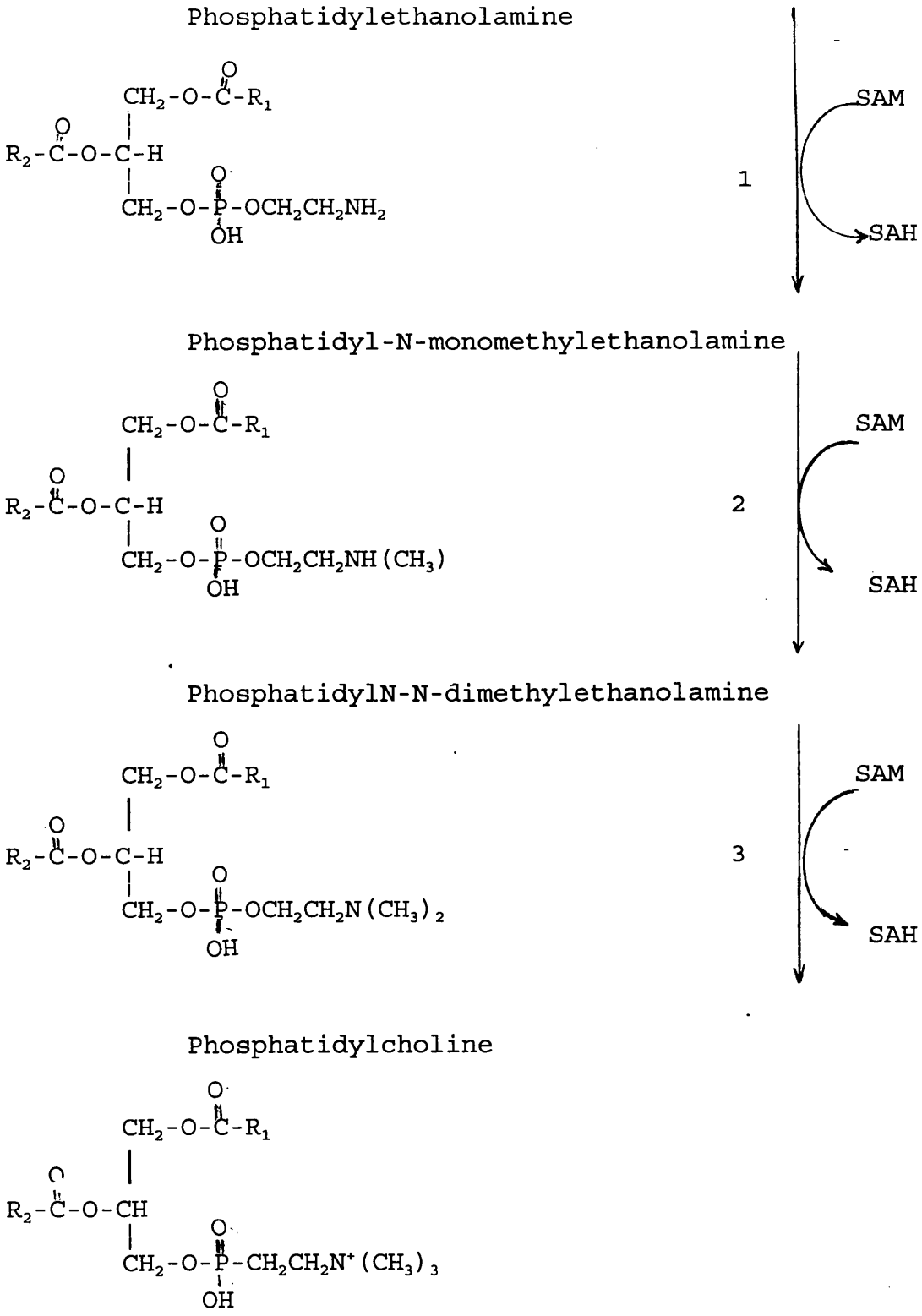


Fig.8a N-Methylation of Phosphatidylethanolamine to Phosphotidylcholine

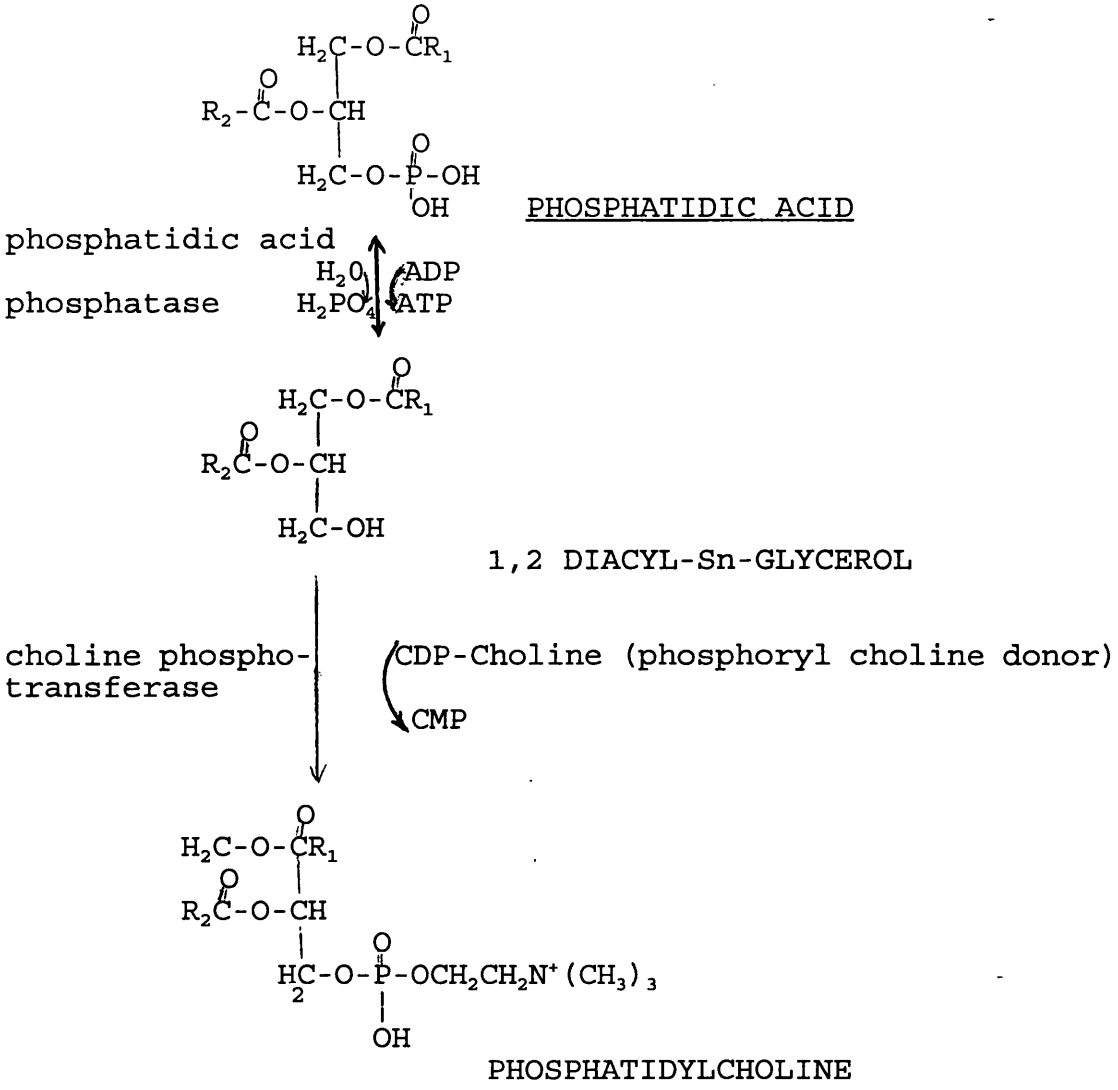


Fig.8b Biosynthesis of phosphatidycholine from 1,2 diacyl-sn-glycerol via the CDP-choline pathway.

1.7.2 Cellular significance of phospholipid methylation

The main physico-chemical effect quoted to be a result of phospholipid methylation is an increase in membrane fluidity. Hirata et al (1978) reported that enzymatic methylation of PE to PC results in an increased erythrocyte membrane fluidity. They suggested that alterations in membrane fluidity due to phospholipid methylation should produce pleiotropic changes in membrane function. In another study, Hirata et al (1980) concluded that the assymetric distribution of phospholipid methylating enzymes in membranes provides an important mechanism for the transmission of biochemical signals in cells. Perhaps the topology of these enzymes makes possible the translocation of phospholipids from the cytoplasmic side to the outer surface of membranes by successive methylation. These findings have been disputed by Vance and Kruijff (1980) who argue that the methylation of very small amounts of PE could not produce significant changes in membrane fluidity. Other workers found that neither phospholipid methylation nor addition of PME caused major changes in membrane fluidity (Chauhan et al, 1982).

Whereas no unequivocal evidence exists to support or completely dispute these views, it has been proposed that coupling of ß-adrenergic receptors to the guanylnucleotide coupling factor and adenyl cyclase is facilitated by phospholipid methylation. The increased fluidity in the membrane is proposed to permit lateral movement of the

receptor so that it can couple with the adenyl cyclase system (Hirata and Axelrod, 1980). In their study of the effect of catecholamine-stimulated ß-adrenergic receptors on phospholipid methylation, membrane fluidity and adenyl cyclase (Hirata et al, 1979b), it was shown that binding of agonists to the ß-adrenergic receptor caused an increase in phospholipid methylation. This increase was not secondary to the activation of adenyl cyclase : cyclic AMP, and propranolol (antagonist) blocked the effect of isoproterenol. Strittmatter et al (1979) found that incubation with SAM increased the number of ß-adrenergic receptors in reticulocytes, and mammary gland membranes showed an increased number of lactogenic receptors. It may be inferred from these observations that phospholipid methylation may to some extent regulate a number of ß-adrenergic and other receptors present in cells; however no established conclusion has been drawn.

Phospholipid methylation has also been shown to play a role in the chemotaxis of neutrophils (Pike et al, 1978), histamine secretion in mast cells, RBL cells and mitogenesis in lymphocytes (Hirata and Axelrod, 1980; Crew et al, 1980; Ishizaka, 1980). The release of arachidonic acid following receptor stimulation has also been shown to be linked to phospholipid methylation in leukemic basophils (Crew et al, 1980).

In RBL cells and mast cells, bridging of cell-surface immunoglobulin E (IgE) receptors produces a rapid transient

activation of phospholipid methylation, followed by histamine release.

McGivney et al (1981) examined RBL cell variants which were deficient in phospholipid methylation and IgE-mediated, but not ionophore-mediated, histamine release. Reconstitution of these variants resulted in normal levels of lipid methylation and histamine release which strongly indicates that phospholipid methylation may be essential for histamine release.

Phopholipase-induced arachidonic acid release was found to correlate with the release of histamine from cells. Phospholipid methylation seemed to play a part in effecting the Ca^{2+} influx required for phospholipase A_2 activity since, in these system, inhibition of methylation blocked the ability of the cell to respond to the external signal.

1.8 Phospholipid-N-methyltransferase(s) : subcellular loction and purification

Most of the phospholipid-N-methyltransferase activity has usually been associated with the rough and smooth endoplasmic recticulum (ER) in most tissues; however, about 20% of ER-associated methyltranferase activity has been reported in the Golgi apparatus (Higgins and Fieldsend, 1987).

In their study of the topology of the lipid methyltransferase(s), by the introduction of 3-[methyl-^{3}H] SAM into right-side-out of erythrocyte ghosts, Hirata and Axelrod (1980) observed the incorporation of methyl groups into phospholipids, whereas negligible incorporation was observed when the radioactive methyl donor was present on the outside of the membrane. Such studies, coupled with a selective proteolytic-digestion with trypsin, led them to conclude that methyltransferase(I) faces the cytoplasmic side of the membrane, together with its substrate, while methyltransferase(II) (which, they asserted, successively methylates phosphatidyl-N-monomethylethanolamine to phosphatidylcholine) faces the outside of the membrane. By using phospholipase C and inside-out and right-side-out erythrocyte ghosts to which [methyl-^{3}H] SAM was added, they found that methylated phospholipids are translocated from the cytoplasmic side of the membrane to the external side. This ordered arrangement of enzymes and substrates was proposed to facilitate a flip-flop of methylated

phospholipds across the membrane, resulting in changes in membrance fluidity (Hirata and Axelrod, 1980). No unequivocal evidence has, however, been established to support these observations. The two methyltransferases, according to Hirata and Axelrod (1980), are localized in the microsomes and mitochondria of the adrenal medulla. Although various purification attempts have been pursued by various investigators, controversy still exists on the exact molecular weight(s) and forms of phospholipid-N-methyltransferase(s). This is partly due to the fact that the enzyme, being an integral protein, catalyses a multi-step reaction. Besides, various investigators have employed different assay conditions for the enzyme based on pH, SAM, divalent ion concentration, presence of detergents, etc, often resulting in almost contradictory and conflicting results. For example, previous assays of phospholipid-N-methyltransferase activity in hepatic microsomes have utilized endogeneous PE or vesicles of PE, PME or PDE as substrates. Activities assayed with endogeneous PE are often inaccurate owing to the rate-limiting nature of the first methylation step and uncertainty in the amount of PE available to the enzyme (Ridgeway and Vance, 1992). Another problem suggested by Paik et al (1980) was that many laboratories appear to use labelled SAM which may be impure. The summary of various purification attempts is shown in Table 5.

Table 5 Purification review of phospholipid-N-methyl-transferase(s)

REFERENCE	SOURCE OF PURIFICATION	STRUCTURAL INFORMATION
Scarborough & Nyc & Nyc (1967)	Genetic variants of the yeast Neurospora crassa	Two separate proteins responsible for the 3-step methylating process
Crews et al (1980)	Rat brain synaptosomes	Two enzymes which catalyze the whole reaction
Makishima et al) (1985)	Murine thymocyte microsomes	Solubilised proteins with two methylating activities
Pajares et al (1986)	Rat liver microsomes	50 Kda protein which existed as a dimer of two 25 KDa subunits with 3 methylating activities
Kodaki et al (1987) (1989) (1991)	Saccharomyces cerevisiae	Two proteins encoded by two structural genes of molecular weights of 101 KDa and 23 KDa, respectively
Ridgeway and Vance (1987)	Rat liver microsomes	A single polypeptide protein with Mr 18.3 KDa with all 3 methylating activities
Tahara et al (1987)	Zymomonas mobilis	A single protein of Mr 42 KDa (SDS) but 120 KDa on sephadex G-200 with a pI of 8.5
Chung et al (1990)	Rat brain synaptosomes	2 enzymes catalyse the 3-step methylating process
Arondel et al (1993)	Rhodobacter sphaeroides	A 22.9 KDa protein encoded by a single gene, pmt A

1.8.1 Properties and characterization review of phospholipid-N-methytransferase(s)

Despite the apparent importance of the methylation pathway for PC biosynthesis in liver and its implication in various cellular mediated events, detailed knowledge on the structure, properties and regulation of the enzyme(s) has still not yet been fully established.

Based on the "single enzyme hypothesis", Schneider and Vance (1979) reported that the partially purified enzyme from liver has a pH optimum of at least 9.5, catalyses all the three methylation steps and has no Mg^{2+} requirement. The first methylation (PE to PMG) was the slow rate-limiting step with subsequent reactions occurring at a much faster rate. They reported that the best condition for solubilization of the enzyme was by ultrasonic disruption in the presence of 0.2% Triton X-100 and the activity of the enzyme was irreversibly inactivated by concentrations of Triton X-100 greater than 0.6%. Ridgeway et al (1989) showed that the enzyme is not regulated except by substrate availability, in choline-deficient hepatocytes. Audubert and Vance (1983), through an elaborate and detailed study of the kinetic parameters of phospholipid methylation and taking into consideration the level of radioactivity present in the steady state intermediates, produced new data for rat liver microsomal phospholipid methyltransferase.

They reported a Km value of 58μm (PE-->PME) and 65μm (PME-->PDE) and 96μm (PDE-->PC) with a pH optimum of 10.25 for each of the reactions. Their results showed that the apparent Km as well as the pH optimum for all the reactions were similar. This evidence was interpreted in terms of a single enzyme catalysing all three methylations. They argued that previously reported data in the literature could not be accurate enough since the obtained data did not take into consideration the steady state intermediate conversions of the substrates at the right pH conditions and optimal concentrations of SAM.

Based on the "two enzyme hypothesis" Hirata and Axelrod (1980) reported that the two phospholipid methyltransferases in adrenal medulla have different properties. The first enzyme which converts PE to PME, requires Mg^{2+} and has an optimal pH about 7.0 and a low Km (about 2μM) for SAM, whilst the second enzyme which catalyses the stepwise methylation of PME to PC does not require Mg^{2+}, has a high Km (about 100μM) for SAM and an optimal pH of 10.0. Hotchkiss et al (1981) reported that pH optima at high and low concentrations of SAM for incorporation of the methyl group into phospholipids were different. At 2μM SAM there was a broad pH optimum from 9-11 for the formation of the monomethyl product, whilst at 200μM SAM, there was a broad pH optimum also from 9-11 for the formation of PDE and PC with the PC predominating. They also observed that in both lysed and intact platelets, S-adenosylhomocysteine (SAH) and 3-deazaadenosine (3-DZA)

blocked phospholipid methylation, indicating that these two agents are inhibitors of SAM-dependent methylation.
Mori et al (1982) reported that in platelets, the pH optimum for the enzyme was around 10.5 under Tris-HCl and glycine-NaOH buffer systems, whilst in a phosphate buffer system the enzyme exhibited a pH optimum of 8.0 with a three-fold increase in methylation. They suggested that since the increase (under phosphate buffer) in the amount of products by the addition of exogeneous PME or PDE did not differ from that under Tris-HCl buffer, phosphate ion may stimulate only the first step from PE to PME and not affect the next steps from PME to PDE and from PDE to PC. They also found out that methylation in platelets was inhibited to 30% basal value with Ca^{2+} (0.2mM). However, they observed that Ca^{2+} tended to show different effects on methylation in various tissues (activation to 150% of the basal value in the adrenal gland, and inhibition of up to 88% in the basal value in the liver). The mechanism involved in these observations is, however, still unknown.

1.8.2 Regulation of phospholipid-N-methylation by drugs and hormones

Mato and Alemany (1983) reported that the addition of isoprenaline to hepatocytes from mature adrenalectomised rats stimulated N-methyltransferase activity in a dose and time-dependent manner; this stimulation was blocked by the non selective ß-blocker propranolol, and by insulin.

N-methyltransferase activity in hepatocytes incubated with cyclic adenosine monophosphate (c-AMP), or with chlorophenylthioadenosine 3',5'-monophosphate, was also elevated in a dose-dependent manner. These data suggested a role for intracellular c-AMP in the activation of phospholipid N-methyltransferase(s). Surprisingly, the addition of chlorophenylthioadenosine 3',5'-monophosphate inhibited the incorporation of the labelled precursors [^{3}H-methyl] methionine or [^{3}H-methyl] ethanolamine into phospholipids, in spite of the increased methyltransferase activity (Mato and Alemany, 1983). No satisfactory explanation for the discrepancy between increased enzymatic methylation and subnormal incorporation of labelled precursor into phospholipid has been offered.

Hirata et al (1979) showed that isoprenaline enhanced phospholipid methylation in reticulocyte ghosts, and attributed this to an interaction between isoprenaline and adrenoceptors. Guanosine 5'-triphosphate (GTP) was found to enhance the effect of isoprenaline. Hashizume et al (1983) showed that GTP acted by inhibiting methyltransferase I and stimulating the activity of methyltransferase II, although the mechanism for this selective effect was not established. However, this stimulation does not involve c-AMP, but does involve the binding of isoprenaline to its specific receptors. A similar observation was reported in rat mast cells. Other pharmacological agents, such as benzodiazepines (Strittmatter et al, 1979) and concanavalin A (Hirata et al, 1980), also stimulate

phospholipid methylation, presumably by an increased expression of methyltranferase.

A number of hormones also appear to regulate phospholipid methylation. Zawad and Brown (1984) reported that catecholamines enhanced the methylation of phospholipids in the brain, although they did not explain the biochemical basis for this effect. Castano et al (1980) showed that glucagon produced a dose and time-dependent increase in methyltransferase activity in isolated rat hepatocytes, which was attributed to the increased Vmax of the enzyme, although its Km for SAM remained unchanged. This effect was mimicked by exogeneous c-AMP. In another study, however, glucagon did not alter the phospholipid methylation of hepatocytes in terms of incorporation of exogeneous methyl [^{3}H] methionine or [^{3}H] ethanolamine. The reason for this discrepancy has not been explained (Mato and Alemany, 1983; Schanche et al, 1982). Prasad et al (1981) have reported increased incorporation of [^{3}H] methyl of SAM into phospholipids of rat pituitary plasma membrane treated with vasopressin. This does-dependent effect was shown by both lysine-vasopressin and arginine vasopressin, but not by oxytocin and arginine-vasotocin. These workers postulated that SAM may have a role in the secretion of the pituitary growth hormone. Estradiol also increased incorporation of the [^{3}H] methyl group of SAM into pituitary phospholipids (Drouva et al, 1986). These investigators also reported a concurrent increase in the Vmax of the pituitary methyltransferases without any change

in their Km for SAM. Kelly et al (1984) have also shown that physio-logical concentrations of insulin enhanced the incorporation of the [^{3}H] methyl group of SAM into the phospholipid moiety of isolated adipocytes. They proposed a relationship between the increased methylation and certain post-binding cellular events, eg. covalent modification of insulin receptors and hyperpolarisation of membranes, although no experimental evidence to support this correlation was offered.

1.8.3 SAM-dependent methylation and other biogenic pathways

S-adenosyl-L-methionine (SAM) is a metabolite in the trans-sulphuration pathway for the metabolism of methionine. This pathway (Fig.9), which occurs chiefly in the liver, utilises over 70% of dietary methionine and is responsible for the synthesis of polyamines (putrescine, spermidine, spermine), cysteine, glutathione and taurine, as well as a large number of methylated molecules (Cantoni, 1975; Stipanuk, 1986). The enzymatic machinery required for the synthesis of SAM is ubiquitous, and occurs in almost all cells. The enzyme SAM synthetase catalyses the transfer of the adenosyl moiety of adenosine triphosphate (ATP) to the sulphur atom of methionine. The resulting sulphonium compound, SAM, acts at the methyl donor, donating its methyl group to a broad range of acceptor molecules. Among

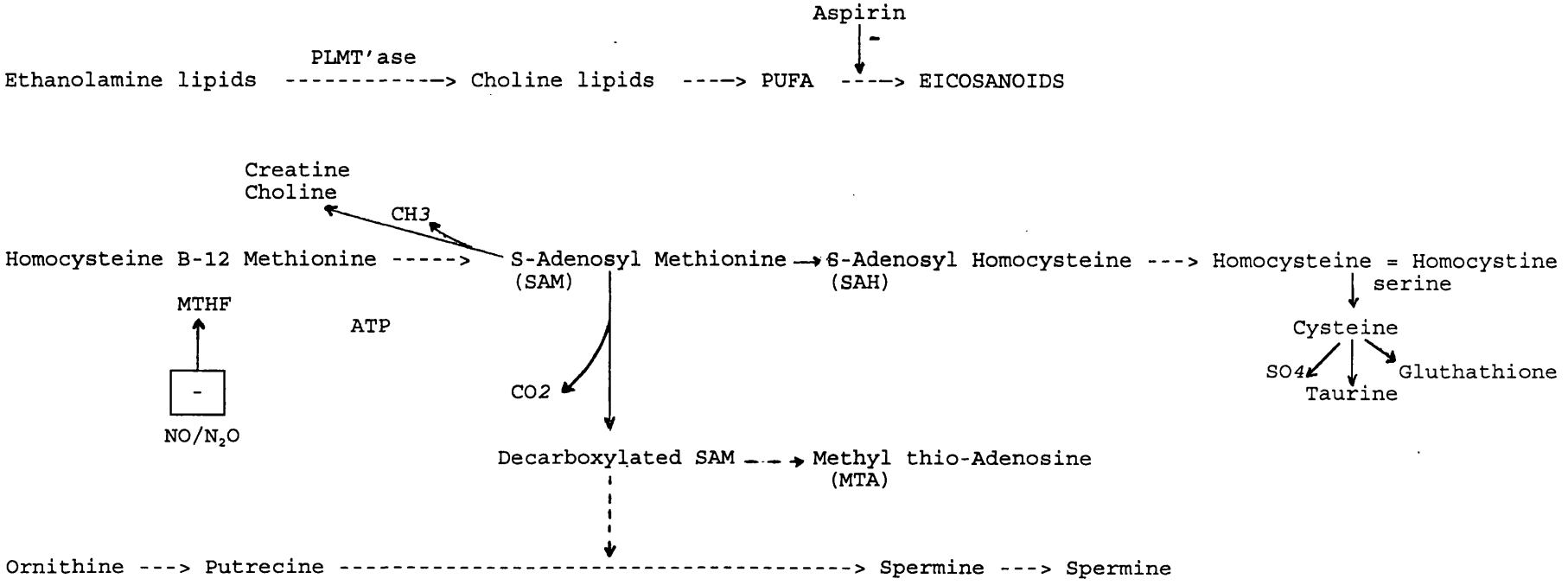


Fig.9 SAM-dependent pathways for phospholipid methylation and other biogenic pathways

the methyl group acceptors are proteins, phospholipids, DNA, RNA, histones, a number of hormones and creatine. While most biological methylating reactions involve SAM as a methyl donor, an important exception is the methylation of homocysteine to methionine, in which the methyl donor is 5'-methyltetrahydrofolate (MTHF). In the process of methylation, SAM is converted to S-adenosylhomocysteine, a competitive inhibitor for all methylation reactions in which SAM is the methyl donor (Cantoni,1975; Eloranta and Kajander, 1984). The decarboxylated analogue of SAM donates its propylamino group to putrescine to form polyamines (Stipanuk, 1986). The SAM content of liver influences whether homoysteine will be recycled to form methionine, or undergo trans-sulphuration to synthesise cystathionine and cysteine (Finkelstein et al, 1982, 1988).

1.8.4 The role of PS decarboxylation in SAM-dependent methylation

The decarboxylation of phosphatidylserine (PS) to phosphatidylethanolamine (PE) (Fig.10b), is catalysed by the enzyme, phosphatidylserine decarboxylase (EC 4.1.1.65), a membrane-bound enzyme which is predominantly located in the inner mitochondria membrane (Zborowski et al, 1983; Percy et al, 1983.) It has been proposed that PE formed through decarboxylation is preferentially transported from the mitochondria to the ER and plasma membrane (Vance, 1991). Although the predominant amount of PE is synthesised *de novo* in many cells via the CDP-ethanolamine

pathway (Kennedy and Weiss, 1956), or through the base-exchange pathway (Fig.10a), in cultured BHK-21 cells PE is most exclusively derived from PS decarboxylation (Voelker et al, 1984). It has also been reported that in cultured neuronal cells, over 30% of the total serine labelled phospholipids were decarboxylated to PE which was a significant substrate for SAM-dependent methylation (Yavin, 1985). The latter observation has been challenged by White et al (1986), who rather confirmed that decarboxylation contributed significantly to the synthesis of ethanolamine glycerophospholipids in the brain. Carlini et al (1993) have suggested that decarboxylated-derived PE may follow different utilisation pathways physiologically. In rat hepatocytes newly made PE is preferentially methylated to PC (Samborski et al, 1958). Vance (1989) proposed that PC and PE derived from PS decarboxylation are rapidly added to very low density lipoproteins (VLDL), whilst PE and PC made via the CDP-ethanolamine pathway are preferentially excluded from VLDL in cultured hepatocytes (Vance and Vance, 1986). This further suggests a specific importance of the PS-decarboxylation pathway.

In other studies various investigators have provided evidence that PS decarboxylase and phospholipid-N-methyltransferase can be co-regulated. Concanavalin A was

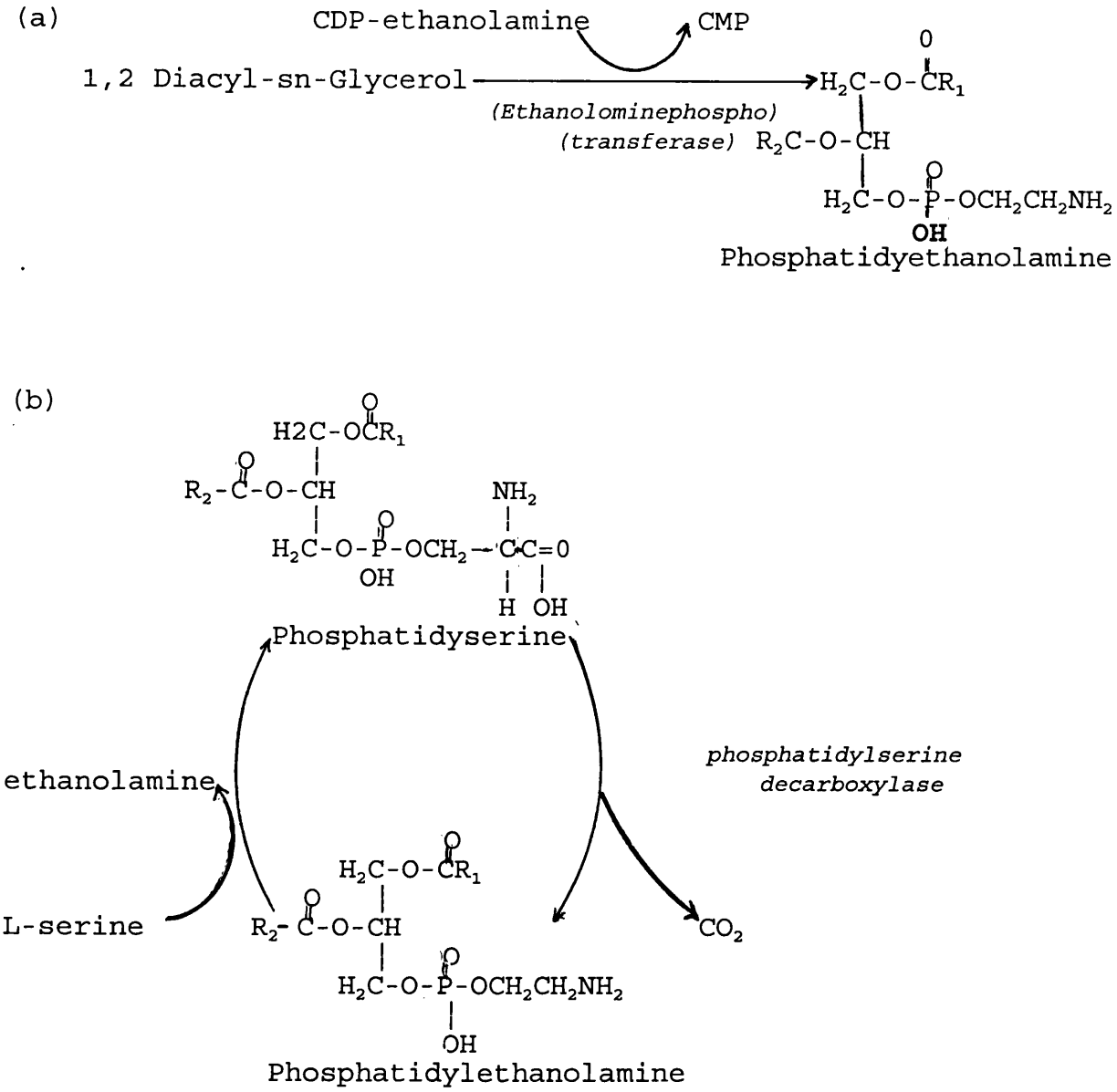


Fig. 10 Biosynthesis of phosphatidyethanolamine via (a) the CDP-ethanolamine pathway and (b) the decarboxylation pathway

In (a) ethanolamine phosphotransferase catalyses the transfer of phosphorylethanolamine from CDP-ethanolamine to 1,2 diacyl-sn-glycerol to form PE. In (b) free serine is exchanged with PE to form PS which can be decarboxylated to give PE. (NB: Free ethanolamine can exchange with CPP-choline to PE via DAG pathway)

observed to stimulate both enzymes in mast cells (Hirata et al, 1979), whilst in yeast the activities of both enzymes were shown to be co-downregulated by inositol and choline (Carson et al, 1984; Lamping et al, 1991, and Overmeyer and Waechter, 1991). It is thus likely that both enzymes could be co-located in cell.

Recent work by Cui et al (1993) indicated that PLMTase resides on a special subcellular fraction associated with mitochondria where the majority of PS decarboxylase activity is also found. They suggested that such co-localization can act as a "bridge" for the synthesis of PC from PS-derived PE. However, no evidence in support of this correlation was offered.

1.9 Research Aims and Objectives

The blood platelet provides an attractive model for this project, first because of its function in blood coagulation and hemostasis and secondly, because of its implicated role in the initiation of thrombosis (Davies et al, 1981).

The first objective was to employ, for the first time, nuclear magnetic resonance (nmr) as a non-invasive, diagnostic technique for the profiling of the lipid content of platelets in both normal and diseased subjects. This could serve as the basis for studying lipid metabolism in platelets and also for monitoring abnormalities in patients with lipid disorders associated with platelet thrombo-

plastic activity or patients with diseases in which platelets have been implicated (Drummond et al, 1987).

It is also well documented that phospholipids play an important role in blood coagulation and membrane function (Chargaff et al, 1936; Papahadjopoulos et al, 1962). The transmethylation pathway is involved in lipid biosynthesis, particularly the conversion of ethanolamine phospholipids to choline phospholipids; however, neither has it been related to the formation of eicosanoids or to blood clotting, nor has the enzyme(s) involved been purified from platelets.

Thus it was also the aim of this project to investigate the metabolism of choline phospholipids via this pathway in platelets, to establish if there exists a cause and effect relationship between phosphatidycholine metabolism in the plasma membrane of platelets and the physiological processes involved in blood clotting. A further aim was to purify and characterise the enzyme(s) involved in the transmethylation.

Finally, since platelets have similar neuroaminergic properties as neurons, we analysed the excitatory sulphur-containing amino acids and polyamine content of platelets and proposed their probable roles in platelet function.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

Whole pack ACD (acid citrate dextrose) anticoagulated blood was obtained from the North London Blood Transfusion Centre; blood from patients with heart disease was obtained from the cardiology department, King's College Hospital, London (UK).

Standard phospholipids were purchased from the Sigma Chemical Company, UK. The following cpmpounds were also purchased.

Deuterated chloroform (99.8%) and perdeuteromethanol (99.8%) from Aldrich. The Spherisorb Silica SW10 (4x250mm) column from Phase Sep. Deeside, UK.

Adenosyl-L-methionine, S-[methyl-^{3}H] (75.9 Ci/mmol) from Dupont 1 and 2-dioleoyl-L-3-phosphatidyl-L-[3-^{14}C] serine (53m Ci/mmol) from Amersham Int. Acrylamide, bis-acrylamide (N, N^{1}-methylene-bis-acrylamide), β-mercaptoethanol, Chaps (3-[(3-Cholamidopropyl)-dimethylammonio]-1-propane sulphon-ate), Comasssie Brilliant Blue R, Glycerol, phosphatidyl-ethanolamine, phosphatidyl-N-monomethylethanolamine, lyso-phosphatidylcholine, phosphatidylcholine, S-adenosyl methionine, sucrose, Trizma base (Tri [hydroxymethyl] aminomethane), Bovine Thrombin, Epinephrine, Concanavalin ensiformis type V, Adenosine 2',5'-Diphosphate were from Sigma.

Sodium dodecyl sulphate, Bio-lyte (Ampholyte) 3/10, IEF Rotofor cell, SDS-preparative cell were from Bio-Rad, and Triton X-100 from BDH.

Allugram SIL G/UV high performance TLC plates were from Camlab. Electrophoretic molecular weight standards and prestained markers were from Bio-Rad. Gelcode silver staining kit was supplied by Pierce, UK. BSA and Folin Ciocalteau Reagent for Lowry protein determination were purchased from Sigma.

Optiphase 'safe' Scintillation fluid was from LKB. DEAE-cellulose, Mono Q Sepharose, fplc columns and Triazine activate agarose were obtained from Pharmacia.

Sulphur amino-acid standards and Norvaline, polyamine standards and 1, 6-diamino-hexane were from Sigma.

All other reagents were of high analytical grade.

2.2 Methods

2.2.1 Platelet preparation from whole blood

Citrated blood obtained from healthy volunteers was dispensed into 50ml centrifuge plastic tubes and centrifuged at 1000 rpm for 15 minutes to obtain a platelet-rich plasma (PRP). Platelets were sedimented by ultracentrifugation at 20,000 rpm at 4°C for half an hour. The platelet pellet was washed twice with Tris-buffered saline (140mM NaCl, 5mM glucose, ImM EDTA and 15mM Tris-HCl, pH 7.4).

2.2.2 Plasma membrane preparation from intact platelets

Washed platelets were suspended in 0.25M sucrose and disrupted by sonication at 4°C for 2 minutes. The platelet suspension was centrifuged at 18,500 rpm for 10 minutes to sediment the cell debris. The resultant supernatant was subjected to centrifugation at 32,000 rpm for one and a half rpm hours to pellet the membrane which was then twice washed with Tris-buffered saline and solubilized either in Tris buffer containing 0.5% triton X100 or in 0.3% chaps in phosphate buffer.

2.2.3 Lipid extraction from platelets

Lipids were extracted according to the method described by Bligh and Dyer (1959), using 10ml of chloroform-methanol (2:1 v/v). Prepared platelets were sonicated in ice for 2 minutes, vortexed and centrifuged. The upper aqueous layer was discarded and the lower organic layer retained. The extracted lipid was washed twice with 0.5M KCl in 50% methanol, filtered through methanol-washed cotton wool and dehydrated using a molecular sieve (crystalline sodium alumino-silicate). The lipid was dried under a stream of nitrogen and redissolved in 0.6ml $CH_3OD:CDCl_3$ (2:1) and transferred to 5-mm nmr tubes. Samples were bubbled with nitrogen, prior to recording their spectra in order to remove oxygen.

2.2.3.a Bond Elut separation of lipids

Bond Elut separations were done in duplicate. All column elutions were performed under low-speed centrifugation (500g) for 5 minutes, and columns were washed with 5ml of hlpc-grade hexane prior to application of the lipid extracts. The extracted lipid residue was redissolved in 1.0ml of chloroform; 0.2ml of dissolved lipids was then applied to each Bond Elut column and another 0.2ml was retained as a control.

According to their different polarities, lipids were separated into four classes as described by Kates et al (1988), by passing different eluants through the columns in the following order: (1) chloroform (eluted non-polar lipids and cholesterol), (2) diethyl ether with 2% acetic acid (eluted non-esterified fatty acids), (3) methanol (eluted non-acidic phospholipids) and (4) 0.05M ammonium acetate in chloroform/methanol (4:1, v/v) plus 2% (v/v) aqueous ammonium solution (eluted acidic phospholipids). A volume of 2 x 4.0ml of eluants was used for all elutions. All eluates and the 0.2ml of unseparated lipid extract were evaporated under a stream of nitrogen, resuspended in 0.6ml of CD_3OD:$CDCl_3$ (2:1, v/v) and transferred to nmr tubes as previously described.

2.2.3.b Tlc separation of lipids

Platelet lipid extracts were separated by tlc on silica gel allugram SIL G/UV high performance plates developed in chloroform/ethanol/triethylamine/water (4:5:4:1, v/v). Chloroform extracts were dried under N_2 and taken up in 40μl of chloroform. After addition of lipid carriers the sample was applied to the plate, developed in solvent and the lipids visualised by Rhodamine G.

2.2.3.c Hplc analysis of extracted lipids

The HPLC procedure was according to the method described by James et al (1989). This was carried out on a Gilson 714 instrument with an IBM compatible computer and monitored by UV detection of 205mm. A gradient profile of 5mM phosphate: acetonitrile buffer (1:4) pH 5.4 into 2.5% acetonitrile was maintained for the elution of lipids. 0.2ml of dissolved lipids (in chloroform/methanol 1:1, v/v) was injected twice onto the column and run for 40 minutes. Peaks were identified by their specific retention times.

2.2.3.d Proton nmr analysis of lipids

Spectra were recorded using a Bruker AM 500 nmr spectrometer. The ID proton spectra were recorded at a temperature of 298K in the Fourier transform (FT) mode with 16*K* data points, using a 45° detection pulse and 2.0s

acquisition time as described by Casu et al (1991), with solvent presaturation during relaxation to remove excess HOD signal. The 2-D COSY experiment was performed on extracted lipids in a non-phase sensitive mode, with solvent presaturation. The 2D spectrum consisted of $2K$ data points obtained from 512 FIDs of 48 scans, with zero-filling in the F_1 dimension. The data were multiplied with a square sine-bell function in both dimensions prior to transformation. Chemical shifts were referenced in both experiments to the residual methanol resonance at 3.31 ppm.

2.2.4 Platelet activation by various agonists

0.5ml of platelet suspension (protein conc 2.5mg/ml) was incubated at 37°C for 2 minutes. The platelet suspension was stirred, aggregating agent added to the desired concentration, and the mixture incubated further for two minutes. Controls were carried out similarly, but without aggregating agent. The incubation was terminated by the addition of 1.0ml of chloroform:methanol (2:1 v/v). The mixture was vortexed for approximately 1 minute and centrifuged at 1000 rpm for 5 minutes. Lipids were subsequently extracted according to the method of Bligh and Dyer (1959) as already described elsewhere, and later analysed by nmr. Aggregation was monitored by measuring the changes in light transmission at 280 nm.

The following aggregating agents were used:-

(1) Thrombin in the presence of 2.5mM CaCl (conc range 1-5 units/ml)
(2) ADP in the presence of 2.5mM CaCl (conc range 5-25 mM)

2.2.5 Protein determination by the Lowry's method

Aliquots (10, 20...100μl etc) of standard 1% BSA protein solution were transferred to individual wells in duplicate on a 96 well micro assay Elisa plate and made up to 200μl with 0.1M NaOH. Similar dilutions were made for the test samples (protein), also in duplicate. 20μl of reagent A comprising 2% Na_2CO_3 in 0.1M NaOH, 2% NaK tartarate, 1% $CuSO_4$ in a ratio of 3:1:1 (v/v) was added to each well and left to stand at room temperature for 5 minutes. 50μl of reagent B comprising 1ml Folin Ciocalteau reagent and 4ml 0.1M NaOH was then added and incubated at room temperature for 15 minutes. Absorbances were taken at 620nm at the end of the incubation period.

2.2.6 Preparation of exogeneous PME and PE

5.0mg of PME and PE were dissolved in 1ml chloroform:methanol (2:1 v/v) separately. The mixtures were dried under N_2 and resuspended in 5ml of 20mM Tris-HCl buffer (pH 8.0) by sonication for 30 seconds to give a final concentration of 1mg/ml of each exogeneous lipid.

2.2.7.a Enzyme assay for phospholipid-N-methyltransferase

The assay of phospholipid methylation was performed according to a modified assay as described by Gibson et al (1961). The standard assay mixture (0.48ml) contained 50mM Tris-HCl buffer, containing 0.5% triton-X100 or 20mM phosphate triton-x100, containing 0.3% chaps, 200uM S-adenosyl-L-[methyl-^{3}H] methionine (2μCi), and 20μg each of exogeneous PME and PE and 400μl of membrane extract. The mixtures (in duplicate) were incubated at 37°C for 60 minutes (except for time-dependent studies). The reaction was terminated at the end of the incubation period by the addition of 2ml $CHCl_3$:CH_3OH:2HCl (6:3:1) and lipids subsequently extracted as previously described. The final extracted lipids were dried and mixed with 4ml of scintillation cocktail, and total ^{3}H-methyl groups incorporated counted on a Beckman LS 5000 CE radioactive counter.

2.2.7.b Enzyme assay for phosphatidylserine decarboxylase

The assay procedure was similar to that described for phospholipid-N-methyltransferase, except 0.05μCi of 1, 2-dioleoyl-L-3-phosphatidyl-L-[3-^{14}C] serine was used instead. The conversion of [^{14}-C]- labelled phosphatidylserine into radio labelled phosphatidylethanolamine (PE) was measured by radiometric scanning of the TLC plate and also by counting the ^{14}C disintegrations per minute (DPM) in 4ml scintillation fluid.

2.2.7.c Product identification by radiometric scanning

Individual phospholipid products were identified by applying an aliquot of the organic phase extract on silica gel plate and developing it in a solvent system made up of chloroform : ethanol : triethylamine : water (4:5:4:1 v/v) in the presence of phospholipid standards. Phospholipids were visualized with 0.12% Rhodamine 6G solution. The distribution of radioactivity in the products was determined by radiometric scanning of the plate using a Berthold Tracemaster model 20.

2.2.7.d Product identification by Hplc

The product(s) area was marked and scraped and finally extracted with chloroform:methanol (2:1 v/v). 0.2ml of the extracted product(s) was injected twice onto the Hplc column. Fractions were eluted using a gradient profile of phosphate buffer into acetonitrile. Eluted fractions were dried under a stream of nitrogen gas and counted in 4ml of scintillating cocktail.

2.2.8 Purification of platelet plasma membrane phospholipid- N-methyltransferase

2.2.8.a Preparative column SDS-PAGE of platelet plasma membranes

This was carried out using a 491 Prep Cell (Bio-Rad). 5ml of solubilised platelet membrane extract was first filtered

with an 0.2μm acro disc filter and loaded on to a 7.5% SDS-gel column in the presence of three prestained protein markers of molecular weights, 18, 21 nd 66 KDa. SDS-PAGE was carried out according to the method adopted by Laemmli (Laemmli, 1970) for 6 hours at 4°C at a flow rate of 2ml/min. Eluted fractions were collected, concentrated and washed free of SDS, employing an ultra concentrator UM-10 membrane (cut-off 10KDa). Concentrator fractions were assayed for the enzyme.

2.2.8.b Preparative iso-electric focusing of platelet plasma membranes

Solubilised platelet membrane was diluted up to 50ml in the presence of 2% ampholyte (Bio-Lyte, pH 3/10) and focused in a Rotofor IEF preparative cell (Bio-Rad) for 4-5 hours at 4°C. Fractions were harvested (at the end of a stabilised voltage) and pH adjusted between 7-8. Harvested fractions were assayed for methyltransferase activity; active fractions were pooled and re-fractionated.

2.2.8.c Q-sepharose ion-exchange chromatography

The solubilised platelet plasma membrane was loaded on to a Q-sepharose packed column (1.6 x 20cm), equilibrated with 20mM phosphate buffer containing 0.3% chaps, pH 8 (Buffer A). Unbound fractions were eluted with this buffer, whilst bound fractions were eluted with a linear salt gradient (0-1M NaCl) in the same buffer (Buffer B) at the flow rate of

2ml/min. Eluted fractions were monitored by absorbance at 280nm, concentrated and assayed for the enzyme activity.

2.2.8.d DEAE-Cellulose (ion-exchange) chromatography

About 5ml of platelet membrane extract, solubilised in 20mM phosphate buffer containing 0.3% chaps, was loaded onto a DEAE-Cellulose (DE52) column previously equilibrated with 50ml of the buffer. Bound samples were eluted with up to 80% NaCl in the same buffer, ultraconcentrated on a YM-10 membrane (cut-off to 10 KDa) and the enzyme activity assayed.

2.2.8.e SAH-agarose affinity chromatography

0.2g of Triazine-activated agarose 4XL was soaked in water for 30 minutes. This was then filtered and suspended in 5ml of 100mM $NaHCO_3$ buffer pH 9.0. To this suspension was added 10mg of S-adenosyl-homocysteine (SAH), followed by a gentle spinning for 15 hours at room temperature. Excess SAH was removed by washing with 20ml of 0.5M KCl. Unreacted groups on the agarose were blocked by a 2 hour incubation with 2M ethanolamine. The gel, approximately $1cm^3$ volume, was finally washed with 20ml of 0.5M KCl and equilibrated with 5mM K_2HPO_4, pH 6.0, containing 5mM EDTA and 10mM 2-mecapto-ethanol in a 2ml-volume column. The sample (2ml) was applied to the column, gently mixed for 10 minutes and the column washed with equilibration buffer to elute non-

specifically bound proteins. Bound fractions were eluted with 10mM K_2HPO_4 (pH 8.0) containing 1m NaCl in the presence of 0.3% chaps.

2.2.9 Analytical SDS-PAGE of purified active fractions

This was carried out according to the method adopted by Laemmli (Laemmli, 1970), employing a Mini-Protean II Cell (Bio-Rad). Purified fractions were diluted at least 1:4 with SDS-reducing buffer comprising 0.5M Tris-HCl (pH 6.8), 0.1% glycerol, 10% (w/v) SDS, 0.04% β-mecaptoethanol and 0.05% (w/v) bromophenol blue. Mixed fractions were heated to about 95°C for 5 minutes and electrophoresed on a 12% T gel (0.75mm) for 45 minutes at a constant voltage setting of 200V. The gels were stained with Coomassie Blue G and then silver stained with the GEL CODE silver staining kit.

2.3.a Extraction of polyamines and sulphur-amino acids from platelets in activated and NO studies

To 5ml of washed platelet suspension in Tris buffer was added 100μl of 0.1 mg/m 1, 6-diamino-hexane (polyamine internal standard) and 10μl of Norvaline (2mg/ml sulphur-aminoacid internal standard). Samples were incubated for 5 minutes at 37°C and subsequently activated with thrombin to a concentration of 10 units/ml (for polyamines only). Samples to be treated with NO, were bubbled through with nitrogen gas and mixed with 1ml of saturated NO solution

prior to activation. Controls were devoid of NO or thrombin. Reactions were terminated after 2 minutes with 1ml of cold 1.5M PCA. Tubes were then sonicated for 2 minutes in ice and centrifuged for 10 minutes at 13,000 rpm. Supernatants from each vial were collected individually and the pH adjusted to 4.0 with 1M KOH. They were further centrifuged at the same speed for 5 minutes. The final supernatants obtained were freeze-dried overnight. The resulting powdered samples were re-dissolved in up to 200μL of 0.1M HCl for subsequent derivatization. Experiments were performed in duplicate.

2.3.b Derivatization of polyamines and sulphur-amino acids

Extracted polyamines, including standards, were derivatised by mixing 100μl of extracted sample, 200μl of saturated Na_2CO_3 and 200μl of dansylchloride reagent (1mg/ml in acetone). The mixture was vortexed, sealed and incubated at 65°C in a preheated oven for 10 minutes. The derivatised sample was kept in an aluminium foil ready for Hplc analysis.

For sulphuramino acids, derivatization was carried out by adding 100μl of OPA reagent in 0.1M potassium borate buffer (pH 12.0) to 100μl of extracted sample. Samples was allowed to incubate at room temperature for 2 minutes and were then ready for Hplc analysis.

2.3.1 Hplc analysis of platelet polyamines and sulphur-amino acids

(a) The polyamines (including standards) were analysed by injecting 20μl (3x) of sample into a column maintained at a flow rate of 1ml/min with a gradient of 70% mobile phase A (10mM phosphate buffer) and 30% mobile phase B (185ml acetonitrile and 15ml methanol), increasing the mobile phase up to 90% over a total run time of 50 minutes. The sensitivity was set at the 0.02 scale using a fluorescence detector.

(b) The sulphuramino acids (standards and extracted sample) were analysed by injecting 50μl (3x) of sample into the column maintained at a flow rate of 1ml/min with a gradient of 75mM phosphate buffer containing 10% (v/v) methanol (mobile phase A) into mobile phase B (100% methanol). The detection was carried out with a Gilson (M121) fluorometer, $lambda_{ex}$ 340nm, $lambda_{em}$ 455nm, with the sensitivity set up to the 0.02 scale. The data acquisition and evaluation were performed in triplicate by Gilson 712 software.

Chapter 3

RESULTS AND DISCUSSION

CHAPTER 3

PROTON NMR LIPID PROFILING OF PLATELET MEMBRANES

3.1 1-D and 2-D nmr analysis of platelet total lipids

The 1-dimensional (1-D) proton nmr spectrum of the total lipid extract of the platelet membrane lipid is shown in Fig.11, while the 2-dimensional (2-D) COSY (homonuclear correlated spectroscopy) spectrum in shown in Fig. 12. The fatty chain, the sterol methyl and the methylene proton resonances occur between 0.65ppm and 2.90ppm, the phospholipid head group and glycerol backbone proton resonances occur between 3.05ppm and 5.25ppm, and the vinyl proton signals occur between 5.30ppm and 5.95ppm.

Choline phospholipids were identified by their characteristic $-N^{+}(CH_3)_3$ proton signals at about 3.20ppm which consisted of two partially overlapping singlets (Fig.11). The proportion of the total choline lipids was estimated from the integrals of the singlets. The two choline head group methylene protons, $-OCH_2-CH_2N^{+}$, resonated at 3.60 ($-C\underline{H}_2N^{+}$) and 4.25ppm ($-OC\underline{H}_2$), and were confirmed by the cross peaks in the 2-D COSY spectrum of the total lipids (Fig.12).

Ethanolamine phospholipids were also identified by their characteristic head group $-CH_2C\underline{H}_2NH_2$ methylene proton resonance at about 3.10ppm. The shape of this signal indicated the presence of a mixture of this class of lipid, which was later confirmed by nmr analysis of the hplc fraction of this lipid. Examination of the 2-D COSY spectrum of the total lipids (Fig.12) showed the cross peak

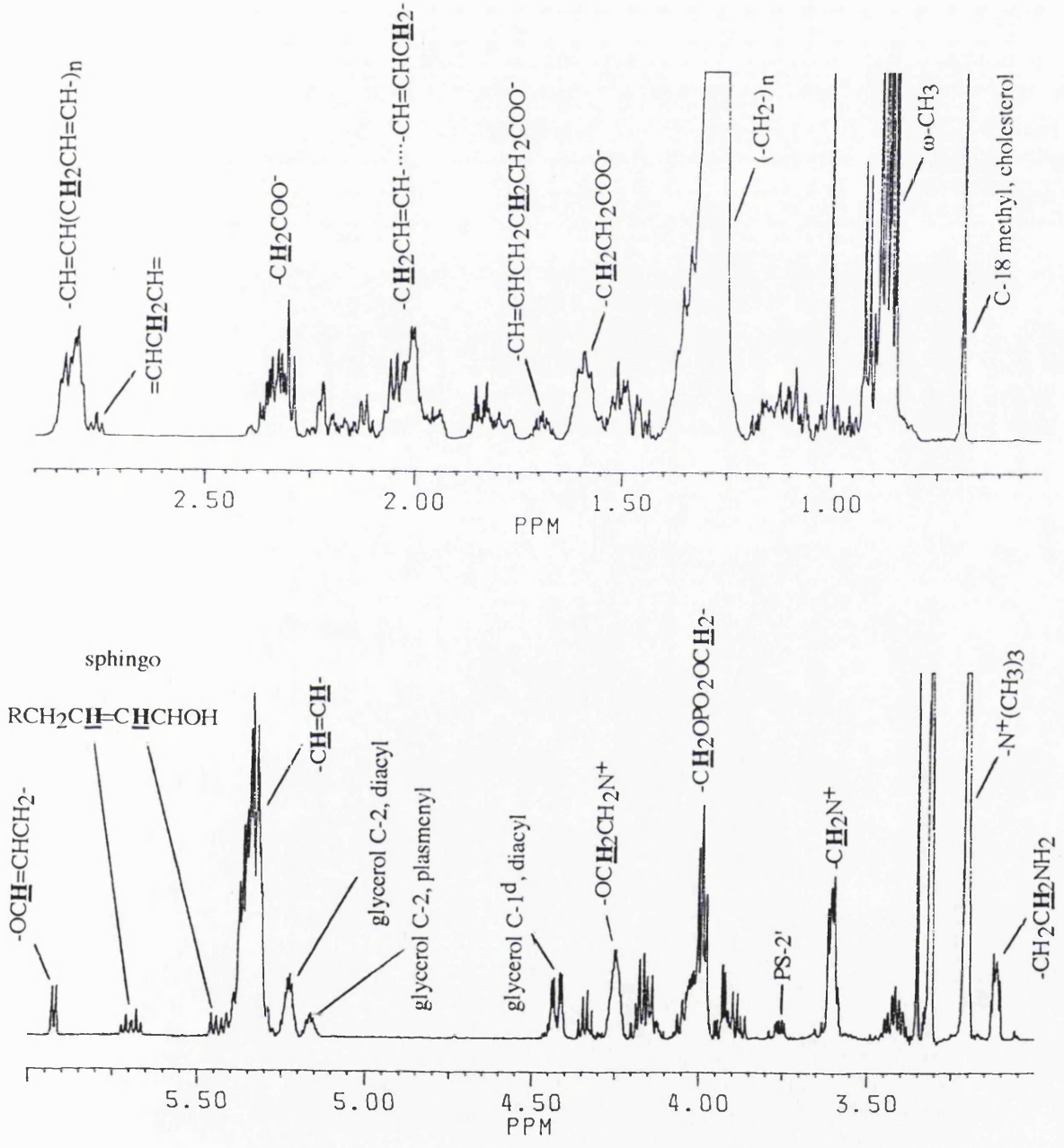


Fig. 11 1-dimensional proton nmr spectrum of platelet total lipids. Spectrum was recorded at a temperature of 298K in Fourier transform mode with 16K data points using 45° detection pulse and 2.0s acquisition time.

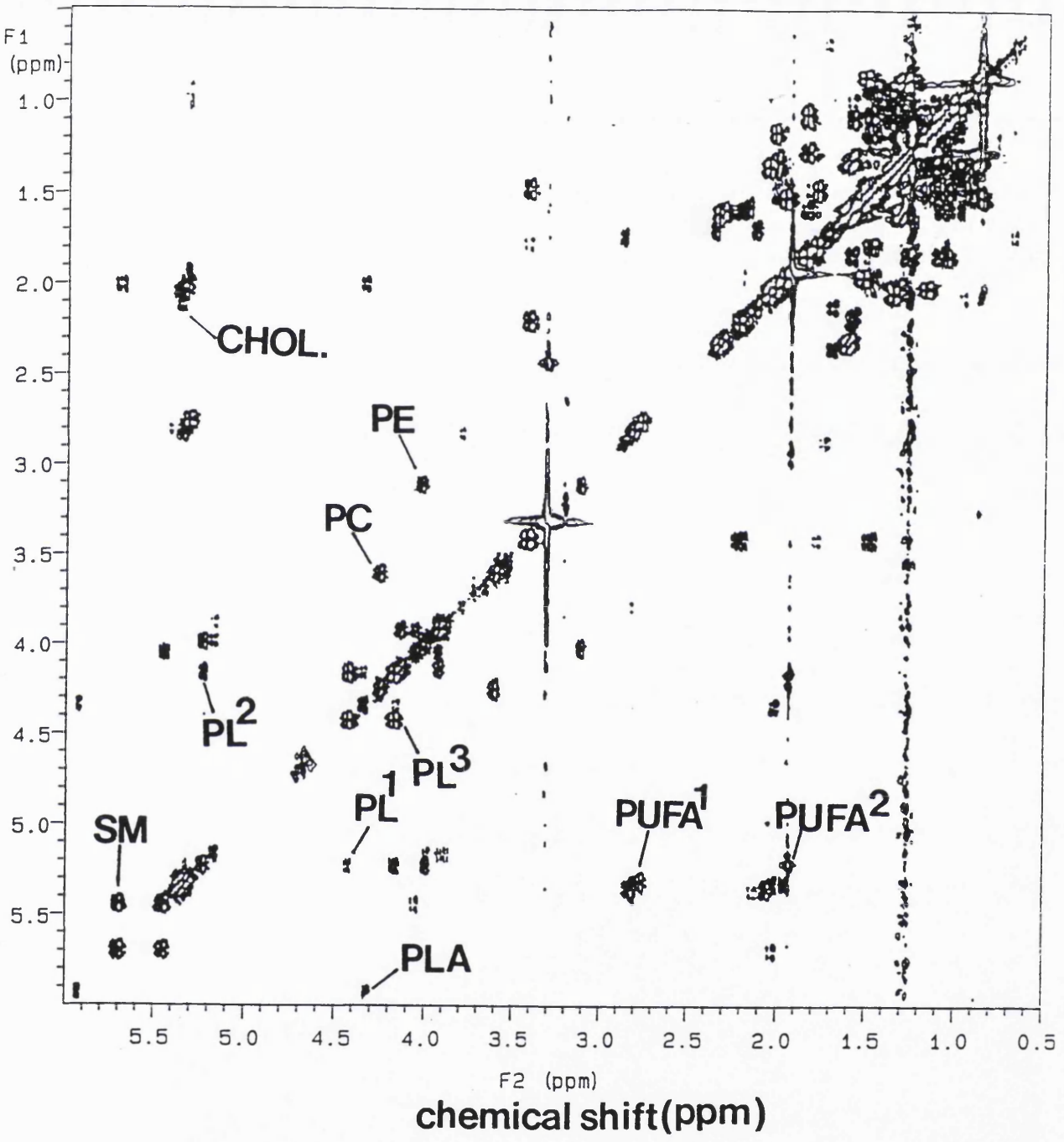


Fig. 12 2-dimensional proton nmr spectrum of platelet total lipids. The 2-D spectrum consisted of 2K data points obtained from 512 FIDSs of 48 scans with zero filling in the F_1 dimensions. SM, sphingomyelin; PLA, plasmalogen; PL1, phospholipid glycerol C-1 (downfield) and C-2 proton; PL2, phospholipid glcyerol C-1 upfield and downfield protons; PC, phosphatidylcholine; PE, phosphatidylethanolamine; CHOL; cholesterol; PUFA, polyunsaturated fatty acid vinyl and allyl protons.

(3.12ppm, 3.98ppm), which identified the -OC$\underline{H}_2$ head group methylene protons at 3.98ppm.

The backbone glycerol Sn_2 proton multiplet at about 5.21ppm represented the diacylglycerolphospholipids present. The magnetically unequivalent glycerol Sn_1 methylene proton resonated at 4.42ppm and 4.15ppm, while both glycerol Sn_3 methylene proton resonances overlapped at about 4.0ppm. Couplings between these glycerol backbone protons were confirmed by cross peaks in the 2-D COSY spectrum of the platelet total lipids and unequivocally give their assignments.

The multiplet at 5.15ppm, arising from the glycerol Sn_2 proton, represented all the ether lipids, both alkylacyl and plasmenyl types. The ratios of the integrals of the glycerol Sn_2 proton signals at 5.21ppm and 5.15ppm gave the levels of diacyl to total ether lipids, respectively. Plasmenyl lipids gave their characteristic vinyl proton resonances at 5.92 (doublet signal) and 4.33ppm (quartet signal). The ratio of aklylacyl to plasmenyl glycerophospholipids was therefore determined by the integral difference at 5.15ppm and 5.92ppm.

Sphingolipids were identified by a choline $\underline{N}^+$ methyl singlet at about 3.196ppm which partially overlapped also with the head group methylene protons of phosphatidylcholine. The multiplets at 5.70ppm and 5.44ppm were diagnostic of the characteristic sphingenine moiety vinyl protons (HO-CH-C$\underline{H}$=C$\underline{H}$) and, therefore, confirmed the presence of sphingomyelin in human blood platelets.

Phosphatidylserine (PS) was the predominant acidic lipid detected in the nmr spectrum of platelet lipids. It was identified by its characteristic signal at about 3.75ppm (Fig.12), resulting from the head group proton, $>C\underline{H}NH^{+}_{3}$. This was estimated to consist of 6.92 $\pm$ 1.5% of the total lipids (Table 6A) and 10.5$\pm$2 of the total phospholipids in platelet membranes (Table 7). Phosphatidylinositol (PI), phosphatidylglycerol (PG) and phosphatidic acid (PA) signals were not detected above the nmr noise level, indicating their low concentrations in the platelet membranes.

Cholesterol was the main steroid component detected in the nmr analysis (Fig.11, 12). It was identified by its characteristic C-18 methyl singlet at around 0.68ppm (Fig.11). Other cholesterol signals included the C-3 proton at 3.42ppm and the vinyl proton at 5.32ppm. The integrals of these peaks were consistent with the number of cholesterol protons giving rise to these signals. No signals characteristic of cholesterol esters (usually a multiplet at about 4.25ppm) were detected, indicating that cholesterol esters were present at concentrations of less than 1% in the platelet membranes. Other common neutral lipids, such as di and triglycerides, were also not detected above the nmr noise levels.

The fatty acid region of the spectrum (0.80ppm-2.90ppm) (Fig.11), contains the component spectra of a diversity of fatty acyl chains, from saturated, mono and polyunsaturated fatty acids. The signal at 5.33ppm represented all the

Table 6 Nmr analysis of the total lipid extract from human platelet membranes

(A)

Lipid identity	Chemical shift (ppm)	Integral area	Composition (mol% ± SD)
Cholesterol	0.68	0.450	34.80 ± 3.0
Total phosphocholine	3.20	0.353	28.21 ± 2.3
Total phosphoethanolamine	3.10	0.255	19.50 ± 0.9
Phosphoserine	3.75	0.110	6.92 ± 1.5
Unsaturated sphingolipids	5.70	0.160	12.90 ± 0.8
Plasmalogen	5.90	0.160	-
Trigylcerides	4.32/4.15	-	-

(B)

Fatty chain	Chemical shift (ppm)	% Total chain
Total chain	0.86, 0.95	100.0
Total unsaturated	2.02	39.8
Total saturated		60.2
Linoleic (C:2)	2.75	5.3 ± 0.38
Arachidonic acid*(C:4)	1.70	12.0 ± 2.7
Docosahexaenoic (C:6)	2.40	2.8 ± 0.5
Monounsaturated		19.70

C:n denotes fatty chains with n double bonds

* The signal at 1.70ppm may also include eicosapentaenoic acid with similar structure ($-CH_2\underline{CH_2}CH_2COO^-$)

(Errors in estimations were due to variations in sample preparations)

Table 7 Platelet membranes phospholipid composition (Values for Nmr estimates are given as means ± SE for 10 subjects)

Phospholipid class	Nmr est'd (mol %)	[1]TLC est'd	[2]GC (mol %)
Phosphatidylcholine	43.3 ± 3.0	41.3 ± 1.1	39.9 ± 0.8
Phosphatidylethanolamine	29.7 ± 1.8	28.1 ± 0.6	23.3 ± 1.1
Phosphatidylserine	10.5 ± 2.0	11.0 ± 0.5	11.1 ± 0.5
Sphingomyelin	19.4 ± 2.4	18.0 ± 0.7	19.6 ± 0.4
Total recovery	102.9 ± 2.3	98.0 ± 3.8	100.0 (PI=6.1±0.4)

1T.L.C. Safrit et al (1971)

2GC-Mahadevapa and Holub (1982)

vinyl (-CH<u>H</u>=C<u>H</u>-) protons in the fatty acids and cholesterol, and thus by subtracting the contribution from cholesterol one obtains the relative measure or degree of unsaturation in the fatty acids. The ratio of saturated to unsaturated fatty acids was calculated to be 1.5:1 (integral of methylene resonance at ca 2.00ppm to that of the w-CH_3 at 0.86ppm). The individual polyunsaturated fatty acids detected included linoleic acid with a characteristic triplet at 2.75ppm arising from the specific methylene protons in the chain (-CH=CH-C<u>H</u>$_2$-CH=CH-CH_2); arachidonic acid as a multiplet at ca 1.70ppm arising from its characteristic beta methylene protons, (=CH-$CH_2CH_2CH_2COO^-$); docosahexanoic acid occurred at 2.40ppm as a multiplet (=$CHCH_2Ch_2COO^-$). The overall nmr estimated fatty acids are shown in Table 6(B).

3.1b 1-D and 2-D nmr total lipid profiling of intact platelet membranes from patients with coronary artery disease (CAD)

The platelet lipid distributions of three patients, compared with a normal subject, is shown in Table 8.
Patient A is a 40 year old man with a history of Ischaemic heart disease (IHD), who was diagnosed with unstable angina, with atrial fibrillation. He was an ex-heavy smoker with a high blood pressure. Medication at the time the blood sample was taken included diltiazem (12mg), aspirin (25mg), digoxin (125mg) and trimethoprin. From Table 8 it can be seen that, compared to the normal, patient A had

Table 8 Comparison of lipid distribution of normal subjects and patients with coronary artery disease (cad)

COMPONENTS	*NORMAL SUBJECTS (mol %)	PATIENT A (mol %)	PATIENT B (mol %)	PATIENT C (mol %)
Cholesterol	34.8±3.0	43.30	56.76	54.34
PC	28.2±2.3	29.30	19.20	22.20
PE	19.5±0.9	17.80	6.50	13.20
PS	6.92±1.5	trace	trace	undetected
SPH	12.9±0.8	9.60	17.50	10.20
Arachidonic Acid	12.0±2.7	4.82	1.6	2.08
Docosahexae-noic Acid	2.8±15	6.53	0.23	0.43
Linoleic Acid	5.3±0.38	4.3	7.2	6.6
OTHER SPECIAL FEATURES	**NORMAL SUBJECTS**	**PATIENT A**	**PATIENT B**	**PATIENT C**
Unsaturation Index	1.02	0.72	0.44	0.55
Diacyl:Ether Lipids	2.8±0.45	3.7	-	2.42
Cholesterol: Phosphlipids	0.53	0.76	1.3	1.2
Cholesterol Esters	undet-ected	undet-ected	10.2% (of total lipids)	trace
Total Phospholipids	65.4±3%	56.70%	43.23%	45.6%
Triglycerides	absent	undet-ected	present	trace

* values shown are mean ± standard deviation in ten normal subjects

an elevated platelet cholesterol and decreased levels of sphingomyelin and fatty acids (mainly arachidonic acid and doco-sahexaenoic acid). Other special features also observed (Table 8) included an unsaturation index less than unity, an elevated cholesterol: phospholipid ratio and an overall decrease in percentage phospholipids. The 1-D nmr total lipid profiling of Patient A is shown in Fig.13a, whilst the 2-D nmr spectrum is shown in Fig.13b. Examination of the 1-D nmr spectrum clearly showed the absence of cholesterol esters since no multiplet at about 4.52ppm was observed, whilst the absence of Sn_1 and Sn_3 proton resonance at around 4.32 and 4.15ppm essentially indicated the absence of triglycerides.

Patient B is a 70 year old man with a primary coronary artery disease. He was an ex-heavy drinker but with no secondary risk CHD (Coronary Heart Disease) factors. Medication at the time when blood samples was collected included 75mg aspirin, athenolol and diltizem (12mg).

Examination of the platelet lipid distribution of this patient (Table 8) reveals an elevated cholesterol content (56.76%), a decrease in phospholipids (mainly PC and PE) content, and also a decrease in the fatty acids (mainly arachidonic acid and docosahexanoic acids). Among other special features observed included a very low unsaturation index (0.44), a slightly higher cholesterol: phospholipid ratio (0.76) and high levels of cholesterol esters (10.2%). The 1-D and 2-D nmr spectra of the total platelet lipids of this patient are shown in Figs.14a and 14b. The most

unique feature of the 1-D spectra was the presence of cholesterol esters at about 4.52ppm, with a characteristic multiplet signal. It is also interesting to note the elevated level of linoleic acid as indicated by the increase in height of its specific signal at 2.75ppm. The presence of triglycerides was shown by the Sn_3 proton resonance at 4.32ppm.

Patient C is a 62 year old man who was diagnosed as having atheroscleorosis with a history of primary coronary heart disease. He was on heparin infusion at the time of sample collection. Total lipid extracted from (250ml of blood) platelets was 1.4mg. The nmr total platelet lipid analysis of this patient is shown in Table 8 and revealed an elevated cholesterol content, a fall in total phospholipids and also a decrease in arachidonic acid and docosahexaenoic acid. Special observed features also included a decreased unsaturation index, a high cholesterol:phospholipid ratio (1.2:1) and traces of cholesterol esters and triglycerides. The 1-D and 2-D nmr spectra are shown in Figs.15a and 15b, respectively.

At this stage, however, it was difficult to attribute the observed differences between the platelet lipid composition of these patients and those of the normal subject as solely due to the diseased state of the patients. However, it is worthwhile to comment on their possible implication, especially in coronary artery disease.

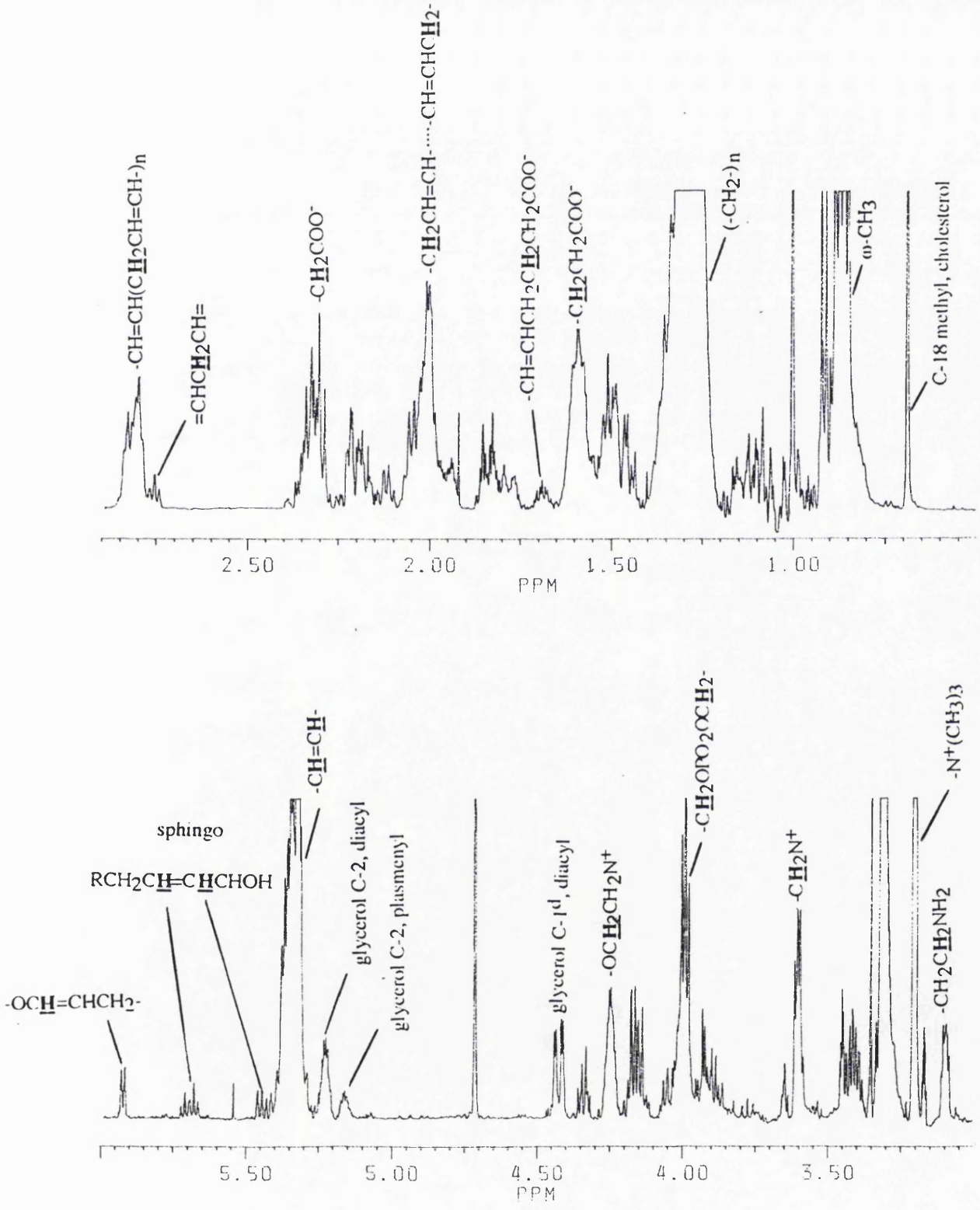


Fig. 13a 1-dimensional proton nmr spectrum of platelet total lipids of Patient A. Procedure as described in Fig. 11.

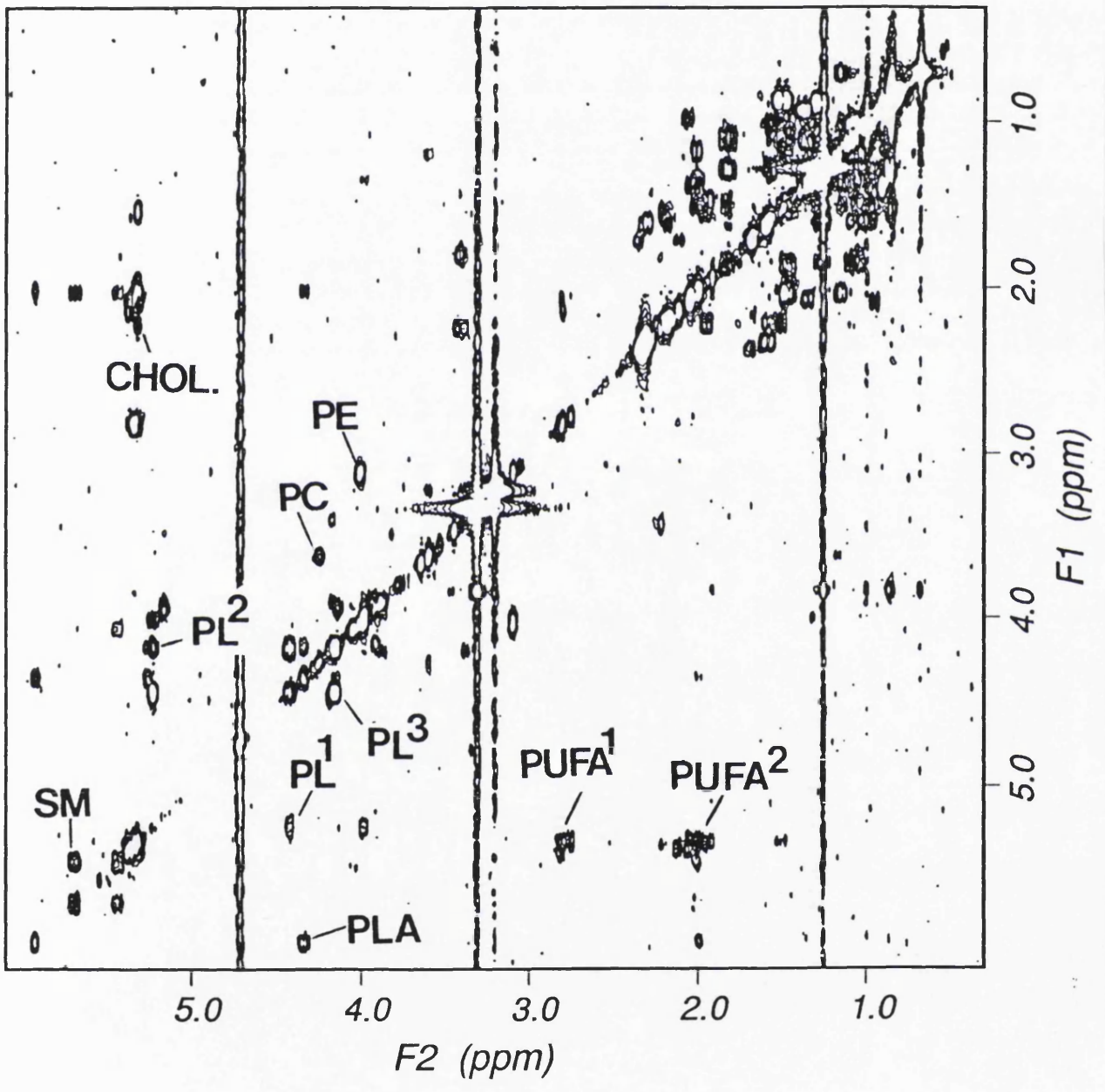


Fig. 13b 2-dimensional proton nmr COSY spectrum of platelet total lipid of Patient A. Procedure as described in Fig. 12.

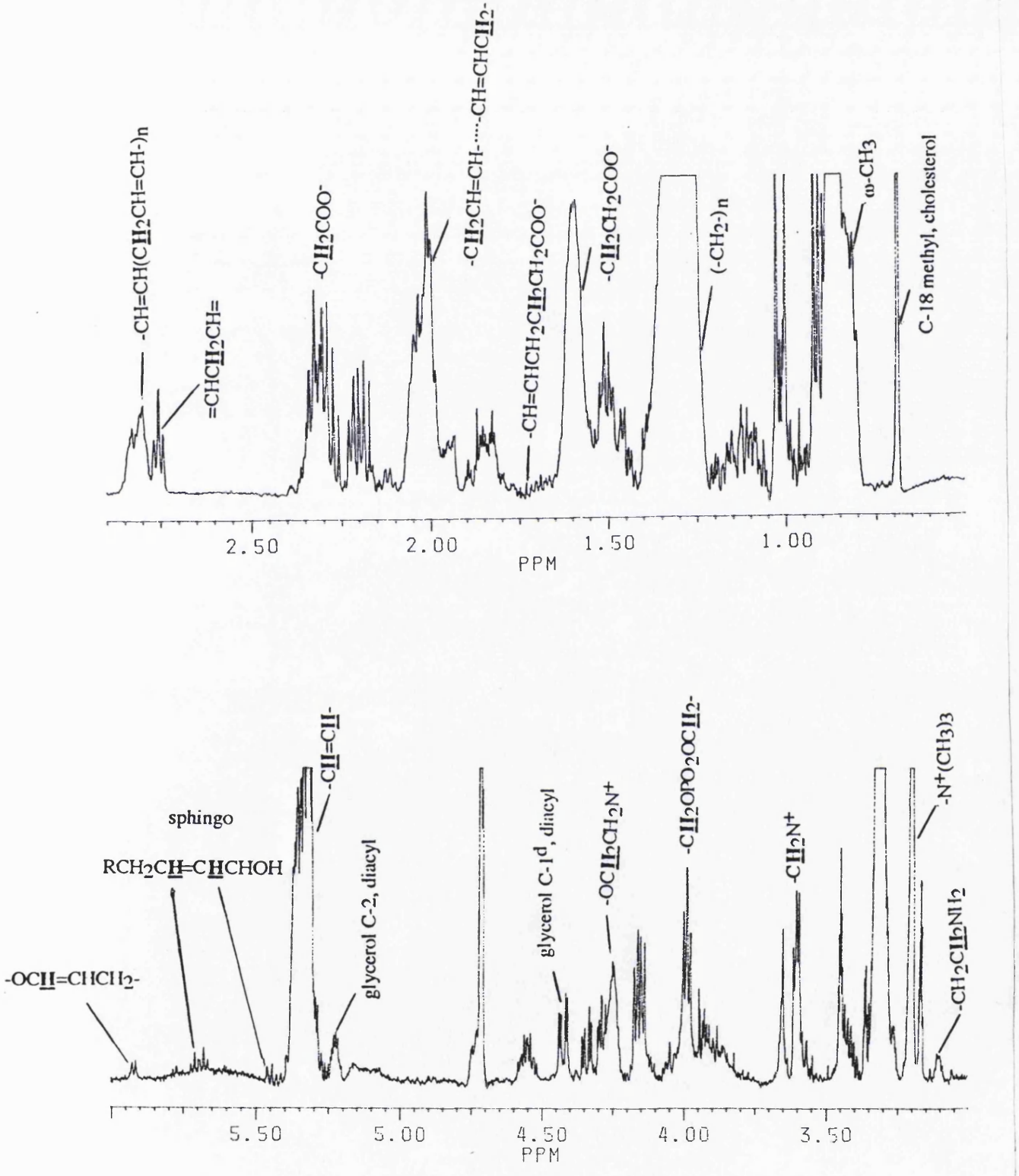


Fig. 14a 1-dimensional proton nmr spectrum of platelet total lipids of Patient B. Procedure as described in Fig. 11.

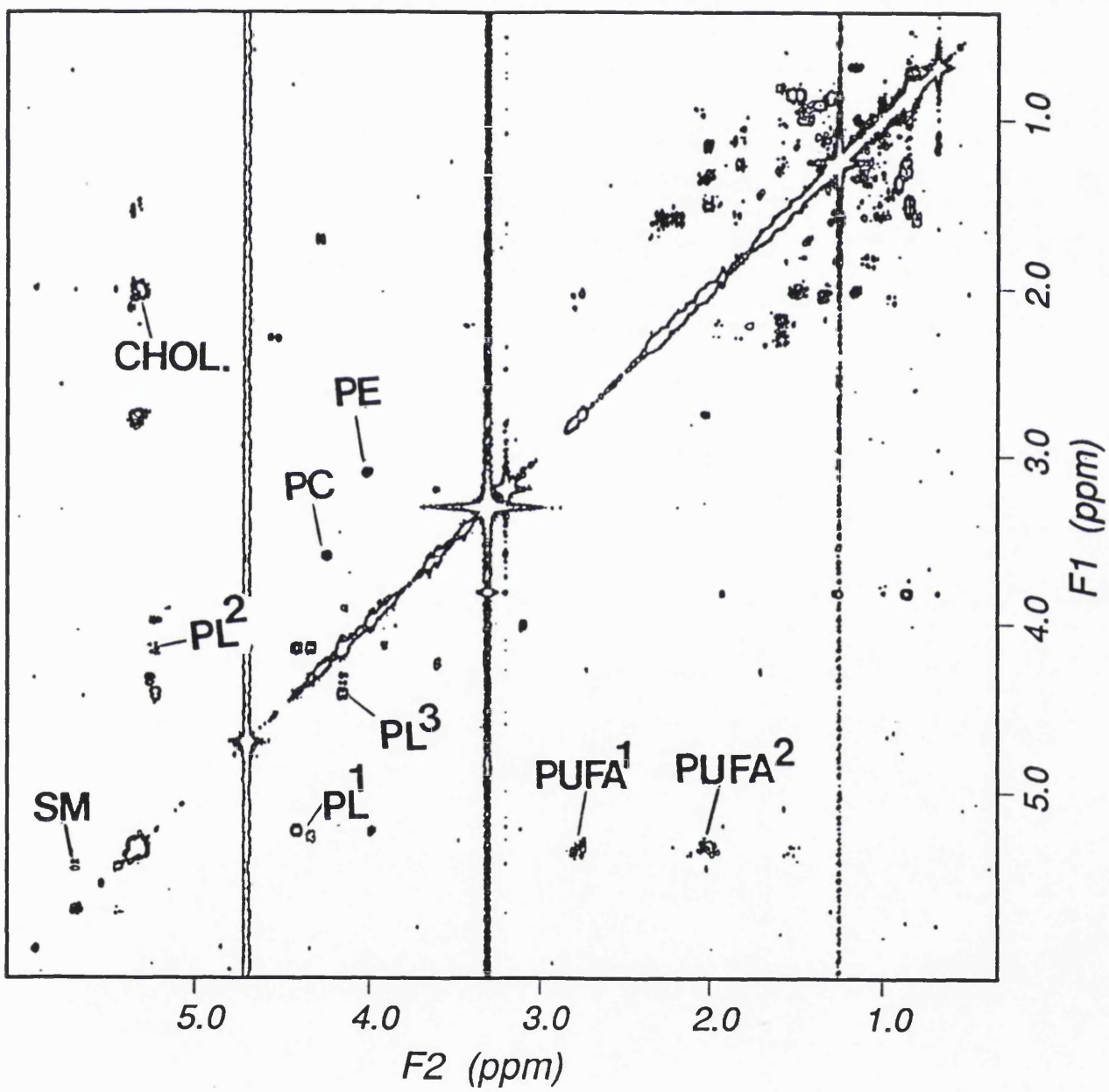


Fig. 14b 2-dimensional proton nmr COSY spectrum of platelet total lipids of Patient B. Procedure as described in Fig. 12.

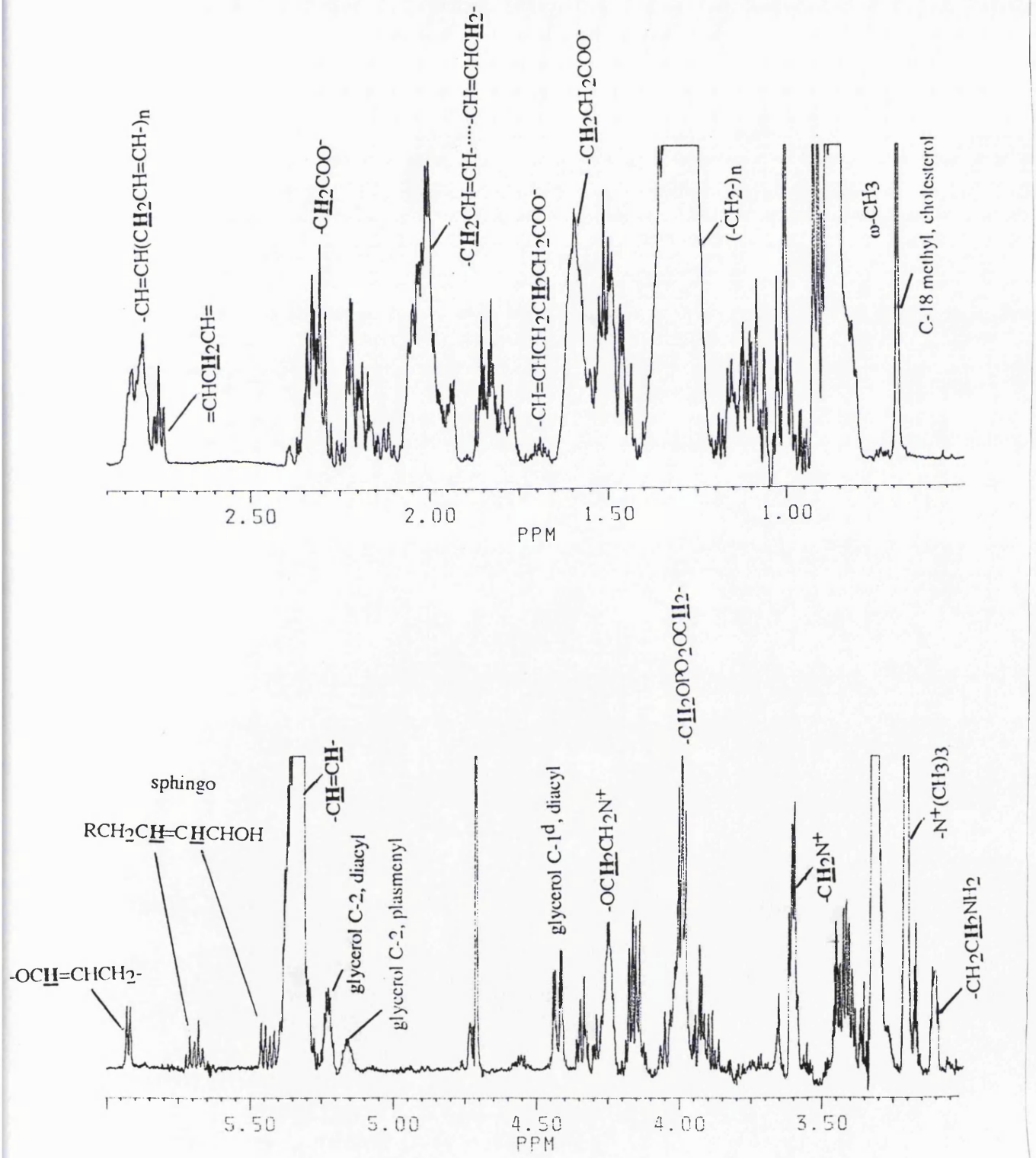


Fig. 15a 1-dimensional proton nmr spectrum of platelet total lipids of Patient C. Procedure as described in Fig. 11.

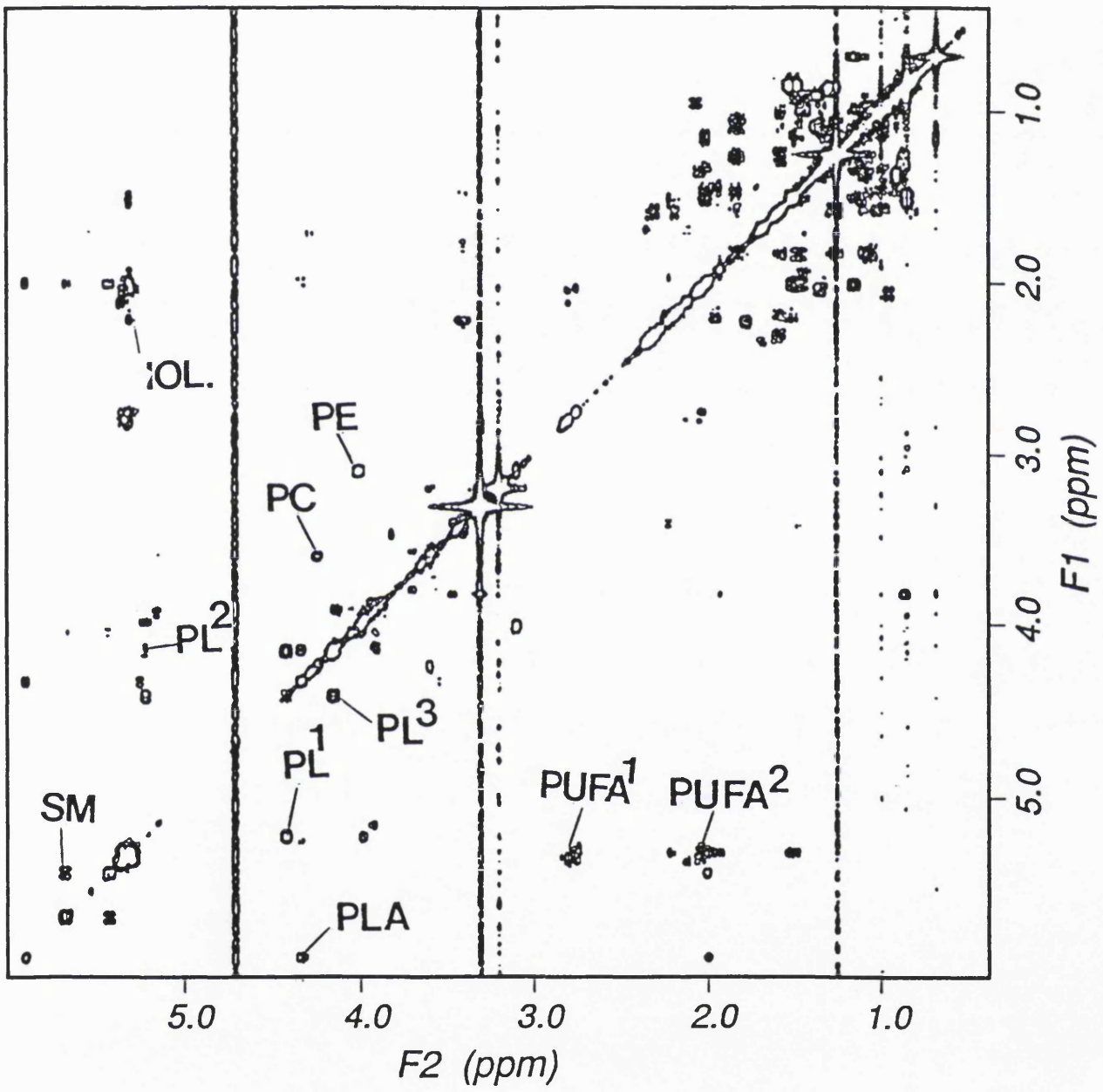


Fig. 15b 2-dimensional proton nmr COSY spectrum of platelet total lipids of Patient C. Procedure as described in Fig. 12.

Coronary artery disease (CAD) is the most common heart disease (Miller et al, 1981) and is almost always the result of atherosclerosis - hardening of the arteries. The primary physiological event leading to the formation of atherosclerotic plaques is the increased deposition of macro molecules (including cholesterol) within endothelial cells of arteries. The fatty deposition causes a reaction within the vessel wall, and scar-like fibrous tissue may build up around the deposit, forming a plaque, which can be calcified. There is a progressive narrowing which in some cases can lead to total obstruction or occlusion of the arteries and interference of blood flow.

Focal, hemodynamically induced endothelial injury with enhanced cell permeability is the probable determinant of the atheromatous process. Additional initiation factors include the release of platelet constituents, hypertension, carbon monoxide, antigen-antibody complexes, and hyperlipidemia. Cigarette smoking may prove to be an important initiating factory that exerts its effect either directly through an immune mechanism, or indirectly through released platelet constituents or carbon monoxide.

Factors that influence the nature or the rate of lesion development at all subsequent stages include hypercholesterolemia, with an associated excess of LDL (low density lipoproteins), and disturbances in platelet function, hemostasis and thrombosis. The accelerating factors exert their influence by stimulating to varying degrees the synthesis by smooth muscle cells of collagen, elastin and

the glycosaminoglycans. In addition, lipoproteins may significantly influence lipid metabolism, including the uptake and accumulation of lipids within the smooth muscle cells. Additionally, platelets may directly contribute to plaque growth by serving as components of mural thrombi (Ross et al, 1974). CAD is much more common among males than among females, affecting five times more males than females under 45 years of age. The sex preference falls off rapidly between 45 and 60 years of age, although males in this group still have about twice as many heart attacks as females do. In the elderly, the incidence is about the same for both sexes. The primary and secondary risk factors associated with CAD are shown in Table 9.

The results of the CAD patients whose platelets were analysed by proton nmr, indicated an overall increase in the cholesterol content compared to those of the normal subjects (Table 8).

Derksen and Cohen (1973) have shown that platelets cannot synthesize cholesterol; therefore, the cholesterol contents observed in these patients reflect the initial composition of the megakaryocyte. It has been shown that exchange of cholesterol with plasma lipoproteins may result in remodelling of the endogeneous cholesterol content of platelets. It therefore stands to reason that the apparent elevated cholesterol levels as estimated in these patients could reflect a contribution of that which is exchanged with those of their plasma lipoproteins. The mechanism for such an exchange is, however, not clear. It may be via a

Table 9 primary and secondary risk factors associated with coronary heart disease*

Primary Factors:

Genetic predisposition to coronary heart disease (CHD)
Hypertension
Cigarette smoking
Elevated total cholesterol (LDC cholesterol)
Decreased HDL cholesterol

Secondary Factors:

Lack of exercise
Obesity
Age
Male sex
Stress
Diabetes mellitus
Gout and hyperuricemia
Renal failure patients receiving hemodialysis
Subjects taking oral contraceptives

*(Kaplan and Pesce, 1989)

receptor-mediated process. Due to the positive correlation between blood cholesterol and increased risk for coronary heart disease, it could be quite likely that the high platelet cholesterol content could be an indication of elevated plasma cholesterol levels among these patients, which may be secondary to high dietary cholesterol.

Platelet membranes of normal individuals have a much lower cholesterol:phospholipid ratio, approximately 1:2 (Marcus et al, 1969). It was clear from the analysis (Table 8) that all the patients showed a higher cholesterol:phospholipid ratio (Patient A - 0.76:1; Patient B - 1.3:1; Patient C -1.2:1). Studies by Carvalko et al (1974), suggested that changing the cholesterol content of the platelet membrane alters the platelet response to aggregating agents. They studied platelet function in patients with type II hyperbetalipoproteinemia and 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 cholesterol and platelet function, but they did not establish the mechanism. Studies also by Shattil et al (1975), using cholesterol-rich liposomes, showed that 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 18-fold reduction in sensitivity to epinephrine. These observations would suggest that the enhanced sensitivity to aggregating agents might well have

been reflected in an increased platelet cholesterol content. Indeed Bennett et al (1974) have reported an increased cholesterol:phospholipid ratio in patients with type II hyperlipoproteinemia whose platelets demonstrate hypersensitivity. It is, however, not certain whether the observed increase in platelet cholesterol levels among the CAD patients in this experiment induced hypersensitivity (secondary to increased thrombatic activity), or whether the cholesterol content influenced the nature or rate of lesion development in the atheromatous plaque. However, it is worthwhile to note that hypercholesterolemia with associated LDL and disturbances in platelet functions play a role in atherosclerosis (Miller et al, 1981).

Blood cholesterol often increases with age and it is quite interesting to observe this trend among patients A (40 years), B (70 years) and C (62 years), with platelet cholesterol levels of 43.30%, 56.76% and 54.34% respectively (Table 8). Of the three patients, only patient B (70 years) showed the presence of cholesterol esters (about 10.2% of total lipids). It is, however, not certain whether this was a reflection of age or diet. Patient A was an ex-heavy smoker, and although cigarette smoking is associated with the development of atherosclerosis (Doyle et al, 1964), the mechanism of action has not been clarified. Levine et al (1973) showed that cigarette smoking increased platelet aggregation in peripheral venous blood in healthy volunteers. Several studies showed that adding nicotine to the perfusion medium

depressed prostacyclin (PGI_2) production in isolated vascular tissue of a number of species, including human (Dadak et al, 1981; Stoel et al, 1980; Wenmalm, 1978). It is, however, not certain, to what extent the previous habit of patient A had on the lipid content and function of his platelets.

Another major feature in the distribution of platelet lipids among the CAD patients analysed by proton nmr was an overall decrease in the phospholipid content (mainly PC and PE, PI and PS undetected), and fatty acid content (arachidonic and decosahexaenonic acid) (Table 8). Mehta et al (1980) have suggested that, in atherosclerotic arteries, hyperactive platelets aggregate and are trapped, whilst Data et al (1981) observed reduced aggregation in healthy humans. Indeed, Foyld et al (1973) have reported that platelet activation is associated with profound changes in lipid metabolism. As platelets aggregate, phospholipases are activated, resulting in increased catabolism of phospholipids to release arachidonic acid, which is further metabolised to prostaglandins and thromboxanes, which can further amplify aggregation. It therefore stands to reason from these studies that the observed decreased level of phospholipids and fatty acids could be associated with the increased thrombotic activity in these patients with an overall increase in metabolic influx.

Chignard et al (1979) had observed an increase in the turnover of ether lipids, particularly PAF (platelet

activating factor). Russel and Deykin (1976), however, found that thrombin does impair the initial binding of arachidonic acid by platelets, but it does not reduce its overall incorporation into complex lipids. At this stage it was difficult to know precisely what did cause the declines in the phospholipids and fatty acids. Since the three patients were also on different drug-regimens, it is not certain whether the drugs did have any effect on the lipid levels or their metabolism.

The presence of trigylcerides in the platelet membranes of patient B (Fig.14a) may reflect a high plasma lipoprotein content, possibly due to diet or the past heavy drinking habit of this patient. Alcohol intake is known to cause hypertriglyceridemia; however, it is more likely to reflect dietary intake than alcohol consumption, since hypertriglyceridemia associated with alcohol is often acute and transient.

The apparent low unsaturation index observed among all the three patients (Table 8) could reflect the underlying high plasma cholesterol state of these patients, possibly associated with diet. It has been recommended (Naito, 1982) that for a therapeutic diet for lower plasma cholesterol in patients with type II hyperlipidaemia, an unsaturation index of 1 to 1.2 is desirable, implying that such patients should consume about 2 tablespoons of polyunsaturated fats or vegetable oil per day.

Despite the observed differences, the overall trend in the composition of the phospholipids were quite similar to

those of the normal subjects, with PC as the predominant phospholipid followed by PE.

The difficulty of obtaining blood samples from such patients was the contributing factor of the limited number of patients in this study.

3.2 Analysis of glycerophospholipids by a combination of hplc and nmr

(a) Nmr analysis of choline phospholipids hplc fractions:

The glycerophosphocholine lipids eluted with an Rf of 27 minutes from the hplc column (Fig.16). The hplc analysis of this class of lipids revealed a broad peak, indicating the presence of more than one type of choline lipid in the platelet membranes, with the possibility of a subclass to each type. The nmr analysis (Fig.17) of this hplc fraction permitted a more detailed analysis and established that only phosphocholines that were conjugated to diacylglycerols were present; ether phosphocholines were either absent or occurred in another hplc fraction. The presence of two main types of this class of lipids, namely phosphatidylcholine and sphingomyelin, were confirmed purely by the nmr analysis. The proportion of each type present was estimated from their integrals.

The overall analysis suggested that phosphatidyl-choline made up 28.21 $\pm$ 2.3% of the total lipid

present in platelet membranes (Table 6[A]) and 43.3 ± 3.0% of phospholipids (Table 7). This confirmed PC as the predominant phospholipid in platelet membranes. The estimates compared favourably with those of Safrit et al (1971). The level of unsaturation, deduced from the ratio of the integrals of the vinyl resonance at 5.34ppm to that of the w-CH_3 at 0.86ppm, was 0.66 and suggested lower levels of polyunsaturated fatty acid in PC. Total unsaturation was calculated to be about 51.2% of fatty acids. Analysis of the fatty acid region on PC showed a greater level of linoleic compared to arachidonic acid. The fatty acid analysis of the choline phospholipids is shown in Table 10. The cholesterol:PC ratio was 0.53:1, similar to the 0.5:1 value reported by Marcus et al (1969) for normal platelets. The ratio of the integral of the N^+-methyl singlet to the w-CH_3 signal (0.43), nearly 0.5, suggested two fatty acid chains per choline headgroup in PC.

(b) Nmr analysis of ethanolamine phospholipids hplc fractions: They eluted with an Rf of 20 minutes from the hplc column (Fig.16). The ethanolamine lipids were present in the diacyl (5.23ppm, glycerol Sn_2 proton) and ether (5.15ppm, glycerol Sn_2 proton) forms. The diacyl lipid made up 38.8% whilst the ether form made up 61.2% of the ethanolamine lipids. The ethanolamine phospholipids constituted 19.5 ± 0.9% of

the total lipids in platelet membranes (Table 6A) and 29.7 ± 1.8% of the total phospholipids (Table 7). The ratio of the integral at 3.10 to the combined glycerol Sn_2 integral at 5.23ppm (diacyl) and 5.15ppm (ether) was calculated to be approximately 2.0 indicating that there was one ethanolamine group per glycerol moiety. The specific vinyl protons at 5.92ppm (doublet) and 4.33ppm (quartet) further characterised the plasmenyl (1-alkenyl-2-acyl glycerolphospholipid) moiety of this class of lipids. From the ratio of the integral of the signal at 5.92ppm (plasmenyl) to that at 5.15ppm (total ether) (Fig.18), the plasmenyl form was estimated at about 90%, whilst the alkylacyl form was present at 10% of the ether form of the ethanolamine lipids. The level of unsaturation in the ethanolamine phospholipids (1.2) was about twice as high as in phosphatidylcholine (0.66). The level of arachidonic acid (29.81%) in PE was three times as high as that of the (9.8%), suggesting that PE was a richer arachidonate source. Again a fatty chain analysis of the ethanolamine lipids was achieved and is shown in Fig. 18 and Table 11.

(c) **Nmr analysis of sphingolipids (ceramide lipids) in hplc fractions:** This lipid eluted as a broad double peak, with Rf value between 32 and 34 minutes, from the hplc column (Fig.16). Nmr analysis of this class of lipid (Fig.19) revealed a choline N± methyl singlet

at about 3.196ppm, a multiplet at 4.25ppm ($-OCH_2CH_2N^{+}$), and a tripet at 3.60ppm, ($-CH_2N^{+}$). The multiplets at 5.70ppm and 5.44ppm were diagnostic of a sphingenine moiety of the amide α-methylene protons ($NHCOCH_2-$) present in both sphingenine and spinganine types of sphingomyelin. The ratio of the sphingosine specific vinyl proton resonance to that of the $N^{+}-CH_3$ was unity indicating that sphingomyelin present in platelet membranes occurred predominantly as the sphingenine analogue. This implied that ceramide ethanolamine was less than 1% of the ceramide lipids of platelet membranes. Sphingomyelin was calculated at 12.9 ± 0.8% of the total lipids in platelet membranes (Table 6[A]) and 19.4 ± 2.4% of the total phospholipids (Table 7). It was not clear from the nmr spectrum whether the two peaks observed in the Hplc fractionation (Figure 4) were different sphingolipids or both choline lipids since they were analysed as a mixture.

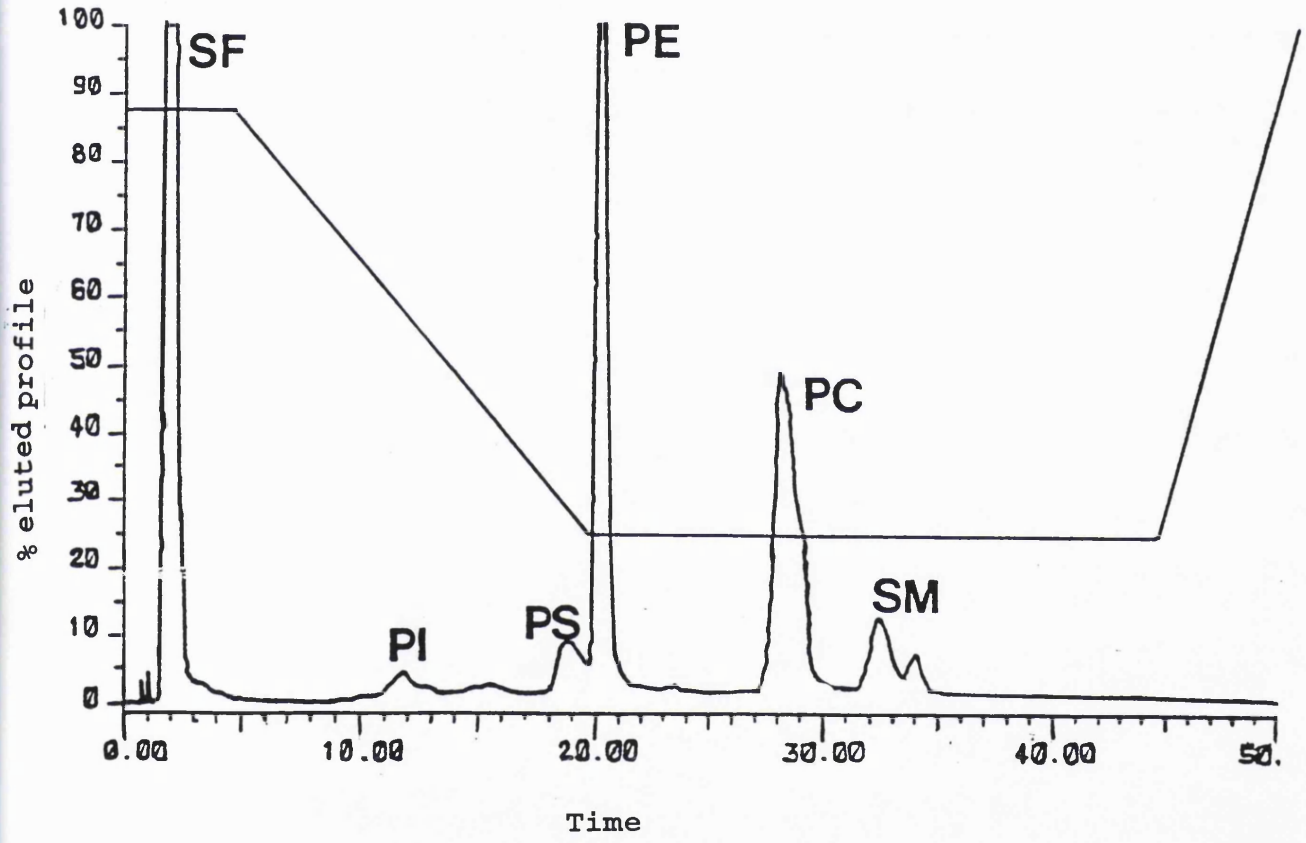


Figure 16: Hplc profile of platelet total lipids. Solvent A, acetonitrile: 5mM KH_2PO_4 (4:1) pH 5.4; Solvent B, acetonitrile. SF, solvent front; PI, phosphatidylinositol; PS, phosphatidylserine, PE, phosphatidylethanolamine; PC, phosphatidylcholine; SM, sphingomyelin.

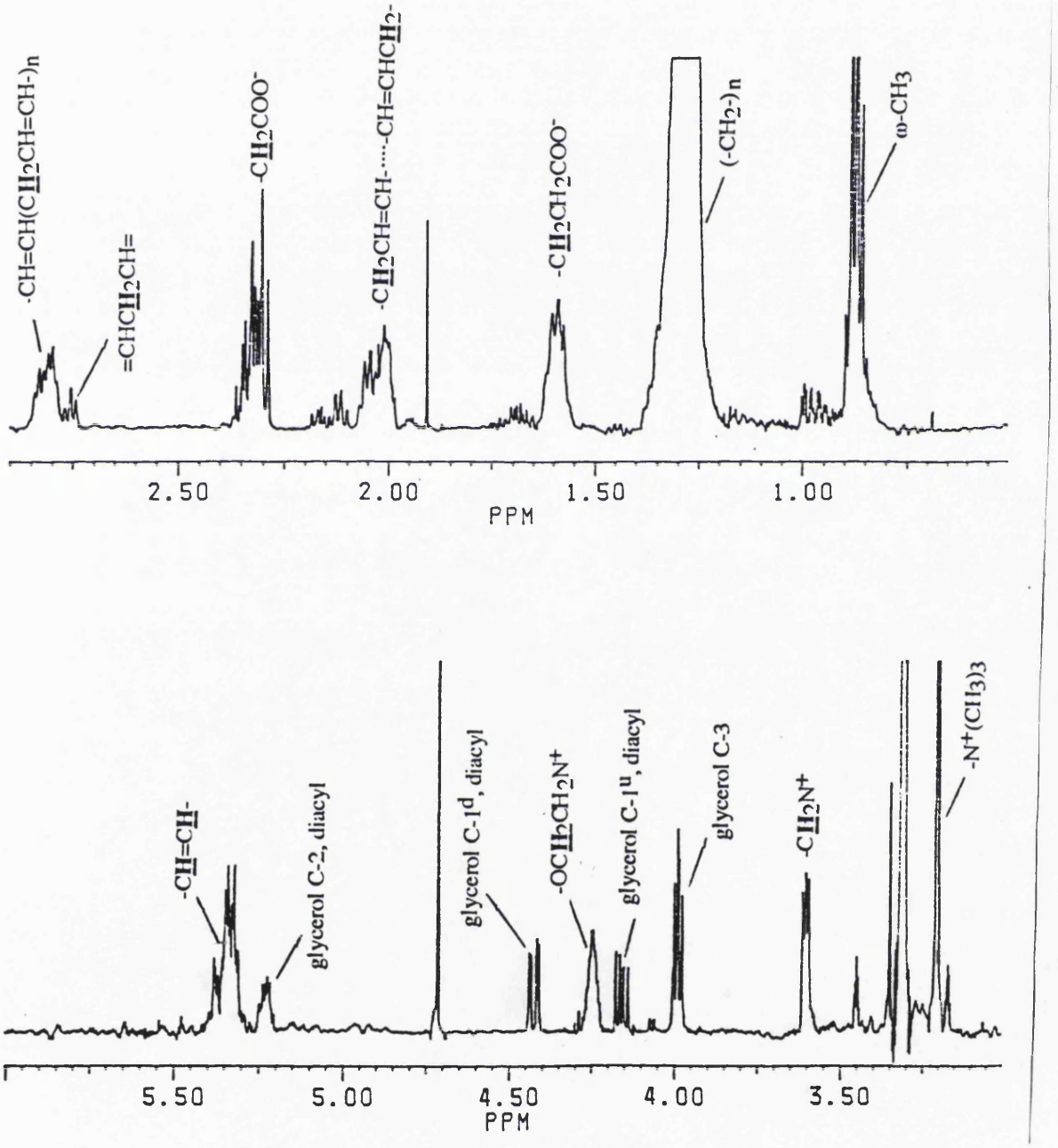


Figure 17: 1-dimensional proton nmr spectrum of platelet choline phospholipids. Procedure as in Figure 11.

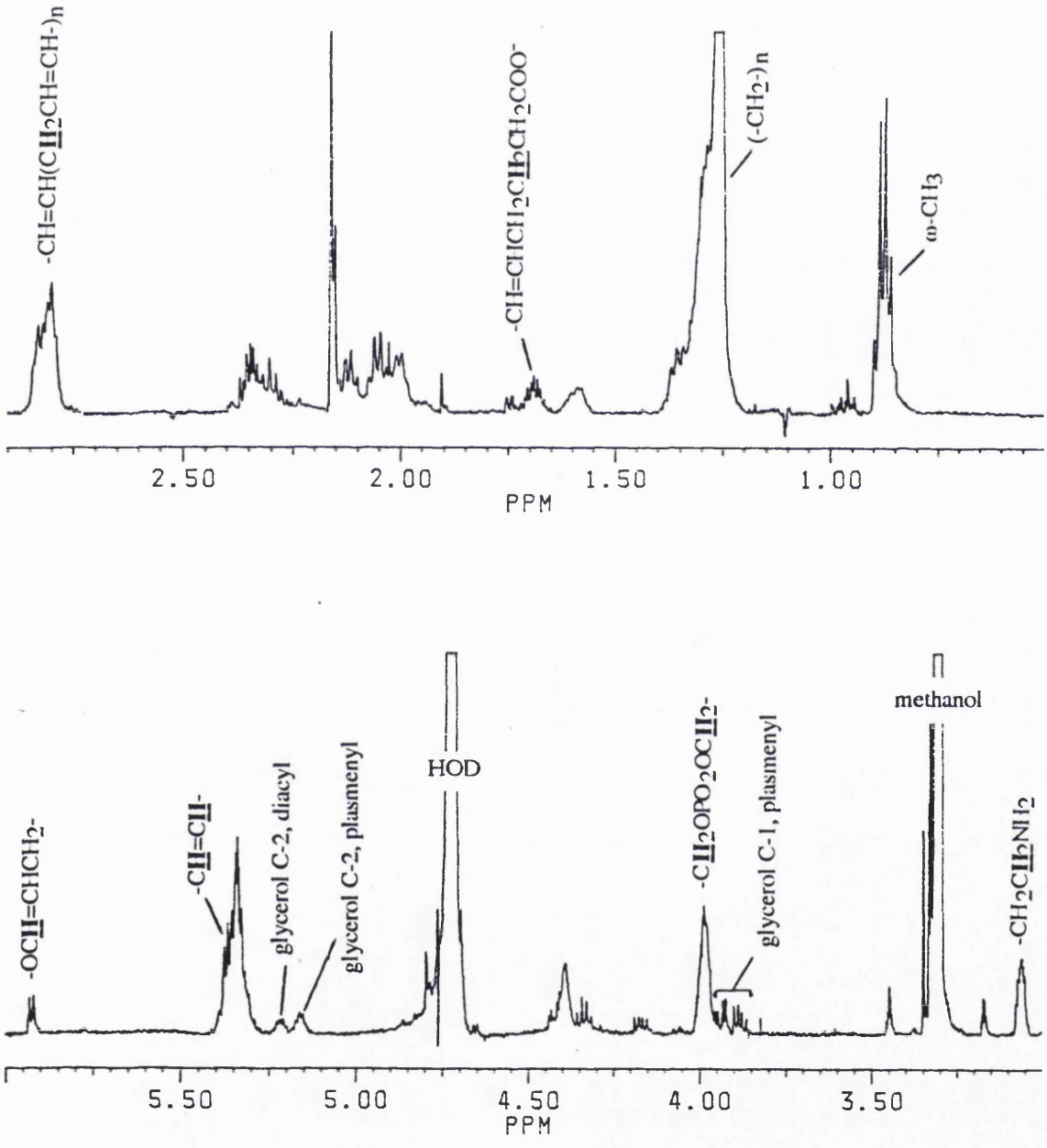


Figure 18: 1-dimensional proton nmr spectrum of platelet ethanolamine phospholipids. Procedure as in Figure 11.

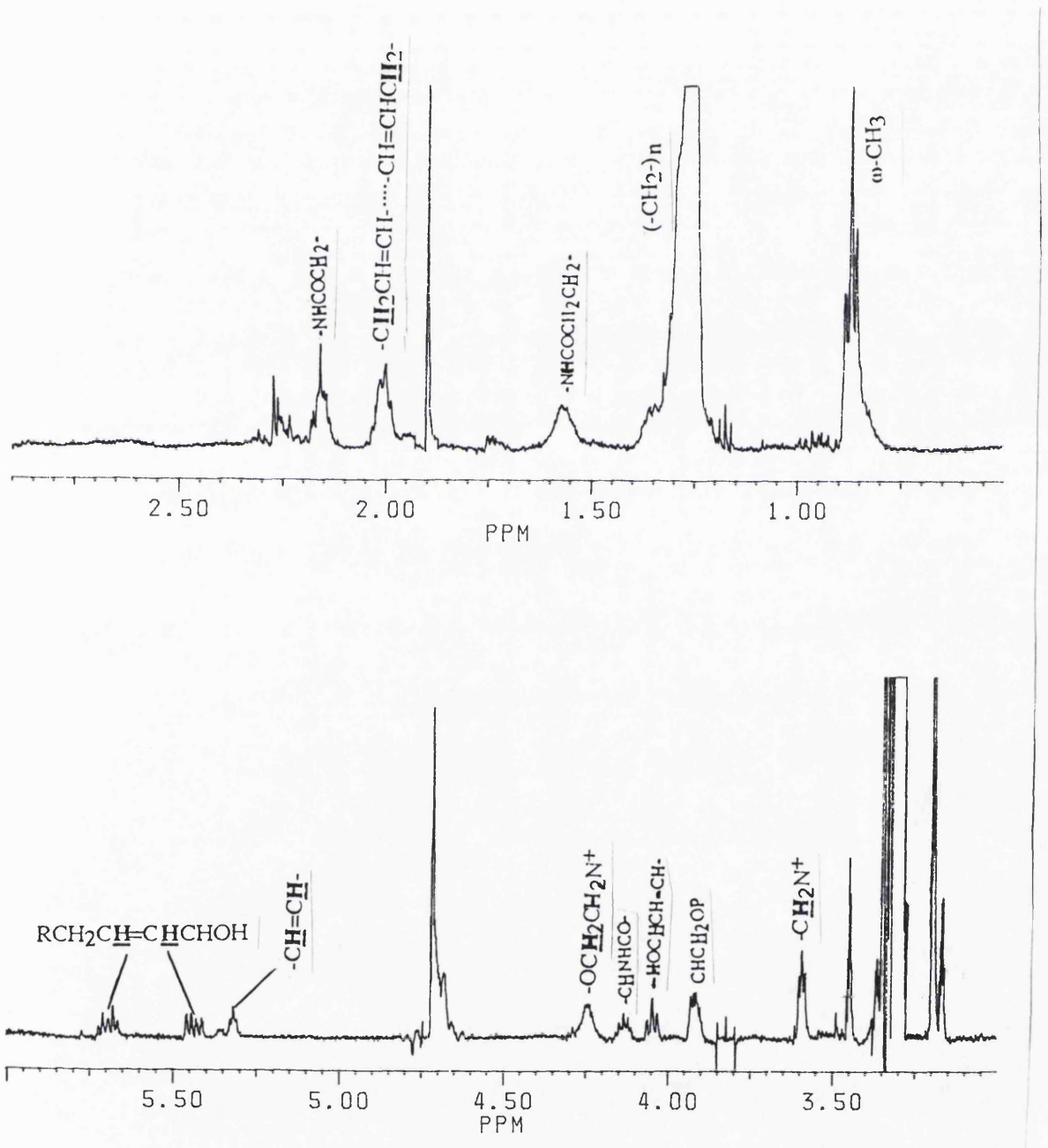


Figure 19: 1-dimensional proton nmr spectrum of platelet sphingolipids. Procedure as in Figure 11.

Table 10 Fatty acid composition of platelet membrane choline phospholipids by nmr analysis.

Fatty chain	Chemical shift(ppm)	% of total chain
Total chains	0.86	100.00
Total unsaturated	2.02	51.20
Total saturated	-	48.80
Linoleic (C:2)	2.75	12.10
Arachidonic (C:4)	1.70	9.18
Hexaenoic (C:6)	2.40	3.53
α-Linoleic (C:3)	0.96	11.95
Monosaturated	-	14.44

C:n denotes fatty chains with n double bonds.

Table 11 Fatty acid composition of platelet membrane ethanolamine phospholipids by nmr analysis.

Fatty chain	Chemical shift(ppm)	% of total chain
Total chains	0.86	100.00
Total unsaturated	2.02	57.60
Total saturated	-	42.40
Linoleic (C:2)	2.75	3.03
Arachidonic (C:4)	1.70	29.81
Hexaenoic (C:6)	2.40	3.82
Linoleic (C:3)	0.95	9.60
Monounsaturated	-	11.35

C:n denotes fatty chains with n double bonds.

3.3 Bond-elut and nmr analysis of the various classes of platelet lipids

Even though the nmr approach for analysing lipids has proved versatile and comprehensive, there are several drawbacks still to be overcome. By applying a simple ion-exchange chromatographic procedure using commercial Bond Elut columns (Kates et al, 1988; Kaluzny et al, 1985) prior to the nmr assay, platelet lipids were separated into four individual fractions according to their polarity. These were;

Fraction A - Neutral lipids and cholesterol (Fig.20a)

Fraction B - Non-esterified fatty acids (Fig.20b)

Fraction C - Non-acidic phospholipids (Fig.20c)

Fraction D - Acidic phospholipids (Fig.20d)

The efficiency of the Bond Elut fractionation was observed by the presence of marker cholesterol resonance at around 0.68ppm in Figure 20a, but not in Figures 20b, 20c and 20d. It was quite obvious by the absence of their characteristic signals from the spectra in Figures 20a and 20b that no phospholipid or acidic phospholipids were present. The analysis of cholesterol (the only observed steroid) was therefore made easier by the absence of overlapping fraction C and fraction D lipid spectra. Table 12 shows that 93.8% of the total platelet cholesterol was recovered during the Bond Elut fractionation. No triclycerides were detected, as indicated by the absence of their glycerol C-1 and C-3 methylene resonance at about 4.16ppm and 4.30ppm (double doublets), respectively.

The extractable non-esterified fatty acids (Fig.20b) were principally saturated fatty acids, as reflected by the resonances at approximately 0.9, 1.36, 2.00ppm etc. As expected, most fatty acids in platelets were tightly bound or associated with the phospholipids.

Fraction C, the non-acidic phospholipids, were readily analysed by the absence of overlapping resonances from fraction A and D lipids (Fig.20c). Unsaturated ether and sphingoid lipid were present in appreciable levels in platelet membranes as shown by their specific signal resonances at 5.90ppm and 5.70ppm, respectively. The sphingoid lipids were accurately quantified by the characteristic resonances at 5.70ppm and 5.44ppm arising from the double bond (CH=CH) of their sphigenine moiety. The unsaturated ether lipids were measured from their doublet resonance at about 5.90ppm and quartet resonance at 4.35ppm.

The existence of two different forms of the choline lipids, as previously observed from the nmr analysis of the Hplc fractions, was further confirmed in the Bond Elut analysis. The singlet at about 3.196ppm was diagnostic for the N^+ methyl singlet of the spingomyelin choline head group. The absence of a third spingoid $N^+(CH_3)_3$ resonance at 3.20ppm indicated that ceramide ethanolamine was the principal spingoid.

The ratio of the total choline to total ethanolamine lipids (2:1) obtained from the integral areas of their respective head groups at 3.20ppm and 3.10ppm, indicated that platelet

membranes were twice as much richer in choline lipids than in ethanolamine lipids. The estimated recoveries of the principal phospholipids are shown in Table 12.

The acidic lipids nmr spectrum is presented in Fig.20d. Prior to the fractionation, the analysis of this fraction of lipids was quite difficult due to their lower concentration in platelets and also due to the overlap of their signal resonances.

However, the characteristic quartet head group resonance of PS at 3.75ppm made its quantitation relatively easier. The absence of the characteristic triplet at 3.85 arising from the CH_1-resonance of PI from the spectrum is an indication of the absence of this type of acidic phospholipid in the spectrum. Similarly, no signals of cardiolipin or phosphatidic acid were observed, indicating their low concentration in platelet membrane.

A complete fatty acid analysis of the non-acidic phospholipids (fraction C) by nmr has not yet been achieved, but a great deal of information was obtained from the fatty acid region of this fraction of lipids (from 2.90ppm-0.80ppm of Fig.20c). For example, docosahexanoic and related acids have a distinctive μ-methyl signal at 2.40ppm, arachidonic acid and related acids have their characteristic β and methylene resonances at 2.20ppm and 1.70ppm, respectively, and linoleic acid gave alkylic CH_2 resonances at 2.75ppm. These structure-specific resonances gave accurate qualitative and quantitative analyses of

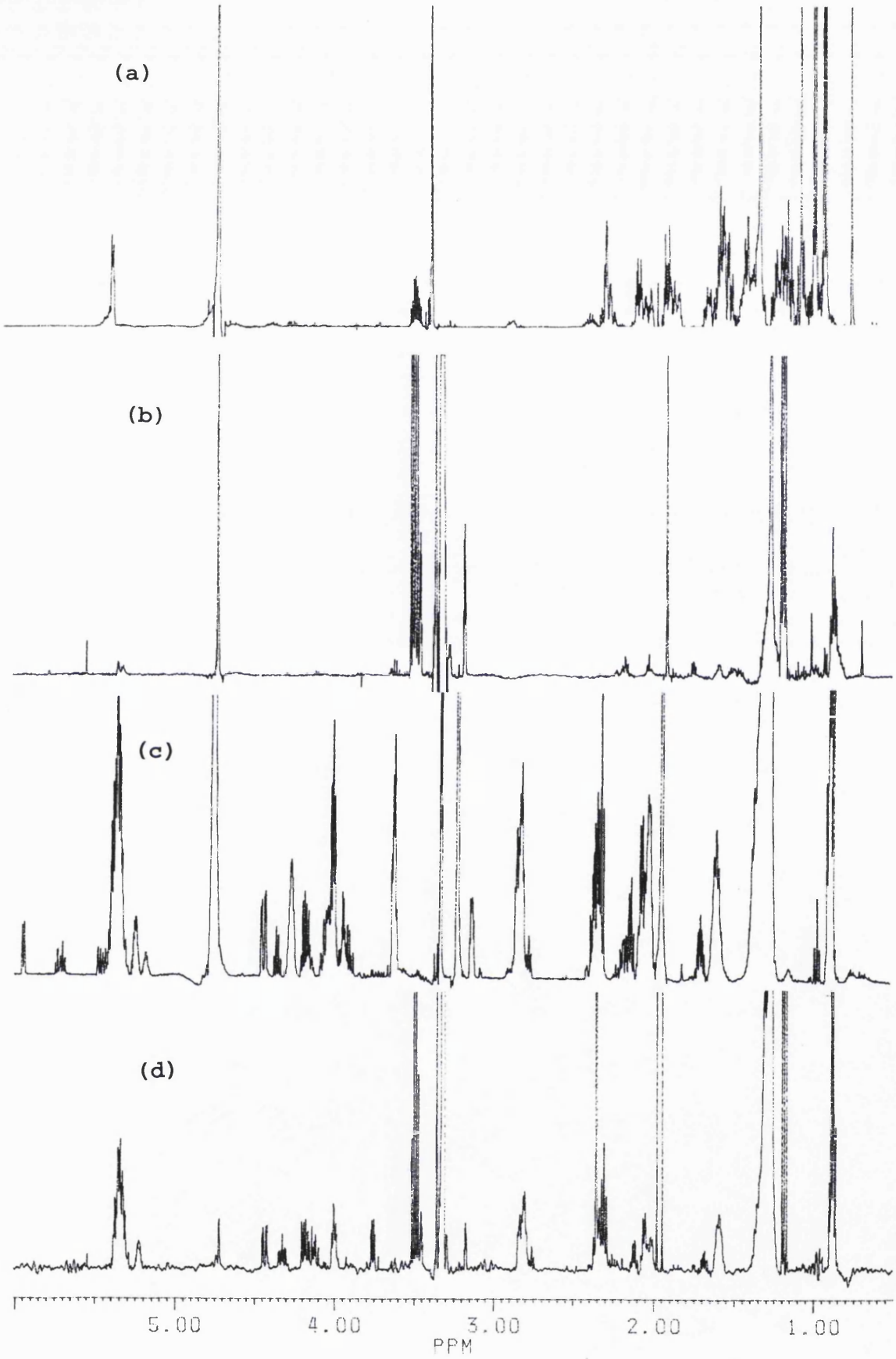


Fig. 20 Bond-Elut and nmr analysis of the various classes of platelet lipids (a) Neutral lipids and cholesterol (b) non-esterified fatty acids (c) non-acidic phospholipids (d) acidic phospholipids. The extraction and nmr procedures are described under Materials and Methods.

Table 12 quantitation of lipids from human blood platelets from nmr spectra of bond-elut fractions

Chemical Species	Chemical Shift (ppm)	Area Before Separation	Area After Separation*			% Recovery
			FRACTION A	FRACTION C	FRACTION D	
PC	3.20	0.690	-	0.483±0.003	-	70.0
PE	3.10	0.330	-	0.285±0.005	-	86.4
PS	3.75	0.220	-	-	0.202±0.002	91.8
PI	3.75	-	-	-	-	-
Cholesterol	0.68	0.465	0.433±0.004	-	-	93.1
Sphingomyelin	5.70	0.200	-	0.165±0.002	-	82.5
Plasmalogen	5.90	0.195	-	0.146±0.002	-	74.9
Triglycerides	4.30	-	-	-	-	-

* Areas are the average of duplicate fractionations.

Table 13 fatty acid analysis of nmr spectra of bond-elut fractions

Group Chemical Shift (ppm)	Areas Before Separation	Areas After Separation			Estimated % Recovery
		FRACTION A	FRACTION C	FRACTION D	
2.80	1.473	0.05	1.229	0.10	93.62
2.75	0.111	trace	0.104	0.012	104.5
2.40	0.036	-	0.0285	-	76.2
2.00	1.068	0.256	0.876	0.15	119.8
1.70	0.426	-	0.245	-	57.5
0.95	2.879	0.577	2.014	0.24	98.2

these fatty acids components. The relative estimates of the fatty acids of the Bond Elut fractions are shown in Table 13.

3.4 Nmr phospholipid analysis and signal transduction

Cellular functions and proliferation are frequently activated by interaction of extracelluar messengers with their specific cell-surface receptors, and the molecular basis of such signal transmission across the membrane has attracted great attention. A wide variety of agonists including muscarinic cholinergic and α-adrenergenic stimulators, chemo-attractants and growth factors, and many other biologically active substances have been shown repeatedly to provoke phospholipid turnover in their target tissues (Michell, 1975, 1979; Hawthorne & White, 1975).

3.4.1 Changes in membrane lipid composition in thrombin-stimulated platelets

The relative integral area ratios of the various lipids in thrombin-stimulated platelets with respect to unstimulated platelets are shown in Table 14. As observed from the Table, the most obvious changes in the integral ratios following stimulation of platelets with thrombin occurred in the total choline lipids and also in the arachidonic acid.

Table 14 relative integral area ratios of various lipids in thrombin-stimulated platelets*

Chemical Species	Chemical Shift (ppm)	Area Ratio Conc of Thrombin 2 units/ml	Area Ratio Conc of Thrombin 3 units/ml	Area Ratio Conc of Thrombin 5 units/ml
Cholesterol	0.68	0.97±0.07	0.97±0.05	1.03±0.06
Total Choline Lipids	3.20	0.92±0.05	0.79±0.06	0.79±0.05
Total Ethanolamine Lipids	3.10	0.94±0.03	0.82±0.06	1.0±0.01
Plasmalogen	5.92	1.0	0.83±0.01	1.04±0/05
Sphingomyelin	5.70	1.3±0.06	0.83±0.01	1.22±0.06
CH=CH	5.35	0.95±0.04	0.85±0.05	1.08±0.05
Arachidonic Acid	1.70	1.16±0.07	0.60±0.04	0.47±0.01
PS	3.75	0.84±0.03	0.86±0.02	0.84±0.02
PI	3.75	-	-	-
Linoleic Acid	2.75	1.0	0.83±0.04	1.03±0.01
Docosahexaenoic Acid	2.40	0.82±0.07	0.82±0.03	0.84±0.02
Total PUFA	2.80	1.0	0.97±0.04	1.04±0.06

* Ratios represent mean values of duplicate experiments.

(Platelet suspensions were incubated at 37°C and stimulated with various thrombin concentrations for 2 minutes.)

Choline lipids gave a relative integral ratio of 0.79:1 (79%) respective to those in control platelets. This was estimated to account for an overall reduction of 11.5±1.2% in choline lipids of platelets stimulated with 5 units/ml of thrombin and 11.7±2% in platelets stimulated with 3 units/ml of thrombin, values similar to the 12.9±4.6% reported by Bevers et al (1983). In both cases platelets were incubated in presence of thrombin for 2 minutes. However, the relative integral ratio of choline lipids stimulated with 2 units/ml thrombin (0.92) represented only a 4.1±1% reduction. The trend in the alteration in choline lipids following stimulation with various concentrations of thrombin is shown in Fig.21. The results indicated that considerable degradation of the plasma membrane choline phospholipids of platelets occurred upon stimulation with thrombin. The losses may reflect either increased hydrolysis of this class of lipids or decreased synthesis following stimulation with thrombin. The apparent losses could be due to the hydrolytic action of phospholipases (particularly phospholipase A_2) on phosphatidylcholine, resulting in the release of arachidonic acid. Indeed, this has been reported extensively in the literature. According to various investigators (Blackwell et al, 1977; Guichardant and Lagarde, 1980), when platelets are stimulated, part of the stimulus-secretion coupling mechanism involves the activation of a phospholipase A_2 activity, which could cleave arachidonic acid from different phospholipids. The released arachidonic acid

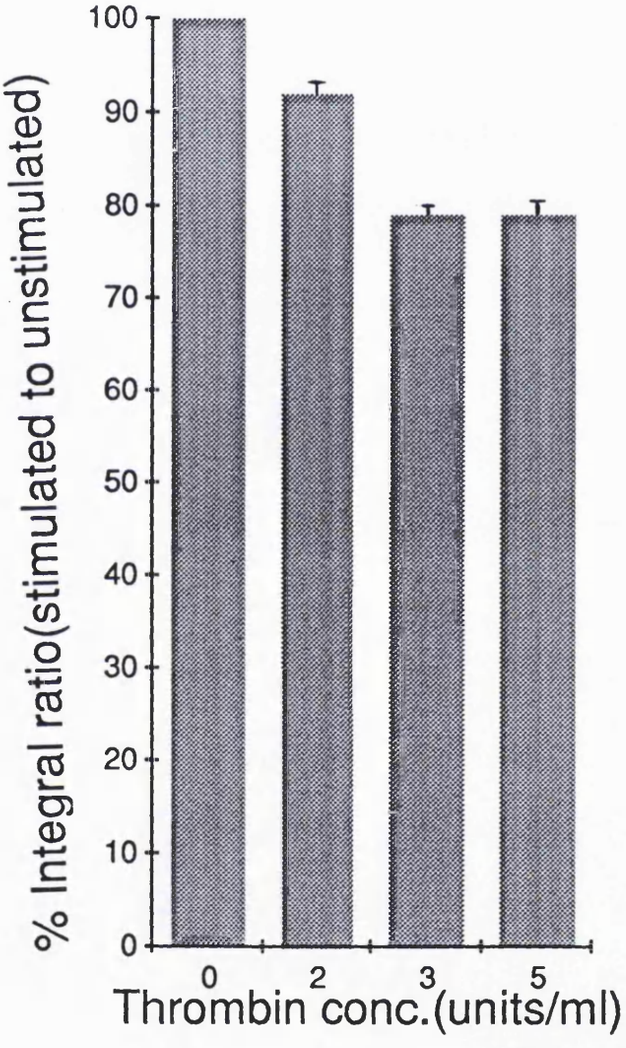


Fig. 21 Alterations in choline lipid levels following thrombin stimulation of platelets. Platelets were stimulated by 2, 3 and 5 units/ml of thrombin at 37°C for 2 minutes. The control sample contained no thrombin.

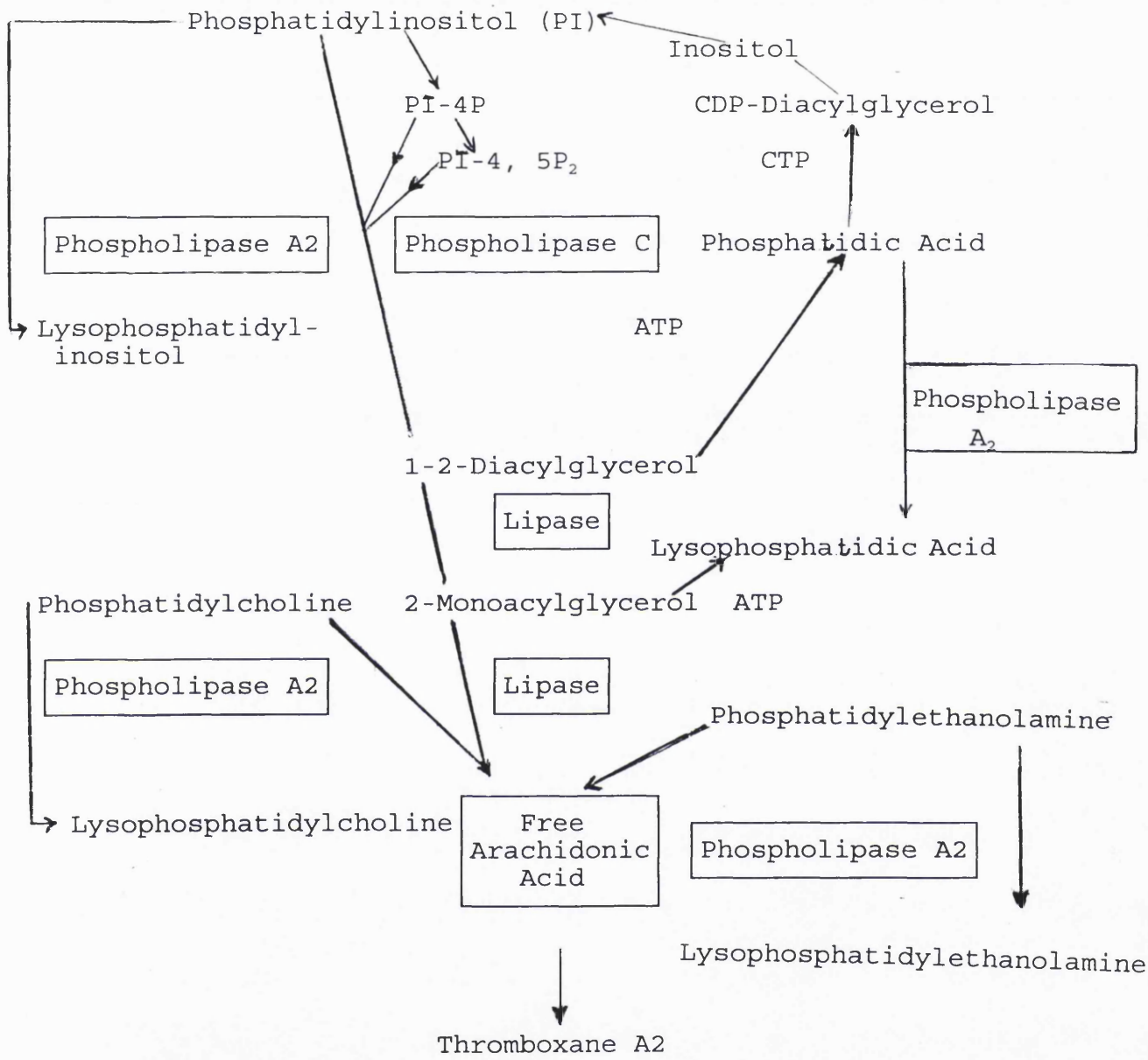


Fig. 22 **Summary of selected biochemical reactions implicated in the turnover of platelet phospholipids and the release of arachidonic acid in thrombin-stimulated platelets.**

becomes a substrate for cyclo-oxygenase in the synthesis of thromboxane (Fig.22). According to Majerus et al (1983), phosphatidylinositol (PI) loses 30 to 60% of its arachidonate, and phosphatidylcholine (PC) appears to lose from 0 to 10% of its arachidonate.

Mahadevappa et al (1983) observed that thrombin-dependent loss of radioactivity from [^{3}H] glycerol-labelled phosphatidylcholine amounted to 14% within 90s. The work of Bills et al (1977), which employed human platelets prelabelled with [^{14}C] oleic, [^{14}C] linoleic or [^{14}C] arachidonic acid, led to the conclusion that the 1-acyl 2-arachidonyl species of phosphatidylcholine is exclusively hydrolysed in stimulated platelets. However, it was not possible by this work at this stage to determine which molecular species of the platelet choline phospholipids was hydrolysed and by which phospholipase.

The absence of any lysophosphatidylcholine (LPC) signal in the nmr analysis could also indicate the loss of this class of lipid during the extraction stage in the soluble portion of the extract. Chemical analysis has revealed that an increase in the level of LPC (Broekman et al, 1980; McKean et al, 1981) enriched in palmitic acid and stearic acid occurs following exposure of human platelets to thrombin. The fact that no characteristic signal resonance of this lipid was observed could also indicate the hydrolysis of this lipid by lysophospholipase activity in the human platelets used in the experiment.

It should also be emphasised that the observed reduction in PC could also be due to the inhibitory effect of thrombin on the *de novo* synthesis of PC. Deykin (1973), observed this inhibitory effect on PC synthesis from labelled glycerol, which was further confirmed by Rittenhouse-Simmons et al (1977). In contrast, the very early entry (15s) of exogenous [^{14}C] arachidonic acid into platelet phosphatidylcholine (Holub et al, 1982) has been observed to be stimulated by thrombin.

Elsbach et al (1971) did not observe any influence of thrombin on the incorporation of exogenous [^{32}P] lysophosphatidylcholine into platelet [^{32}P]-phosphatidylcholine. From these observations, and also from the fact that the bulk of PC often reflects the composition preformed from the megakaryocyte in the bone marrow (Shick et al, 1981), it is quite unlikely that the nmr reductions in PC were due to the inhibitory effect of thrombin on its de novo synthesis.

The differences in the reduction of PC observed for the platelets stimulated with 2 units/ml (4.1%), 3 units/ml (11.7%) and 5 units/ml (11.5%) could reflect a thrombin-dependent concentration effect or incomplete stimulation, particularly in the case of that stimulated with 2 units/ml thrombin.

Since a very pure form of thrombin was used in the experiment, it is also quite unlikely that the observed changes were due to some substance other than thrombin. It has been previously reported that substances present in

crude thrombin preparations could elicit a pseudo-thrombin stimulation (Yin et al, 1968). It is, however, difficult to define from this work the exact mechanism for the PC degradation or to ascertain the proportion of this lipid which was hydrolysed to arachidonic acid.

The reduction in the integral area ratios of arachidonic acid was quite striking, but not unexpected. Platelets stimulated with 5 units/ml gave a massively reduced ratio of 0.47:1, corresponding to a 35 $\pm$ 1.2% reduction of arachidonic acid levels, whilst the platelets stimulated with 3 units/ml thrombin gave a ratio of 0.60:1, corresponding to 25 $\pm$ 1.1%. These results suggested a rapid mobilization of arachidonic acid during the platelet stimulation. It is quite interesting to note the rate at which arachidonic acid was utilized. Majerus et al (1983), noted that, within a matter of seconds, the process (arachidonic acid utilization) is several fold faster at 37°C, and no free arachidonate can be detected under these conditions when platelets from an aspirin-free donor were used. The fact that after 2 minutes some free arachidonate was still detected in the nmr analysis during these experiments might indicate that stimulation in platelets was either not complete, or possibly reflected by the fact that not all the platelets were functional at the time of the experiment, even though all the platelets were obtained from aspirin-free donors. The degree of arachidonate utilization might thus reflect the extent of the stimulation.

The metabolism of arachidonic acid is of particular importance, since a novel class of prostaglandins (PG) formed in platelets from arachidonic acid plays a central role in platelet function. Smith and Willis (1971) demonstrated that thrombin-aggregated platelets form and release PGE_2 and $F_{2\alpha}$ and further showed that aspirin inhibits PG formation and release. As already discussed, the arachidonic acid released from phospholipids is rapidly metabolised via the cyclooxygenase pathway into prostaglandin G_2 (PGG_2), a cyclic endoperoxide that has a half-life of less than 5 minutes (Fig.2). PGG_2 is a powerful initiator of platelet aggregation, but it is further transformed to an even more potent aggregating agent, thromboxane A_2. This oxane compound is very unstable and persists for less than 40s in plasma before decomposing to a stable, inert compound thromboxane B_2. In addition to transformation by cyclooxygenase, arachidonic acid is also metabolised by a separate enzyme, platelet lipoxygenase, which converts arachidonic acid into 12L-hydroxy-5,8,10,14-eicosatetraenoic acid, or HETE. This compound has no apparent effect on platelet function, but it has been shown to be a chemoattractant for human polymorphonuclear leukocytes *in vitro* (Turner et al, 1975). Hamberg and co-workers (1974b) have reported that aspirin and indomethacin inhibit platelet cyclooxygenase, but HETE formation is actually enhanced in aspirin-treated platelets. The existence of two pathways of arachidonic acid has been established (Russell and Deykin, 1976).

Since this experiment was not designed to study the arachidonic acid metabolic pathways, it was quite difficult to ascertain the levels of arachidonate metabolites that were derived from the utilized arachidonic acid observed in this experiment; besides it could also not be established which cellular phospholipids liberated the arachidonate on stimulation with thrombin. It is, however, interesting to note that no PI was detected at all which could indicate its complete hydrolysis via a combination of phospholipase C, phospholipase A_2 and PI Kinase in the stimulated platelets. The trend of the alterations in arachidonic acid levels following stimulation with thrombin is shown in Fig.23, whilst Fig.24 illustrates the overall trend among the various chemical species analysed by the proton nmr, following stimulation with 5 units/ml thrombin. The small changes in the integral ratios of total ethanolaminelipids (Table 14) make it difficult to discern whether there was any susceptibility of this class of lipid towards phospholipase action in the thrombin-stimulated platelets.

3.4.2 Changes in membrane lipid composition of ADP-stimulated platelets

The nmr integral area ratios of the various lipid species of ADP-stimulated platelets, relative to unstimulated platelets, are shown on Table 15. The data indicate a reduction in proportion of PE at various ADP concentra-

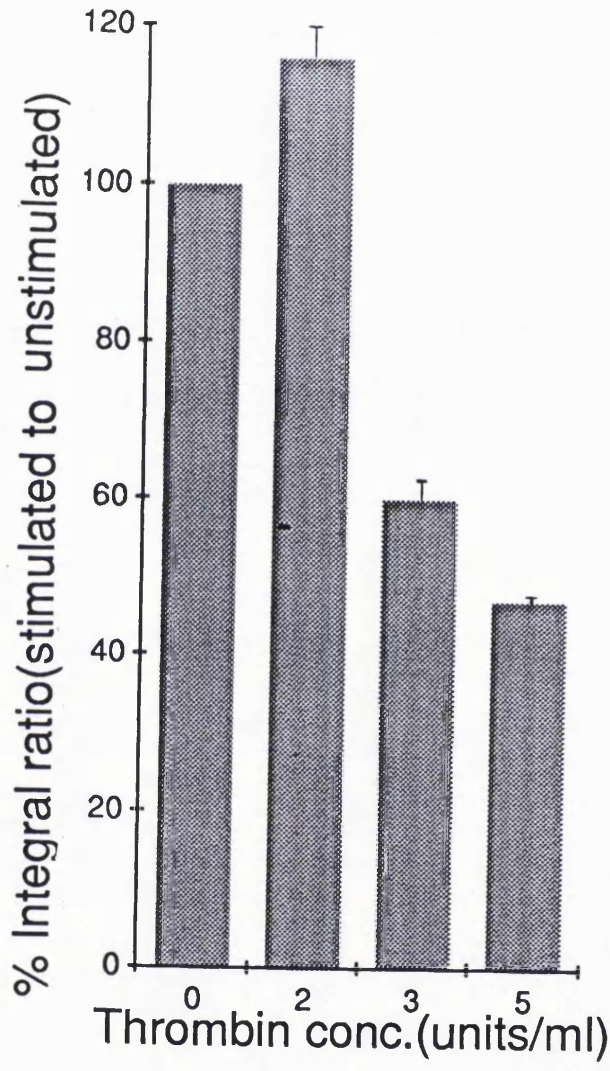


Fig. 23 Alterations in arachidonic acid composition during platelet stimulation with various concentrations of thrombin. Platelet suspensions (protein conc. 2.5mg/ml) was incubated at 37°C for 2 minutes and stimulated with various thrombin concentrations as indicated. Lipids were extracted after incubation had been terminated and subsequently analysed by nmr as described under Materials and Methods.

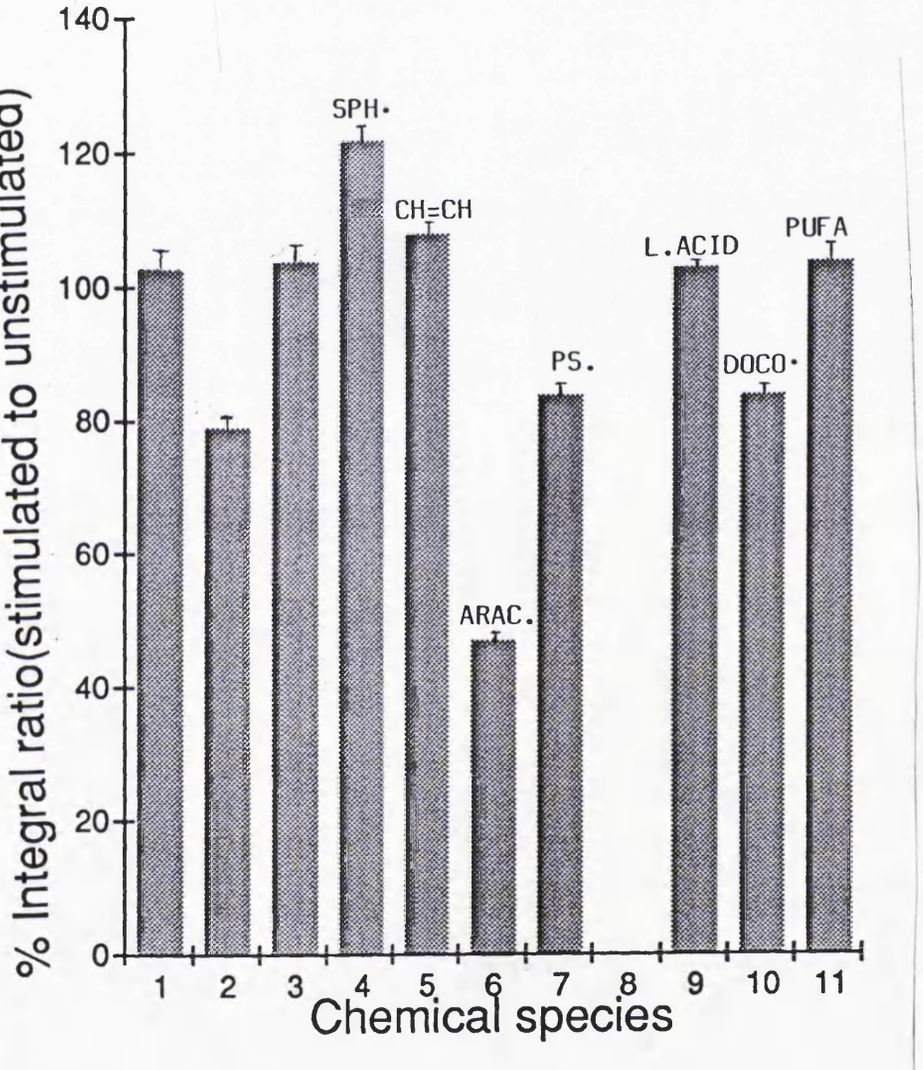


Fig. 24 Patterns of platelet lipid composition upon stimulation with 5 units/ml thrombin at 37°C. Lipids were extracted in chloroform:methanol (2:1 v/v), dried and 1-D proton nmr spectra recorded in Fourier transformation mode after re-suspension in D-6ml of CD_3oD:$CDCl_3$ (2:1, v/v). Errors were mainly due to variations during sample preparations and lipid extraction.

Table 15 Relative integral area ratios of various lipids in adp-stimulated platelets with respect to those of untreated platelets

Chemical Species	Chemical Shift (ppm) Integral	Area Ratio Conc of ADP 5μM	Area Ratio Conc of ADP 15μM	Area Ratio Conc of ADP 20μM	Area Ratio Conc of ADP 25μM
Cholesterol	0.68 1.24	1.0 ±0.03	0.91±0.04	0.91±0.02	0.90 ±0.02
Total Choline Lipids	3.20 1.84	0.91±0.04	0.85±0.01	0.82±0.02	0.89 ±0.03
Total Ethanolamine Lipids	3.10 0.31	0.75±0.01	0.72±0.02	0.74±0.01	0.76 ±0.03
Plasmalogen	5.92 0.44	1.0 ±0.06	0.82±0.02	0.92±0.02	0.647±0.03
Sphingomyelin	5.70 0.42	0.80±0.02	0.72±0.01	0.84±0.04	1.0 ±0.04
CH=CH	5.35 6.55	0.86±0.02	0.82±0.03	0.70±0.01	0.87 ±0.02
Aracidonic Acid	1.70 1.27	0.8 ±0.01	0.84±0.01	0.86±0.02	0.90 ±0.03
PS	3.75 0.27	1.2 ±0.08	0.97±0.01	0.94±0.02	0.94 ±0.01
PI	3.75 -	-			
Linoleic Acid	2.75 0.33	1.1 ±0.05	1.05±0.04	1.01±0.06	1.02 ±0.03
Docosahexaenoic Acid	2.40 0.076	1.1 ±0.04	1.0 ±0.02	1.1 ±0.05	0.8 ±0.06
Total PUFA	2.80 3.83	0.79±0.01	0.74±0.02	0.72±0.02	0.8 ±0.01

*Platelet suspensions incubated at 37°C and stimulated with various concentrations of ADP. Extracted lipids were analysed by proton nmr in the 1-dimensional mode. (Nmr spectra of platelet lipids as a function of ADP concentrations are shown in the appendix.)

tions. The average integral ratio was 0.75:1 corresponding to an overall reduction of 14.32 ± 1.7% in PE levels.

The results also indicated a reduction in the total PUFA content (0.76), estimated to be a 13.5 ± 2.2% reduction, whilst the arachidonic acid levels showed a comparatively lower reduction (8.14 ± 2%) compared to a massive average fall of 30% in the thrombin-stimulated platelets.

It seems from these results that PE was the chemical species which underwent greatest hydrolysis, whilst the fall in PUFA seems somewhat to decrease in parallel with PE.

The concentration dependent effect of ADP was not pronounced as reflected by the area ratio of various ADP concentration. Indeed Haslam (1964) had previously reported that addition of external ADP causes appreciable aggregation only at concentrations of 50μM or above and the concentration range employed in this work was between 5-25μM.

The degree of stimulus-linked decrease in the amount of PE, as reflected by the ratios, could be via the phospholipase activity to give rise to free arachidonic acid. By means of quantitative analyses Broekman et al (1980) have observed the enhanced release of lysophosphatidylethanol-amine within 15s following thrombin addition to platelets. Fatty acid and aldehyde analyses of the "lyso" derivative showed the presence of a considerable amount of stearic acid, which indicated that the hydrolysis was reflective of phospholipase A_2 activity, with a preference for diacyl

rather than plasmogenic phosphatidylethanolamine. The nmr analysis of platelet membrane PE identified these two forms of PE, namely the diacyl and plasmogenic (ether) forms, distinguished by their glycerol Sn_2 proton resonances at 5.23ppm (diacyl) and 5.15ppm (plasmogenic). The fact that the integral ratio of plasmalogen at 5.92 showed no change, suggested that the diacyl rather than the plasmogenic PE might have been the preferred hydrolytic species for phospholipase activity.

Mahadevappa et al (1983) observed a percentage loss (thrombin-dependent) of radioactivity from [^{3}H] glycerol-labelled PE of <5% in 90s as compared to 14% and approximately 50% in the case of [^{3}H] phosphatidycholine and [^{3}H] phosphatidylinositol, respectively. This seems to agree with the results from this experiment in which virtually no reduction was observed in PE in the thrombin-stimulated platelets. However, when ADP was used as agonist PE becomes rather more susceptible to hydrolysis. This seems to be a direct influence of the type of agonist. This difference in availability of phospholipids for phospholipase action in platelets activated with thrombin or ADP may reflect differences in the structural organisation of these lipids in the membrane towards the agonist. From the data obtained, however, it is difficult to establish this view or to ascertain whether the exposure of these phospholipids resulted in significant organisational changes of increased phospholipase action upon activation. The mechanism underlying this "substrate-

switch" upon stimulation with the agonist remains uncertain.

The decline in PE in the ADP-stimulated platelets could also be a reflection of ADP inhibition on PE *de novo* biosynthesis. Lewis and Majerus (1960) and Rittenhouse-Simmons et al (1977) have reported that the exposure of human platelets to thrombin inhibits the entry of the de novo precursor, radiolabelled glycerol, into phosphatidyl-ethanolamine. Deykin (1973) had earlier compared the effects, on platelet lipid synthesis, of aggregation induced by thrombin and poly-1-lysines and observed that incorporation of glycerol into all classes of phospholipids was inhibited by both agents, thus suggesting that the impaired phospholipid synthesis was not a thrombin-mediated event, but rather reflected the consequences of aggregation itself. This makes it difficult to conclude whether such an effect existed or not in the ADP-stimulated platelets. It would appear, however, that the degradation of PC and PE are both important pathways for the release of arachidonic acid, in addition to the breakdown of PI (by phospholipase A_2 activity directly) and by phospholipase C activity on PI or its phosphorylated derivatives, combined with diacyl glycerol lipase-monoacyl glycerol lipase activity and (or) diacyl glycerol kinase-phospholipase A_2 activity via deacylation. The available information does not yet, however, confirm this. On the other hand, it cannot be excluded that during stimulation specific phospholipids are

indeed degraded over the time course of incubation with the agonists.

Rittenhouse-Simmons et al (1977) have reported a limited loss of [^{3}H] arachidonic acid from diacyl phosphatidyl ethanolamine in response to thrombin, which could explain why a comparatively lower (8.14 $\pm$ 2.0%) fraction of arachidonic acid was lost. The observed reduction may thus reflect the extent of its release from PE and hence the degree of its hydrolysis. Previous nmr analysis has indicated that PE was enriched in arachidonic acid more than the other phospholipids in platelets. It seems reasonable, however, from these observations to suggest that the nature of agonist and type of cellular phospholipid can influence the degree of release and utilization of arachidonic acid.

It is also quite possible that some form of transbilayer movement of phospholipids did occur during the aggregation. Although the exact mechanism of such movements cannot be deduced from this work, alterations in the phospholipid metabolism could nevertheless, represent early and important biochemical events in the response of human platelets to agonist stimulation as previously suggested by other investigators (Lupin et al, 1986; Zwaal and Bevers, 1989). It is increasingly apparent that further research in this area (using nmr) is needed to consider the subcellular localisation for these membrane-mediated processes, and elucidate the involvement of individual molecular species of various phospholipids in them, for a

better physiological understanding of these changes as far as the role of phospholipids is concerned in platelet function.

CONCLUSION

The present study demonstrates that the analysis of cellular lipids can be achieved quite rapidly and comprehensively and just as accurately by using proton nmr in combination with other previously used methods.

The overall phospholipid composition of platelet membranes by the nmr analysis amounted to 65.4±3%. The rest, mainly cholesterol, making up 34.8%.

The nmr analysis showed that PC constituted the major class of platelet membrane phospholipid making up 28.2±2.3%, followed by PE (19.5±0.9%) and PS also accounting for 6.92±1.5% of the total. These values were highly reproducible and comparable to those reported by Mahadevapa and Holub (1982) and Safrit et al (1971). The choline phospholipids was mainly in the diacyl form with a relatively lower degree of unsaturation index (0.66) compared with the unsaturation index of 1.2 of ethanolamine phospholipids.

The nmr approach facilitated the simultaneous quantitative analysis of cholesterol (the only steroid detected) which allowed the estimation of the cholesterol:phospholipid ratio, a value significant in the diagnosis of type II

hyper-proteinemia. A value of 0.53:1 obtained was highly comparable to the 0.5:1 value reported by Marcus et al (1969).

Unsaturated fatty acyl compounds, mainly linoleic, arachidonic and docosahexaenoic acid, were the main PUFAs detected, amounting to a total of nearly 20% of the total fatty acid chains present in platelet membranes.

A combination of other methods with nmr permitted a more detailed analysis of the individual class of phospholipids. Thus, for example, the nmr analysis of the choline hplc fraction established that only phosphocholines that were conjugated to diacylglycerols were present. The presence of two main types of choline phospholipids, namely phosphatidylcholine and sphingomyelin in platelet membranes were confirmed purely by the nmr approach. The fatty acid composition of the majority of lipids were also determined. In PC, linoleic acid (C:2) makes up 12.1% of the total acid chain while α-linoleic acid (C:3) makes up 11.95%. Arachidonic accounted for 9.3% of the total fatty acid chain in PC.

In PE, the nmr analysis of the hplc fraction indicated that this class of phospholipid existed in the diacyl and ether forms in platelet membranes. The diacyl made up 38.8% while the ether form made up 61.2%. The level of arachidonic acid in PE was estimated at 29.81, almost three times as high as that in PC (9.8%).

The nmr analysis of the sphingolipids in the hplc fraction indicated that this class of lipid occurred predominantly as the sphingenine analogue in platelet membranes.

The combination of nmr and the Bond-elut method also facilitated the separation of platelet membrane lipids individually into natural lipids, non-esterified fatty acids, non-acidic phospholipids and acidic phospholipids, with a high degree of efficiency. Recovery values between 70-93% were achieved (Table 12). The nmr/Bond-elut approach provided a great deal of information on the fatty acid region of the non-acidic phospholipids. For example, docosahexanoic and related acids were observed to have a distinctive w-methyl triplet at 2.40ppm; arachidonic and related acids have their characteristic ß and methylene resonances at 2.20ppm and 1.70ppm, respectively, and linoleic acids gave alkylic CH_2 resonances at 2.75ppm. These structure-specific resonances permitted accurate quantification of these fatty acid components. Although the Bond-elut fractionation was adopted to facilitate the separation of the acidic phospholipids, namely CL (cardiolipin), PS (phoshatidylserine), PA (phosphatidic acid), PI (phosphatidylinositol) and PG (phosphoglycerol), great difficult was encountered in adequate qualitative and quantitative analysis of most of these lipids. It is not certain whether this was an inherent drawback of the nmr approach; however the platelet content of these acidic phospholipids is significantly very low (Mahadevapa and Holub, 1982).

The usefulness of the nmr methodology as an investigative diagnostic tool was reinforced by the results obtained from the lipid analysis of three (3) cardiac patients. Compared to those of normal individuals, the striking features observed included elevated cholesterol between 43-56 mol % (normal individuals value obtained was 34.8±3 mol %), a decrease in the arachidonic fraction in all patients (1.6-4.8 mol %) compared to the 12 mol % obtained for normal individuals. Despite the fact that the sample size of study was very small (a problem due to great difficulty of getting access to such patients for research purposes), the pathological and diagnostic significance of these results cannot be over-emphasised. Hypercholesterolemia with an associated excess low density lipoprotein and disturbances in platelet function are among the risk factors of coronary artery disease (Kaplan and Pesce, 1989).

It was also not possible by this nmr approach to investigate phospholipid metabolism in terms of measuring metabolic levels or fluxes associated with platelet aggregation following stimulation with thrombin and ADP at 37°C. The qualitative changes of various classes of lipids was accomplished by the estimation of their intensities at their characteristic chemical shifts using deuterated chloroform, as the external platelets are platelets activated with various concentrations of thrombin and ADP displayed changes in the composition of specific phospholipids. In particular, the choline and ethanolamine phospholipids estimated from their N-methyl ($-CH_3$) and

methylene proton ($-CH_2\underline{C}H_2NH_2$) resonance at 3.20ppm and 3.10ppm, respectively, showed reductions averaging between 10-15% following thrombin and ADP stimulation. These nmr results suggest a correlation between mobilization of these classes of lipids and platelet aggregation, and also confirm that specific choline and ethanolamine lipids were hydrolysed by these agonists via phospholipase(s) action. At this stage, however, it could not be concluded whether the mobilization resulted from phospholipase A, C, D or whether the lipids were either saturated or unsaturated. It should now be also possible with the data obtained from this work and the methodologies adopted to study lipid cellular metabolism in platelets for application in solving medical problems involving platelets.

CHAPTER 4

PLATELET PHOSPHOLIPID-N-METHYLATION

4.1 Phospholipid-N-methylation in crude platelet membrane preparations

When a crude homogenate of platelets was incubated with S-adenosyl-L-[methyl-^{3}H] methionine in 25mM Tris buffer containing 0.5% triton X100, a significant incorporation of ^{3}H-methyl groups into the membranes was observed. The methylated products were examined by thin-layer chromatography of chloroform-methanol extracts as described under Materials and Methods. The spots, as visualized by spraying with 0.06% Rhodamine 6G solution, are shown in Fig.25. Each profile of interest was determined by serial scrapes of the plate. Distinct peaks of radioactivity associated with PME, PDE and PC were expressed as a percentage of the total incorporated radioactivity. The peaks of the radioactivity corresponded with those of authentic phospholipid standards. The percentage incorporation of ^{3}H-methyl group into each phospholipid at various incubation times is shown in Fig.26.

The results indicated that the crude platelet membrane extracts incubated with S-adenosyl-L-[methyl-^{3}H] methionine synthesized three lipid extractable products; PME, PDE and PC. The incorporation of methyl groups into all the three lipids was essentially linear for the first 30 minutes at 37°C, with the greatest radioactivity occurring in the monomethylated product (over 60%), followed by PDE (ca 30%) and PC (under 10%). After 45 minutes of incubation the rate of incorporation of methyl groups into PME had fallen to 46%, whilst PDE had increased to 49%, with PC showing

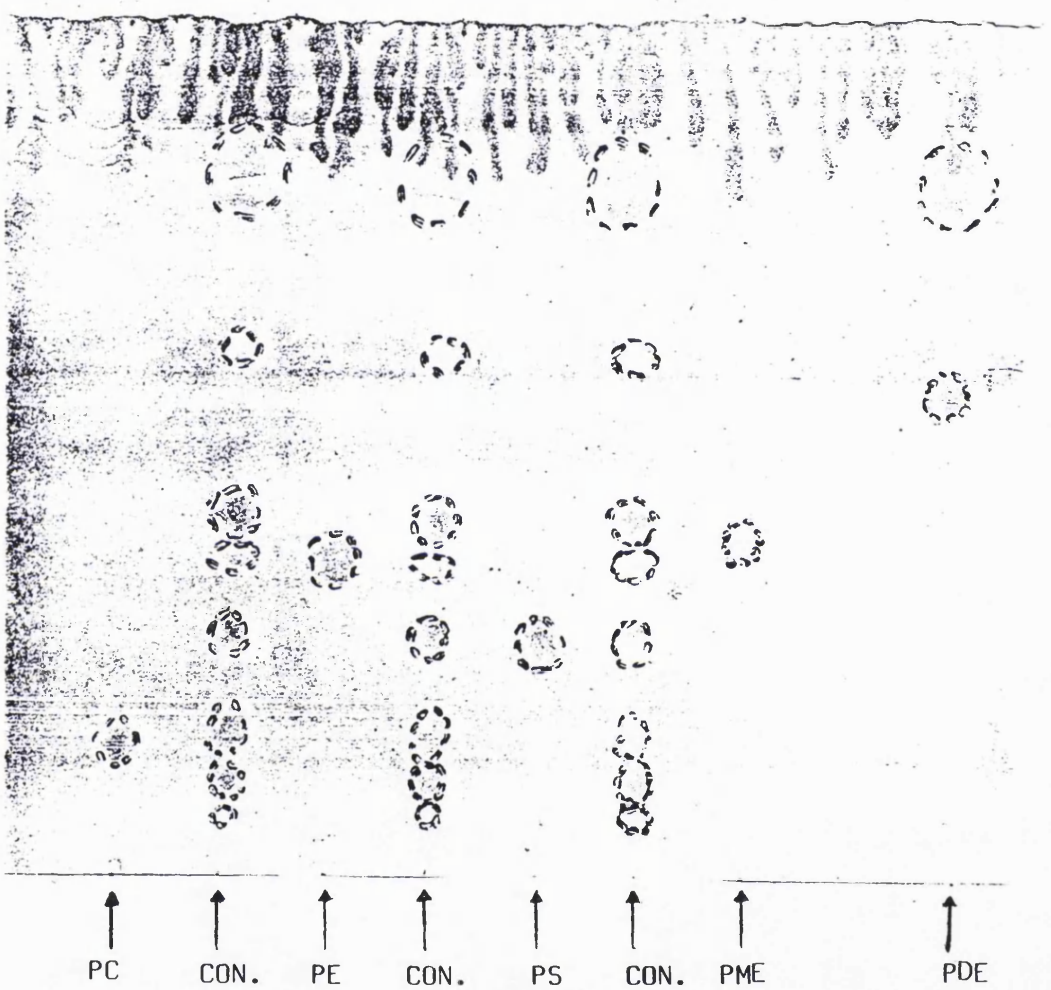


Fig. 25 Tlc identification of methylated products.

An aliquots of extracted platelet lipids in $CHCl_3$:CH_3OH (2:1, V/V) was spotted onto a TLC plate and developed in a solvent system comprising chloroform:ethanol:triethylamine:water (4:5:4:1) and visualised by staining with Rhodamine G.

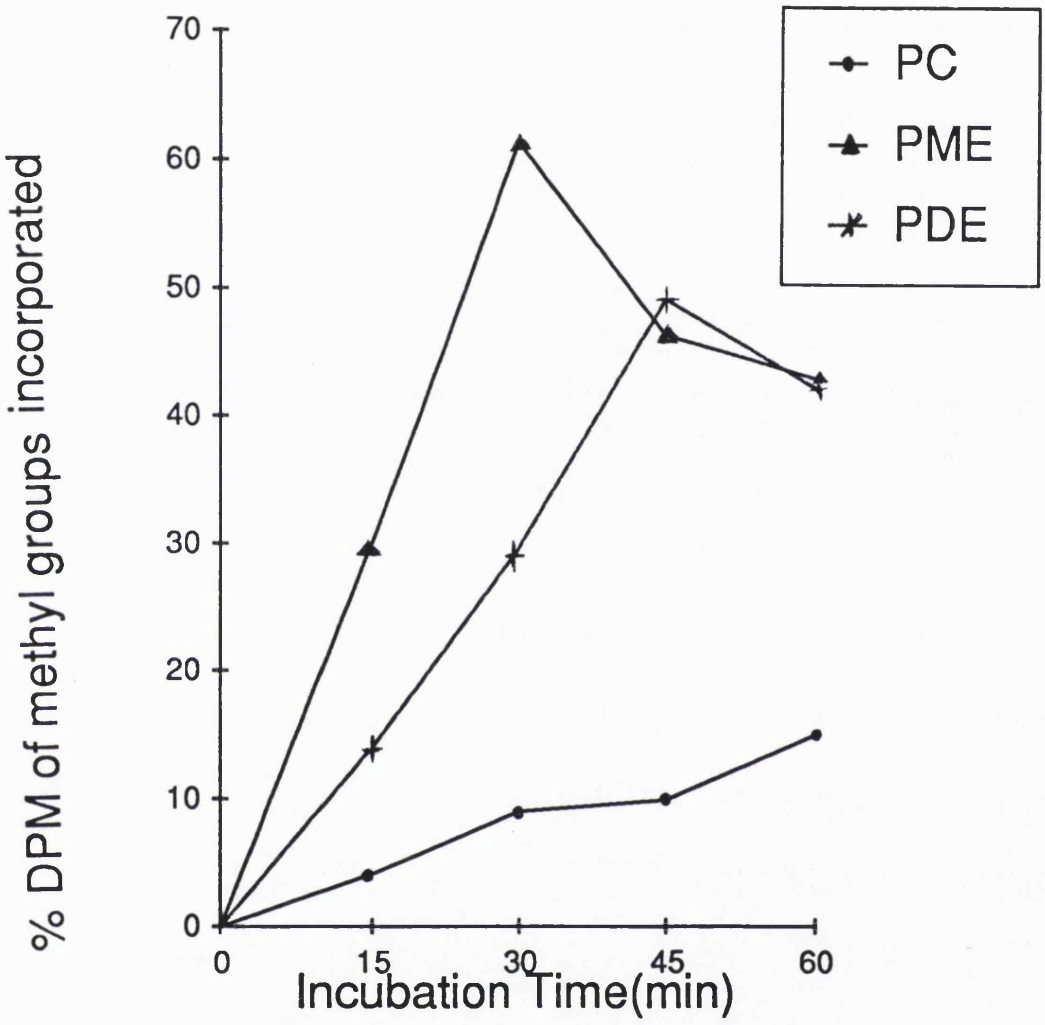


Fig. 26 Percentage incorporation of ^{3}H-methyl groups into phospholipids by crude platelet membrane preparations. Membrane extracts (1.0mg protein) were incubated at 37°C at various time intervals in the presence of 50mM Tris-HCl buffer (pH 7.4), containing 0.5% Triton-X100, 200μM of ^{3}[H]-SAM and 20μg each of exogenous PME and PE in a total volume of 400μl. The incubation was stopped by the addition 0f 2ml $CHCl_3$:CH_3OH:2HCl (6:3:1). The extraction and separation were performed as described under "Materials and Methods". The incorporation of the radioactivity into PC, PME and PDE is shown.

just a slight increase of incorporation; however, after one hour of incubation, PC was the only methylated product showing an increased incorporation of methyl groups (15%), with PME and PDE showing a fall to about 42% each. Nevertheless, the overall incorporation of methyl groups was higher in the monomethylated product, followed by the di-methylated and finally PC. The subsequent changes after 30 minutes of incubation may reflect the interconversion of one phospholipid form to the other; however, it is not clear from our experiments whether this reflects a direct effect on enzyme kinetics or subcellular distribution of the radioactive products in the crude membrane preparations.

Cordasco et al (1981) have reported that, in platelets, each of the three products of phospholipid methylation is evenly distributed between the cytoplasmic and the extracellular membrane surfaces, and that ^{3}H-labelled PME, PDE and PC do not exhibit the sidedness found in erythrocytes (Hirata and Axelrod, 1978), and synaptosomes (Crews et al, 1980), where ^{3}H-PME is predominantly localized on the cytoplasmic side of the membrane and ^{3}H-PDE and ^{3}H-PC are on the exterior surface of the membrane. Hirata and Axelrod (1978) have postulated that this asymmetric product distribution is established by a flip-flop mechanism whereby ^{3}H-PME, synthesized on the inner membrane surface, is translocated through the membrane and methylated to ^{3}H-PDE and ^{3}H-PC on the outer membrane surface.

Although the results did not necessarily establish whether a single enzyme or multiple enzymes caused the observed methylation, they did demonstrate that the enzyme(s) system present in the crude preparation was capable of synthesizing PC from PE via the successive methylation pathway. Data have been published which indicate that two N-methyltransferases are responsible for the methylation (Scarborough and Nyc, 1967, Crews et al, 1980).

Unstimulated platelets are capable of renewing their pool of phosphatidylcholine by at least three routes: (1) de novo synthesis from glycerol, free fatty acids and choline (Lewis and Majerus, 1969, Williams and Call, 1972 and Deykin and Desser, 1968); (2) reacylation of lysophosphatidylcholine (Elsbach et al, 1971); (3) methylation of phosphatidylethanolamine (Shattil et al, 1981). Comparative data from various studies have, however, indicated that in platelets the N-methylation pathway for phosphatidycholine synthesis is a quantitatively minor one. This therefore may explain the comparatively lower levels of ^{3}H-methyl group incorporation in the PC component in this experiment.

Although the exact requirements for renewal of PC during the platelet's lifespan *in vivo* are unknown, the maintenance of membrane composition would be expected to preserve the functional integrity of platelet membranes, as well as preserving membrane fluidity. Perturbations of membrane lipid fluidity are known to affect the function of platelet membranes (Shattil and Cooper, 1978).

The conditions of assay employed in these experiments were similar to those reported elsewhere in the literature; however, like many others (Hotchkiss et al, 1981), our preparation did not show any dependence on Mg^{2+} for activity, nor could the requirement for any other co-factor be established.

4.2 Phospholipid-N-methylation of purified platelet methyltransferase

The schematic flow-chart in Fig.27 shows how the purified platelet plasma membrane enzyme was obtained. Phospholipid-N-methyltransferase activity in the purified platelet fractions was assayed in a similar way as for the crude fraction preparations, in the presence of 0.5 mg/ml exogenous PME/PE mixture and 200μM of cold SAM (S-adenosyl methionine). Control experiments were run concurrently without purified membrane extracts. Phosphate or Tris buffer systems containing either 0.3% chaps or 0.5% Triton X-100 were used in the assay.

The ^{3}H-methylated products under formed in the phosphate or tris buffer systems were analyzed by the Berthold tlc radio label scanner. As shown on Fig.28a, the major phospholipid into which methyl groups were incorporated was PME, which

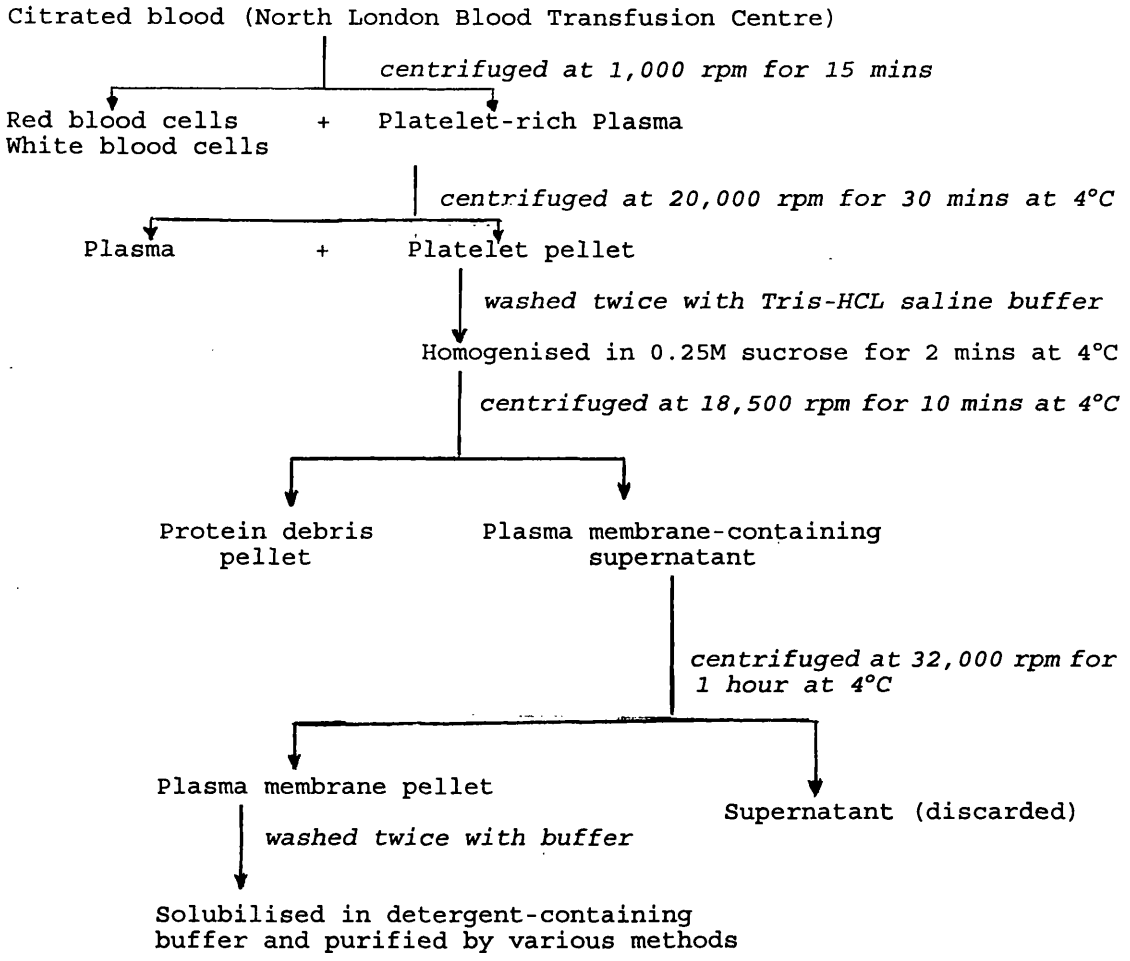


Fig.27 **Schematic flow-chart for the preparation and purification of platelet plasma membranes**

accounted for over 90% of the total methylated products with less than 10% representing PDE. PC, which is the final product of the methylation process, was not detected by the scanner (PC usually travels at a distance of 4cm on the tlc plate).

Even though PC formation via the N-methylation pathway has been known to be quantitatively a minor one, particularly in platelets (Shattil and Cooper, 1978), it was expected that a purified fraction would have enhanced PC formation compared to the crude membrane preparations. Various explanations could account for this surprising but not unexpected result. It should be mentioned that the tlc radioactive scanning procedure used in the identification of the methylated products has a very low sensitivity (less than 3% of total radioactive counts); thus low PC formation could probably not be detected. The fact that a radioactive peak was observed in scraped portions of PC, when these were counted in a scintillation cocktail in the crude preparations, makes the above explanation more convincing.

Hotchkiss et al (1981) reported that the distribution of the methylated products is dependent on the SAM concentration. Using platelet membrane fragments, they observed that at low SAM concentration (2μM), the methyl group was predominantly incorporated into phosphatidyl-monomethylethanolamine (PME), whereas at high concentration of SAM (200μM), phosphatidylcholine was the major product.

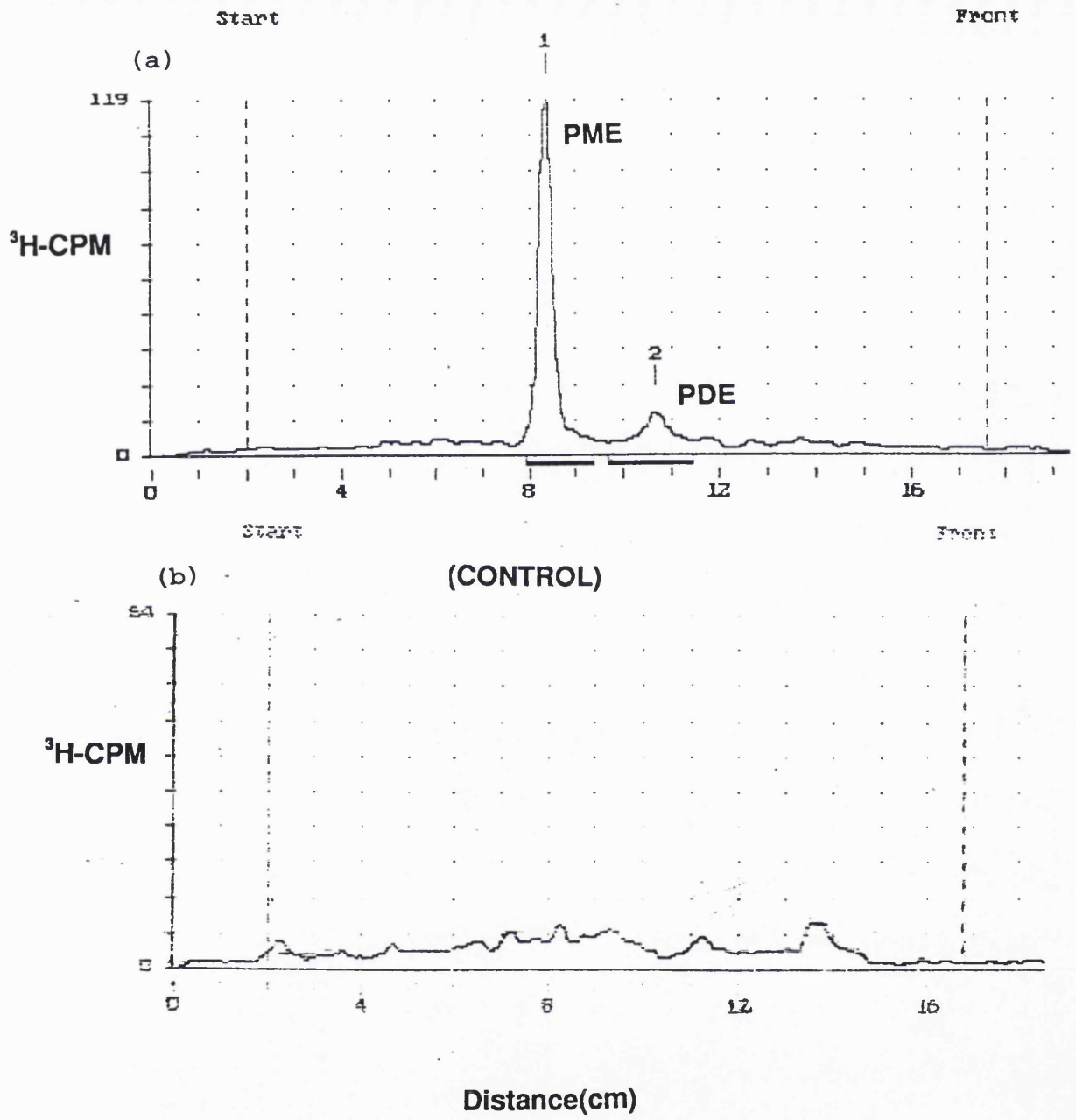


Fig. 28 (a) ^{3}H-methyl group incorporation into phospholipids by purified platelet methyltransferase. Purified platelet membrane extracts were incubated at 37°C for 1 hour in the presence of 20m0 pH phosphate buffer (pH 8.0),containing 0.3% chaps, 20μM each of exogenous PME and PE and 2μCi^{3}H-SAM. The reaction was terminated by the addition of $CHCl_3$:CH_3OH:2HCl (6:3:1, V/V). The radioactivity in the final extracted lipids as described under "Materials and Methods" was scanned on a Berthold Tracemaster Model 20.

(b) Control.

It was therefore surprising that using a SAM concentration of 200μM and similar assay conditions (pH and temperature) no PC was detected. Based on their work, they suggested that two enzymes may be involved in the three successive methylation steps of PE to PC in platelets. It is thus quite possible that the second enzyme system which is responsible for the methylation of PME to PC might have lost activity or been lost entirely during the various purification stages.

Hirata and Axelrod (1978) have proposed that the two enzymes and their substrates are asymmetrically distributed in the membranes; the first methyltransferase and phosphatidylethanolamine, according to Hirata and Axelrod (1978), are localized on the cytoplasmic side, whereas the second methyltransferase and phosphatidycholine are on the exterior surface of the membrane. It is therefore most probable that such spatial segregation of the methyltransferases could result in one of them having been lost during any purification procedure, as already asserted.

Another possible cause for the undetectable levels of PC could be the absence of certain co-factor(s) in the assay medium. Phospholipid methyltransferase activity in beef adrenal microsomes has been shown to have a strong Mg^{2+} dependence, specifically for the formation of PME (Hirata et al, 1978a); however, like Hotchkiss and associates (1981), Mg^{2+} dependence in the purified platelet extracts could not be demonstrated in buffer containing this cation. Moreover, Mori et al (1982), demonstrated that most

divalent cations including Ca^{2+}, Cu^{2+}, Mg^{2+}, Mn^{2+}, Ni^{2+}, Co^{2+} (conc 0.2mM) had an inhibitory effect on methylation in platelets. It is therefore apparent from these investigations, that lack of co-factor could not be the primary cause of the incomplete methylation to PC.

The hydrolytic action of phospholipases (particularly phospholipase A_2) on PC that might have been formed could also not be ruled out as a possible cause. The fact, however, that no lysophosphatidylcholine nor its derivatives were detected, makes this assertion very remote.

The physiological role of this intermediate (PME) in platelet function is still unknown, although it has been demonstrated that it is capable of increasing the fluidity of rat red blood cell membrane lipids (Hirata and Axelrod, 1978). Whether maintenance of a trace of this lipid intermediate might play a role in maintaining the function of platelet membranes or their fluidity within a narrow concentration range, remains to be elucidated.

In another study, Hirata et al (1979) also suggested that the accumulation of PME within the membrane, resulting in a decrease of membrane viscosity, should make possible a more rapid lateral movement of a ß-adrenergic receptor-isoproterenol complex to facilitate adenylate cyclase coupling in rat recticulocyte ghosts. It is not certain, however, whether a similar function of this intermediate does exist in platelets.

4.2.1 HPLC product identification of phospholipid-N-methylation of purified platelet methyltransferase

The authentic identity of the methylated product was further confirmed by running a chloroform-methanol extract of the product on a high performance liquid chromatography (HPLC) column. The procedure is described under Materials and Methods. Various investigators have successfully employed this procedure to separate as well as to identify glycerophospholipids (Christie, 1985).

The radioactive profile of the HPLC run is shown on Fig.29. As can be seen, the major peak of radioactivity eluted with a retention time of 20 minutes, which correspondence with the retention time of an authentic PME standard. This confirmed that, under the conditions of the assay employed, the predominant methylated product formed was phosphatidyl-N-monomethylethanolamine.

4.3 Comparison of liver and platelet plasma membrane methylations

The results obtained from the methylation assays of the purified platelet plasma membrane enzyme, prompted us to further investigate the methylation from a partially purified enzyme from liver microsomes, employing the same assay conditions.

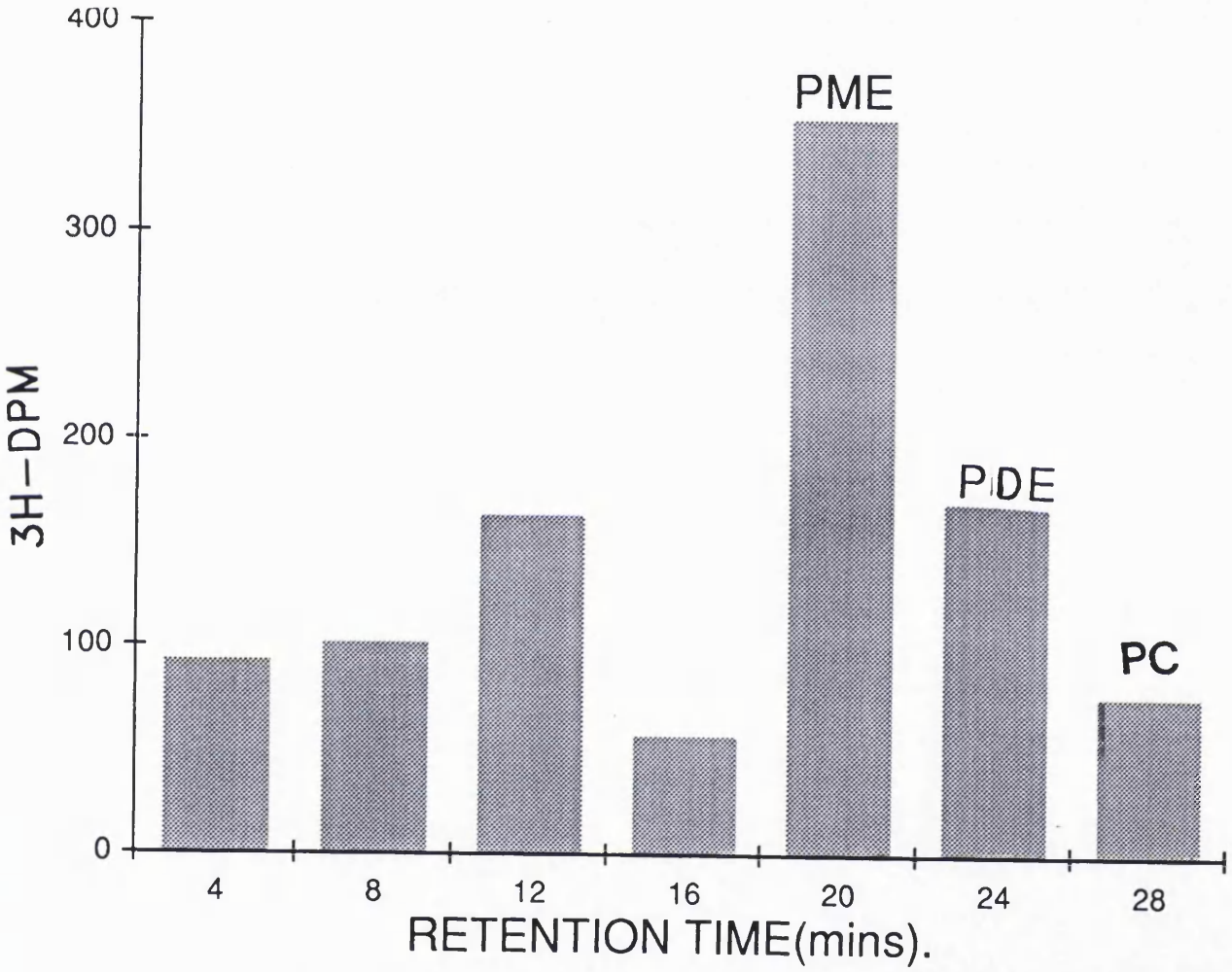


Fig. 29 Hplc analysis of PME-methylated products. The scraped product was re-extracted with chloroform:methanol (2:1 V/V). 0.2ml of the extracted product was injected twice on a Silica SW10 (4x250mm) column and eluted with a gradient profile of phosphate into acetonitrile (1:4). Eluted fractions were dried and radioactivity counted in 4ml scintillation cocktail on a Beckman LS 5000 CE radioactive counter.

Indeed, the importance of the methylation pathway in rat liver hepatocytes has already been assessed and it is reported to account for about 20% of the PC found in the liver (Sundler and Äkesson, 1975).

The results of the N-methylation assay from the partially purified liver enzyme are shown on Fig.30. It can be seen from the results that incubation of the partially purified enzyme with labelled SAM in the presence of exogenous substrates resulted in the labelling of phospholipids. Over 95% of the radioactivity was localized in PC and less than 5% in PDE (phosphatidyldimethylethanolamine); no PME was detected. This indicated a complete and effective successive methylation from PE through PME and PDE to PC. These results in the liver further confirm that the conditions of the assay previously employed for the platelets were adequate for complete methylation; however, compared to the liver, PME was the predominant product of methylation (Fig.28a). The differences between the methylation in blood platelets and the liver microsomes, may suggest a high degree of tissue specificity for the methylation process, and confirms a greater PC turnover via this pathway in the liver. This high level of PC formation was detectable by the radioactive scanner, even at its low level of sensitivity.

Several authors have considered the question of the relative importance of the sequential methylation mechanism to the synthesis of hepatic phosphatidylcholine. Originally Balint et al (1967), Tinoco et al (1967), and Lyman et al

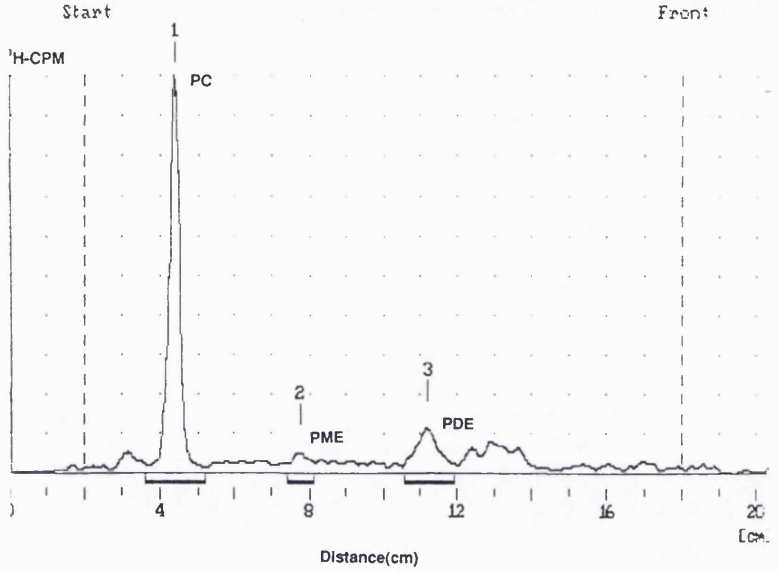


Fig. 30 Incorporation of ^{3}H-methyl groups into phospholipids by partially purified liver methyltransferase. The assay was performed at 37°C as described under "Materials and Methods".

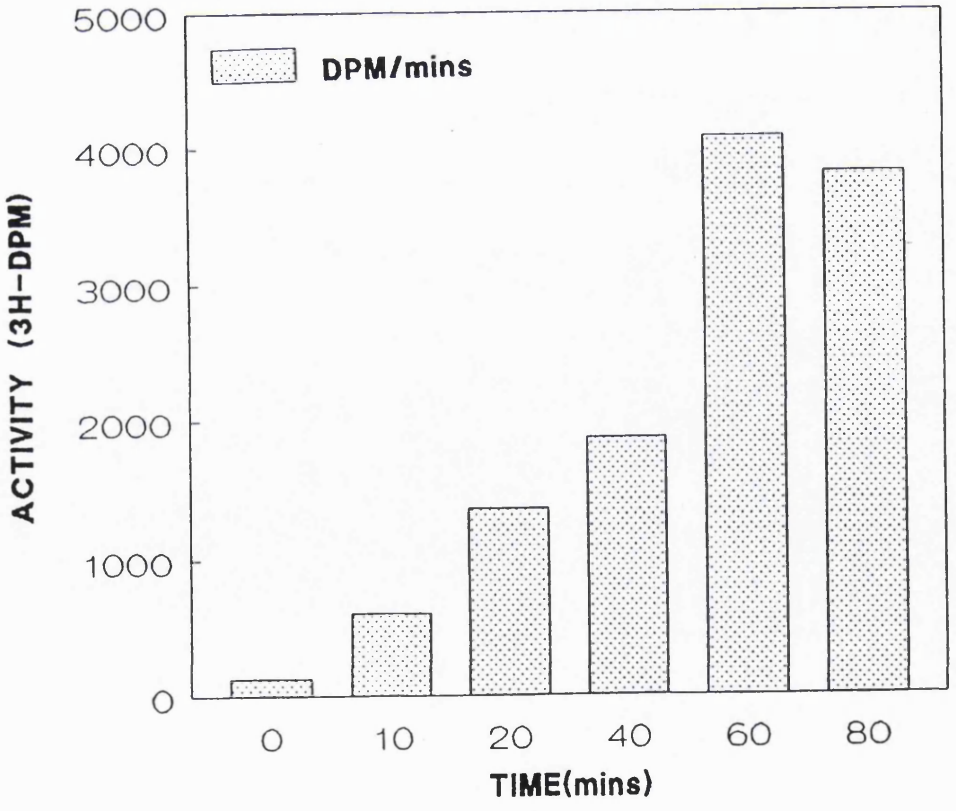


Fig. 31 Time-dependent total methylation by purified platelet membrane methyltransferase. The procedure is similar to the one described under "Figure 26".

(1968) proposed that this pathway was mainly used to synthesize phosphatidylcholines containing arachidonic acid. However, Arvidson (1968b), Rytter et al (1968), Lyman et al (1969), and Kanoh (1969) supplied convincing evidence that docosa hexaenoic, and particularly 1-palmitoyl-2-docosahexaenoyl-sn-glycerol-3-phosphorylethanolamine, were preferred substrates for the methylation reactions. There is no agreement, however, on the contribution of this pathway to the synthesis of tetraenoic phosphatidylcholines. Several groups (Trewhella and Collins, 1973; Salerno and Beeler, 1973; Lyman et al, 1973) believe that the *N*-methylation pathway might contribute significantly to the synthesis of these phosphatidylcholines with arachidonic acid. It is somewhat difficult to reconcile this view with the rather strong evidence for the predominant role of the acylation exchange pathway in the formation of this species of phosphatidylcholine.

Hotchkiss et al (1981) have concluded that phospholipid methylation is not required for the initial activation of platelets, but the inhibition of this methylation pathway may activate platelet function at low levels of agonist; however, the basis of this view has not yet been established.

It seems quite likely that the contribution of the diacylglycerol (DAG) pathway to PC synthesis in platelets (in the megakaryocytes) may far exceed that obtained via the *N*-methylation pathway.

4.4 Time-dependent methylation of purified platelet methyltransferase

Figure 31 depicts the incorporation of ^{3}H-methyl groups into total phospholipids, as a function of time, by purified platelet methylating enzyme(s) (protein conc. 0.13mg/ml). The results demonstrate that the incorporation of methyl groups into phospholipid(s) was essentially linear over a 60 minute incubation period with radioactive SAM (200μM) at 37°C. The incorporation corresponded with a specific enzyme activity of 25.3nmol/min/mg protein. The methylation almost doubled up between 40-60 minutes incubation time, indicating a rapid formation of the methylated products over this period.

A similar time-dependent methyltransferase activity has been demonstrated by other investigators (Cordasco et al, 1981; Shattil et al, 1981).

4.5 Cellular phospholipid decarboxylation and methylation

In mammals, *in vivo* studies have indicated that liver contains phosphatidyserine decarboxylase (Bremer et al, 1960; Yeung and Kuksis, 1976), and *in vitro* studies have localized the enzyme to the inner membrane of mitochondria (Dennis and Kennedy, 1972; Van Golde et al, 1974). Bremer and collaborators (1960) suggested that in liver this enzyme is part of a pathway for *de novo* synthesis of ethanolamine and choline. According to them, some of the

ethanolamine glycerophospholipids formed through this pathway in liver are subsequently methylated to form a quantitatively significant amount of phosphatidylcholine. The methylation of ethanolamine glycerophospholipids to form phosphatidylcholine has also been demonstrated in brain tissue *in vivo* (Mozzi and Porcellati, 1979) and *in vitro* (Blusztajn et al, 1979; Crews et al, 1980).

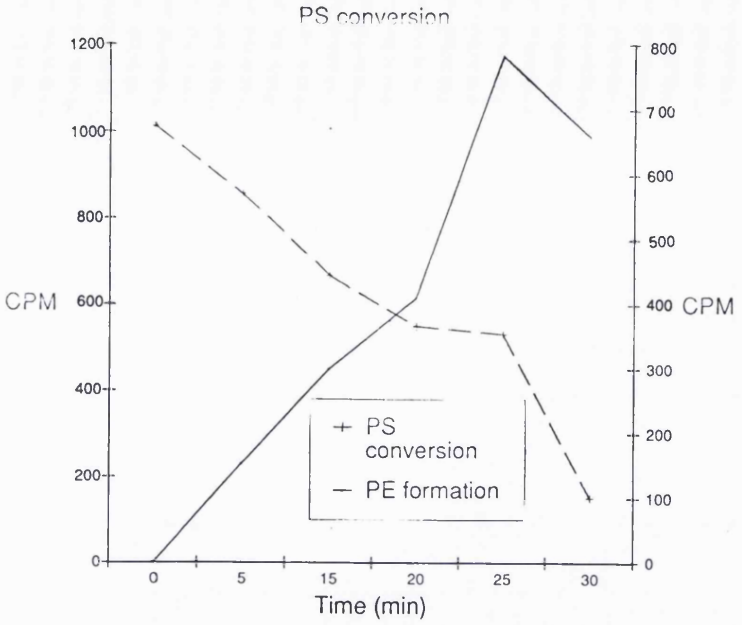
Hirata and Axelrod (1980) reported that upon the addition of [^{14}C]-phosphatidylserine to mast cells, Con A stimulated the incorporation of radioactivity into mono-, di- and tri-methylated phospholipids as well as lysophosphatidyl-choline. These experiments indicated a cascade of bio-chemical reactions in mast cells as follows: phosphat-idylserine $\xrightarrow{-\ CO_2}$ phosphatidylethanolamine $\xrightarrow{+\ CH_3}$ phosphatidyl-N-monomethylethanolamine $\xrightarrow{+\ 2CH_3}$ phosphatid-ylcholine $\xrightarrow{\text{-fatty acid}}$ lysophosphatidylcholine. Work in our laboratory (Auchi et al, 1993) has also shown the presence of phosphatidylserine decarboxylase in liver plasma membranes which seemed to be coupled to the methy-lation process. It was therefore expedient to investigate whether such a pathway does exist in platelets. Hitherto, no unique phospholipid nor this pathway of phospholipid metabolism has as yet been identified in human platelets. It was therefore thought that such an investigation would serve to promote further understanding of membrane biogen-esis and fluidity in platelet membranes. It has indeed been suggested that in the course of such reactions (as already described) the substrates, phosphatidylserine and phosphat-

idyethanolamine, which are usually localized on the cytoplasmic side of the membranes (Chap et al, 1977), are probably translocated to the outer surface.

4.5.1 Platelet phosphatidylserine decarboxylation

A preparation of crude platelet membrane-extract was utilized in a bid to investigate the interconversions of phosphatidylserine (PS) to phosphatidylethanolamine (PE), and ultimately to phosphatidycholine (PC). The partially purified platelet plasma membranes (0.95mg protein) were solubilised in a Tris-buffer containing 0.5% Triton-X100. [^{14}C]-labelled PS was incubated at 37°C with the solubilised membrane in presence of SAM at various time intervals. The chloroform-extractable radioactive products were separated by tlc and determined quantitatively by radiometric scanning as described under Materials and Methods.
Surprisingly, the radiolabelled initially present in PS was quantitatively converted to PE during the entire incubation period (Fig.32(a) & (b) and Table 16(A) & (B)), confirming a precursor-product relationship between PS and PE via the decarboxylation pathway. Fig.32 shows the radiometric scan of the results, whilst Fig.34 shows an hplc identification of the product to be PE. These results for the first time demonstrate the presence of phosphatidylserine decarboxylase in platelet plasma membranes and provide compelling evidence that PE synthesis via PS does exist in platelets as in the liver. However, since crude prepara-

(a)



(b)

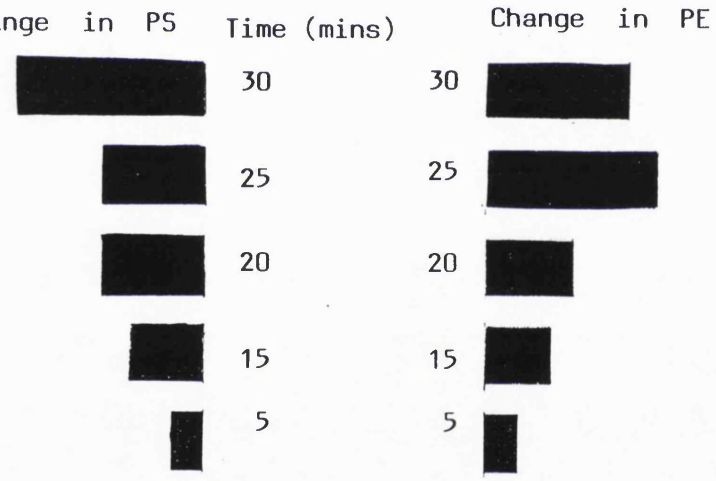


Fig. 32(a) & (b) Time-dependent PS decarboxylation in platelet plasma membranes. Platelet membrane extracts (0.95mg protein) were equilibrated for 5, 15, 20, 25 and 30 minutes in 20ml phosphate buffer (pH 8.0) in the presence of 0.05μCi-^{14}[PS]. The incubation was stopped at various time intervals by the addition of CHl_3:CH_3OH:2HCl (6:3:1). The product extraction and separation were performed as described under "Materials and Methods". The conversion of ^{14}[PS] to ^{14}[PE] was measured by counting exxtracted lipid in 4ml scintillation cocktail on a Beckman radioactive counter.

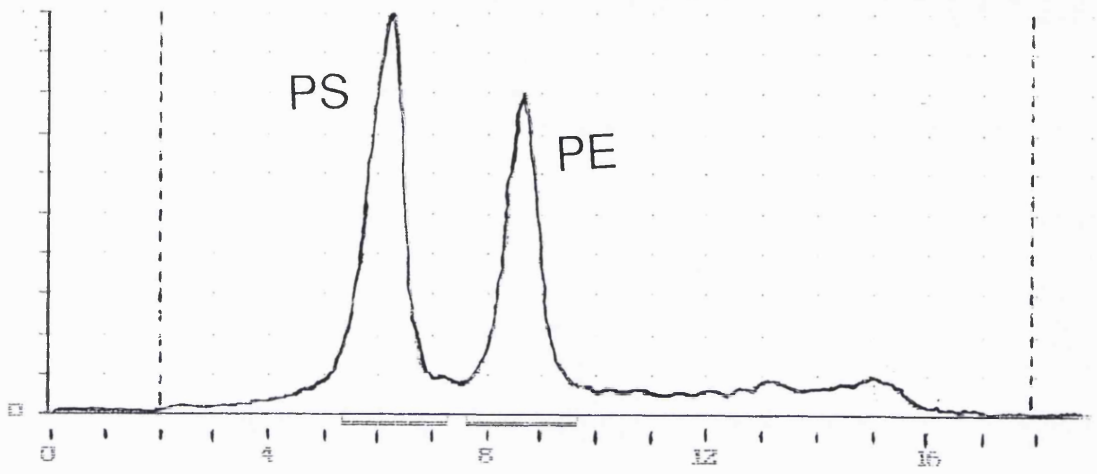


Fig. 33 Radiometric scanning of PS-decarboxylation in platelets. Membrane extracts were incubated at 37°C in the presence of radiolabelled PS, as previously described and counted on a Berthold Tracemaster Scanner, model 20.

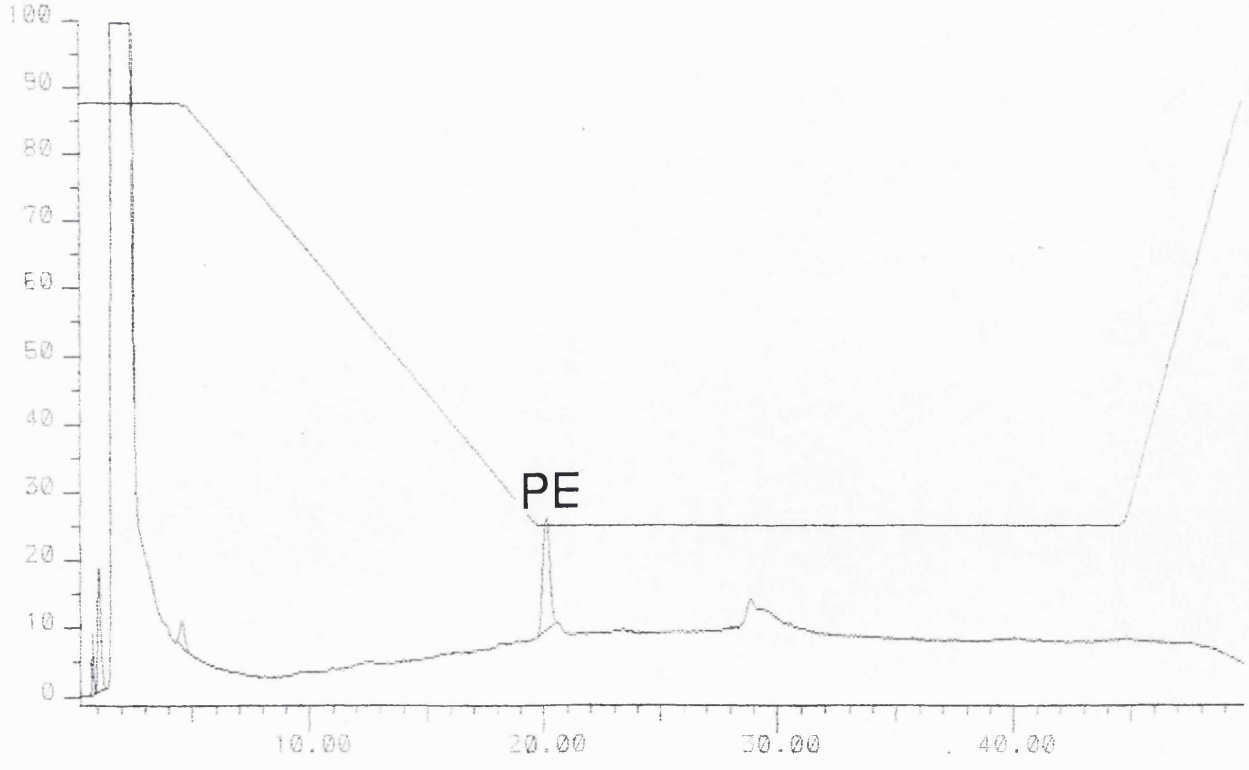


Fig. 34 Hplc product identification of PS-decarboxylation in platelet plasma membranes. Conditions of the Hplc run are similar to that described under Figure 29.

Table 16 Time-dependent conversions of radio-labelled [^{14}C]-PS to PE in platelet membrane preparations as detected by radiometric scanning.

(A) Time (min)	CPM in PS	% SD	CPM in PE	% SD
0	1014	1.14	0	-
5	859	0.76	154	1.80
15	667	0.87	300	1.29
20	550	0.96	411	1.10
*25	532	0.97	785	0.80
*30	152	1.81	660	0.87

(B) Time (min)	ΔPS (CPM)	ΔPE (CPM)
5	154	154
15	347	300
20	464	411
*25	482	785
*30	867	660

*Unexpected results could be due to pipetting errors during assay.

ations were used, it was not possible to establish the subcellular location of this enzyme in platelets. The results, however, indicate that the activity of the enzyme was not coupled to methylation, since no detectable PC was observed. It is also not certain whether the enzyme was obtained from contaminable sources in the preparation (for eg white blood cells, plasma etc). This does, however, seem quite unlikely, since none of such sources has been reported to exhibit the enzyme activity.

Consistent with this finding is the observation that phosphatidylserine decarboxylase activity is markedly enhanced when the enzyme is solubilised by non-ionic detergents (Dygas and Zborowski, 1989).

At this stage, however, the significance of this enzyme in platelet membrane function could not be predicted. Nevertheless, as with the erythrocyte membrane, the decarboxylation process may play a role in platelet membrane fluidity, and possibility facilitate lateral mobility and clustering of platelet surface receptors. Further work in this area is thus necessary to establish these findings.

4.6 Purification of phospholipid-N-methyltransferase from platelet plasma membranes

Although phospholipid-N-methyltransferase(s) activities have been demonstrated in platelets by various investigators (Mori et al, 1983; Hotchkiss et al, 1981;

Cordasco et al, 1981; Shattil et al, 1981), no purification of this enzyme has up to date been carried out. We therefore attempted for the first time to purify this enzyme using various classical methods of protein purification described in the literature.

4.6.1 Purification by preparative column SDS-gel electrophoresis

The underlying principle of this method is that specific proteins from a complex mixture are purified by continuous-elution electrophoresis. During a run, proteins are electrophoresed vertically through a cylindrical sieving gel. As individual bands migrate off the gel, they pass directly into an elution chamber consisting of a thin frit. A dialysis membrane (molecular weight cut-off 6,000d) directly underneath the elution frit, traps proteins within the chamber which are then subsequently eluted (on the basis of weight) by the elution buffer flowing evenly and radially through the elution tube.

The procedure is described under Materials and Methods section 2.2.8a. This method was used as a preliminary means to locate the molecular weight range of phospholipid-N-methyltransferase from human platelet plasma membranes. The results are shown on Fig.35 and indicate that the major phospholipid-N-methyltransferase(s) activity (over 75%) eluted at M_r 60-70KDa and 5-20% of activity occurring between 20KDa and >70KDa. Since higher molecular fractions (>100KDa) were not collected, it is not certain

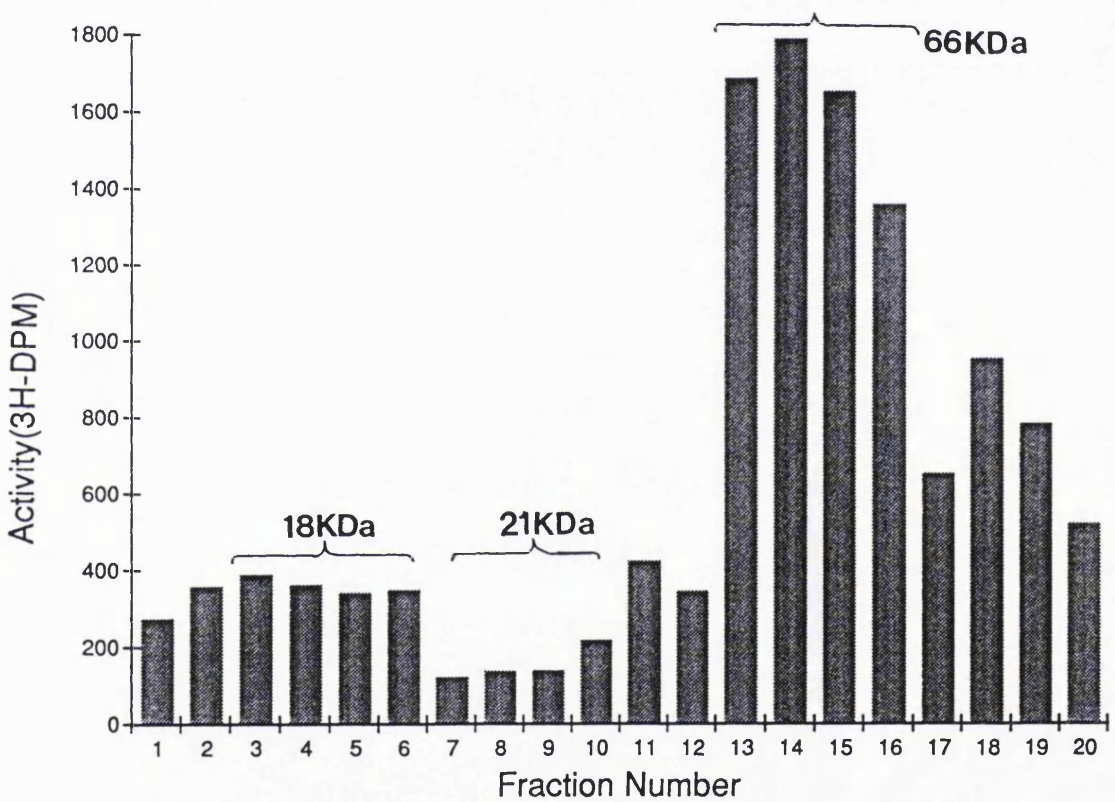


Fig. 35 Preparative SDS-PAGE of platelet plasma membranes. Solubilised membrane extract was loaded onto a 7.5% gel and fractions collected at a flow rate of 2 ml/min. Methyltransferase activity was assayed in eluted fractions as described in the text.

whether any activities did occur in those fractions. PME was the predominant product formed when the fractions were assayed for the enzyme activity after being washed free of SDS with 20% ethylene glycol.

Similar results had been obtained in our laboratory by Tsvetnitsky (1994), who showed that the major forms of myelin methyltransferase activity eluted at M_r 60-70 and about 100KDa. It should be stressed that, even though the procedure strongly predicted the molecular weight size of the eluted enzyme, the activity recovered was quite low. This could be due to the denaturating effect of SDS, some of which might have still been retained in the purified extract after washing. Besides, an ineffective cooling system might have greatly reduced the enzyme activity. Nonetheless, the results provided a springboard for the verification of the molecular weight by other procedures later on adopted.

It is reasonable to suggest from these results that the molecular weight of the protein isolated from the platelet membrane proteins with methyltransferase activity has a molecular weight size of the order of 60-70KDa.

4.6.2 Purification by isoelectric focusing (IEF)

This technique, sometimes called *electrofocusing*, is based on moving boundary electrophoresis. Proteins are separated in an electric field across which there are both *voltage* and *pH gradients*. The anode region is at a lower pH than

the cathode region and a stable pH gradient is maintained between the electrodes. Proteins which are initially at pH regions below their isoelectric point will be positively charged and will migrate towards the cathode, but as they do so the surrounding pH will be steadily increasing until it corresponds to their isoelectric points. They will then be in the zwitterion form with no net charge so further movement will cease. Likewise, proteins which are initially at pH regions above their isoelectric points will be negatively charged and will migrate towards the anode until they reach their isoelectric points and become stationary. The high resolving power of IEF applied to the separation of proteins in free solution gives qualitative and reproducible means of protein separation, whilst at the same time maintaining them in their biologically active forms.

Platelet membranes were either solubilized in Triton X-100 (0.5%) or Chaps (0.3%) and isoelectrically focused over a pH range of 3-10, as already described under Materials and Methods.

The results of the first and second fractionations are shown on Fig.36(a) & (b). The first fractionation shows two broad regions of activity in both the acidic and basic regions. Following the refractionation of the active fractions, an improved resolution was obtained (Fig.36b). The results clearly indicated two peaks of activity corresponding to an acidic form of the enzyme with a pI of approximately 3.5 and a basic form with a pI of approxim-

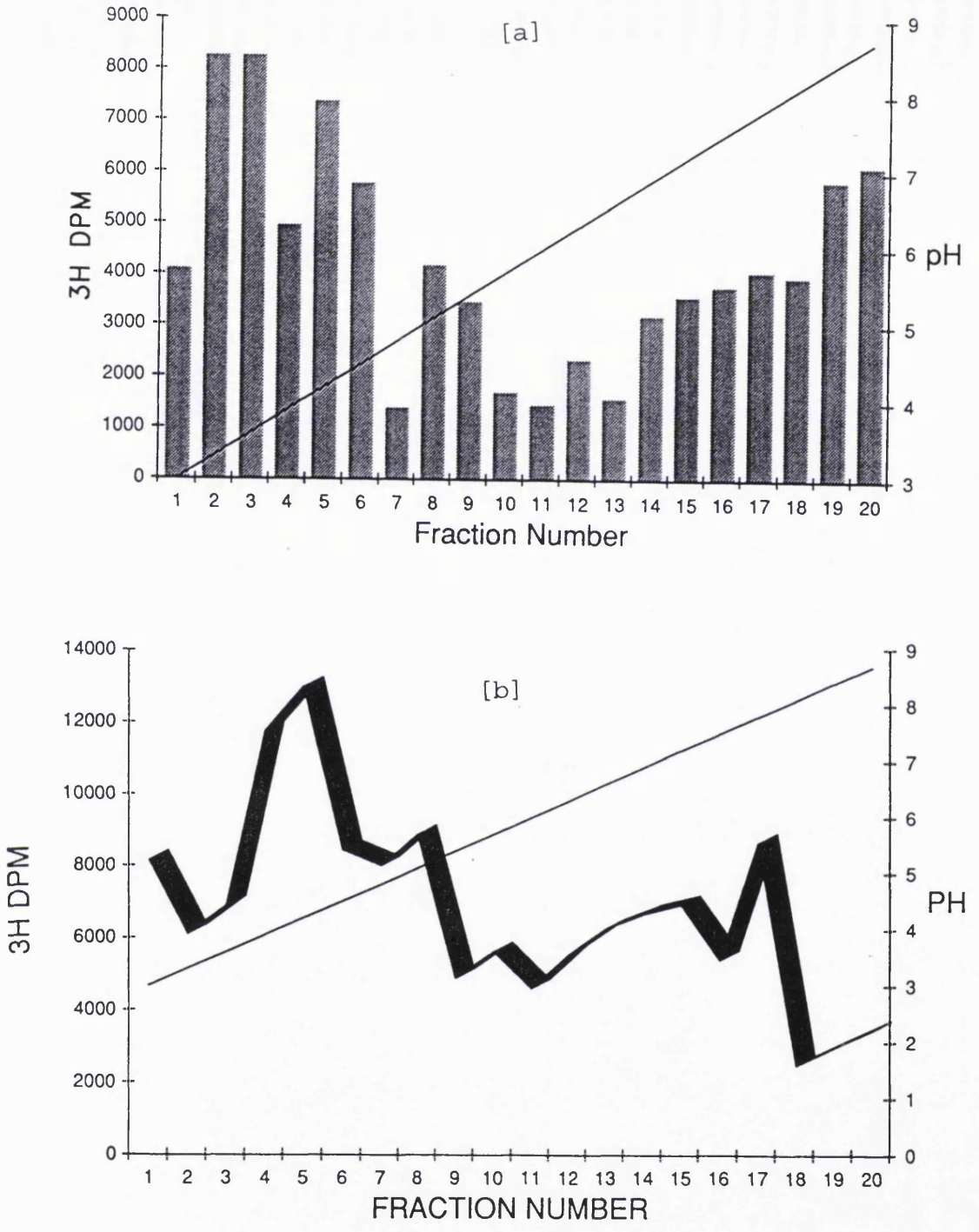


Fig. 36 (a) First IEF fractionation of platelet plasma membranes. Solubilised platelet membrane was diluted up to 50ml in the presence of 2% ampholyte and focused in a Rotofor IEF preparative cell for 4-5 hours at 4°C. Fractions were harvested and pH adjusted to 7-8.

(b) Second IEF fractionation of platelet plasma membranes

ately 8.5. SDS-PAGE of both forms is shown in Fig.37. Interestingly, the acidic fractions (5-7) gave 3 bands corresponding to M_r of ~ 67, 50, 35KDa, whilst the basic fractions (17-19) gave essentially a single deeply stained band at 67KDa.

When the acidic fractions (4-7) were pooled and refractionated over a narrow pH range, 3 peaks of activity were obtained spreading over a narrow pI range of <3.0-5.0 (Fig.38a). Analysis of the product formation after incubation of extracts of these fractions with SAM indicated that PME was the predominant methylated product (Fig.38b). At this stage, however, it was not certain whether these close peaks of activity represented isoforms of the "acidic enzyme" or phosphorylated forms of the same enzyme. Tsvetnitsky (1994) had obtained similar results on solubilised myelin extracts and concluded that the very reproducibility of the separation profiles was a direct reflection of the real ratio of differently charged isoforms of the acidic form. Ridgway and Vance (1989) have reported the existence of at least one of these enzymes from liver which can be phosphorylated. It is, therefore, not impossible that within the narrow range of the observed pIs, different phosphorylated forms of the acidic enzyme could occur in platelets. The fact should also not be ruled out that the apparent "isoforms" could also be due to incomplete refractionation caused by a heterogeneity of micelles formed during solubilization of aggregated proteins. Indeed a precipitate was observed in one of the

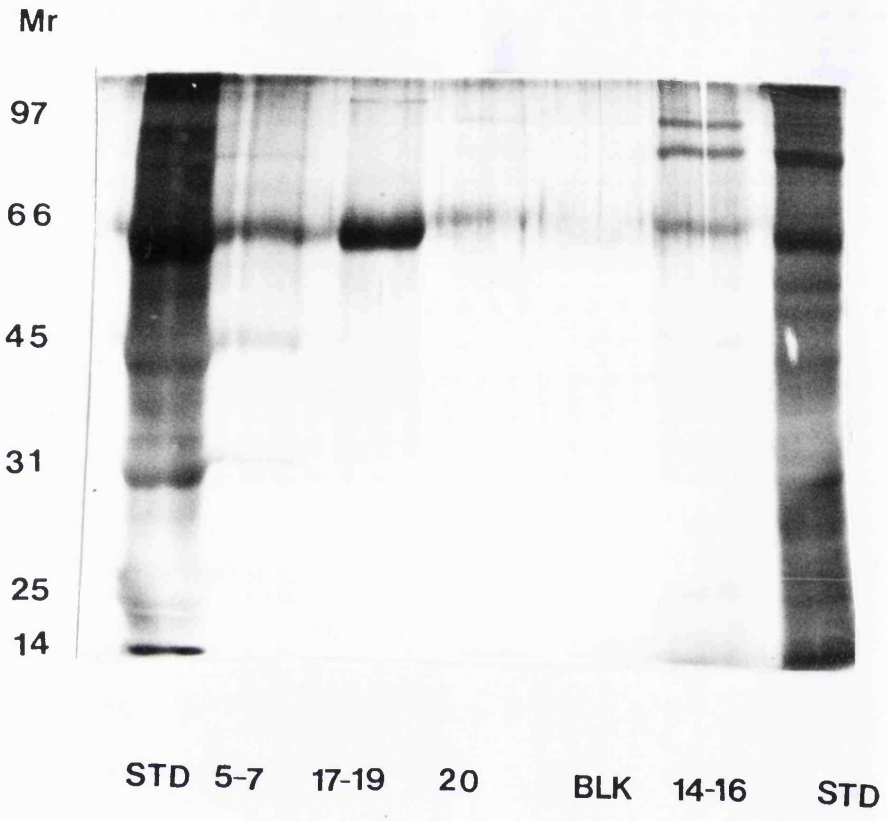


Fig. 37 SDS-PAGE of IEF-purified platelet plasma membranes. Purified fractions obtained from the second refractionation of the platelet membrane extracts (Fig.36b) were diluted (1:4) with SDS-reducing buffer comprising 0.5m Tris-HCl, 0.1% glycerol, 10% (w/v) SDS, 0.04% ß-mecaptoethanol and 0.05% (w/v)-bromophenol blue. Mixed fractions were heated to about 95°C for 5 minutes and electrophoresed on 12% T gel (0.75mm) and silver stained. Fractions 5-7 represent active conc. acidic fractions eluted with a pI between 3.4-4.5, whilst fractions 17-19 are active conc. basic fractions eluted with a pI of between 8.5-9.5. The acidic fractions gave 3 bands corresponding to M_r of 67, 50 and 35 KDa, whilst the basic fractions gave essentially a single band of M_r 67 KDa. Fractions 14-16, 20 showing some activity also showed the 67 KDa band.

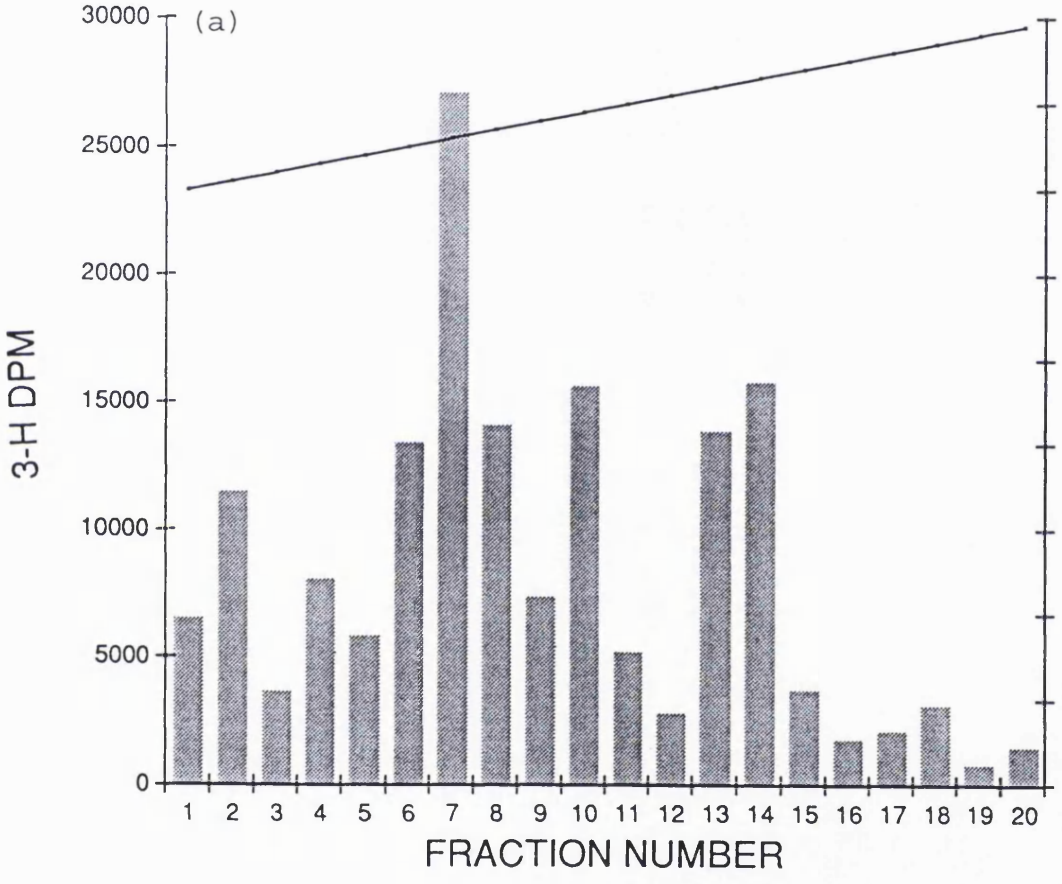
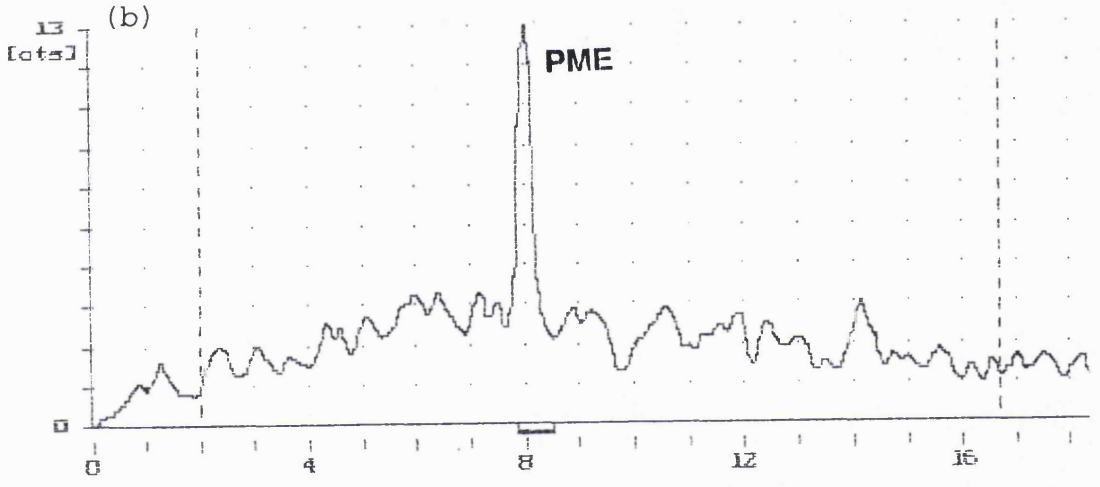


Fig. 38 (a) IEF refractionation of acidic active fractions of platelet methyltransferase. Fractions 4-7 from the second refractionation (Fig.36b) were pooled and diluted in water to 50ml and refractionation over a narrow pH range (3-5) at 4°C for 4 hours. Fractions were harvested and pH adjusted to neutral, and methyltransferase activity assayed as described under "Materials and Methods".



(b) Tlc product analysis of active acidic fraction. An aliquot of the extracted product in $CHCl_3$: CH_3OH (2:1, v/v) was spotted on a Tlc plate and developed in a chloroform:ethanol:triethyl-amine: water (4:5:4:1, v/v) solvent system and scanned on a Berthold Tracemaster radioactive scanner.

separating compartments. It is thus necessary that further work in this area is undertaken, in order to establish these observations and possibly characterise the "isoforms". Work by Cui et al (1993) seems to support the existence of isoforms of phospholipid-methyltransferase(s).

4.6.3 Purification by ion-exchange chromatography and IEF

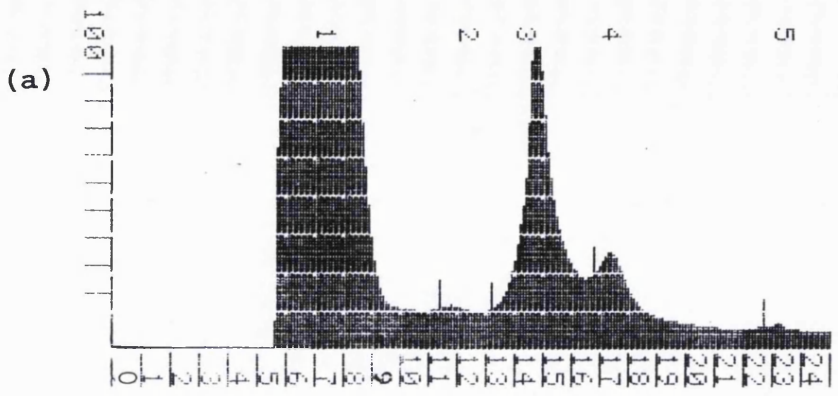
It was thought that based on the results from the purification studies obtained purely by IEF, a more comprehensive purification of platelet plasma phospholipid-N-methyltransferase could be achieved by the combination of ion-exchange chromotography and IEF methods.

The anion-exchange procedure using Q-sepharose was based on a modification of that adopted by Pajares et al (1984). Prepared platelet membranes were solubilized in 20mM phosphate buffer pH 8.0 in the presence of 0.3% chaps. It has been noted by McBride (1988), that the presence of chaps in the buffer stabilizes and enhances the enzyme activity.

The protein profile from the Q-sepharose column is shown in Fig.39a. Most of the methyltransferase activity was recovered in fractions 13-19 (profile 3), which eluted with about 70% salt (acidic proteins). The activity/protein profile is shown on Fig.39b, whilst the SDS-PAGE of the active fractions is shown on Fig.39c. Fractions 13-14 (acidic) gave 4 major bands ranging from M_r 90-50KDa, with a couple of tiny light bands above >90KDa. Fractions 15

and 16 gave rise to a heterogeneity of proteins ranging from >100KDa to <20KDa. It was observed that the separation profile was quite reproducible, and the activity of the enzyme recovered was satisfactorily stable. The whole active fractions were pooled and diluted to 50ml in presence of 0.2% ampholyte (a tenth of the usual ampholyte concentration was used) to match the lower protein concentration of the pooled fractions.

An IEF profile of the active fractions is shown on Fig.39d. Essentially, one peak of acidic activity was recovered, confirming the results from the ion-exchange chromotography. The pI of this enzyme was 3.5. The SDS-PAGE of this protein revealed two bands at 67KDa and 50KDa respectively, Fig.39e. These results were consistent with the results earlier obtained from both preparative gel electrophoresis and IEF, and indicate that the enzyme exists as an isozyme with acidic and basic pI of approximately 3.5 and 8.5, respectively, with a molecular size of the order of 67KDa. Attempts to examine whether there was any enzyme activity associated with the 50KDa band by running an assay with cut portions of the band from a native gel proved unsuccessful. However, subsequent claim by Varela et al (1984) that lipid methyltransferase consisted of 50KDa and 25KDa sub units and also by McBride (1988) in our own laboratory, makes it plausible that the 50KDa protein detected on the SDS-PAGE could also be associated with some methyltransferase activity in platelet plasma membranes.



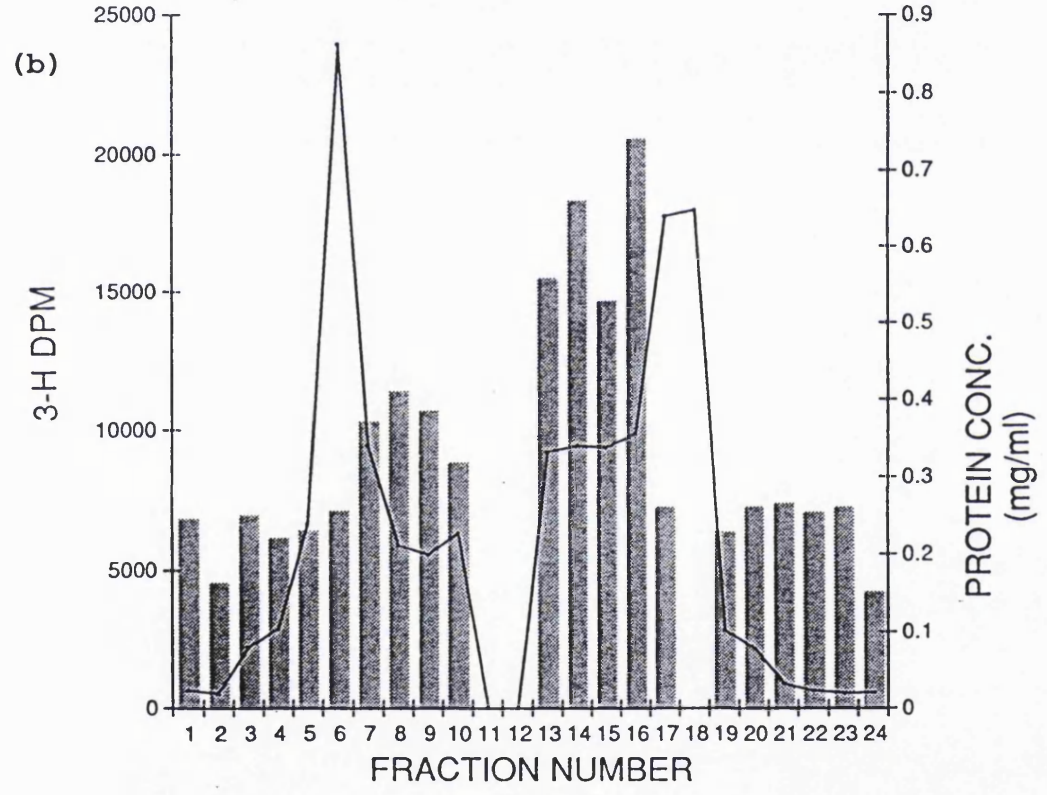


Fig. 39 (a) Q-sepharose protein separation profile of platelet plasma membranes. Solubilised membrane was loaded on a Q-Sepharose packed column (1.6x20cm), equilibrated with 20mM phosphate buffer containing 0.3% chaps. Eluted fractions were monitored by absorbance at 280nm. Most activity was recovered in peak 3 which eluted with 70-80% salt.

(b) Activity/protein profile of Q-sepharose purified platelet methyltransferase. Enzyme was assayed as described under "Materials and Methods".

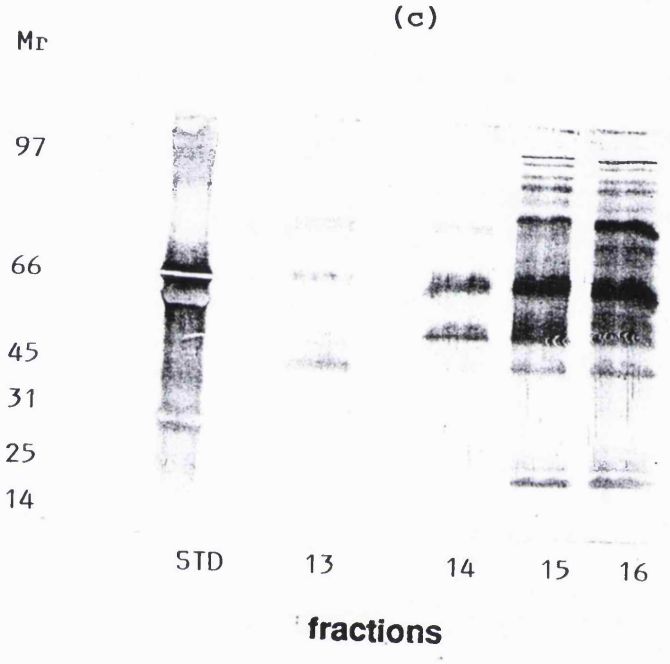
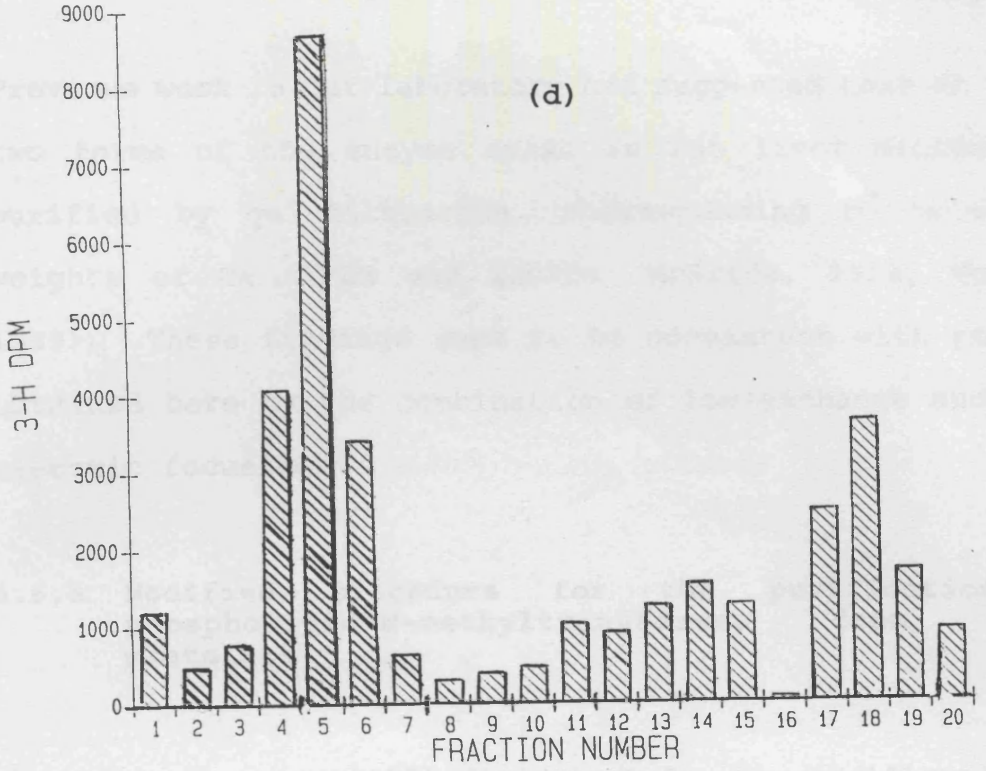
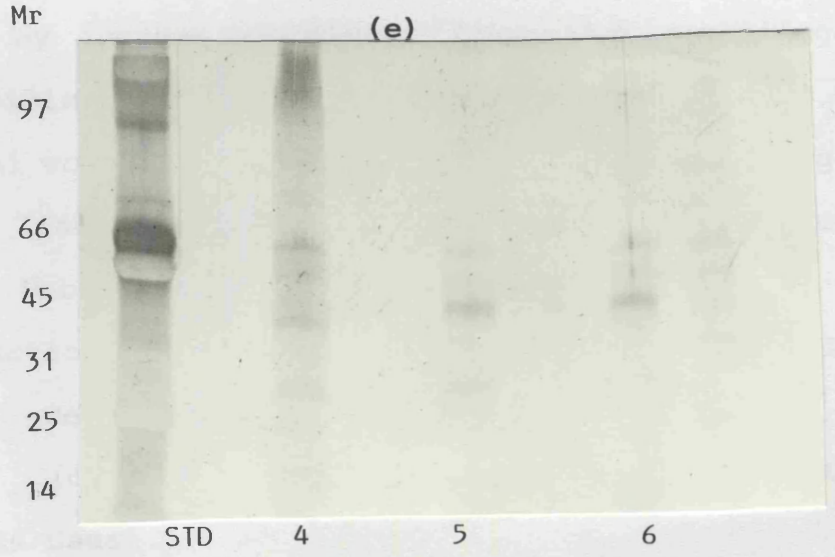


Fig. 39 (c) SDS-PAGE of Q-sepharose partially purified platelet plasma membranes. Frctions 13-16 were individually ultra concentrated (procedure, under Materials and Methods) and electrophoresed on a 12% T gel and Coomassie Blue. Fractions 13 and 14 were most active (ref Fig. 39a & b), followed by fractions 15 and 16.





(d) IEF fractionation of Q-sepharose acidic fractions. Fractions 13 and 14 were pooled and diluted in the presence of 2% ampholyte and fractionated on an IEF Rotofor cell as previously described.

(e) SDS-PAGE of acidic fractions from (d). Fractions 4, 5 and 6 from Fig.39 were individually concentrated and analysed.

Previous work in our laboratory had suggested that at least two forms of the enzyme exist in rat liver microsomes, purified by gel-filtration, corresponding to molecular weights of Ca 67KDa and 200KDa (McBride, 1988; Fonteh, 1989). These findings seem to be consistent with results obtained here by the combination of ion-exchange and iso-electric focusing.

4.6.4 Modified procedure for the purification of phospholipid-N-methyltransferase from human platelets

The steps for the modified approach for the purification of the enzyme are shown on Fig.40. Platelet plasma membranes prepared by sucrose gradient differential centrifugation was solubilised in a phosphate buffer containing 0.3% chaps in a total volume of 5ml (protein concentration 2.5mg/ml). This was loaded onto an equilibrated DEAE anion-exchange column. Unbound proteins were eluted with buffer while bound fractions were eluted with up to 80% of NaCl. Eluted fractions were collected and concentrated on a YM-10 membrane (cut off 10KDa) ultraconcentrator at 4°C and assayed as usual.

The DEAE profile is shown on Fig.41. The figure presents two major areas of activity; one in the unbound fractions (fractions 4-20), presumably the basic protein with an activity of $13x10^3$DPM/mg protein, whilst the acidic protein (fractions 32-44), which eluted with 80% salt, gave an activity of $28x10^3$DPM/mg protein. It was decided at this

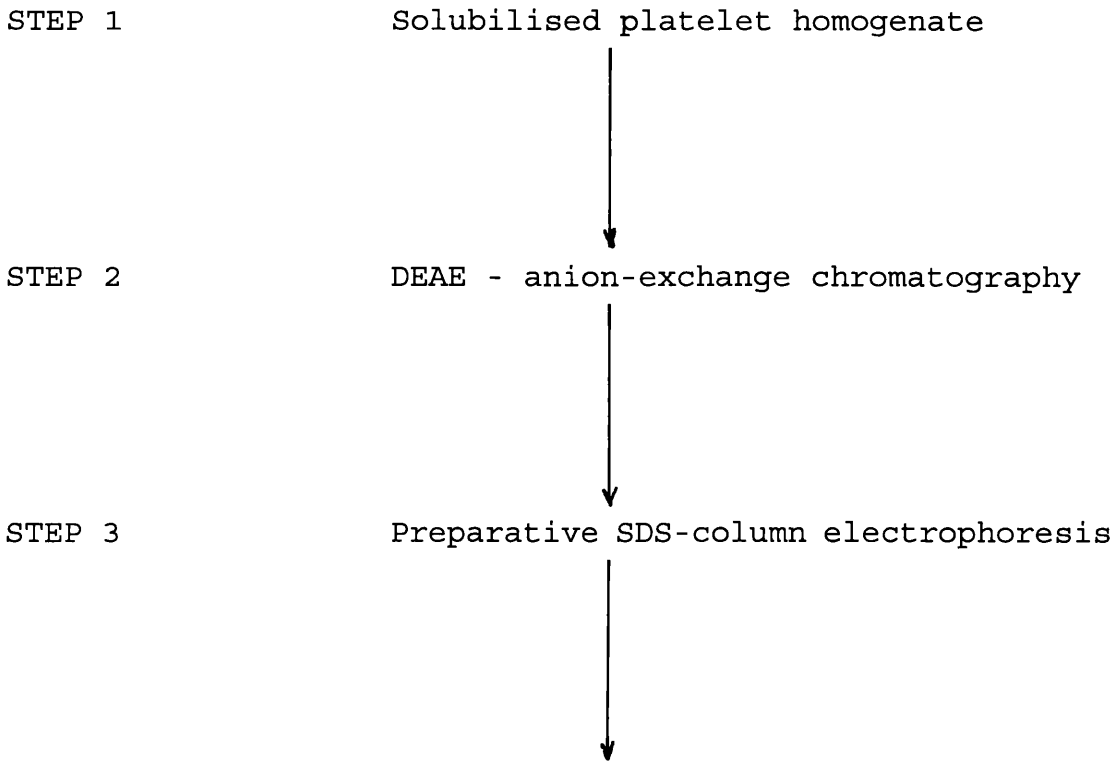


Fig. 40 Modified procedure for the purification of phospholipid-N-methyltransferase from human blood platelets.

stage that in order to avoid further complication of mixing the acidic and basic fractions, we should rather concentrate on purifying the acidic enzyme which, after all, had proved stable in the previous purifications. Thus fractions 32-44 were pooled and concentrated to give a final protein concentration of 0.34mg/ml in a total volume of 5ml. This was then loaded onto a preparative SDS-gel and electrophoresed as described under Materials and Methods, with the slight modification of a decreased separating gel size, to reduce the elution time (usually 6 hours). Besides, the elution/running buffer was also twice diluted to reduce the salt concentration. A high salt concentration had been realised to prolong the time required by the sample to enter the gel.

Eluted active fractions from the preparative gel electrophoresis (step 3) were similarly concentrated (protein concentration 0.13mg/ml) and finally loaded onto a triazine activated SAH-agarose affinity column, and subsequently eluted as described under section 2.2.8e. The overall purification fold and individual yields is presented on Table 17. From step 1 to step 2 a purification fold of 5.83 and activity of 1.9mmol/min/mg were achieved. Step 3 gave, by far, the most remarkable fold of purification (77.45). This represented over 13 times purification enhancement from step 2, with a yield of 181%. Steps 4 and 5 represent unbound and bound proteins, respectively, as eluted from the agarose gel. Although similar conditions of run as described by Kim et al (1978) were employed, it is not

certain what contributed to the apparently lower degree of purification than expected. This could be due to loss of enzyme activity after step 3 (likely due to the presence of some SDS), or possibly overloading of the column (2ml volume) could have resulted in lack of effective binding of the proteins to the column. This could be a factor since a lot of activity was retained in the unbound fraction (refer to Table 17). Similar problems had been encountered by Fonteh (1989). The purification trend on the agarose affinity column was, however, very similar to those obtained by Tsvetnitsky (1994), (data not shown). The overall trend of purification by this modified approach is illustrated on Fig.42.

The SDS-PAGE of the active fractions from step 3 (Fig.43) was consistent with the molecular weight of 65 $\pm$ 2KDa, as observed previously by the other means of purification. These results further confirmed that in platelet plasma membranes, a protein of molecular weight of the order of 65 $\pm$2KDa does exist, which exhibits methyltransferase activity. However, the intriguing question as to why this enzyme only catalyses the first step of the methylation process with a predominant PME product formation remains to be answered.

Fig. 44 demonstrated the stability of an IEF-purified platelet enzyme after six weeks of storage at -70°C. After six weeks of storage at -70°C, both the acidic and basic forms of the enzyme in platelets lost above 50% of their activities. This is, however, quite typical of most

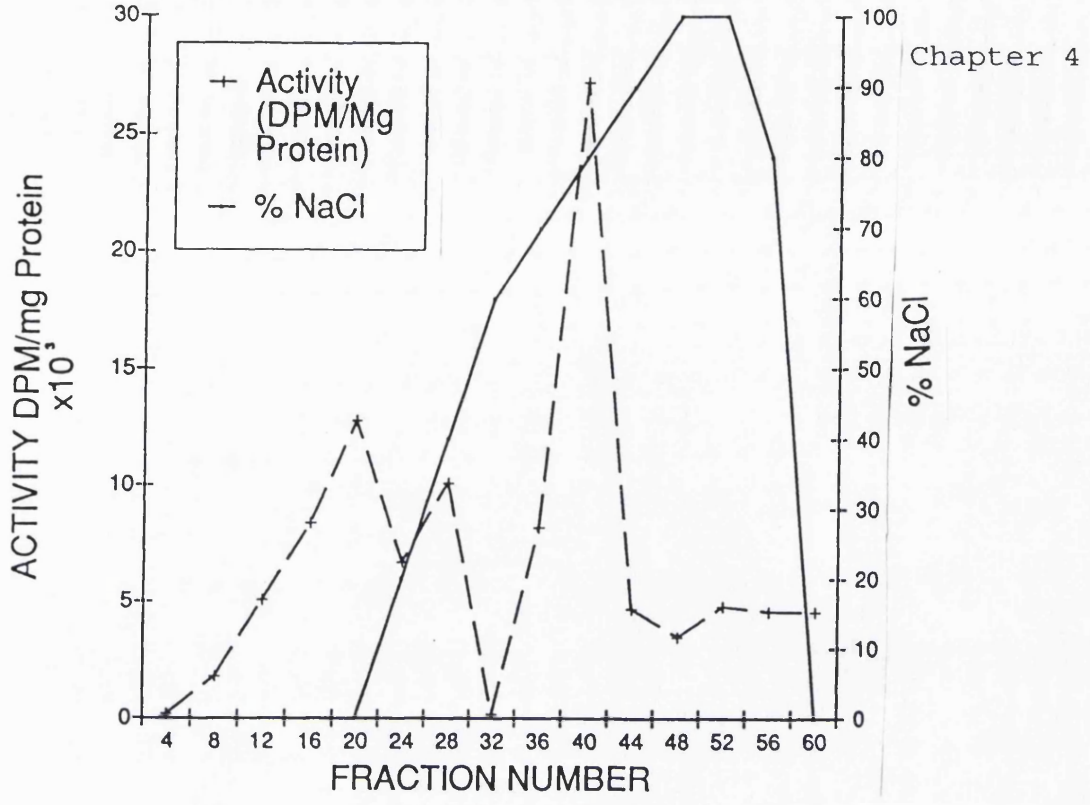


Fig. 41 DEAE protein separation profile of platelet plasma membranes. 5ml of solubilised platelet membrane (0.34mg protein) was loaded on a DE52 column. Bond fractions were eluted with 70-80% NaCl.

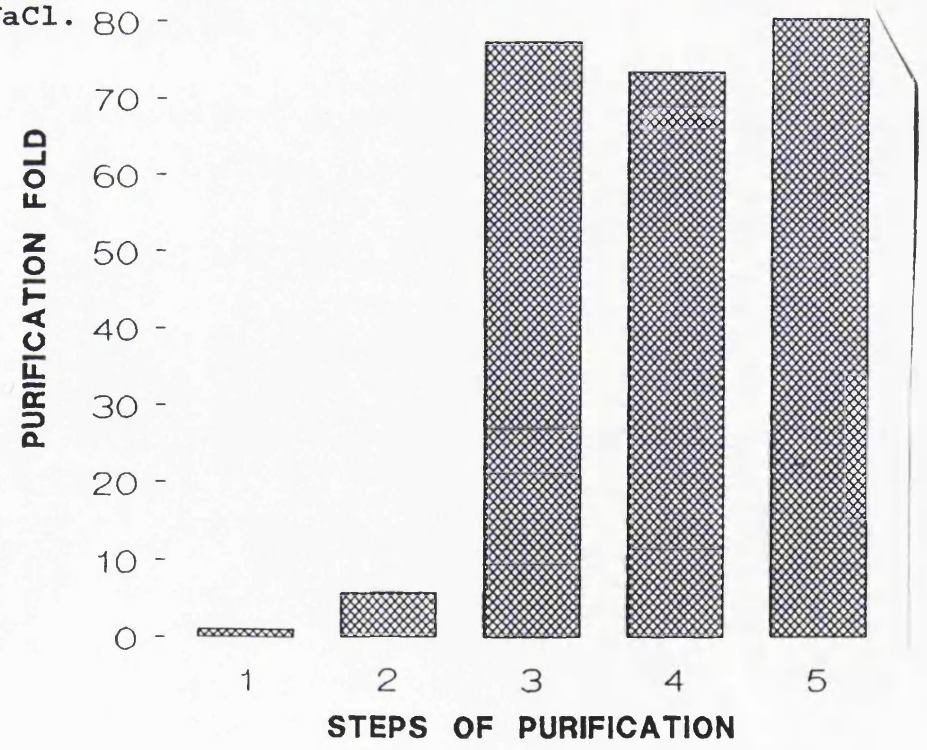


Fig. 42 Purification factor versus steps of purification of platelet phospholipid methyltransferase. Steps of purification: 1) crude solubilised membrane homogenate; 2) purification on DEAE; 3) purification on preparative SDS column; 4) & 5) purification by affinity chromatography.

Table 17 Purification results of phospholipid-N-methyl-transferase from platelet plasma membranes.

PURIFICATION STEP	VOLUME (ml)	PROTEIN CONC (mg/ml)	ACTIVITY*	PURIFICATION FACTOR	TOTAL DPM	YIELD %
Solubilised platelet membrane	5	2.5	0.33	1	4078	100
DEAE:cation-exchange	5	0.34	1.9	5.83	3233	79.2
Preparative column gel electrophoresis	2.2	0.13	25.3	77.45	7394	181.3
Affinity chromatography						
a) unbound fraction	1.0	0.13	24.3	73.6	3328	81.6
b) Bound fraction	0.8	0.09	26.6	80.6	1707	41.8

*Activity = nmol of ^{3}H-methyl group transferred per minute per mg protein.

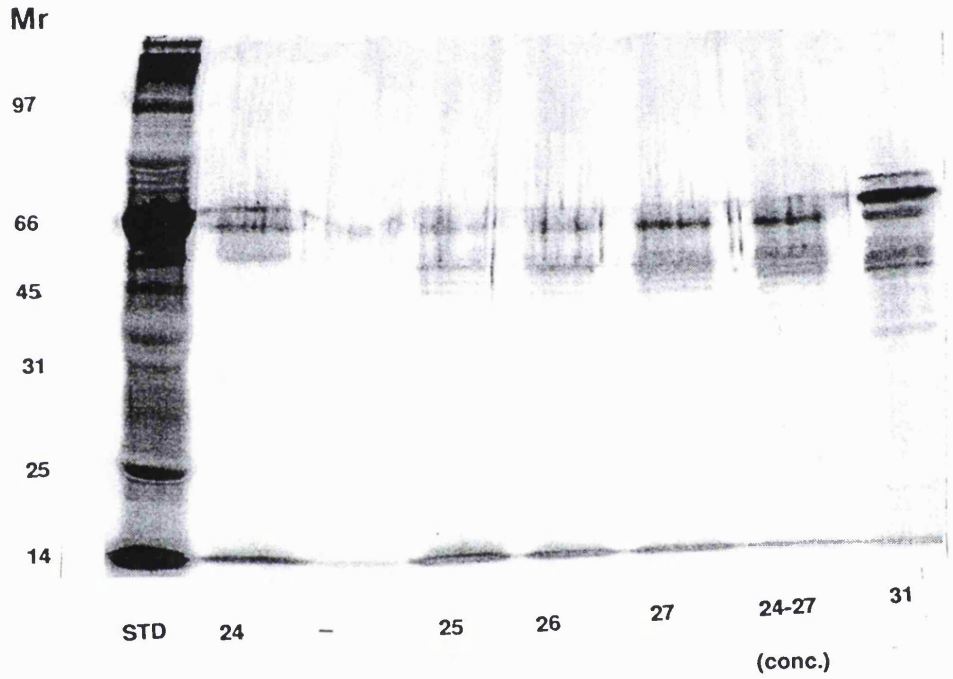


Fig. 43 SDS-PAGE of purified platelet plasma membranes from the modified purification procedure. Purified fractions were diluted (1:4) with SDS-reducing buffer and heated to 95°C for 5 minutes and electrophoresed on 12% T polyacrylamide gel. Bands were made visible by silver staining. Fractions 24, 25, 25, 27 and 31 are the active fractions as obtained from third stage of purification (preparative column electrophoresis), whilst fractions 24-27 are the pooled and concentrated active fractions. The major band common to these active fractions in this analytical gel was 67±2 KDa.

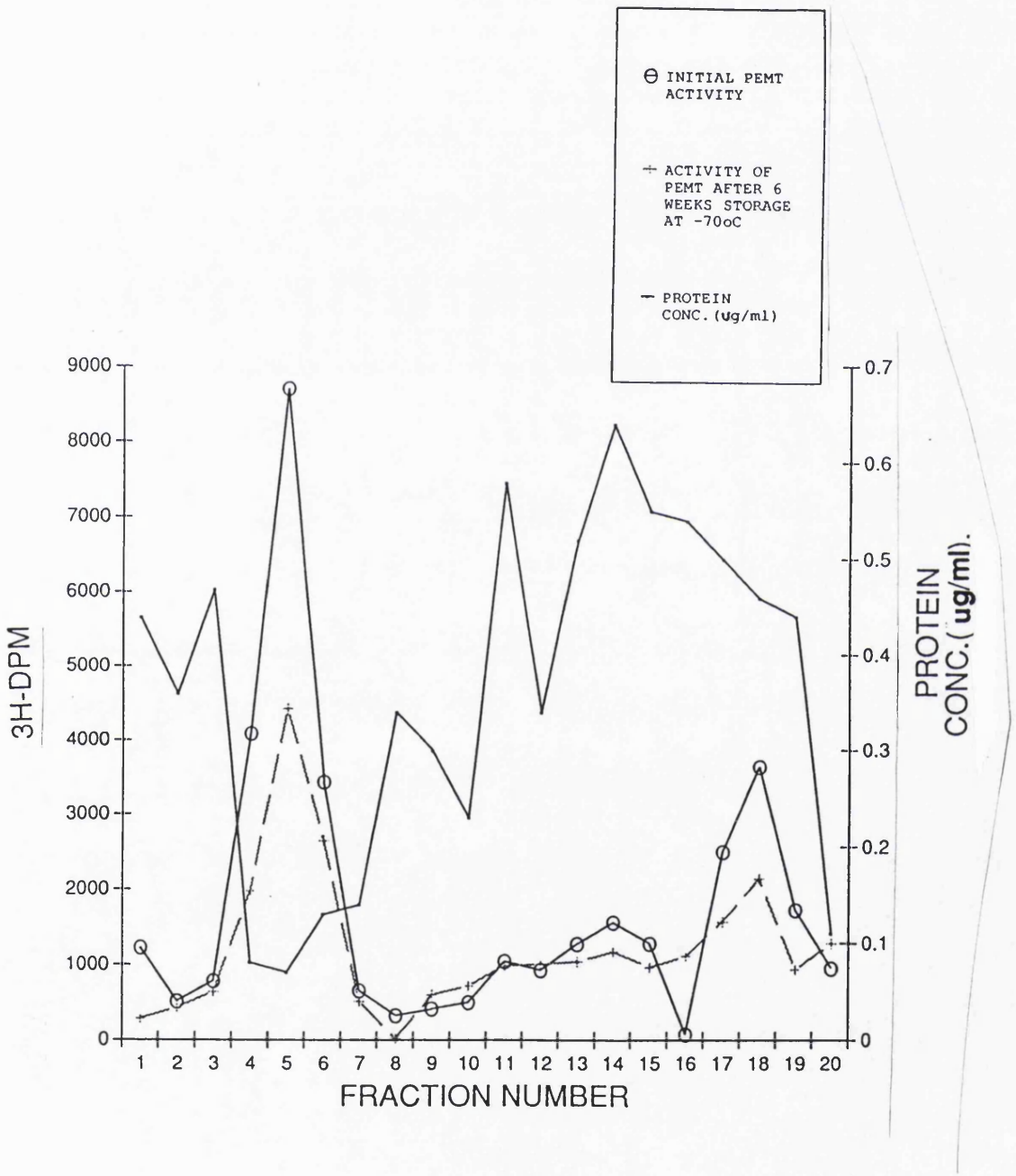


Fig. 44 Stability studies of purified platelet methyltransferase after six weeks of storage at -70°C. Individual fractions were assayed as described under "Materials and Methods".

enzymes, although this also gives a strong indication that the enzyme is fairly stable.

CONCLUSION

A membrane-bound protein with phospholipid methyltransferase activity was identified and isolated from human blood platelet plasma membranes. The plasma membranes were prepared and purified by velocity and sucrose gradient centrifugation and solubilised in either phosphate buffer (pH 8.0) containing 0.3% chaps or Tris buffer (pH 7.4 containing 0.5% Triton-X100. A combination of procedures including ion-exchange chromatography, preparative IEF, preparative SDS-column electrophoresis, ultra concentration and affinity chromatography were adopted.

The preparative rotorfor electrofocusing using pH 3-10 ampholyte at 4°C yielded activities at both acidic and basic pHs of approximately 3.5 and 8.5, respectively. The basic activity yielded predominantly a single 67KDa band on SDS-PAGE gels and the acidic activity also gave a similar band with other minor bands at 50KDa and 35KDa. Both the acidic and basic active fractions catalysed the methylation of PE in the presence of S-adenosyl methionine, with a predominant formation of PME. The fraction purified by preparative column electrophoresis also yielded a major methylating activity that eluted between 60-70 KDa. This latter molecular weight protein was filtered and washed

free of SDS and its activity measured; it too yielded predominantly the monomethylated PE. The identity of the product was confirmed by Tlc and Hplc separations.

A comparative assay of the purified platelet enzyme with that from partially purified liver microsomes indicated an almost complete methylation to PC in the latter, strongly suggesting tissue-specificity of this enzyme(s).

An overall purification factor of 77.45 and a basal yield of 181% was achieved of the purified enzyme from the protein membrane source, which also exhibited a linear time-dependent methylation over a 60 minute incubation period at 37°C. The enzyme was also observed to be stable over six weeks of storage. However, the initial activity had been reduced to 50% at the end of this period. By incubating C^{14}-labelled PS with solubilised platelet membranes and determining the radioactive product(s) formed quantitatively by Tlc and radiometric scanning, the amount of PS converted to PE was determined. These results for the first time provide compelling evidence that PE synthesis via PS does exist in platelets. The results, however, indicated that the activity of the detected enzyme was not coupled to the methylation process in platelets.

CHAPTER 5

PLATELET POLYAMINES AND SULPHUR AMINO-ACIDS; ANALYSIS AND SIGNIFICANCE

Chapter 5

5.1 Biosynthesis and cellular significance of polyamines

The polyamines putrescine, spermidine and spermine represent a group of naturally occurring compounds exerting a bewildering number of biological effects, yet despite several decades of intensive research work, their exact physiological function remains obscure. Chemically these compounds are organic, aliphatic cations with two (putrescine), three (spermidine) or four (spermine) amino groups that are fully protonated at physiological pH values. Their biosynthesis is accomplished by a concerted action of four different enzymes: ornithine decarboxylase, adenosylmethionine decarboxylase, spermidine synthase and spermine synthase. Out of these four enzymes, the two decarboxylases represent unique mammalian enzymes with an extremely short half life and dramatic inducibility in response to growth promoting stimuli.

The primary carbon and nitrogen sources for putrescine, spermidine and spermine are the amino acids L-methionine and L-ornithine. In animal cells, the latter compound is formed from L-arginine in a reaction catalysed by arginase. Even though arginase is not usually included as a real polyamine biosynthetic enzyme, recent experimental evidence suggests that some forms of arginase exclusively function to generate ornithine for the biosynthesis of the polyamines. Ornithine is converted to putrescine in a seemingly simple decarboxylation reaction catalysed by ornithine decarboxylase, a highly inducible enzyme with a

half life of less than 0.5hr (Russel and Snyder, 1968, 1969). Putrescine serves as a precursor for spermidine synthesis being coupled to a propylamine moiety derived from decarboxylated adenosylmethionine. The latter compound is generated by the action of adenosylmethionine decarboxylase on adenosylmethionine (a methyl donor in biological methylations). The coupling of putrescine to the propylamine group to yield spermidine is catalysed by spermidine synthase. Similar coupling of spermidine to the same propylamine moiety to yield spermine is catalysed by spermine synthase, which resembles spermidine synthase, but is an entirely different enzyme. The entire enzyme-system involved in the biosynthesis of the polyamines is irreversible in practice; thus to convert spermine back to putrescine (which is what occurs in a living mammalian cell), a completely different set of enzymes is required. In brief, spermine or spermidine is first acetylated by a polyamine acetylase and oxidized by a polyamine oxidase to yield spermidine from spermine and putrescine from spermidine. The biosynthesis of the polyamines is summarised in Fig.45.

Early studies showed that the polyamines are closely connected to the proliferation of animal cells (Cohen, 1971). As expected, the polyamines interact with whole cells, cell organelles and nucleic acids, finally influencing the rates of innumerable metabolic reactions. Following the discovery of mammalian ornithine decarboxylase in re-

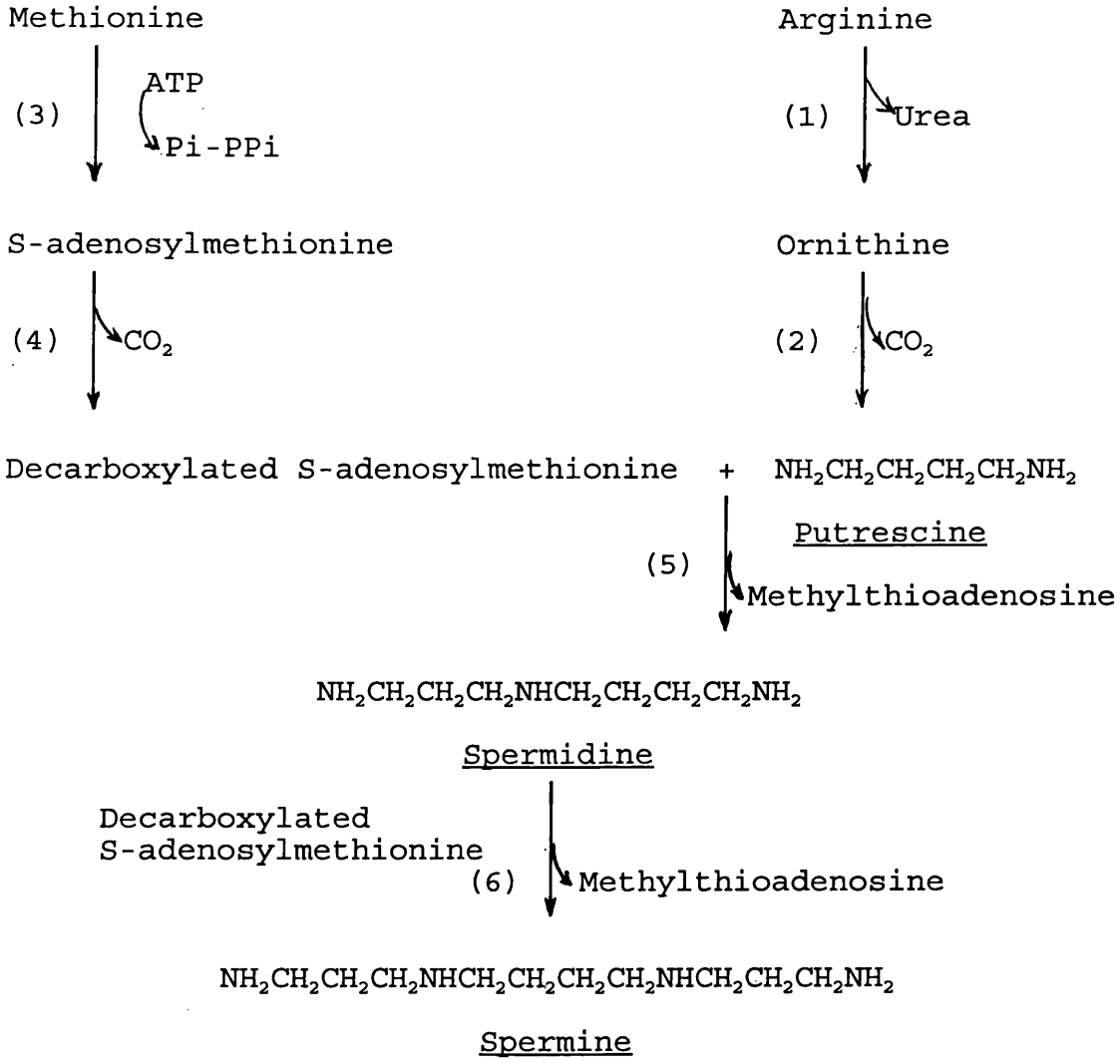


Fig. 45 **Biosynthesis of putrescine, spermidine and spermine from arginine and methionine. The reactions are catalysed by the following enzymes: (1) arginase; (2) ornithine decarboxylase; (3) S-adenosylmethionine synthetase; (4) S-adenosylmethionine decarboxylase; (5) spermidine synthase; (6) spermine synthase.**

generating rat liver (Russel and Snyder, 1968; Jänne and Raina, 1968), it became evident that an early accumulation of putrescine and spermine was a sign of accelerated growth (Jänne and Raina, 1978). In tumour formation, two antagonistic processes occur in the tumour itself and in the normal surrounding tissue: active destruction and active proliferation. There are also increases in the concentration of polyamines in blood and urine of cancer patients and tumour-bearing animals (Berdynskikh et al, 1976; Jänne et al, 1978), and it is possible that an increase in the concentration of putrescine in the blood reflects active cell proliferation, whereas high levels of spermidine and spermine reflect mass cell death. Polyamines have also been implicated in leukemics (Rennert et al, 1976) and in psoriasis (a hyperproliferative skin disease) patients (Henry et al, 1981).

Pharmacologically, the polyamines, especially spermidine and spermine, are toxic substances. The toxicity is manifested as nephrotoxicity, hypothermia and sedation (Jänne et al, 1978). One of the most exotic actions ascribed to the polyamines is the initiation of the burial of dead nonspecifics in rats (Pinel et al, 1981). This phenomenon suggests that the polyamines may act as pheromone-like substances in rodents.

Some scientists have proposed that polyamines may act as second messengers, modulating the activity of protein kinase and nuclease, and have significant effects on cellular ionic homeostasis (Cochet and Chambaz, 1983; Tabor

and Tabor, 1984). At the same time, spermine and spermidine have insulin-like effects on metabolism in adipose tissue (Amatruda and Lockwood, 1974). Shelepov et al (1990) have suggested that increased levels of polyamines in the blood might produce disturbances in the link between carbohydrate and lipid metabolism in cancer patients and tumour-bearing animals.

In their study Dieh et al (1992), concluded that biosynthesis of the polyamines, putrescine, spermidine and spermine is required for DNA synthesis and liver regeneration after partial hepatectomy, and suggested that putrescine itself acts to restore hepatic DNA synthesis in ethanol-fed rats. It has also been shown that polyamines can act as glucocorticoid mediators of the synthesis of the postulated vascular permeability inhibitory protein that does not inhibit phospholipase A_2 activities (Azuma et al, 1990). They suggested that polyamines behave as glucocorticoid-type anti-inflammatory drugs.

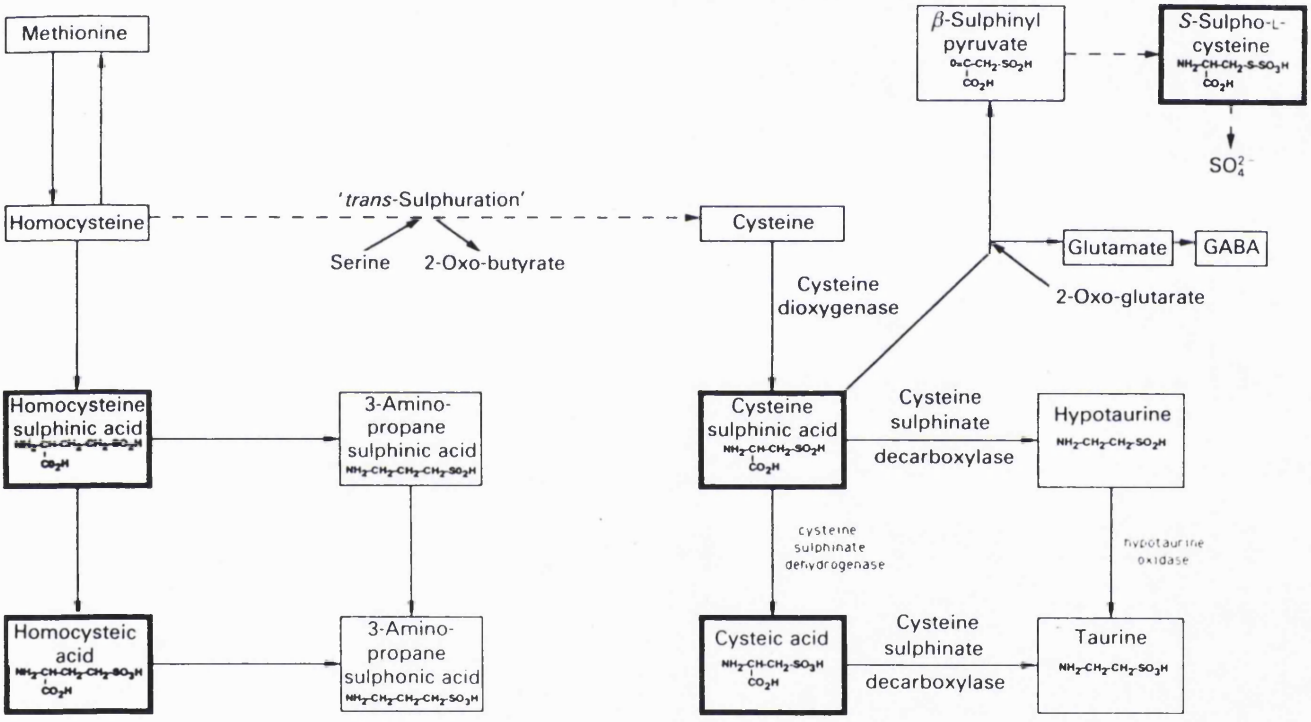
Other studies (Tyrns et al, 1988; Bacchi et al, 1980) have also indicated that polyamine antimetabolites may have potential as antiviral and antibacterial drugs, suggesting that polyamines may play a role in viral and bacterial-borne diseases. Indeed, it has been shown that the curative effect of DFMO on trypanosomiasis was a result of polyamine depletion, as the therapeutic effect could be blocked by a concomitant administration of putrescine and spermidine (Nathan et al, 1981).

The molecular biology of the natural polyamines seems to be a fascinating sector of basic research, offering a viable approach to a better understanding of the functions of polyamines.

5.2 Sulphur-amino acids; biosynthesis and cellular significance

The sulphur-amino acids (SAAs), comprise the excitatory acidic compounds, namely L-cysteine sulphinate (CSA), L-cysteic acid (CA), L-homocysteine sulphinate (HSA), L-homocysteic acid (HCA) and S-sulpho-L-cysteine (SC) and the inhibitory compounds, taurine and hypotaurine.

The biosynthetic route of neuroactive SAAs in mammalian brain originates from catabolism of the essential amino acid methionine, as shown in Fig.46. In a series of enzymic reactions, methionine is demethylated to homocysteine, which lies at a metabolic point. The only other source of homocysteine in vertebrates is the hydrolysis of S-adenosyl homocysteine, an inhibitor and product of S-adenosyl methionine-dependent transmethylation. The fate of intracellular homocysteine is either salvage to methionine through remethylation, or conversion to cysteine via the trans-sulphuration pathway. In brain, cysteine is oxidized by the enzyme cysteine dioxygenase to CSA, which in turn undergoes rapid further metabolism either by decarboxylation to hypotaurine, oxidation to CA (which is further decarboxylated to



The excitatory SAAs are highlighted by the bold boxes.

Fig. 46 Biosynthetic pathway of sulphur-containing amino acids.

taurine), or transamination to ß-sulphinyl pyruvate. The neuroactive SAA, SC lies along the metabolic pathway between ß-sulphinyl pyruvate and its end-product, inorganic sulphate. Transamination of CSA also represents a minor biosynthetic route of glutamate [and -amino butyric acid (GABA)]. The endogenous route from homocysteine is obscure and is represented on Fig.47 only by analogy to the metabolism of cystine. In most tissues, the remeth-ylation reaction is catalysed by the ubiquitous enzyme, methionine synthetase. This enzyme requires vitamin B_{12} [methyl(1) cobalamin] as a cofactor and 5-methyltetra-hydrofolate as methyl donor; thus 5-methyltetra-hydrofolate enters the pool of reduced folates, and homocysteine is remethylated to methionine. Homocysteine remethylation is also catalysed by an alternative enzyme, betaine-homocysteine methyltransferase, requiring betaine as methyl donor. However, this enzyme is generally confined to the liver. The metabolism of homocysteine along the trans-sulphuration pathway is catalysed by two B_6-dependent enzymes. The first step is the cystathione ß-synthetase reaction, where homocysteine is condensed with serine to form α-keto butyrate and cysteine, catalysed by cystathionine lyase.
A number of observations have prompted considerable recent interest in the study of neuroactive SAAs which have generated an increased awareness of a possible functional role of these compounds.

The study of neuroactive SAAs on platelets in our laboratory was particularly prompted by the fact that in addition to their role in haemostasis, platelets are often utilized in neuropsychiatric research as a model of central nervous system (CNS) biochemistry (Stahl, 1977). They have properties in common with monoaminergic neurons.

The SAAs are potent agonists at excitatory amino acid receptors and are considered to be neurotransmitter candidates at one or more of these receptor classes in mammalian CNS (Mewett et al, 1983).

Indeed HCA, and in particular CSA, fulfil several important criteria of neurotransmitter function. For example, both have been reported to be released from (Do et al, 1986) and actively transported into rat brain tissue (Cox et al, 1977; Recasens et al, 1982).

The SAAs, or a disturbance in the metabolism of their precursors, have been implicated in the pathogenesis of various neurological and neurodegenerative disorders (Andine et al, 1991; Galjaard, 1971) and they also appear to produce a similar range of excitotic properties of L-glutamate-induced cell damage (Pullan et al, 1987; Olney et al, 1972), possibly mediated by glutamate receptor subtypes (Kim et al, 1987). Epidemiological and experimental evidence has shown that homocysteine may provoke vascular lesions, and moderate homocysteinemia is an independent risk factor for premature vascular disease (Refsum and Ueland, 1990).

5.3 Hplc separation of platelet polyamines

Figures 47a and 47b depict the Hplc profiles of standard and platelet polyamines, respectively. The conditions employed in the analysis are described under Materials and Methods. The dansyl derivatization provided sufficient fluorescent detection of individual polyamines as shown on the chromatograms. The recovery of the polyamines was estimated from the recovery values of the known amount of internal standards added. The concentrations of polyamines expressed in nmol/mg of protein from platelets treated in different ways are represented on Table 18. It could be seen from the data presented that of the polyamines from the non treated (control) platelets, the most abundant polyamine was spermidine, followed by putrecine, followed by spermine and then cadaverine. A similar trend of polyamine distribution in platelets has also been demonstrated by Villanueva and Adlakha (1978), and also by Copper et al, 1976.

The data also indicated that even though there were no significant differences in the concentrations of polyamines from platelets treated with thrombin and NO, compared to the controls, the differences appear to be significant. For example, spermine concentration of 0.12 ± 0.02 nmol/mg protein has reduced to 0.06 ± 0.02 in the thrombin treated platelets, and to 0.08 ± 0.03 in the NO treated platelets. A similar pattern of reduction was observed in the other polyamines as well.

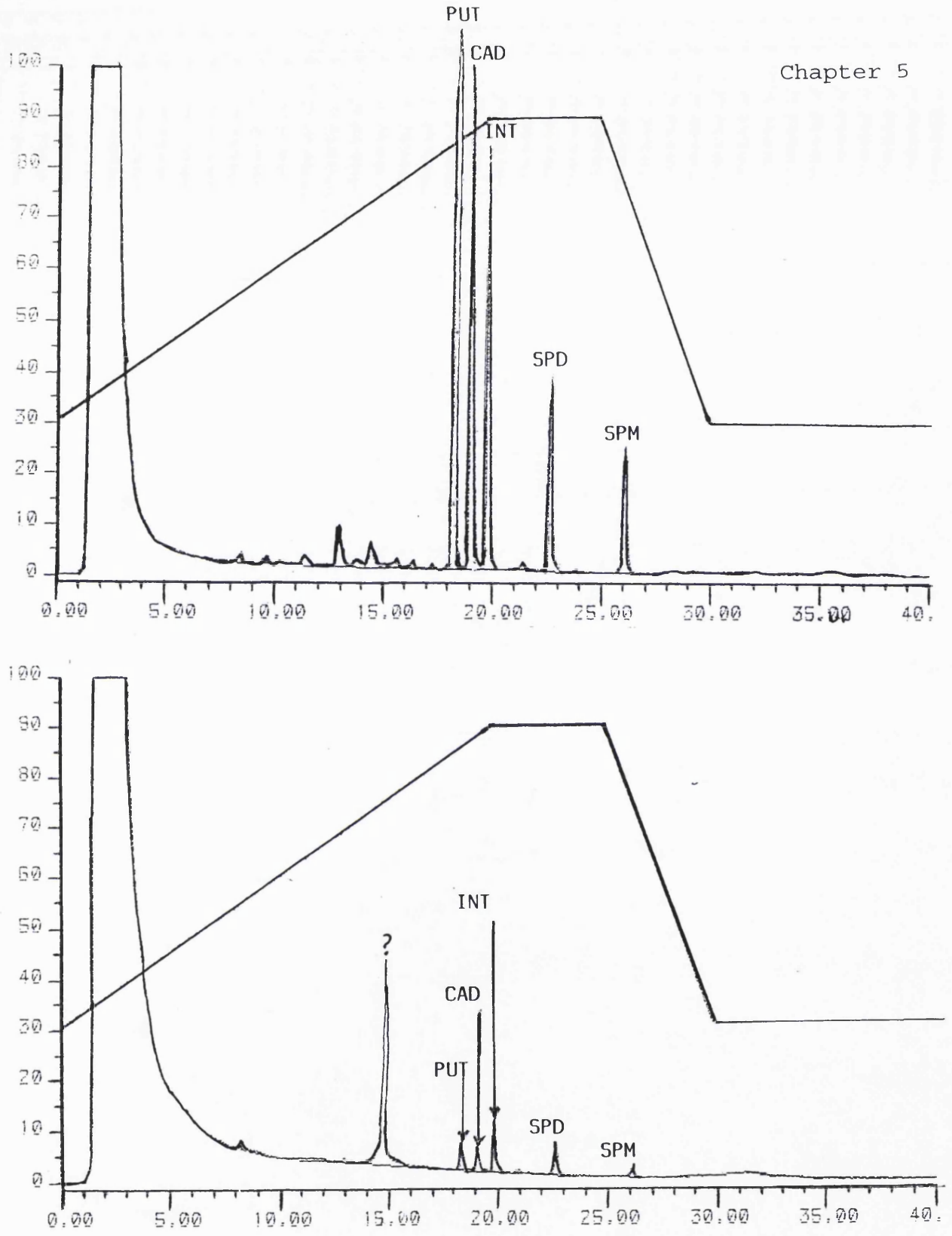


Fig. 47 (a) Hplc separation profile of standard polyamines
(b) Hplc separation profile of platelet polyamines

Extracted polyamines were derivatised with dansylchloride. 20μl (3x) of derivatised samples were injected into a column maintained at a flow rate of 1ml/min with 70% mobile phase A (10mM phosphate buffer) and 30% mobile phase B (acetonitrile and methanol).

Table 18 **Polyamine concentrations of differently treated platelets from a normal subject. *Polyamine concentrations are expressed in nmols/mg protein.**

	Control	Thrombin-treated	NO-treated
Putrescine	0.153±0.02	0.124±0.04	0.124±0.02
Cadaverine	0.10 ±0.03	0.04 ±0.01	0.06 ±0.03
Spermidine	0.19 ±0.03	0.13 ±0.02	0.16 ±0.03
Spermine	0.12 ±0.02	0.06 ±0.02	0.08 ±0.03

*Values represent the mean of three different analyses.

It is not clear at this stage whether the trend of the polyamine distribution in the platelets (control) was a reflection of platelet age. Changes in polyamine content due to age of erythrocytes has been reported by Copper et al (1976). The polyamine content as observed for the non-treated platelets may be important in understanding platelet cell ageing in the characterization of polyamine function in human platelets. In the light of the evidence that the preponderance of red cell constituents (including polyamines) lost during ageing are membrane associated (Weed and Reed, 1966), the results from platelets may also reflect membrane-associated polyamines lost during fragmentation of platelets from the megakaryocytes. In fact, polyamines have been shown to be highly associated with phospholipid and fatty acid content, and membrane stabilization of rat erythrocytes (Tabor et al, 1969; Chun et al, 1976). Platelet polyamine content may thus be clinically useful as an indicator of the presence of a young or defective platelet population in the work-up of an undiagnosed abnormality in the blood.

The changes in polyamine content after treatment with thrombin and NO is worth commenting upon. This could be important in the light of the discovery that polyamines have effects on the inhibition of drug-induced platelet aggregation. The state of aggregation of platelets is thought to be controlled by the cyclic AMP level of the platelet, with low cyclic AMP levels associated with increased platelet aggregation (William, 1972). That

polyamines are intimately related to the cAMP/cGMP balance has been demonstrated by their effects on adenylate cyclase (Rennerts et al, 1976). The content of serotonin (5-hydroxytryptamine), a biogenic amine concentrated and stored by the platelet as a major compound to be released during aggregation, amounts to 100 times that of putrescine. It is not known whether the release of such high concentrations of serotonin does in any way modulate any effect on the less abundant polyamines, or whether the release of serotonin results in any inhibition of the enzymes associated with polyamine synthesis causing a reduction in their levels.

It should, however, be emphasised that one of the peculiarities of polyamine biosynthesis is that feedback inhibition of ornithine decarboxylase, the most highly regulated enzyme, has never developed in any organism (Davis, 1990). Thus, this makes it highly unlikely that an allosteric response was responsible for the observed reductions in the platelet polyamine content after treatment with thrombin. An intriguing possibility may be that during the thrombin stimulation, polyamine content might have decreased via conjugation, degradation or cellular compartmentation. It is known that mammals have an elaborate polyamine interconversion system, which adjusts the ratios of polyamines, and degrades excess polyamines (Pegg, 1988).

Nitric oxide (NO), is part of a multifactorial, synergistic, down-regulatory system which controls platelet

haemostasis (Radomski and Moncada, 1991). Thus the possibility that it can exert a direct or indirect effect on polyamine synthesis is quite likely. Besides, NO has been shown in our laboratory to be a potent inhibitor of methionine synthetase and its substrate, methionine, acts as a precursor for polyamine biosynthesis (Fig.45).
At this stage, however, no firm conclusions could be made on these observations. This mandates further investigations to elucidate the mechanisms of the effects of thrombin and NO on platelets and to ascertain their physiological importance on platelet function.

5.4 Hplc separation of platelet sulphur-containing amino acids

Figures 48(a) and 48(b) show the hplc profiles of standard and platelet SAAs respectively, whilst Table 19 presents the concentrations of PCA-extracted platelet sulphur amino acids subsequently derivatized by O-phthaldehyde (OPA) reagent containing mercaptoethanol. The recovery of the amino acids was estimated from the recovery of a known amount of added internal standard (nor-valine) which was calculated at 92.24$\pm$1.3%.
The application of the suggested mobile phase composition and gradient profile, as described under Materials and Methods, made possible the separation of all the investigated sulphur-containing amino acids. In order to overcome

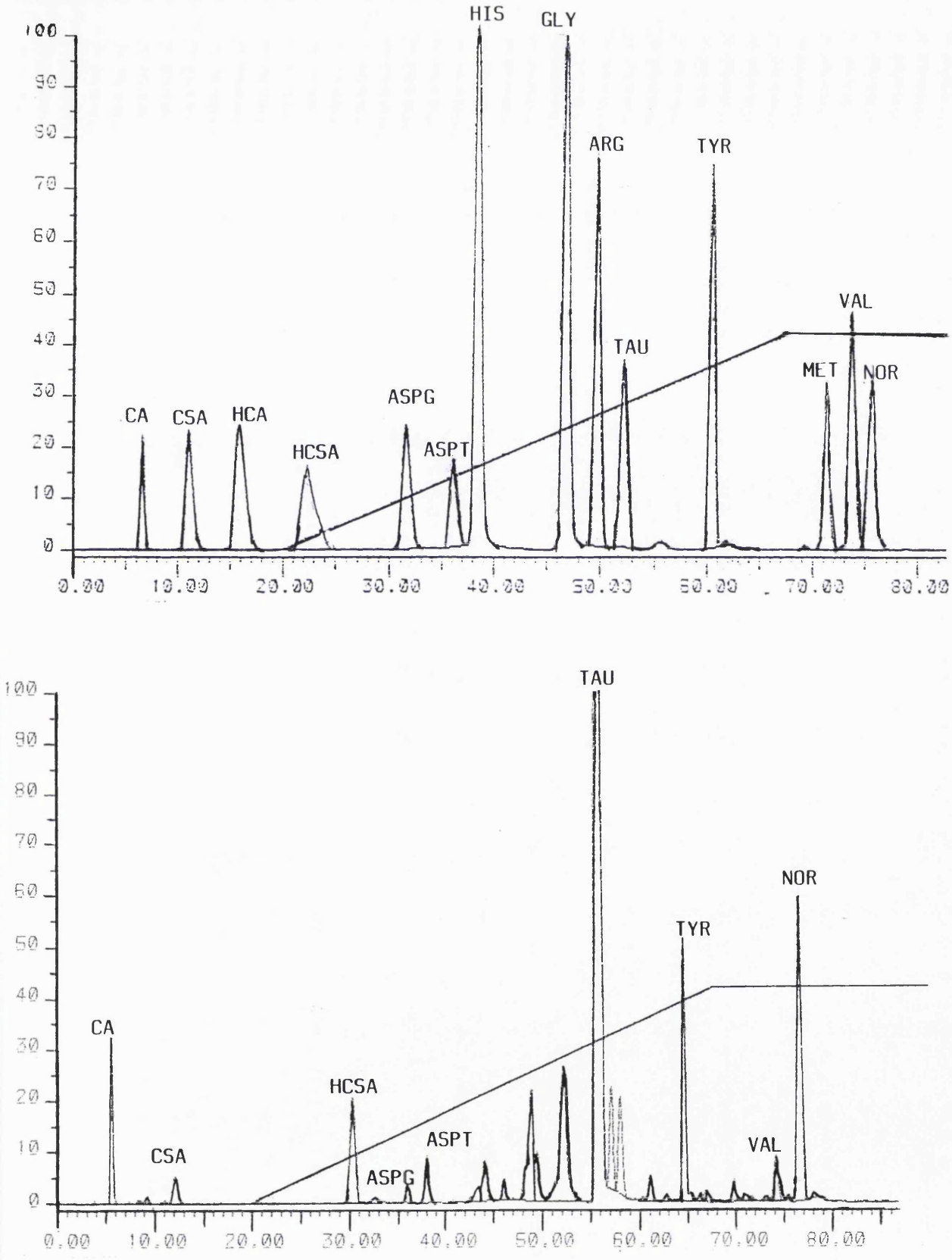


Fig. 48 (a) Hplc separation profile of standard SAA
(b) Hplc separation profile of platelet SAA

50μl of OPA-derivatised sample was injected (3x) onto a column maintained at a flow rate of 1ml/min with a gradient of 75mM phosphate buffer containing 10% (v/v) methanol (mobile phase A) into 100% methanol (mobile phase B).

the instability of the OPA derivatives (a problem encountered in the initial stages), the derivatized extracts were often prepared and analysed on the same day. The investigated human blood platelets contained 0.15±0.06ng/mg protein CA, 0.15±0.03 ng/mg protein CSA, 0.69±0.22 ng/mg protein HSCA, 4.2±0.73ng/mg protein TAU, and 0.1±0.04 ng/mg MET. These results may reflect the dietary intake of plasma-exchanged SAA or indicate the existence of an active enzyme-system responsible for the biosynthesis of SAA in platelets. However, the basic question which arises concerning the role of SAA in platelets, namely the mechanism of their release, action and uptake, is yet to be answered. It is not readily apparent from these results how such extra-CNS localization of SAAs can be reconciled with their proposed role as endogenous excitatory transmitters, although the monoaminergic properties of platelets being similar to those of neurons (Stahl, 1977) could be a plausible explanation.

The neuroexcitatory action of certain SAAs was first demonstrated coincidentally with that of L-glutamate in the first electrophysiological description of the excitatory actions of acidic amino acids on single neurons in the mammalian CNS (Curtis and Watkins, 1960). Of particular interest was the finding that S-sulpho-L-cysteine (SSC), CA and HSCA were mixed agonists exhibiting only moderate selectivity for N-methyl-D-aspartate (NMDA) receptors. In contrast, both HCA and CSA exhibited markedly greater

selectivity for NMDA receptors compared with non-NMDA receptors (Griffiths, 1993). Thus, the existence of SAAs in platelets will further enhance the use of platelets as ideal models for neuropsychiatric research, especially in studies designed to determine the structural features required for agonist and antagonist binding to neuro-receptors, and also to the study of the topology of transmitter recognition sites.

SAAs may also have a functional role in platelet physiology and biochemistry. It appears that SAAs reproduce a similar range of excitotoxic properties to those characteristic of L-glutamate-induced cell damage (Pullan et al, 1987; Olney et al, 1972). It is therefore possible that SAAs may be involved in removing aged platelet populations from circulation via their excitotoxic action. This is, however, not certain.

Biochemical studies related to the neurochemical actions of SAAs as extracellular signals and the signal transduction mechanisms by which they exert these effects have only recently been undertaken. For example, a SAA-evoked, inotropic excitatory amino acid (EAA) receptor-mediated, calcium-dependent release has been demonstrated for [^{3}H]D-aspartate and endogenous L-glutamate from primary cultures of mouse cerebellar granule cells (Griffiths, 1992). The physiological implication, if any, of a Ca^{2+}-independent mode of release is still the subject of much debate.

It has also been shown that SAAs evoke receptor-mediated production of inositol phosphate in primary cultures of

striatal neurons (Griffiths, 1992). These observations may suggest a similar and important role of SAAs in cellular signalling in platelets.

It should also be mentioned that the physiological and biochemical significance of the high levels of taurine (TAU) detected in platelets (Table 19) is quite uncertain, although a reduced excretion (elevated plasma taurine) has been observed in cystinuric patients (King, 1968).

Although the exact role of SAAs in platelets has not yet been fully established, the results from this work strongly indicate that they form part of the metabolic milieu of the platelet and may be of physiological and pathological importance in platelet function. Further work is, however, necessary to establish the mechanism of release, action and uptake of these important excitatory compounds in human blood platelets.

Table 19 Concentrations of sulphur-amino acids in human blood platelets expressed in ng/mg protein.

Sulphur Amino Acid	Detection Response	Concentration (ng/mg protein)
CA	1.05±0.01	0.15±0.06
CSA	0.91±0.02	0.15±0.03
HCA	0.81±0.01	0.69±0.22
HSCA	1.09±0.02	0.69±0.22
TAU	1.16±0.01	4.2 ±0.73
MET	1.12±0.03	0.10±0.04

CHAPTER 6

SUMMARY

6.0 Summary

Lipids were extracted from normal individual's blood platelets according to the method adopted by Bligh and Dyer (1959), and analysed by 1-D and 2-D nmr. The relative amounts detected agreed with similar data obtained purely by chrom-atographic means, but the nmr method proved to be not only more rapid, comprehensive and just as accurate as any single chromato-graphic method, but also yielded information on the fatty acid composition of the intact, non-hydrolysed lipids. To extend the information obtained by the nmr approach, the latter was combined with hplc fractionation. Nmr analysis of specific phospholipid classes yielded, for example, the relative composition of the various choline and ethanolamine containing lipids. Specifically, analysis was achieved of the diacylglycerophosphocholines and ethano-lamines, the ether lipid ethanolamines and cholines, and the corresponding ceramide (sphingoid) phospholipids. It was also possible to analyse for the C-1 unsaturated and saturated lipids attached to the alkylacyl ether lipids and the sphinganine and sphingenine lipids.

The overall phospholipid composition of platelet membranes by the nmr analysis amounted to 65.4 $\pm$ 3% of the total lipids of which the choline phospholipids were the most abundant, followed by the ethanolamine phospholipids. The nmr estimates of the distribution of individual platelet phospholipids agreed closely with the values reported by

Safrit et al (1971) for normal subjects. The choline phospholipids (43.3 ± 3% of the total phospholipids) were mainly in the diacyl form, with a relatively lower degree of unsaturation compared to the ethanolamine phospholipids. Cholesterol was the main steroid lipid detected, accounting for a third of the overall lipid distribution in platelet membranes. It should, however, be mentioned that the cholesterol content, as estimated by nmr, reflects the initial composition of the megakaryocytes, since it has been shown that platelets cannot synthesize cholesterol (Derksen and Cohen, 1973). The cholesterol:phospholipid ratio was calculated from nmr to be 0.53:1 which agreed closely with the 0.5:1 value reported by Marcus et al (1969). Values exceeding this ratio have been reported in patients with type II hyperproteinemia whose platelets demonstrate hypersensitivity (Bennett et al, 1974).

Unsaturated fatty acids, i.e. linoleic, arachidonic and docosahexaenoic acids, were the main PUFAs detected, amounting to a total of about 20% of the total fatty acid present. Thus the rest, mainly palmitic, stearic and oleic fatty acids as reported elsewhere (Marcus et al, 1969), accounted for approximately 80%.

To extend the usefulness of the nmr methodology as an investigative diagnostic tool, the lipid profiles of three patients with varying degrees of coronary artery disease (CAD) were investigated by nmr. The most striking features observed in these patients compared with that of a normal individual lipid profile were, (i) an elevated cholest-

erol, (ii) a high cholesterol:phospholipid ratio, (iii) an overall decrease in the choline and ethanolamine phospholipids and fatty acids, and finally (iv) a low unsaturation index.

Although the sample size of this study was small (because of the difficulty of getting ready access to patients), the above observations could be of pathological significance since they may either directly or indirectly influence the nature or the rate of lesion development of atherosclerotic plaque in coronary artery disease. Indeed hypercholesterolemia, with an associated excess of LDL (low density lipoproteins) and disturbances in platelet function, are among the risk factors in CAD. It has been shown (Derksen and Cohen, 1973) that exchange of cholesterol with plasma lipoproteins may result in remodelling of the endogenous content of platelets, possibly via a receptor mediated process. Due to the positive correlation between blood cholesterol and increased risk for coronary heart disease, it is quite likely that the observed elevated platelet cholesterol is a reflection of hypercholesterolemia among patients which may also be secondary to dietary or genetic predeposition. Bennett et al (1974) reported an increased cholesterol:phospholipid ratio in patients with type II hypersensitivity. An increase of hyperactive platelets as found in atherosclerotic arteries (Mehta et al, 1980) would inevitably increase phospholipid turnover (degradation) and that of some fatty acids. This could explain the observed decreases in phospholipids and fatty acids. Chignard et al

(1979) had observed an increase in the turnover of ether lipids, particularly of platelet activating factor (PAF). At this stage, the effect of various anti-thrombotic drugs on the lipid metabolism or distribution in these patients could not be speculated upon. However, the decrease in the fatty acids was ascertained to be the primary cause of the low unsaturation index. A low unsaturation index has been observed in patients with type II hyperlipidaemia and other lipid related diseases (Naito, 1982). It has been recommended in the same study by Naito (1982) that for a therapeutic diet for such patients, an unsaturation index of 1-1.2 is desirable.

It was also possible by this nmr approach to investigate phospholipid metabolism in terms of measuring metabolite levels or fluxes associated with platelet aggregation following stimulation with thrombin and ADP at 37°C. The quantitative changes of various classes of lipids was accomplished by the estimation of their intensities at their characteristic chemical shifts using deuterated chloroform as the external reference at about 7.60ppm.

In comparison with unstimulated platelets, platelets activated with various concentrations of thrombin and ADP displayed changes in the composition of specific phospholipids. In particular, the choline and ethanolamine phospholipids as estimated from their N-methyl ($-CH_3$) and methylene proton ($-CH_2\underline{CH_2}NH_2$) resonances at 3.20ppm and 3.10ppm, respectively, showed reduction averaging between 10-15% following thrombin and ADP stimulation. These nmr

results suggest a correlation between mobilization of these classes of lipids and platelet aggregation, and also confirm that specific choline and ethanolamine lipids were hydrolysed by these agonist via phospholipase(s) action. At this stage, however, it could not be concluded whether the mobilization resulted from phospholipase A, C, D or whether the lipids were either saturated or unsaturated.

A membrane-bound protein with N-methyltransferase activity, associated with phospholipid metabolism, has been isolated from human blood platelet plasma membranes. The plasma membranes were purified by velocity and sucrose gradient centrifugation and solubilized in buffer containing either 0.5% Triton-X 100 or 0.3% chaps. The partially purified solubilized extracts were further purified using a combination of ion-exchange chromatographic procedures, preparative IEF and preparative SDS-PAGE column electrophosesis.

The preparative rotofor electrofocusing using pH 3-10 ampholyte at 4°C yielded activities at both acidic and basic pHs of approximately 3.5 and 8.5, respectively. The pH 8.5 activity yielded predominantly a single 67KDa band on SDS-PAGE gels and the pH 3.5 activity also gave a similar band and a few lighter bands at 35KDa and 50KDa. The acidic and basic fractions catalysed the transfer of methyl groups from S-adenosyl methionine to PE with a predominant formation of PME (ca 90%). The fraction purified by preparative SDS-PAGE column electrophoresis also yielded a major methylating activity that eluted between 60-70KDa.

This latter protein was filtered and washed free of SDS and its activity measured; it, too, yielded predominantly the monomethylated PE.

An overall purification factor of 77.45 and a yield of 181% was achieved of the purified enzyme. This isolated protein exhibited a time-dependent change over a 60 minute incubation period at 37°C, and was stable for over six weeks at -70°C. However, the activity was reduced to 50% of its original level after this storage at -70°C.

These data are consistent with the hypothesis that several SAM-dependent isozymes exist that convert PE to PC, but other explanations for the various activities exist including the presence of separate enzymes for the methylation of ether lipids and diacylglycerophospholipids or separate enzymes for mono, di and trimethylation (Gibbons et al, 1993) which may be tissue-specific. Hirata and Axelrod (1978) have proposed that there are two enzymes responsible for the methylation whose substrates are asymmetrically distributed in the membranes. This proposed spatial segregation of the enzyme(s) further supports the observation from this work. The explanation of the differences in methylation observed between platelets and liver (the latter has been reported to have functional and biological links with phospholipid methylation) may lie in the structural distribution of this enzyme(s) in these cells. Whatever the role of the phospholipid methylating enzyme(s) in platelet function, it is not clear whether this enzyme(s) is biochemically assoc-iated with the supply

of PC which is subsequently cleaved by phospholipases to release arachidonic acid. Another type of pathway or substrate may be a major source of arachidonic acid as also suggested by Hotchkiss et al, 1981. This was based on their conclusion that the supply of PC from the methylation pathway in human platelet is not tightly linked to the presence of the phospholipases which are activated following stimulation with thrombin. It is thus unlikely that the apparent "incomplete" methylation to PC observed in this work was due to the hydrolytic action of phospholipases.

The physiological role of the detected monomethylated intermediate PME is still uncertain. The demonstration that it is capable of increasing the fluidity of rat red blood cell membrane lipids (Hirata and Axelrod, 1978c), could also apply to platelet cell membrane lipids. Further work in this area may still be necessary to establish the biochemical relationship between this enzyme(s) and the proteolipid domains of platelets, which include functional complexes of receptors, ion channel proteins and associated proteins (as found in other systems too), and also to elucidate the mechanisms of such relationships.

By incubating C^{14}-labelled PS with solubilised platelet membranes and determining the radioactive product(s) formed quantitatively by TLC and radiometric scanning, it was also observed that PS was quantitatively converted to PE, strongly indicating the presence of phosphatidylserine decarboxylase in platelet plasma membranes. This enzyme

has previously not been detected in platelet plasma membrane, although Auchi et al (1993) have partially purified the enzyme from liver plasma membrane and observed that 90% of the total activity occurred between 60-70KDa with 90% of PC formation. It was, however, clear from this work that the activity of the platelet membrane phosphatidylserine decarboxylase was not coupled to that of phospholipid-N-methyltransferase.

Finally, by employing hplc fluorescent OPA derivatization, the polyamine and sulphur containing amino acid contents of platelets were qualitatively and quantitatively analysed and their possible roles in platelet cellular function suggested.

It is hoped that the findings of this work will contribute further in enhancing the understanding of the role(s) of phospholipids in platelet function and particularly afford a novel methodological approach for the elucidation of the action of these cellular components in platelet functions. It should now be also possible, with the data obtained from this work and the methodologies adopted, to study important biological and medical problems involving platelets. These include lipid-mediated platelet cell signalling and lipid metabolic fluxes in pathological conditions involving platelets.

REFERENCES

Akesson, B. Structural requirements of the phospholipid substrate for phospholipid N-methylation in rat liver. *Biochim. et Biophys. Acta.* **752:** 460-466, 1983.

Amatruda, J.M., and Lockwood, D.H. Insulin-like effects of polyamines. Spermidine binding to fat cells and fat cell membranes. *Biochim. et Biophys. Acta.* **372:** 266-273, 1975.

Andine, P., Orwar, O., Jacobson, I., Sandberg, M., and Hagberg, H. *J. Neurochem.* **57:** 230-236, 1991.

Arvidson, G.A.E. Biosynthesis of phosphatidylcholines in rat liver. *Eur. J. Biochem.* **5:** 415-421, 1968b.

Auchi, L., Tsvetnitsky, V., Yeboah, F.A., and Gibbons, W.A. Purification of plasma membrane rat liver phosphatidylserine decarboxylase. *Biochem. Soc. Trans.* **21:** 488**S**, 1993.

Azuma, Y., Ohtani, S., Matsunaga, Y., Ueda, Y., Tajima, K., Takagi, N. Possible mechanisms of the anti-inflammatory action of polyamines. *Gifu-Shika-Gakkai-Zasshi (Japanese Science Journal)* **2:** 509-515, 1990.

Bacchi, C.J., Nathan, H.C., Hunter, S.H., McCann, P.P., Sjoerdsma, A. Polyamine metabolism: A potential therapeutic target in Trypanosomes. *Science* **210:** 332-334, 1980.

Babette, B.W., Hajjar, D.P., Eldor, A., Falcone, D.J., Tack-Goldman, K., and Minick, R.C. Interactions between Prostacyclin metabolism and cholesterol ester metabolism in vascular wall. *Advances in Prost Throm. of Leak. Res.* **11:** 429-433, 1983.

Balint, J.A., Beeler, D.A., Trebble, D.H., and Spitzer, H.L. Studies on the biosynthesis of hepatic and biliary lecithins. *J. Lipid Res.* **8:** 486-493, 1967.

Bell, R.L., Kennerly, D.A., Stanford, N., and Majerus, P.W. *Proc. Natl. Acad. Sci. USA.* **76:** 3238-3241, 1979.

Bennett, J.S., Shattil, S.J., Copper, R.A., and Colman, R.F. Platelet hypersensitivity in familial hyperbetalipoproteinemia. The role of platelet lipid composition. *Blood.* **44:** 918, 1974.

Benveniste, J., Tencé, M., Varenne, P., Bidault, J., Boullet, C., and Polonsky, J. Semi-synthese et structure proposée du facteur activant les plaquettes (PAF): PAF-acether, un alkyl ether analogue de la lysophophatidylcholine. *C.R. Acad. Sci.* **289:** 1037-1040, 1979.

Berdynskikh, N.K., Zaletok, S.P., Khomenko, A.I., Slynchak, S.M., Kasianenko, I.V., Evtushenko, A.V., Erusalimskii El. Excretion of polyamines in cancer patients. *Voprosy Onkologii (Russia)* **22(7):** 3-6, 1976.

Bevers, E.M., Comfurius, P., Van Rijn, J.L.M.L., Hemker, H.C., and Zwaal, R.F.A. General of prothrombin-converting activity and the exposure of phosphatidylserine at the outer surface of platelets. *Eur. J. Biochem.* **122:** 429-436, 1982.

Bevers, E.M., Comfurius, P., and Zwaal, R.F.A. Changes in membrane phospholipid distribution during platelet activation. *Biochim. et Biophys. Acta.* **736:** 57-66, 1983.

Bills, T.K., Smith, J.B., and Silver, M.J. Selective release of arachidonic acid from the phospholipids of human platelets in response to thrombin. *J. Clin. Invest.* **60:** 1-6, 1977.

Bjornstad, P., and Bremer J. In vivo studies on pathways for the biosynthesis of lecithin in the rat. *J. Lipid Res.* **7:**38-45, 1966.

Blackwell, G.J., Duncombe, W.G., Flower, R.J., Parsons, M.F., and Vane, J.R. The distribution and metabolism of arachidonic acid in rabbit platelets during aggregation and its modification by drugs. *Br. J. Pharmacol.* **59:** 353-366, 1977.

Bligh, E.G., Dyer, W.J. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37:** 911-917, 1959.

Blusztajn, J.K., Zeisel, S.H., and Wurtman, R.J. Synthesis of lecithin (phosphatidylcholine) from phosphatidyl-thanolamine in bovine brain. *Brain Res.* **179:** 319-327, 1979.

Brasitus, T.A., Davidson, N.O., Sohacter, D. Variation in dietary triacylglycerol saturation alters the lipid composition and fluidity of raw intestinal membranes. *Biochim. et Biophys. Acta.* **812:** 460-472, 1985.

Braquet, P., Tonqui, L., Shen, T.Y., and Varagaftig, B.B. Perspectives in platelet-activating factor research. *Pharmacol. Rev.* **39:** 97-145, 1987.

Bremer, J., Fogard, P.H., and Greenberg, D.M. The synthesis of choline and its relation to phospholipid metabolism. *Biochim. et Biophys. Acta.* **43:** 477-488, 1960.

Bremer, J., and Greenberg, D.M. Methyl transferring enzyme system of microsomes in the biosynthesis of lectin (phosphatidylcholine). *Biochim. et Biophys. Acta.* **46:** 205-216, 1961.

Broekman, M.J., Phosphatidylinositol 4, 5-biphosphate may represent the site of release of plasma membrane-bound calcium upon stimulation of human platelets. *Biochem. Biophys. Res. Comm.* **120:** 226-231, 1984.

Broekman, M.J., Handin, R.I., Derksen, A., Cohen, P. Distribution of phospholipids, fatty acids and platelet factor 3 activity among subcellular fractions of human platelets. *Blood* **47:** 963-971, 1976.

Broekman, M.J., Ward, J.W., Marcus, A.J. Phospholipid metabolism in stimulated human platelets. Changes in phosphatidylinositol, phosphatidic acid, and lysophopholipids. *J. Clin. Invest.* **66:** 275-283, 1980.

Cantoni, G.L. Biological methylation: selected topics. *Annual Review of Biochemistry* **44:** 435-451, 1975.

Carlini, E., Corazzi, L., Camici, O., and Arienti, G. The fate of phosphatidylethanolamine formed by decarboxylation in rat brain mitochondria. *Biochem. Molec. Biol. Int.* **29:** 821-829, 1993.

Carson, M.A., Emala, M., Hogsten, P., and Waechter, C.J. Co-ordinate regulation of phosphatidylserine decarboxylase activity and phospholipid N-methylation in yeast. *J. Biol. Chem.* **259:** 6267-6273, 1984.

Carvalho, A.C.A., Colman, R.W., and Lees, R.S. Platelet function in hyperlipoproteinemia. *New Eng. J. Med.* **290:** 434-438, 1974.

Castano, J.G., Alemany, S., Nieto, A., Mato, J.S. Activation of phospholipid methyltransferase by glucagon in rat hepatocytes. *J. Biol. Chem.* **255:** 9041-9045, 1980.

Casu, M., Anderson, G.J., Choi, G., and Gibbons W.A. 1D and 2D proton NMR of lipids from rat liver. *Magn. Res. Chem.* **29:** 594-02, 1991.

Carrol, R.C., Gerrard, J.M. Phosphorylation of platelet actin-binding protein during platelet activation. *Blood* **59:** 466-471, 1982.

Chap, H.J., Zwaal, R.F.A., Van Deenen, L.L.M. Action of highly purified phospholipases on blood platelets. *Biochim. et Biophys. Acta.* **467:** 146-147, 1977.

Chargaff, E., Bancroft, F.W., and Stanley-Brown, M. Studies on the chemistry of blood coagulation III. The chemical constituents of blood platelets and their role in blood clotting; with remarks on the activation of clotting by lipids. *J. Biol. Chem.* **116:** 237, 1936.

Charo, I.F., Feinman, R.D., and Detwiler, T.C. Inter-relations of platelet aggregation and secretion. *J. Clin. Invest.* **60:** 866-873, 1977.

Chaunan, V.P.S., Sikka, S.C., and Kalra, V.K. *Biochim. Biophys. Acta.* **688:** 357, 1962.

Chingnard, M., Le Couedic, J.P., Tencé, M., Vargaftig, B.B., and Benveniste, J. The role of platelet-activating factor in platelet aggregation. *Nature* **279:** 799-800, 1979.

Chingnard, M., Le Couedic, J.P., Vargaftig, B.B., and Benveniste, J. Platelet-activating factor (PAF-acether) secretion from platelets: effect of aggregating agents. *Br. J. Haematol.* **46:** 455-464, 1980.

Christie, W.W. Rapid separation and quantitation of lipid classes by high performance liquid chromatography and mass (light-scattering) detection. *J. Lipid Res.* **26:** 507-512, 1985.

Chun, P., Rennert, O., Staffen, E., and Taylor, W. Effect of polyamines on the electrokinetic properties of red blood cells. *Biochem. Biophys. Res. Commun.* **69:** 1095-1101, 1976.

Cochet, C., chambaz, E.M. Polyamine-mediated protein phosphorylation: a possible target for intracellular polyamine action. *Molecular and Cellular Endocrinology* **30:** 247-266, 1983.

Cohen, S.S. Introduction to the polyamines. Prentice-Hall, Englewood Cliffs N.J., 1971.

Cohen, P., Derksen, A., and van den Bosch, H. Pathways of fatty acid metabolism in human platelets. *J. Clin. Invest.* **49:** 128-139, 1970.

Cooper, K.D., Shukla, J.B., and Rennert, O.M. Polyamine distribution in cellular compartment of blood and in ageing erythroyctes. *Clinica Chimica Acta.* **73:** 71-88, 1976.

Cordasco, D.M., Segarnick, D.J., and Rotrosen, J. Human platelet phospholipid methylation. *Life Sciences* **29:** 2299-2309, 1981.

Cox, D.W.G., Headley, P.M., and Watkins, J.C. Actions of L- and D-homocysteate in rat CNS: a correlation between low affinity uptake and the time courses of excitation by micro electrophoretically applied L-glutamate analogues. *J. Neurochem.* **29:** 579-588, 1977.

Crew, F.T., Hirata, F., and Axelrod, J. Identification and properties of methyltransferases that synthesize phosphatidylcholine in rat brain synaptosomes. *J. Neurochem.* **34:** 1491-1498, 1980.

Crew, F.T., Hirata, F., and Axelrod, J. Identification and properties of methyltransferases that synthesize phosphat-idylcholine in rat brain synaptosomes. *J. Neurochemistry.* **34:** 1491-1498, 1980.

Crew, F.T., Morita, Y., Hirata, F., Axelrod, J., and Siraganian P. Phospholipid methylation affects immuno-

globulin E-mediated histamine and arachidonic acid release in rat leukemic basophils. *Biochem. Biophys. Res. Commun.* **93:** 42-49, 1980.

Cui, Z., Vance, J.E., Chen, M.H., Voelker, D.R., and Vance, D.E. Cloning and expression of a novel phosphatidyl-ethanolamine-N-methytransferase: a specific biochemical and cytological marker for a unique membrane fraction in rat liver. *J. Biol. Chem.* **268:** 16655-16663, 1993.

Curtis, D.R., and Watkins, J.C. The excitation and depression of spinal neurons by structurally related amino acids. *J. Neurochem.* **6:** 1117-141, 1960.

Cusack, N.J., Hickman, M.E., and Born, G.V.R. Effects of D- and L-ehantiomers of adenosine, AMP and ADP and their 2-chloro- and 2-azido-analogues on human platelets. *Proc. R. Soc. London B.* **206:** 139-144, 1979.

Cusack, N.J., and Hourani, S.M.O. Adenosine, adenine nucleotides and platelet function. *In* Purinergic Regulation of Cell Function, ed. by J. Phillis, CRC Press, New York, pp.121-131, 1991.

Dadak, C.H., Leithner, C.H., Sinzinger, H., and Silberbauer, K. Diminished prostacyclin formation in umbilical arteries of babies born to women who smoke. *Lancet.* **i:94**, 1981.

Data, J.L., Molony, B.A., Meinzinger, M.M., and Gorman, R.R. Intravenous infusion of prostacyclin sodium in man: Clinical effects and influence on platelet adenosine diphosphate sensitivity and adenosine 3'-5' cyclic monophosphate levels. *Circulcation* **64:** 4-12, 1981.

Davies, T., Davidson, M.M.L., McClenaghan, M.D., Say, A., and Haslam, R.J. Factors affecting platelet cyclic GMP levels during aggregation induced by collagen and by arachidonic acid. *Thromb. Res.* **9:** 387-405, 1976.

Davies, M.J., and Thomas, T. The pathological basis and microanatomy of occlusive thrombus formation in human coronary arteries. *Philos. Trans. R. Soc. Lond. B.* **294:** 225-229, 1981.

Davies, R.H. Management of polyamine pools and the regulation of ornithine decarboxylase. *Journal of Cellular Biochemistry* **44:** 199-205, 1990.

De Chaffoy de Courcelles, D., Roevens, P., and Van Belle, H. Stimulation by serotonin of 40KDa and 20KDa protein phosphorylation in human platelet. *FEBS Lett.* **171:** 289-292, 1984.

Dennis, E.A., and Kennedy, E.P. Intracellular sites of lipid synthesis and the biogenesis of mitochondria. *J. Lipid. Res.* **13:** 263-267, 1972.

Derksen, A., and Cohen, P. Extensive incorporation of (2-^{14}C) mevalonic acid into cholesterol precursors by human platelets in vitro. *J. Biol. Chem.* ***248:*** *7396-7403, 1973.*

Deykin, D., and Dresser, R.K. The incorporation of acetate and palmitate into lipids by human platelets. *J. Clin. Invest.* **47:** 1590-1602, 1968.

Deykin, D. Altered lipid metabolism after primary aggregation. *J. Clin. Invest.* **52:** 483-492, 1973.

Deykin, D., and Snyder, D. The effect of epinephrine on platelet lipid metabolism. *J. Lab. Clin. Med.* **82:** 554-559, 1973.

Diehl, A.M., Yang, S.Q., Brown, N., Smith, J., Gordon, R., Casero, R. Ethanol-associated alterations in the kinetics of putrescine uptake and metabolism by regenerated liver. *Alcohol - Clin. Exp. Res.* **16(1):** 5-10, 1992.

Do, K.Q., Mattenberger, M., Streit, P., and Cuenod, M. In vitro release of endogenous excitatory sulfur-containing amino acids from various brain regions. *J. Neurochem.* **46:** 779-786, 1986.

Doyle, J.T., Dawber, T.R., Kannel, W.B., Kinch, S.H., and Kahn, H.A. The relationship of cigarette smoking to coronary heart disease. *JAMA* **190:** 886-890, 1964.

Drouva, S.V., Laplante, E., Le Blanc, P., Bechet, J. Estradiol activates methylating enzyme(s) involved in the conversion of phosphatidylethanolamine to phosphatidyl-choline in rat pituitary membranes. *Endocrinology* **119:** 2611-2622, 1986.

Drummond, A.H., and MacIntyre, D.E. *In* Platelets in Biology and Pathology, ed. 3, ed. by D.E. MacIntyre and J.L. Gordon. Elsevier, Amsterdam, 1987.

Dygas, A., and Zborowski, J. Effects of Triton-X100 on the activity and solubilization of rat mitochondrial phosphatidyserine decarboxylase. *Acta Biochim. Polonica.* **36:** 131-141, 1989.

Eloranta, T.O., Kajander, E.O. Catabolism and lability of S-adenosyl-L-methionine in rat liver extracts. *Biochem. J.* **224:** 137-144, 1984.

Elsbach, P., Pettis, P., Marcus, A. Lysolecithin metabolism by human platelets. *Blood* **37:** 675-693, 1971.

Erne, P. and Pletscher, A. Rapid intracellular release of calcium in human in platelets by stimulation of 5-HT_2 receptors. *Br. J. Pharmacol.* **84:** 545-549, 1985a.

Fenn, G.C., Caberos, L.P., and Littleton, J.M. Effect of ethanol on thrombin-induced platelet phospholipid breakdown and release of [^{3}H]-5 hydroxytryptamine. *Alcohol.* **2:** 177-182, 1985.

Feinstein, M.B. Platelets, macrophages and neutrophils. *In* Inositol Lipids in Cell Signalling, ed. by R.H. Michell, A.H. Drummond, and C.P. Dowens, pp.247-281. Academic Press, London, 1989.

Flores, N.A., Sheridan, D.J. The pathophysiological role of platelets during mycocardial ischaemia. *Cardiovascular Res.* **28:** 295-342, 1994.

Flores, N.A., Sheridan, D.J. Electrophysiological and arrhythmogenic effects of platelet activating factor during normal perfusion myocardial ischaemia and reperfusion in guinea-pigs. *Br. J. Pharmacol.* **101:** 734-738, 1990.

Finkelstein, J.D., Kyle, W.E., Harris, B.J., Martin, J.J. Methionine metabolism in mammals: concentration of metabolites in rat tissue. *Journal of Nutrition* **112:** 1101-1018, 1982.

Finkelstein, J.D., Martin, J.J., and Harris, B.J. Methionine metabolism in mammals: The methionine sparing effect of cystine. *J. Biol. Chem.* **263:** 11750-11754, 1988.

Fisher, S.K., Lucio, A., Rooijen, A., Agranoff, B.W. Renewed interest in the polyphosphoinositides. *Trends in Biochem. Sci.* **9:** 215-219, 1984.

Fonteh, A.N. Lipid methyltransferase: purification, anaesthetic and drug effects. Ph.D. Thesis, Univ. of London, 1989.

Fox, J.E.B., Reynolds, C.C., Phillips, D.R. Calcium-dependent proteolysis during platelet aggregation. *J. Biol. Chem.* **258:** 9973-9981, 1983.

Funder, J., Herschco, L., Rothstein, A., and Livne, A. Na^+/H^+ exchange and aggregation of human platelets activated by ADP: The exchange is not required for aggregation. *Biochim. et Biophys. Acta.* **938:** 425-433, 1988.

Fuster, V., Cohen, M., and Halperin, J. Asprin in the prevention of coronary disease. *N. Eng. J. of Med.* **231:** 183-185, 1989.

Galjaard, H. *In* Genetic Metabolic Disease, pp.366-379, Elsevier/North Holland Biomedical Press, Amsterdam, 1971.

Gibbons, W.A., Auchi, L., McBride, K., Fonteh, A.N., Larijani, B., Tsvetnitsky, V., Anderson, M.M., Yeboah, F.A. The purification and molecular weight determination of rat liver microsomal phospholipid N-methyltransferase. *Biochem. Soc. Trans.* **22:** 449, 1993.

Gibson, K.D., Wilson, J.D., and Udenfriend, S. The enzymatic conversion of phospholipid ethanolamine to phospholipid choline in rat liver. *J. Biol. Chem.* **236:** 673-679, 1961.

Griffiths, R. The biochemistry and pharmacology of excitatory sulphur-containing amino acids. *Biochem. Soc. Trans.* **21:** 66-71, 1993.

Griffiths, R. *In* Alfred Benzon Symposium 32; Drug research related to neuroactive amino acids. (Schousboe, A., Diemer, N.H., and Kofod, H. eds.), pp.64-81, Munksgarrd, Copenhagen, 1992.

Hamaberg, M., Svensson, J., and Samuelsson, B. Prostaglandin endoperoxides: A new concept concerning the mode of action and release of prostaglandins. *Proc. Natl. Acad. Sci. USA.* **71:** 3824-3828, 1974b.

Hashizume, K., Kobayashi, M., Ichikawa, K. Guanosine 5'-triphosphate modulation of S-adenosyl-L-methionine mediated methylation of phosphatidylethanolamine in rat liver plasma membrane. *Biochem. Biophys. Res. Comm.* **114:** 425-430, 1983.

Haslam, R.J. Role of adenosine diphosphate in the aggregation of human blood platelets by thrombin and by fatty acids. *Nature* **202:** 765-768, 1964.

Haslam, R.J. Interaction of the pharmacological receptors of blood platelets with adenylate cyclase. *Ser. Haematol.* **6:**333-350, 1973.

Haslam, R.J., and Rosson, G.M. Aggregation of human blood platelets by vasopressin. *Am. J. Physiol.* **223:** 958-967, 1972.

Hawthorne, J.N., and White, D.A. *Vitamin's Hormones* **33:** 529-573, 1975.

Hennes, A.R., Awai, K., Hammerstand, K., and Duboff, G. Carbon-14 in carboxyl carbon of fatty acid from normal and diabetic subjects. *Nature* **210:** 839-844, 1966.

Henry, M., Juillard, J., Tell, G., Schechter, P.J.S., Grove, J., Koch-Wesser, J., Grosshans, E. Comparative effects of topical anthralinine and difluoromethyl ornithine (alpha DFMO) on epidermal polyamine concentrations in psoriasis. *Br. J. Dermatol.* **105:** Supplement 20, pp.33-34, 1981.

Higgins, J.A. Biogenesis of endoplasmic reticulum phosphatidylcholine. Translocation of intermediates across the membrane bilayer during methylation of phosphatidylethanolamine. *Biochem. et Biophys. Acta.* **640:** 11-15, 1981.

Higgins, J.A., and Fieldsend, J.K. Phosphatidylcholine synthesis for incorporation into membranes or for secretion as plasma lipoproteins by golgi membranes of rat liver. *J. Lipid Res.* **28:** 268-278, 1987.

Hirata, F., Axelrod, J. Enzymatic methylation of phosphatidylethanolamine increases erythrocyte membrane fluidity. *Nature* **275:** 219-220, 1978.

Hirata, F., Axelrod, J., and Crews, F.T. Concanavalin A stimulates phospholipid methylation and phosphatidylserine decarboxylase in rat mast cells. *Proc. Natl. Acad. Sci. USA* **76:** 4813-4816, 1979.

Hirata, F., and Axelrod, J. Phospholipid methylation and biological signal transmission. *Science* **209:** 1082-1909, 1980.

Hirata, F., Axelrod, J. Enzymatic synthesis and rapid translocation of phosphatidylcholine by two methyltransferases in erythrocytes. *Proc. Natl. Acad. Sci. USA* **75:** 2348-2352, 1978.

Hirata, F., Strittmatter, W.J., Axelrod, J. ß-Adrenergic receptor agonists increase phospholipid methylation, membrane fluidity, and ß-adrenergic receptor-adenylate cyclase coupling. *Proc. Natl. Acad. Sci. USA* **76:** 368-372, 1979.

Hirata, M., Hayashi, Y., Ushikubi, F., Yokota, Y., Kageyama, R., Nakanishi, S., and Narumiya, S. Cloning and expression of CDNA for a human thromboxane A_2 receptor. *Nature* **349:** 617, 1991.

Hoak, J.C., Spector, A.A., Fry, G.L., and Barnes, B.C. Localization of free fatty acids taken by human platelets. *Blood* **40:** 16-22, 1972.

Holmsem, H. Platelet energy metabolism in relation to function. *In* Platelets and Thrombosis, ed. by D.C.B. Mills and F.I. Pasetti, pp.45-62, Academic Press, New York, 1977.

Holmsen, H., Weiss, H.J. Secretable storage pools in platelets. *Annu. Rev. Med.* **30:** 119-134, 1979.

Holmsen, H., Karpatkin, S. Metabolism of platelets. *In* Williams W.J., Beutler, E., Erslev, A.J., Lichtman, M.A. eds. Haematology, 3rd ed. New York. McGraw-Hill, 1157, 1983.

Hotchkiss, A., Jordon, J.V., Hirata, F., Shulman, N.R., and Axelrod, J. Phospholipid methylation and human platelet function. *Biochem. Pharmacol.* **30:** 2089-2095, 1981.

Hourani, S.M.D. and Cusack, N.J. Pharmacological receptors on blood platelets. *Pharmacological Reviews.* **43:** 244-285, 1991.

Hutton, R.A., and Deykin, D. The effect of ADP on phosphatidylinositol synthesis in normal and storage pool deficient human platelets. *Proc. 16th Annual Meeting, Am. Soc. Hematol.* p.116. Abstract No. 238.

Ichohen, P., Derksen, A., and van den Bosch, H. Pathways of fatty acid metabolism in human platelets. *J. Clin. Invest.* **49:** 128-139, 1970.

Imai, A., Yano, K., Kameyama, Y., Nozawa, Y. Evidence for predominance of phospholipase A_2 in release of arachidonic acid thrombin-activated platelets. *Jpn. J. Exp. Med.* **52:** 99-105, 1982.

Ishizaka, T., Hirata, F., Ishizaka, K., Axelrod, J. Phospholipid methylation, Ca^{2+} influx, and histamine release by bridging of IgE receptors on rat mast cells. *Proc. Natl. Acad. Sci. USA.* **77:** 1903-1906, 1980.

Jänne, J., Raina, A. Stimulation of spermidine synthesis in the regenerating rat liver: relation to increased ornithine decarboxylase activity. *Acta. Chem. Scand.* **22:** 1349-51, 1968.

Jänne, J., Raina, A. Polyamines in rapid growth and cancer. *Biochim. et Biophys. Acta.* **473:** 241-293, 1978.

Junl-Moler, S., Edvardsson, N., Jahnmatz, B., Rosen, A., Sorensen, S., Omblus, R. Double-blind trial of aspirin in primary prevention of myocardial infarction in patients with stable chronic angina pectoris. The Swedish Angina Pectoris Aspirin Trial (SAPAT) Group. *Lancet.* **340:** 1421-1425, 1992.

Kanoh, H. Biosynthesis of molecular species of phosphatidylcholine from radioactive precursors in rat liver slices. *Biochim. et Biophys. Acta.* **176:** 756-763, 1969.

Kaplan, L.A. and Pesce, A.J. in Clincal Chemistry, theory, analysis and correlation. pp.454-483, 1989.

Karpartkin, S., Charmatz, A., Langer, R.M. Glycogenesis and gluconeogenesis in human platelets. Incorporation of glucose, pyruvate, and citrate into platelet glycogen synthetase and fructose-1, 6-diphosphatase activity. *J. Clin. Invest.* **49:** 140-149, 1970.

Kennedy, E.P., and Weiss, S.B. The function of cytidine coenzymes in the biosynthesis of phospholipids. *J. Biol. Chem.* **222:** 193-214, 1956.

Kerry, R., and Scrutton, M.C. Platelet adrenoceptors. *In* The Platelets: Physiology and Pharmacology ed. by G.L. Longenecker, pp.113-157, Academic Press, Orlando, FL, 1985.

Kerry, R., Scrutton, M.C., and Wallis, R.B. Mammalian platelet adrenoceptors. *Br. J. Pharmacol.* **81:**91-102, 1984.

Kelly, K.L., Kiechle, F.L., Jarret, L. Insulin stimulation of phospholipid methylation in isolated rat adipose plasma membranes. *Proc. Natl. Acad. Sci. USA.* **81:** 1089-1092, 1984.

Kim, J.P., Koh, J., and Choi, D.W. L-Homocysteate is a potent neurotoxin on cultured cortical neurons. *Brain Res.* **437:** 103-110, 1987.

Kim, S., Nochumson, S., Chin, W., and Paik, W.K. A rapid method for the purification of S-adenosylmethionine : protein-carboxyl-D methyltransferase by affinity chromatography. *Anal. Biochem.* **84:** 412-422, 1978.

King, J.S., Jr. Treatment of cystinuria with α-mercapto-propionylglycine: A preliminary report with some notes on column chromatography mercaptans. *Proc. Soc. Exp. Bio. Med.* **129:** 927-932, 1968.

Laemmli, U.K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* **222:** 680-685, 1970.

Lakher, M.B., Nurtman, R.J. Molecular composition of phosphatidylcholines produced by the phospholipid methylation pathway in rat brain in vivo. *Biochem. J.* **244:** 325-330, 1987a.

Lakher, M.B., Wurtman, R.J. *In* vivo synthesis of phosphat-idylcholine in rat brain via the phospholipid methylation pathway. *Brain Research.* **419:** 131-140, 1987b.

Lamping, E., Kohlwein, S.D., Henry, S.A., and Paltauf, F. Co-ordinate regulation of phosphatidyserine decarboxylase in Saccharomyces cerevisae. *J. Bacteriol.* **173:** 6432-6437, 1991.

Launay, J.M., Vittet, D., Viclaud, M., Rondot, A., Mathieu, M.N., Lalau-Keraly, C., Cantau, B., and Cherillard, C. Via vasopressin specific receptors on human platelets : pontentiation by ADP and epinephrine and evidence for homologous down-regulation. *Throm. Res.* **45:** 323-331, 1987.

Levine, P.H. An acute effect of cigarette smoking on platelet function. A possible link between smoking and arterial thrombosis. *Circulation.* **48:** 619-623, 1973.

Lewis, N., Majerus, P.W. Lipid metabolism in human platelets : De novo phospholipid synthesis and the effect of thrombin on the pattern of synthesis. *J. Clin. Invest.* **48:** 2114-2123, 1969.

Lupu, F., Calb, M., Scurei, C., and Simionescu, N. Changes in the organization of membrane lipids during human platelet activation. *Lab. Invest.* **54:** 136-145.

Lyman, R.L., and Hopkins, S.M., Sheehan, G., and Tinoco, J. Effects of estradiol and testosterone on the incorporation of [Me-^{14}C] methionine in rat liver lecithins. *Biochim. et Biophys. et Acta.* **152:** 197-207, 1968.

Lyman, R.L., and Hopkins, S.M., and Sheehan, G., and Tinoco, J. Incorporation and distribution of [Me-^{14}C] methionine methyl into liver phosphatidylcholine, fractions from control and essential fatty acid deficient rats. *Biochim. et Biophys. Acta.* **176:** 86-94, 1969.

Lyman, R.L., Sheeham, G., Tinoco, J. Phosphatidylethanolamine metabolism in rats fed on a low methionine, choline-deficient diet. *Lipids*. **8:** 71-79, 1973.

MacFarlane, D.E. Agonists and receptors : adenosine diphosphate. *In* Platelet Responses and Metabolism, Vol. II: Receptors and Metabolism, ed. by H. Holmsen, pp.19-36, CRC Press, Boca Raton, FL, 1987.

MacIntyre, D.E., Bushfield, M., Gibson, I., Hopple, S., MacMillan, L., McNicol, A., and Rossi, G. Human platelet receptors and receptor mechanisms for stimulatory and inhibitory lipid mediators. *In* Biology of Icosanoids, ed. by M. Lagarde, pp.321-334, Colloque Inserm, Vol. 152, 1987.

Mahadevappa, V.G., and Holub, B.J. The molecular species composition of individual diacyl phospholipids in human platelets. *Biochim. et. Biophys. Acta*. **713:** 73-79, 1982.

Mahadevappa, V.G., and Holub, B.J. Degradation of different molecular species of phosphatidylinositol in thrombin-stimulated human platelets. *J. Biol. Chem*. **258:** 5337-5339, 1983.

Majerus, P.W., Prescott, S.M., Hofmann, S.L., and Neufeld, E.J., and Wilson, B.W. Uptake and release of arachidonate by platelets. *Advances in Prostaglandin, Thromboxane and Leukotriene Research*. **11:** 45-50, 1983.

Majerus, P.W., Smith, M.B., and Clamon, G.H. Lipid metabolism in human platelets I. Evidence for a complete fatty acid synthesizing system. *J. Clin. Invest*. **48:** 156-164, 1968.

Marcus, A.J., Ullman, H.L., and Safier, L.B. Lipid composition of subcellular particles of human blood platelets. *J. Lipid. Res*. **10:** 108-114, 1969.

Marcus, A.J., Ullman, H.L., and Safier, L.B. Studies on human platelet gangliosides. *J. Clin. Invest*. **51:** 2602-2612, 1972.

Marcus, A.J., Safier, L.B., Ullman, H.L. Interactions between 5-hydroxy-tryptamine and platelet lipid fraction. *In* Biochemistry and Pharmacology of Platelets. pp.309-326. CIBA Foundation Symposium 35 (New Series) American Elsevier, N.Y., 1975.

Marcus, A.J., Ullman, H.L., Safier, L.B., Ballard, H.S. Platelet phosphatides. Their fatty acid and aldehyde composition and activity in different clotting systems. *J. Clin. Invest*. **41:** 2198-2212, 1962.

Mark, P.A., Gellhorn, A., and Kidson, C. Lipid synthesis in human leukocytes, platelets, and erythrocytes. *J. Biol. Chem*. **235:** 2579-2583, 1960.

Massini, P., and Lüscher, E.F. Some effects of ionophores for divalent cations on blood platelets. Comparison with the effects of thrombin. *Biochim. et Biophys. Acta.* **372:** 109-121, 1974.

Mato, J.M., and Alemany, S. What is the function of phospholipid methylation? *Biochem. J.* **213:** 1-5, 1983.

McBride, K. S-adenosylmethionine-dependent methylation of proteins and lipids. Ph.D., thesis (Univ. of London), 1988.

McGivney, A., Crews, F.T., Hirata, F., Axelrod, J., and Siraganian, R.P. Rat basophilic leukemia cell lines defective in phospholipid methyltransferase enzymes, Ca^{2+} influx and histamine release: Reconstruction by hybridization. *Proc. Natl. Acad. Sci. USA.* **78:** 6176-6180, 1981.

McKean, M.L., Smith, J.B., and Silver, M.J. Formation of lysophosphatidylcholine by human platelets in response to thrombin. *J. Biol. Chem.* **256:** 1522-1524, 1981.

Mehta, J., Mehta, P., Pepine, C.J., and Conti, C.R. Platelet function studies in coronary artery disease. VII. Effect of aspirin and tachycardial stress on aortic and coronary venous blood. *Am. J. Cardiol.* **45:** 945-951, 1980.

Mewelt, K.N., Oakes, D.J., Olverman, H.J., Smith, D.A.S., and Watkins, J.C. Pharmacology of the excitatory actions of sulphonic and sulphuric acid. *In* P. Mandel and F.V. Defendis (Eds) CNS Receptors From Molecular Pharmacology to Behaviour, Raven Press, New York, pp.163-174, 1983.

Meyer, F.A. Determinants for platelet adhesion and aggregation on collagen. *In* Platelets : Cellular Response Mechanisms and their Biological Significance, ed. by A. Rotman, F.A. Meyer, C. Gitler, and A. Silberberg, pp.51-66, Wiley, Chichester, England, 1980.

Michell, R.H. Inositol phospholipids and cell surface receptor function. *Biochim. et Biophys. Acta.* **415:** 81-147, 1975.

Michell, R.H. Inositol phospholipid membrane function. *Trends in Biochem. Sci.* **4:** 128-131, 1979.

Miller, M.E., and Lewis, B., eds. Lipoprotein, atherosclerosis and coronary heart disease, New York, American Elsevier Publishing Co. Inc., 1981.

Mori, K., Taniguchis, S., Kumada, K., Nakazawa, K., Fujiwara, M., and Fujiwara, M. Enzymatic properties of phospholipid methylation in rabbit platelets. *Throm. Res.* **29:** 215-224, 1983.

Mozzi, R., and Porcellati, G. Conversion of phosphatidyl-ethanolamine to phosphatidylcholine in rat brain by the methylation pathway. *FEBS. Lett.* **100:** 363-366, 1979.

Mustard, J.F., and Packham, M.A. Factors influencing platelet function: adhesion, release and aggregation. *Pharmacol. Rev.* **22:** 97-187, 1970.

Mustard, J.F., Perry, D.W., Kinlough-Rathbone, R.L., and Packham, M.A. Factors responsible for ADP-induced release reaction of human platelets. *Am. J. Physiol.* **228:** 1757-1765, 1975b.

Naito, H.K., editor. Nutrition and heart disease, New York. Spectrum Publication Inc., 1982.

Nathan, H.C., Bacchi, C.J., Hunter, S.H., Rescigno, D., McCann, P.P., Sjoerdsma, A. Antagonism by polyamines of the curative effects of alpha-difluoromethylornithine in Trypanoso brucei infections. *Biochem. Pharmacol.* **30:** 3010-3013, 1981.

Nishizuka, Y. The role of protein kinase C in cell surface signal transduction and tumour promotion. *Nature.* **308:** 693-698, 1984.

Olney, J.W., Ho, O.L., and Rhee, V. and Schainker, B. Cysteine-induced brain damage in infant and fetal rodents. *Brain Res.* **45:** 117-126, 1972.

Overmeyer, J.H., and Waechter, C.J. Regulation of phosphatidylserine decarboxylase in Saccharomyces cerevisiae by inositol and choline; Kinetics of repression and depression. *Arch. Biochem. Biophys.* **290:** 511-516, 1991.

Packham, M.A., Bryant, N.L., Guccione, M.A., Kinlough-Rathbone, R.L., and Mustard, J.F. Effect of the concentration of Ca^{2+} in the suspending medium on the responses of human and rabbit platelet to aggregating agents. *Thromb. Haemost.* **62:** 968-976, 1989.

Paik, W.K., and Kim, S. Protein methylation, pp.82-97. J. Wiley and Sons, New York, 1980.

Pajares, M.A., Alemany, S., Varela, I., Marin Cao, D., Mato, J.M. Purification and photoaffinity labelling of lipid methytransferase from rat liver. *Biochem. J.* **223:** 61-66, 1984.

Papahajopoulos, D., Hougie, C., and Hanahan, D.J. Influence of surface charge of phospholipids on their clot promoting activity. *Proc. Soc. Expt. Biol. Med.* **111:** 412, 1962.

Peace, P.H., Wright, J.M., Egan, C.M., and Scrutton, M.C. Interaction of human platelets with the 2,3-dialdehyde and 2,3-dialcohol derivatives of adenosine 5'-diphosphate and adenosine 5-triphosphate. *Eur. J. Biochem.* **88:** 543-554, 1978.

Pennington, D.G. Formation of platelets. *In* Platelets in Biology and Pathology 2, ed. by J.L. Gordon, pp.19-41, Elsevier, Amsterdam, 1981.

Pegg, A.E. Polyamine metabolism and its importance in neoplastic growth and as a target for chemotherapy. *Cancer Res.* **48:** 759-774, 1988.

Percy, A.K., Moore, J.F., Carso, M.A., and Waechter, C.J. Characterization of brain phosphatidylserine decarboxylase: localization in the mitochondria inner membrane. *Arch. Biochem. Biophys.* **223:** 484-494, 1983.

Pike, M.C., Kredich, N.M., Snyderman, R. Requirement of S-adenosyl-L-methionine mediated methylation for human monocyte chemotaxis. *Proc. Natl. Acad. Sci. USA.* **75:** 3928-3932, 1978.

Pinel, J.P.J., Gorzalka, B.B., Ladak, F. Cadaverine and putrescine initiate the burial of dead conspecifics by rats. *Physiol. Behav.* **27:** 819-824, 1981.

Prasad, C., Edwards, R.M. Stimulation of rat pituitary phospholipid methyltransferase by vasopressin but not oxytocin. *Biochem. Biophys. Res. Comm.* **103:** 559-564, 1981.

Pullan, L.M., Olney, J.W., Price, M.T., Compton, R.P., Hood, W.F., Michael, J., and Monahan, J.B. Excitatory amino acid receptor potency and subclass specificity of sulfur-containing amino acids. *J. Neurochem.* **49:** 1301-1307, 1987.

Radomski, M.W., and Moncada, S. Role of nitric oxide in endothelial cell - platelet interactions. *Antithrombotics.* **27:** 48, 1991.

Recasens, M., Varga, V., Nanapoulos, D., Saadoun, F., Vincedon, G., Benavide, J. Evidence for cysteine sulphinate as neurotransmitter. *Brain Res.* **239:** 153-173, 1982.

Refsum, H., Ueland, P.M. Clinical significance of pharmacological modulation of homocysteine metabolism. *Tips Reviews.* **11:** 411-416, 1990.

Rennert, O.M., Buehler, B., Miale, T. Polyamines and platelet aggregation. *Life Sciences.* **19:** 257-264, 1976.

Rennert, O., Midle, T., Shukla, D., Lawson, D., and Friars, J. Polyamines concentration in bone marrow aspirates of children with leukemia and other malignances. *Blood.* **47:** 695-701, 1976.

Ridgway, N.D., and Vance, D.E. Phosphatidylethanolaine N-methyltransferase from rat liver. *Methods in Enzymology.* **209:** 366-374, 1992.

Ridgway, N.D., Yao, Z., Vance, D.E. Phosphatidyl-ethanolamine levels and regulation of phosphatidyl-ethanolamine-N-methyltransferase. *J. Biol. Chem.* **264:** 1203-1207, 1989.

Rink, T.J., Smith, S.W., and Tsien, R.J. Cytoplasmic free Ca^{2+} in human platelets: Ca^{2+} thresholds and Ca-independent activation for shape-change and secretion. *FEBS Lett.* **148:** 21-26, 1982.

Rittenhouse, S.E. Inositol lipid metabolism in the responses of stimulated platelets. *Cell Calcium.* **3:** 311-322, 1982.

Rittenhouse-Simmons, S. Production of diglyceride from phosphatidylinositol activated human platelets. *J. Clin. Invest.* **63:** 580-588, 1979.

Ross, R.G., Kariya, B., Harker, L. A platelet-dependent serum factor that stimulates the proliferation of arterial smooth muscle cells in vitro. *Proc. Natl. Acad. Sci. USA.* **71:** 1207-1210, 1974.

Rudnick, G., Bencuya, R., Nelson, P.J., Zito, R.A. Inhibition of platelet serotonin transport by propranold. *Mol. Pharm.* **20:** 118-123, 1981.

Russel, D., Snyder, S.H. Amine synthesis in rapidly growing tissue: ornithine decarboxylase activity in regenerating rat liver, chick embryo, and various tumours. *Proc. Natl. Acad. Sci. USA.* **60:** 1420-1427, 1968.

Russel, D., Snyder, S.H. Amine synthesis in regenerating rat liver: extremely rapid turnover of ornithine decarboxylase. *Mol. Pharmacol.* **5:** 253-263, 1969.

Russel, F.A., and Deykin, D. The effect of thrombin on the uptake and transformation of arachidonic acid by human platelets. *Am. J. Hematol.* **1:** 59-70, 1976.

Rytter, D., Miller, J.E., and Cornatzer, W.E. Specificity for incorporation of choline and ethanolamine into rat liver microsomal lecithins. *Biochim. et Biophys. Acta.* **152:** 418-421, 1968.

Safrit, H.F., Weiss, J.W., Phillips, G.B. The phospholipid and fatty acid composition of platelets in patients with primary defects of platelet function. *Lipids.* **7:** 60-67, 1971.

Sage, S.O., and Reast, R., and Rink, T.J. ADP evokes biphasic Ca^{2+} influx in fura-2-loaded human platelets. *Biochem. J.* **256:** 675-680, 1990.

Salerno, D.M., and Beeler, D.A. The biosynthesis of phospholipids and their precursors in rat liver involving de novo methylation, and base-exchange pathways, in vivo. *Biochim. et Biophys. Acta.* **326:** 325-338, 1973.

Sambooski, R.W., Ridgway, N.D., and Vance, D.E. Evidence that only newly made phosphatidylethanolamine is methylated to phosphatidycholine and that phosphatidylethanolamine is significantly deacylated-reacylated in rat hepatocytes. *J. Biol. Chem.* **265:** 18322-18329, 1990.

Sano, K., Takai, Y., Yamanishi, J., and Nishizuka, Y. A role of calcium-activated phospholipid-dependent protein kinase in human platelet activation. *J. Biol. Chem.* **258:** 2010-2013, 1983.

Schick, B.P., Schick, P.K., and Chase, P.R. Lipid composition of guinea-pig platelets and megakaryocytes. *Biochim. et Biophys. Acta.* **663:** 239-248, 1981.

Schneider, W.J., Vance, D.E. Conversion of phosphatidyl-ethanolamine to phosphatidylcholine in rat liver. *J. Biol. Chem. 254:* 3886-3891, 1979.

Shattil, S.J., Anaya-Galindo, R., Bennett, J., Colman, R.W., and Cooper, R.A. Platelet hypersensitivity induced by cholesterol incorporation. *J. Clin. Invest.* **55:** 639-643, 1975.

Shattil, S.J., Copper, R.A. Role of membrane lipid composition, organization and fluidity in human platelet function. *Prog. Hemostas. Thrombos.* **4:** 59-86, 1978.

Shattil, S.J., McDough, M., and Burch, J.W. Inhibition of platelet phospholipid methylation during platelet secretion. **57:** 537-544, 1981.

Shelepov, V.P., Chekulaev, V.A., Pastia-Zade, G.R. Effect of putrescine on carbohydrate and lipid metabolism in rats. *Biochem. Soc.* **1:** 591-596, 1990.

Siess, W. Molecular mechanisms of platelet activation. *Physiol. Rev.* **69:** 58-178, 1989.

Siess, W., Cuatrecasas, P., Lapetina, E.G. A role for cyclooxygenase products in the formation of phosphatidic acid in stimulated human platelets. *J. Biol. Chem.* **258:** 4683-4686, 1983.

Siffert, W., Siffert, G., Scheid, P., and Akkerman, J.W.N. Na^+/H^+ exchange modulates Ca^{2+} mobilization in human platelets by ADP and the thromboxane mimetic U46619. *J. Biol. Chem.* **264:** 719-725, 1990.

Skaer, R.J., Peters, P.D., and Emmines, J.P. The localization of calcium and phosphorus in human platelets. *J. Cell Sci.* **15:** 679-692, 1974.

Smith, J.B., and Willis, A.L. Aspirin selectively inhibits prostaglandin production in human platelets. *Nature New. Biol.* **231:** 235-237, 1971.

Snyder, P.D., Deswick, R.S., and Krivit, W. The glycosphingolipids and glycosyl hydrolases of human blood platelets. *Biochem. Biophys. Res. Comm.* **46:** 1857-1865, 1972.

Spector, A.A., Hoak, J.D., Warner, E.D., and Fry, G.L. Utilization of long chain free fatty acids by human platelets. *J. Clin. Invest.* **49:** 1489-1496, 1970.

Stahl, S.M. The human platelet. A diagnostic and research tool for study of biogenic amines in psychiatric and neurological disorders. *Arch. Gen. Psychiatry.* **34:** 509-516, 1977.

Stipanuk, M.H. Metabolism of sulphur containing amino acids. *Annual Reviews of Nutrition.* **6:** 179-209, 1986.

Stone, J.V., Singh, R.K., Horak, H., and Barton, P.G. Sulfhydryl analogues of adenosine diphosphate: chemical synthesis and activity as platelet aggregating agents. *Can. J. Biochem.* **54:** 529-533, 1976.

Strittmater, W.J., Hirata, F., and Axelrod, J. Phospholipid methylation unmasks cryptic ß-adrenergic receptors in rat reticulocytes. *Science.* **204:** 1205, 1979.

Sundler, R., and Äkesson, B. Regulation of phospholipid biosynthesis in isolated rat hepatocytes: effects of different substrates. **250:** 3359-3367, 1975.

Sussman, I.I. The role of platelets in atherogenesis/ atheroschscherosis . *In* The Platelets: Physiology and Pharmacology, ed. by G.L. Longenecker, pp.383-405, Academic Press Inc., Orlando, FL, 1985.

Sweat, J.D., Johnson, S.L., Gragoe, E.J., and Limbird, L.E. Inhibitors of Na^+/H^+ exchange block stimulus-provoked arachidonic acid release in human platelets. *J. Biol. Chem.* **260:** 12910-12919, 1985.

Tabor, C.W., Tabor, H. Polyamines. *Annual Review Biochemistry.* **53:** 749-790, 1984.

Takahara, K., Murray, R., Fitzgerald, G.A., and Fitzgerald, D.J. The response to thromboxane A_2 analogues in human platelets. *J. Biol. Chem.* **265:** 6836-6844, 1990.

Tinoco, J., Hopkins, S.M., McIntosh, D.J., Sheehan, G., and Lyman, R.L. Fractionation and analysis of rat liver $^{14}CH_3$-lecithin labelled in vivo. *Lipids.* **2:** 479-483, 1967.

Trewhella, M.A., and Collins, F.D. Pathways of phosphatidylcholine biosynthesis in rat liver. *Biochim. et Biophys. Acta.* **296:** 51-61, 1973.

Tsvetnitsky, V. Phospholipid-N-methyltransferase in brain myelin. Ph.D. thesis. University of London, 1994.

Turner, S.R., Tainer, J.A., and Lynn, W.S. Biosynthesis of chemotatic molecules by arachidonate bipoxygenate of platelets. *Nature.* **257:** 680-681, 1971.

Tyms, A.S., Williamson, J.D., Bacchi, C.J. Polyamine inhibitors in antimicrobial chemotherapy. *J. Antimicrob. Chemother.* **22:** 403-429, 1988.

Valone, F.H. Interrelationships among platelet-activating factor binding and metabolism and induction of platelet activation. *In* Platelet Membrane Receptors: Mol. Bio., Immunol; Biochem. and Pathol. ed. by Jamieson, G.A., pp.319-340, Alan R. Liss Inc., New York, 1988.

Vance, J.E., Pan, D., Vance, D.E., and Campenot, R.B. Biosynthesis of membrane lipids in rat axons. *J. Cell. Biol.* **115:** 1061-1068, 1991.

Vance, J.E. Newly made phosphatidylserine and phosphatidylethanolamine are preferentially translocated between rat liver mitochondria and endoplasmic recticulum. *J. Biol. Chem.* **266:** 89-77, 1991.

Vance, J.E. The use of newly synthesized phospholipids for assembly into secreted hepatic lipoproteins. *Biochim. et Biophys. Acta.* **1006:** 59-69, 1989.

Vance, J.E., and Vance, D.E. Specific pools of phospholipids are used for lipoprotein secretion by cultured rat hepatocytes. *J. Biol. Chem.* **261:** 4486-4491, 1986.

Vance, D.E., Kruijff de Ben. The possible functional significance of phosphatidylethanolamine methylation. *Nature.* **288:** 277-278, 1980.

Vickers, J.D., Kinlough-Rathbone, R.L., Mustard, J.F. Charges in phosphatidylinositol-4, 5-biphosphate 10 seconds after stimulation of washed rabbit platelets with ADP. *Blood.* **60:** 1247-1250, 1982(a).

Vickers, J.D., Kinlough-Rathbone, R.L., Mustard, J.F. Changes in platelet phosphoinositide during the first minute after stimulation on washed rabbit platelets with thrombin. *Biochem. J.* **219:** 25-31, 1984.

Vickers, J.D., Kinlough-Rathbone, R.L., Mustard, J.F. The effect of prostaglandin E_1, I_2 and F_2 on the shape and phosphatidylinositol, 4,5-bisphosphate metabolism of washed rabbit platelets. *Thromb. Res.* **28:** 7331-740, 1982(b).

Villanueva, V.R., Adlakha, R.C. Automated analysis of common basic amino acids, mono-, di, and polyamines, phenolic amines, and indoleamines in crude biological samples. *Anal. Biochem.* **91:** 264-275, 1978.

Voelker, D.R. Phosphatidylserine functions as a major precursor of phosphatidylethanolamine in cultured BHK-21 cells. *Proc. Natl. Acad. Sci. USA.* **81:** 2669-2673, 1984.

Weiss, H.J. Platelet physiology and abnormalities of platelet function (2 parts). *N. Eng. J. Med.* **293:** 531-580, 1975.

Wennalm, A. Effects of nicotine on cardiac prostaglandin and platelet thromboxane. *Br. J. Pharmacol.* **64:** 559-563, 1978.

White, J.G. Effects of colchicine and vinca alkaloids on human platelets. I. Influence on platelet microtubules and contractile function. *Am. J. Pathol.* **53:** 281-291, 1968.

White, J.G., and Krivit, W. An ultrastructural basis for the shape changes induced by chilling. *Blood.* **30:** 625-635, 1967.

White, J.G. Interaction of membrane systems in blood platelets. *Am. J. Pathol.* **66:** 295-312, 1972.

White, J.G., Rao, G.H.R., and Gerrard, J.M. Effect of the ionophose A23187 on blood platelets. I. Influence on aggregation and secretion. *Am. J. Pathol.* **77:** 135-150, 1974.

White, F.V., Toews, A.D., and Morrel, P. Developmental pattern of phosphatidylserine decarboxylase in rat brain. *J. Neurochem.* **46:** 1200-1206, 1986.

Williams, W.J., Call, F.L. Phospholipid metabolism in human platelets. *Trans. Am. Clin. Climatol. Assoc.* **83:** 71-80, 1972.

William, W. Haematology. McGraw Hill, New York, NY, 1972.

Yavin, E. Polar head group decarboxylation and methylation of phospholipids: an alternate route for phosphatidylcholine formation in cultured neuronal cell. *J. Neurochem.* **44:** 1451-1458, 1985.

Yeung, S.K.F., and Kuksis, A. Utilization of L-serine in the in vivo biosynthesis of glycerophospholipids by rat liver. *Lipids.* **11:** 498-505, 1976.

Yin, E.T., and Wessler, S. Bovine thrombin and activated factor X: separation and purification. *J. Biol. Chem.* **243:** 112, 1968.

Zawad, J.S., Brown, F.C. ß-Adrenergic coupled phospholipid methylation. A possible role in withdrawal from chronic ethanol. *Biochem. Pharmacol.* **33:** 3799-3803, 1984.

Zborowski, J., Dygas, A., and Wojtczak, L. Phosphatidylserine decarboxylase is located on the external side of the inner mitochondrial membrane. *FEBS Lett.* **157:** 179-182, 1983.

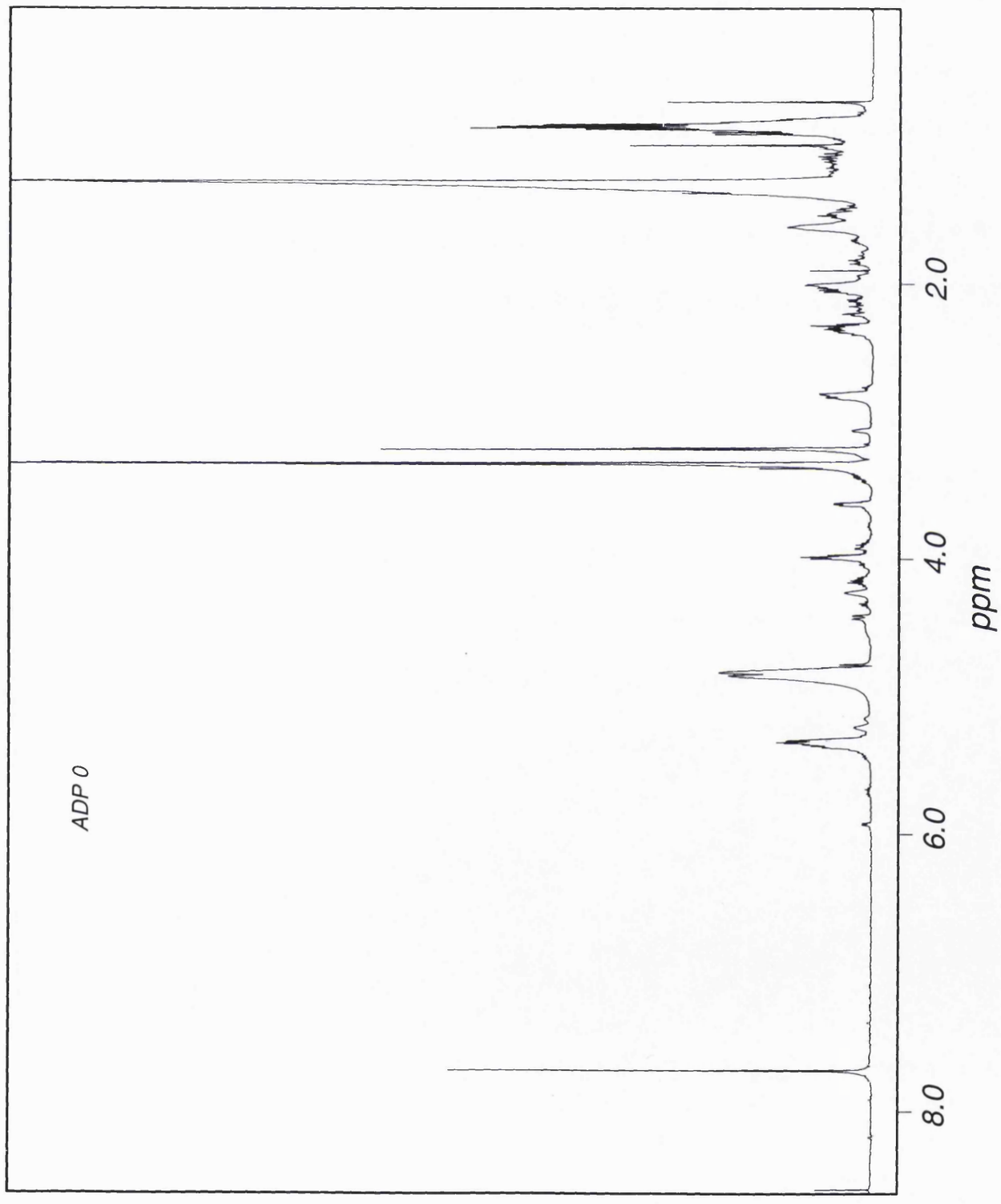
Zucker, M.B., and Nachmias, V.T. Platelet activation. *Arteriosclerosis.* **5:** 2-18, 1985.

Zucker, M.B., Peterson, J. Inhibition of adenosine diphosphate-induced secondary aggregation and other platelet functions by acetysalicyclic acid ingestion. *Proc. Soc. Exp. Biol. Med.* **127:** 547-551, 1968.

Zwaal, R.F.A., Bevers, E.M. Flip-flop of phospholipids in stimulated platelets; mechanism and physiological significance. *Colloque INSERM.* **195:** 47-56, 1989.

Zwaal, R.F.A. Membrane and lipid involvement in blood coagulation. *Biochim. et Biophys. Acta.* **515:** 163, 1978.

APPENDICES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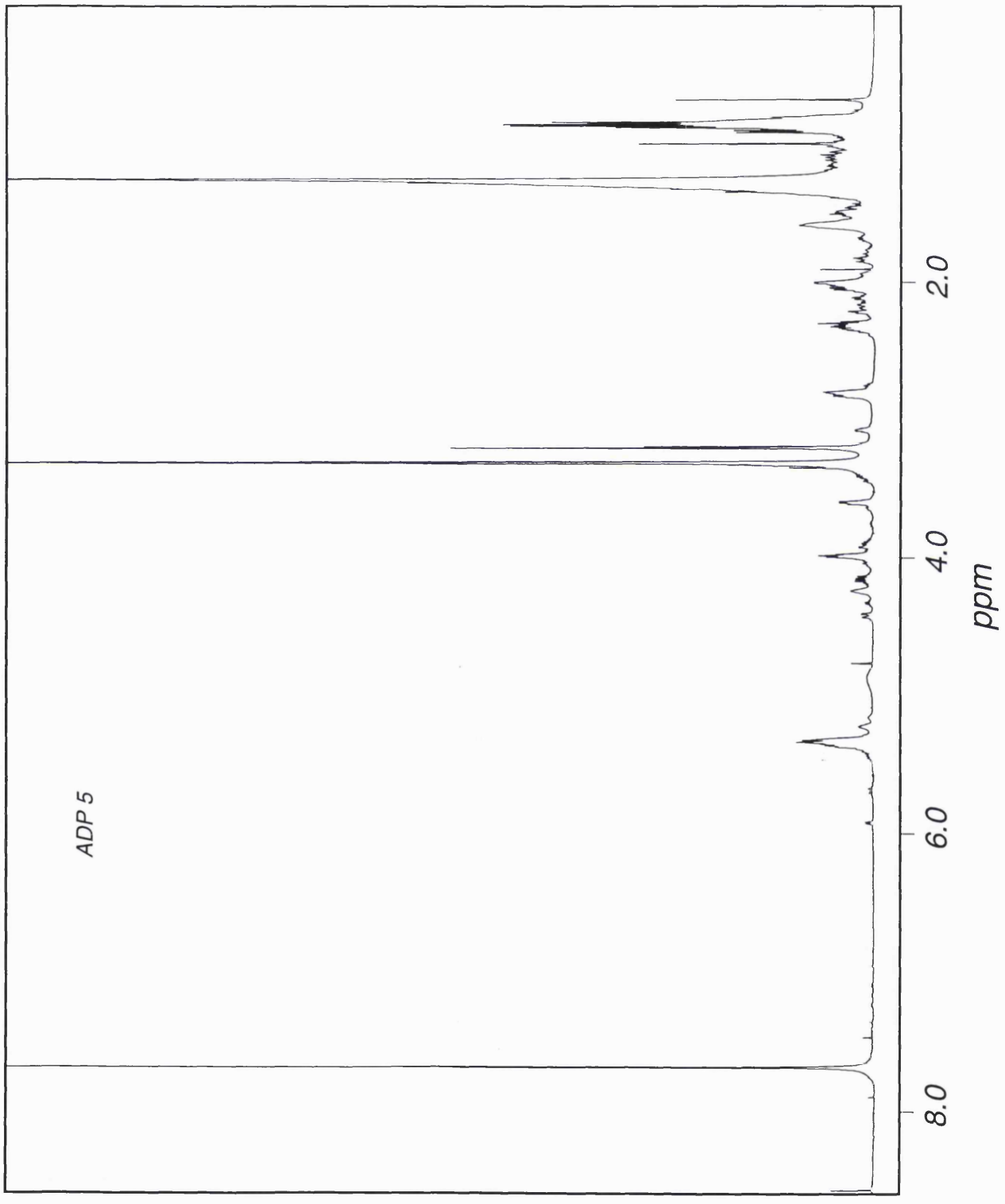
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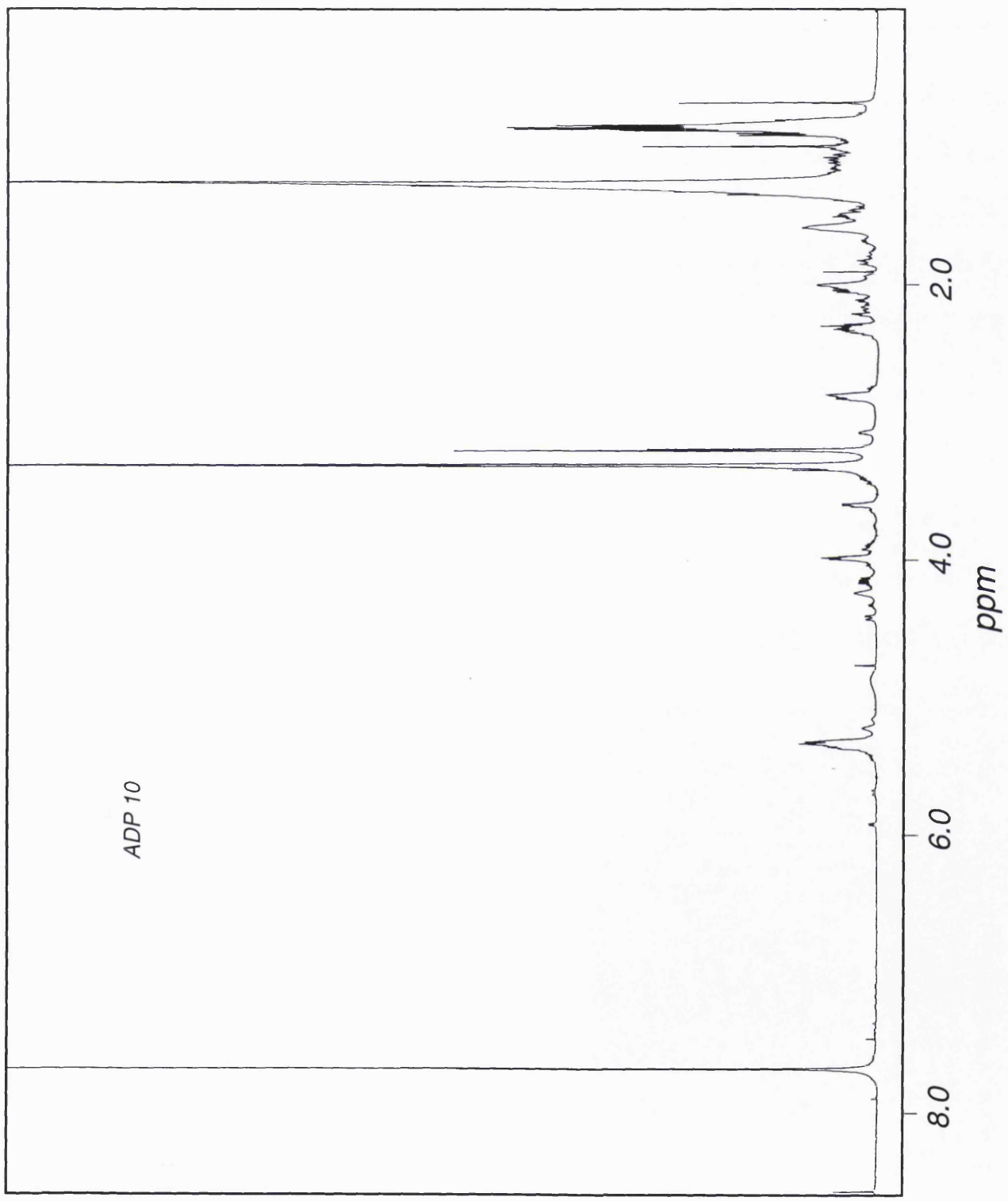
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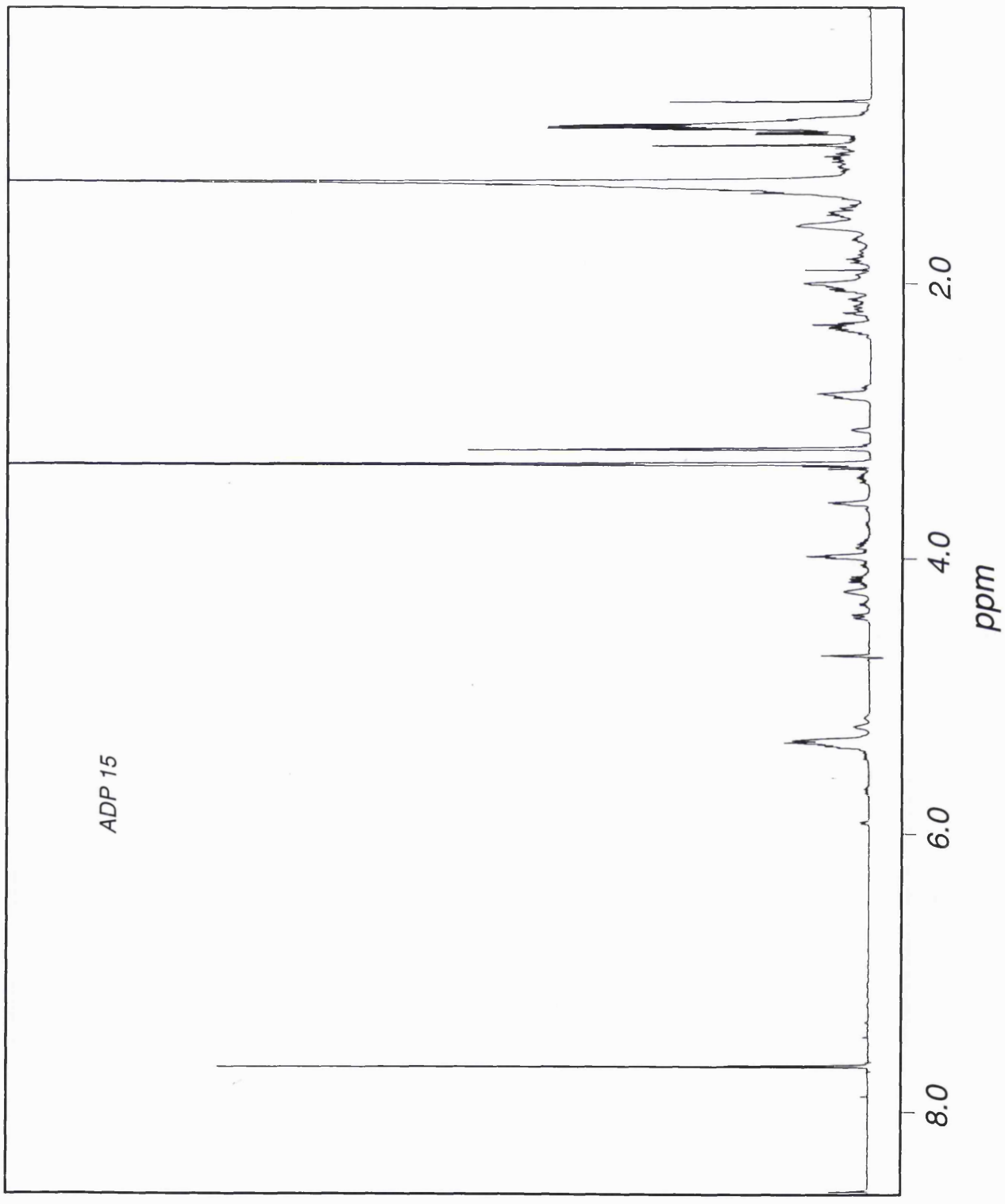
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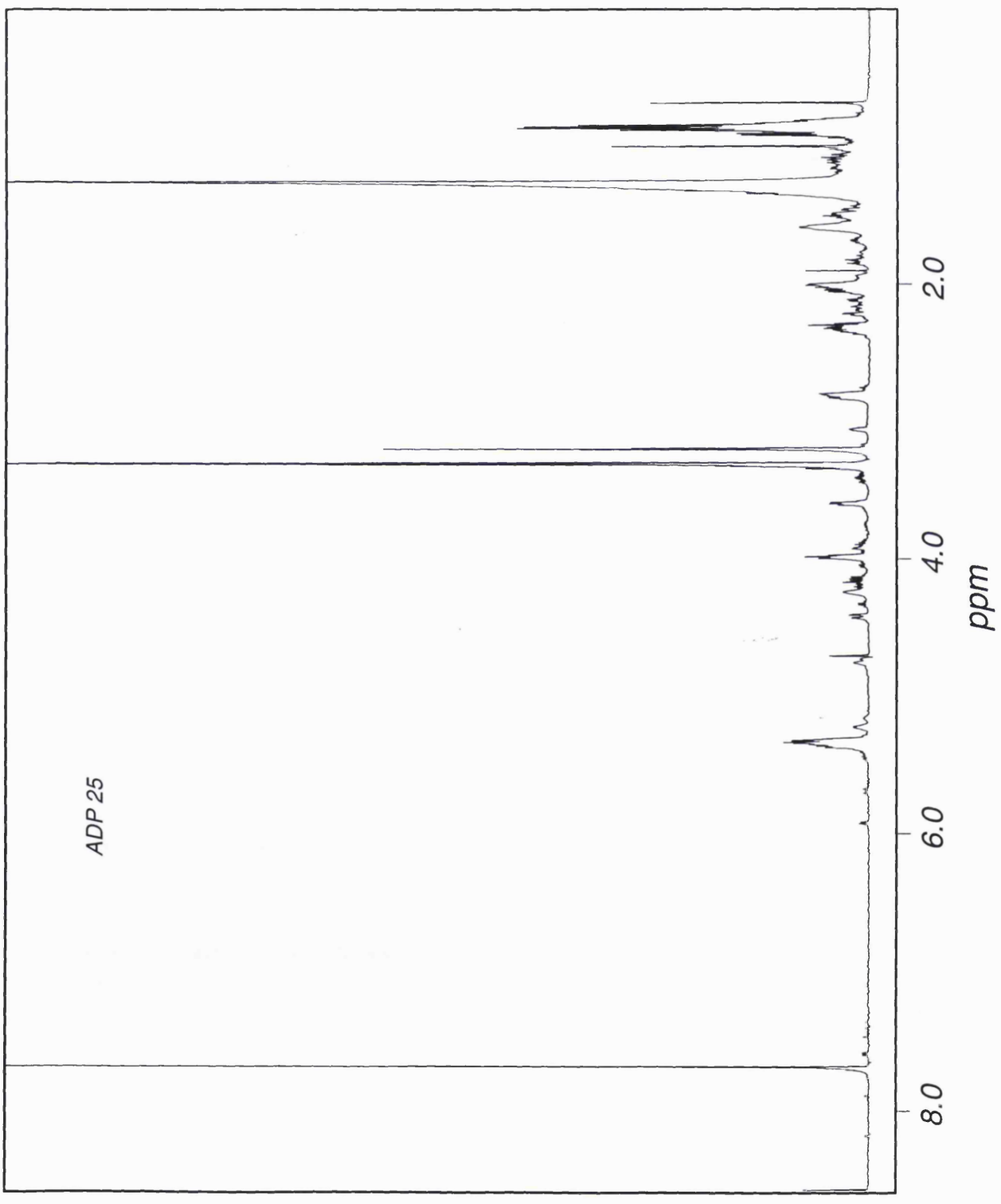
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Purification of plasma membrane rat liver phosphatidylserine decarboxylase.

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The genes and proteins of phosphatidylserine decarboxylase (PSDase) have been characterised in microorganisms [1, 2] and the enzyme partially purified from rat liver mitochondria [3]. This enzyme catalyses formation of phosphatidylethanolamine (PE) from PS. It has not been reported from platelets or liver plasma membranes. The latter were characterised by enzyme markers and shown to contain less then 5 percent mitochondria or other organelle contaminants.

The purified liver plasma membranes were solubilised using one percent CHAPS and diluted to 0.3% prior the PSDase assay. C^{14}-labelled PS was incubated with the solubilised membranes and the chloroform-extractable radioactive products were separated by TLC and determined quantitatively by radiometric scanning. Surprisingly, the principal product was radiolabelled phosphatidylcholine (PC) with small amounts of mono (PME) and dimethylated PE (PDE), corresponding to the four steps of Equation 1

PS---->PE----->PME----->PDE----->PC (Eq. 1)

At incubation times up to 10 minutes for every mole of PS that disappeared greater then 90% of the radioactivity was recovered as PC (Fig. 1).

The CHAPS-solubilised membranes were subjected to native PAGE and the gel divided into 15 x 1 cm slices. Proteins eluted from each slice were assayed for PSDase activity. Ninety percent of total activity occurred between 60 and 70 kDa and the product was 90% PC.

This evidence is consistent with either a single enzyme of *ca* 65kDa that has both PSDase and phospholipid N-methyltransferase activities or two enzymes that co-electrophorese.

Preliminary experiments have shown that PSDase occurs in platelet plasma membranes [4].

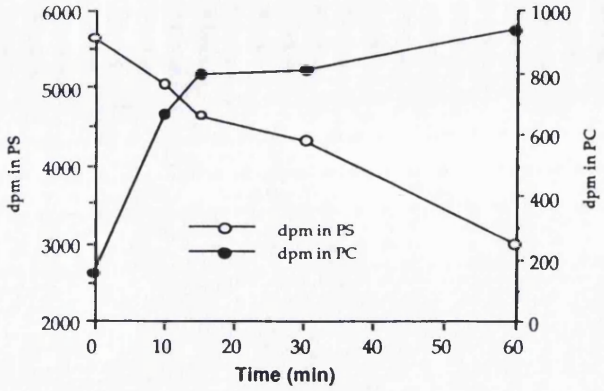

Figure 1. Time course for PS decarboxylation by CHAPS-solubilised rat liver plasma membranes.

References

1. Dowhan, W., Wickner, W. T. & Kennedy, E. P. (1974) J. Biol. Chem. **249**, 3079-3084

2. Li, Q.-X. & Dowhan, W. (1988) J. Biol. Chem. **263**, 11516-11522

3. Voelker, D. R. & Golden, E. B. (1992) in Methods in enzymology (E. A. Dennis and D. E. Vance eds.), **209**, 360-365, Academic Press, Inc., San Diego, California

4. Yeboah, F.A. & Gibbons, W.A. Unpublished results

Isozymes of rat brain myelin phospholipid-N-methyltransferase.

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Myelin is a specialised extension of the plasma membrane of oligodendrocytes in the CNS which wraps around the axon and provides insulation for and facilitation of axonal conduction. Its composition is 70-75% lipid with six major proteins accounting for more than 95% of total protein. The earlier concept of myelin as an inert membrane has been revised and myelin is known to contain numerous enzymes, more than half of which metabolise lipids [1].

Phospholipid methyltransferase (PLMTase) catalyses transfer of CH_3- groups from the methyl donor S-adenosyl-methionine (SAM) to the amino head-group of phosphatidylethanolamine (PE) to yield monomethyl-PE (PME), dimethyl-PE (PDE) and phosphatidylcholine (PC). PLMTase was reported from rat liver [2] to be a single polypeptide, M_r 18.3 kDa, which possessed all three methylating activities. Others suggested PLMTase had two activities [3][4][5]. The first one catalysed formation of PME and the second either completed PC formation from PME or catalysed all three steps [6]. Rat brain myelin PLMTase [7] was also reported to be a two-enzyme methylating system.

Here we report that iso-electric focusing (IEF) of Triton X-100-solubilised myelin proteins resulted in separation of acidic (pI~5) and basic (pI~9) PLMTase activities. The acidic activity yielded three distinct peaks upon further re-focusing.

Myelin was isolated according to the classical procedure [8] with the additional sucrose gradient [9] and solubilised in phosphate buffer (pH 8.0) containing 0.5% (w/v) Triton X-100. The solubilised proteins were subjected to IEF on Preparative IEF Cell (Bio-Rad) over pH gradient from 3 to 10. The harvested fractions were assayed for PLMTase activity after adjusting pH to 8 by incubating the aliquot from each IEF fraction for 30 min at 40°C with 200μM S-adenosyl-L-(^{3}H-*methyl*)-methionine (12.5 mCi/mmol) and 10μl of the mixture of PE (8mg/ml) and PME (1mg/ml). After the phospholipids were extracted and dried, the amount of (^{3}H-*methyl*) incorporated into phospholipids was measured by liquid scintillation counting. The active fractions were pooled and subjected to re-focusing. The aliquots of each fraction were analysed by 12% SDS-PAGE and the gels silver-stained.

The results indicated that iso-electric focusing over the pH range 3-10 separated detergent-solubilised myelin PLMTase into two broad peaks of activity, one in the pI 4-7 and another in the basic region respectively. Upon re-focusing (pH 3-7) the former revealed three distinct methylating activities with pI 4.5, 5.5 and 6.5 (Fig. 1). Re-fractionation of the basic activity (pH 7-10) did not result in tighter focusing but activity centred around pI~9.

TLC separation and radiometric analysis of the products of PLMTase showed predominant formation of PME both from the pI 4.5 and 5.5 activities whilst the pI 6.5 activity yielded 30% PC and ≥60% PME. We cannot yet attribute these differences as evidence of separate mono-methylating and di/tri-methylating activities.

SDS-PAGE of each active acidic fraction unveiled the presence of one major band at M_r 65±2 kDa plus a few minor bands while active basic fractions contained the *ca* 65 kDa band and one at ~200 kDa (not shown).

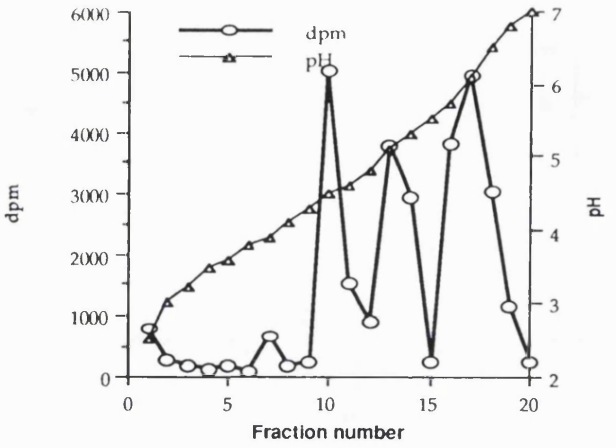

Fig. 1 PLMTase assay of the IEF fractions of Triton X-100-solubilised myelin.

The predominant activity was confirmed to occur at *ca* 65 kDa by preparative column SDS-PAGE with lower activity detected at *ca* 20 kDa.

The most probable but not the only explanation of these data is that rat brain myelin contains three acidic and at least one basic phospholipid methylating isozymes and that the major isozyme in each case is between 60-70 kDa in size.

References

1. Ledeen, R. W. (1984) J. Lipid Res. **25**, 1548-1554
2. Ridgway, N. D. &Vance, D. E. (1987) J. Biol. Chem. **262**, 17231-17239
3. Crews, F., Hirata, F. &Axelrod, J. (1980) J. Neurochem. **34**, 1491-1498
4. Gaynor, P. M. &Carman, G. M. (1990) Biochim. Biophys. Acta **1045**, 156-63
5. Dudeja, P., Foster, E. &Brasitus, T. (1986) Biochim. Biophys. Acta **875**, 493-500
6. Tanaka, Y., Amano, F., Maeda, M., Nishijima, M. &Akamatsu, Y. (1990) Jap. J. Med. Sci. & Biol. **43**, 59-73
7. Chung, K., Yu, B. S. &Choi, M.-U. (1990) Korean Biochem. J. **23**, 103-109
8. Norton, W. &Poduslo, S. (1973) J. Neurochem. **21**, 749-757
9. Haley, J. E., Samuels, F. G. &Ledeen, R. W. (1981) Cell. Mol. Neurobiol. **1**, 175-187

The purification and molecular weight determination of rat liver microsomal phospholipid N-methyltransferase.

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Despite the fact that thirty to forty percent of all phosphatidylcholine (PC) is made by the S-adenosyl-methionine (SAM)-dependent transmethylation pathway in liver [1] and that the conversion of phosphatidylethanolamine (PE) to PC by this pathway (Equation 1) correlates with several signal transduction processes [2],

PE-------> PME-------> PDE-------> PC (Eq. 1)

uncertainty still exists concerning the purification, molecular weight, subunit composition, isozymes and mechanisms of the responsible enzyme(s) phospholipid N-methyltransferase (PLMTase). The latter has been reported to be a single 18.3kDa polypeptide chain that possesses all three methylating activities depicted in equation 1 [3], or an 50kDa enzyme possessing a 25kDa catalytic subunit [4] Additional forms varying up to *ca* 200-300kDa have also been detected [5].
Fig. 1 demonstrated that three molecular weight forms of phospholipid methylating activity can be detected from CHAPS-solubilised liver microsomes at *ca* >200kDa, 66kDa and <40kDa on a Superose 6 gel filtration column. At least 90% of PLMTase activity from both the 200kDa and 66kDa gel filtration fractions was eluted with 1M NaCl from MonoQ columns as showed in Figs. 2 and 3. Ion-exchange chromatography of CHAPS-solubilised microsomes using DEAE-cellulose followed by MonoQ chromatography using a shallower salt gradient (Fig. 4) revealed four peaks of SAM-dependent ethanolamine lipid methylating activity.

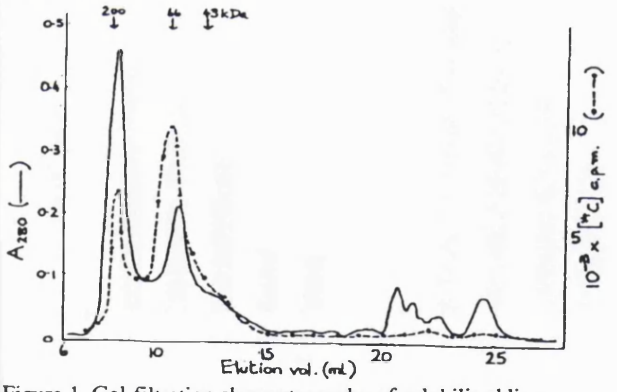

Figure 1. Gel-filtration chromatography of solubilised liver microsomes on Superose 6.

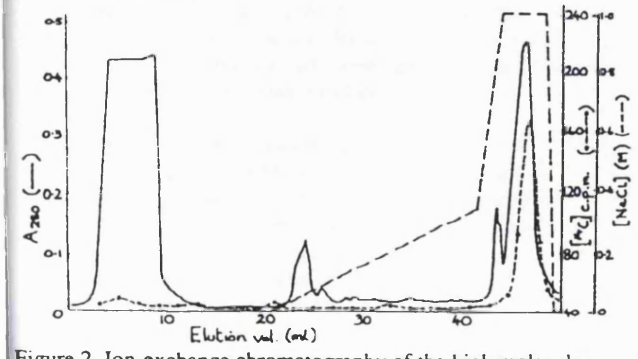

Figure 2. Ion-exchange chromatography of the high molecular weight activity from gel filtration.

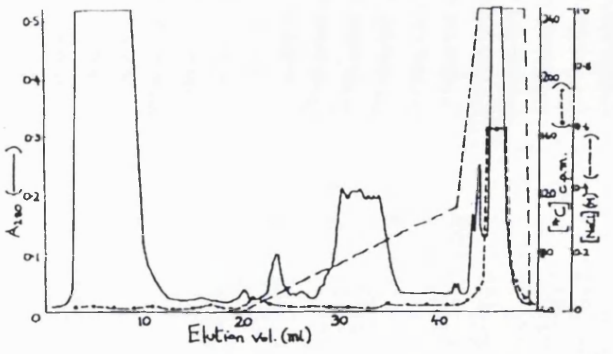

Figure 3. Ion-exchange chromatography of the low molecular weight PLMTase activity from gel-filtration.

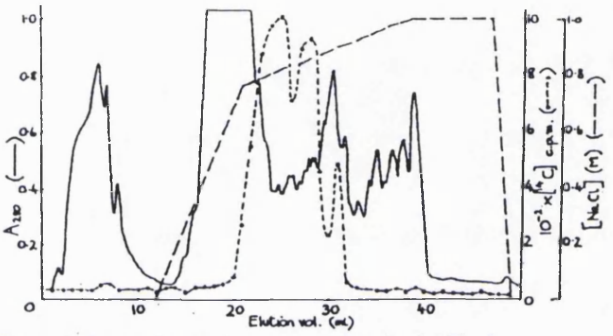

Figure 4. Ion-exchange chromatography of solubilised microsomes eluted from MonoQ with modified salt gradient.

These data are consistent with the hypothesis that several SAM-dependent isozymes exist that convert PE to PC but other explanations for the various activities exist including separate enzymes for the methylation of ether and diacylglycero-phospholipids or separate enzymes for mono, di and trimethylation. The major lipid methylating enzymes, according to the above experiments were *ca* 65±2kDa in size and acidic in nature. Preliminary isoelectic focusing results are consistent with several acidic isozymes plus at least one basic isozyme.

References

1. Vance, D. E. & Ridgway, N. D. (1988) Prog. Lipid Res. **27**, 61-79
2. Hirata, F. & Axelrod, J. (1980) Science **209**, 1082-1090
3. Ridgway, N. D. & Vance, D. E. (1987) J. Biol. Chem. **262**, 17231-17239
4. Pajares, M., Villalba, M. & Mato, J. (1986) Biochem. J. **237**, 699-705
5. Pajares, M. A., Alemany, S., Varela, I., Marin Cao, D. & Mato, J. M. (1984) Biochem. J. **223**, 61-66

ROLE OF VITAMIN B12 ENZYMES IN PLATELET CELL SIGNALLING, ADHESION AND AGGREGATION

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Platelet aggregation is inhibited by nitric (NO) and nitrous (N_2O) oxides. The former has been proposed to stimulate guanyl cyclase and hence to increase the intracellular cGMP concentration. Different mechanisms for N_2O have been proposed from studies in E. coli, liver and brain[1] where it inhibits both the cytosolic and purified forms of vitamin B12-dependent methionine synthase[2] (B12-MS), equation (1).

$$\text{Homocysteine} \xrightarrow{\text{B12-MTHF}} \text{Methionine} \xrightarrow{\text{ATP}} \text{S-adenosyl methionine} \quad (1)$$

Figure 1 shows that ***both*** N_2O and NO inhibit platelet cytosolic B12-MS in a dose-dependent manner as measured[3], by transfer of the radiolabelled methyl group from the cofactor [$^3CH_3$]-methyltetrahydrofolate to homocysteine; NO, from Fig. 1, is a stronger inhibitor B12-MS than N_2O.

The two facts, (a) N_2O and NO inhibition of B12-MS and (b) N_2O and NO inhibition of platelet aggregation [3],strongly suggest that B12-MS and the metabolic pathways that depend upon it (Fig. 2) could be involved in cell signalling, cell excitation and platelet

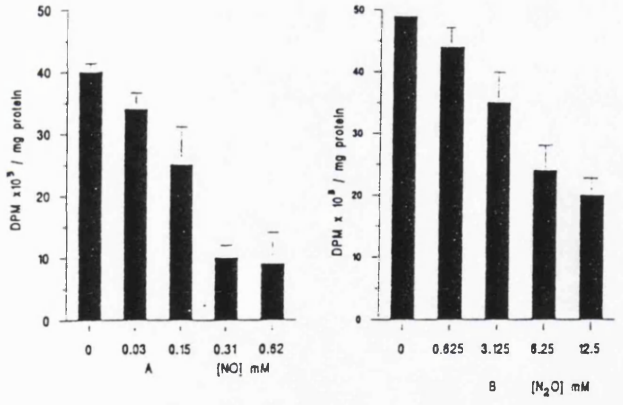

Figure 1: Dose dependence study of the inhibition of platelet methionine synthase by N_2O (A) and NO(B). Authentic gas (N_2O/NO) was bubbled for 30 min through buffer deoxygenated with N_2O to produce saturated gas solutions. Aliquots were introduced into the assay mixture.

aggregation? This hypothesis is strengthened by: (a) the known cellular effects of N_2O, which decreases cellular folate levels in liver and microorganisms and hence is cytostatic and cytotoxic. These can be overcome by addition of methionine and other metabolites from Fig. 2 pathways, (b) The sensitivity of platelet aggregation to a variety of metabolites in Fig. 2. As a corrollary an explanation is provided for the modulation of platelet aggregation by exogenously administered homocysteine (substrate), methionine (product), taurine, polyamines, arachidonate, eicosanoids, phospholipid metabolites [4,5].

Modulation of platelet functions by synthetic inhibitors of enzymes of Fig. 2 that are involved in polyamine biosynthesis (DFMO), phospholipid methylation (deaza-adenosine) phospholipases (indomethacin) cyclooxygenases and lipoxygenases (aspirin), can also be explained by this hypothesis. The above data can therefore be construed as supporting the proposal that vitamin B12-dependent methionine synthase, an intracellular cytosolic enzyme, and the metabolites whose cellular concentrations depend upon B12-MS activity and the formation of S-adenosyl methionine, are involved in platelet cell signalling and aggregation. The principal pathways are trans-sulphuration, transmethylation, aminopropyl transfer and folate production.

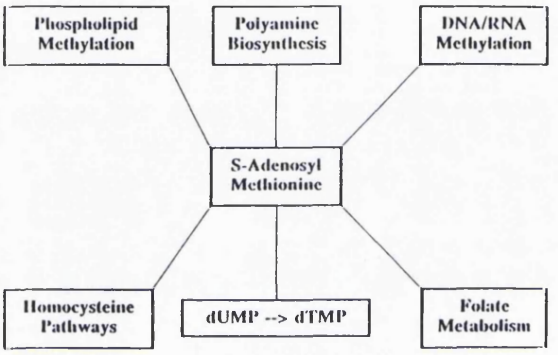

Figure 2: Metabolic pathways and signal transduction metabolism related to B12-MS.

It has not escaped our notice that polyamines, oxidized sulphur amino acids, SAM metabolites and choline phospholipids are now being proposed as intracellular signalling molecules.

References

1. Banerjee, R.V. and Matthews, R.G. (1990) FASEB J. **4**(5) 1450-1459 .
2. Ast, T., Nicolaou, A., Anderson, M.S., James, C. and Gibbons, W.A. (1993) Biochem.Soc. Trans. .
3. Radomski, M.W., Palmer, R.M.J. and Moncada S. (1991) Trends in Pharmacol.Sci. **12**(3) 87-88 .
4. Pales, J.L., Lopez, A. and Vidal, S. (1984) Scand. J. Haematol. **32**(3), 241-244.
5. Miceli, M., Bennardini, F., Mattana, A., Covarrubias, J., Seghieri, G. andFranconi, F. (1992) Platelets **3**(5) 265-9