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ABSTRACT

Five-day stored, human platelets are routinely used in transfusion medicine. Although it has been shown that significant deterioration of platelet function takes place during storage, very little information has been available about the effects of storage on platelet phospholipid (PL) metabolism.

Biosynthesis and remodelling of platelet phospholipids were studied by incubating platelets with trace amounts of radioactive glycerol and/or arachidonic acid (AA) in a non-plasma medium at 22°C. The distribution of radioactivity was monitored for periods varying from 0 to 5 days. Platelet concentrates were washed and total lipids extracted. The glycerophospholipids (GPLs) were separated by HPLC.

The maximum uptake of AA into platelet (75%) was reached after 10 minutes. In contrast, a maximum glycerol uptake was 20% and was attained very slowly (after 6 hours). Glycerol incorporation demonstrated that human platelets can synthesize phosphatidyl choline (PC), phosphatidyl ethanolamine (PE) and phosphatidyl inositol (PI). However, there was very limited de novo synthesis of phosphatidyl serine (PS). The initial rate of AA incorporation into GPLs was 100 times faster than de novo synthesis, at 22°C. During the 5 day storage period, the AA continuously left PC and appeared in PE.

DISTRIBUTION OF RADIOACTIVITY IN PLATELETS

	[^{14}C]glycerol		[^{3}H]AA	
	day 0	day 5	day 0	day 5
PE	13%	17%	14%	42%
PC	63%	65%	55%	27%

In contrast, no changes in the glycerol incorporation pattern were detected suggesting that the mechanism for the AA-remodelling pathway does not involve polar head group exchange but may occur via arachidonoyl transacylase activities. This pathway explains why radioactive AA does not equilibrate with all the GPL pools during short-term labelling.

The present study allowed us to demonstrate that the AA remodelling of GPLs present in many mammalian cells, also exists in human platelets. This long-term shuttle of AA from PC into PE constitutes a resonable explanation for the high AA content of human platelet plasmalogen PE.

A ma petite fille Elyane

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The financial support received from the Medical Research Council of Canada during my study is also gratefully acknowledged.

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ABBREVIATIONS

AA	Arachidonic acid
ACD	Anticoagulant citrate-dextrose solution
ADP	Adenosine diphosphate
Alb	Albumin
BHT	Butylated hydroxy toluene
cAMP	Cyclic adenosine monophosphate
CL	Cardiolipin
CoA	Coenzyme A
COLL	Collagen
CPDA-1	Citrate-phosphate-dextrose-adenine anticoagulant
DEHP	Di(-2-ethylhexyl)phthalate
DHAP	Dihydroxy acetone phosphate
DAG	Diacylglycerol
FA	Fatty acid
GPL	Glycerophospholipids
HEPES	N-2-hydroxyethylpiperazine-n'-2-ethanesulfonic acid
HPLC	High - performance(pressure) liquid chromatography
MAG	Monoacylglycerol
n	Number of analysis
NL	Neutral lipids
P	Probability
PA	Phosphatidic acid
PC	Phosphatidyl choline
PCD	Non-plasma medium (Plasmalyte A/citrate/dextrose)
PE	Phosphatidyl ethanolamine

PG	Phosphatidyl glycerol
PI	Phosphatidyl inositol
PL	Phospholipid
PLA_2	Phospholipase A_2
PRP	Platelet rich plasma
PS	Phosphatidyl serine
PU	Unit of platelet concentrate
PVC	Polyvinyl chloride
RBC	Red blood cells
R.T.	Retention time
SD	Standard deviation
SE	Standard error
SEM	Standard error of mean
SM	Sphingomyelin
TAG	Triacylglycerol
THR	Thrombin
TL	Total lipid
TLC	Thin layer chromatography
WBC	White blood cell

1. INTRODUCTION

It has been more than a hundred years since Bizzozero (1882) published his remarkable article about blood platelets. He was the first to recognize the platelet as a unique cell separate from white (WBCs) and red blood cells (RBCs). Today we know that platelets, in addition to their participation in blood coagulation, have a wide variety of activities. These include: the upkeep of the vascular integrity and the provision of material released directly from storage organelles during platelet activation. The released material and other liberated agents, can be biologically active or they can simply serve as precursors to other biologically active substances such as prostaglandins and thromboxane A_2 (Luscher and Crawford, 1985).

Platelets are anucleated cells with a diameter of 2-4 μm and a thickness of about 1 μm, circulating as discoid or lentiform cells and arising from the introversion of the plasma membrane of megakaryocytes (Luscher and Crawford, 1985). Their number is approximately 2-4 x 10^8 cells/mL of human blood (Weiss, 1975). There is evidence associating platelets with the pathological process of the arterial thrombosis associated with arterosclerotic cardiovascular disease (Holub, 1984).

1.1. Lipid Metabolism in Resting Human Platelets

1.1.1. Lipid Composition of Platelet Membrane

Lipids constitute approximately 17% of the dry weight of the platelet. They are localized in the plasma and organelle membranes. The neutral lipids (NL) constitute 18% of the total lipid content of whole platelets, whereas the phospholipids (PLs) contribute 82% (Marcus et al., 1969).

The NL are mainly composed of cholesterol (> 85%, cholesterol esters

account for only a small portion of platelet cholesterol (<0.4%)) and fatty acids (FAs, mainly palmitate (16:0), stearate (18:0), oleate (18:1) and linoleate (18:2)). There are only trace amounts of free arachidonic acid (20:4), (Bills et al., 1977), triacylglycerol (TAG) and diacylglycerol (DAG) in human platelets (Nordoey et al., 1974).

The PLs contained in the platelets are composed of 40 mole% phosphatidyl choline (PC), 23-28 mole% phosphatidyl ethanolamine (PE), 9-11 mole% phosphatidyl serine (PS), 3-6 mole% phosphatidyl inositol (PI), and 18-20 mole% sphingomyelin (SM), (Cohen and Derksen, 1969; Owen et al., 1981; Mahadevappa and Holub, 1982; Mueller et al., 1983; Vishnubhatla, 1986). Trace amounts of phosphatidic acid (PA), cardiolipin (CL) and the lysophosphoglycerides: lyso-PC and lyso-PE are also present (Marcus et al., 1969; Cohen and Derksen, 1969; Broekman et al., 1980). Arachidonic acid (AA), which is esterified to PL, is the most prevalent FA in human platelets, accounting for 29% of total PL fatty acids (Broekman et al., 1976). AA is known to play a major role in the regulation of some platelet functions by acting as a precursor of various biologically active molecules such as thromboxane A_2 and prostaglandins (Marcus, 1978; Holub, 1984).

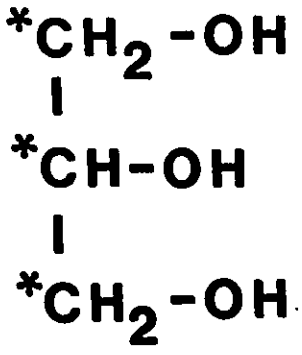
The glycerophospholipids (GPLs) may be grouped into three major molecular classes, according to the nature of the linkage of the hydrocarbon chain to the sn-1 position of the glycerol backbone (trihydroxypropane molecule, Structure A):

1) The alkyl-acyl PLs, which have a saturated alkyl-ether linkage at the sn-1 position and an ester linkage at the sn-2 position (Structure B).

2) The alk-1'-enyl PLs or plasmalogens, which have a vinyl-ether linkage at the sn-1 position and a saturated ester linkage at sn-2 position (Structure C).

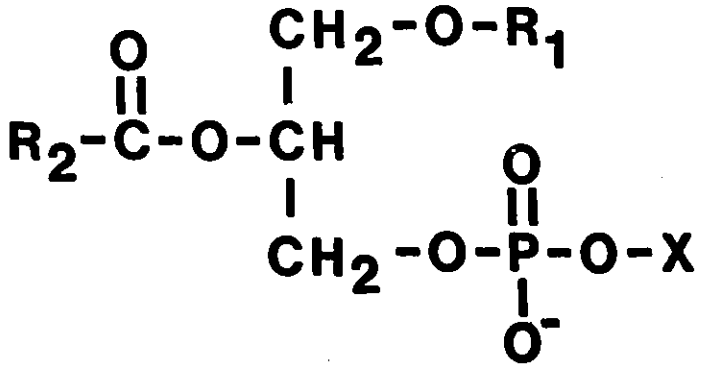
Structure A

$[^{14}C]^{*}$Glycerol



Structure B

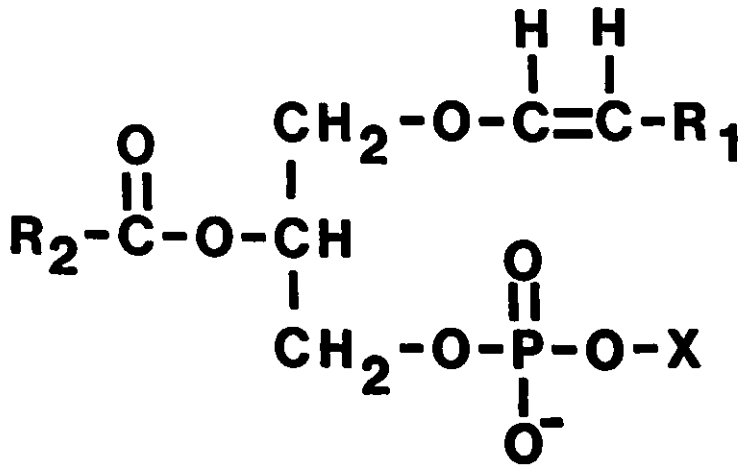
Alkyl-acyl PLs



X: CHOLINE
ETHANOLAMINE
SERINE
INOSITOL

Structure C

Alk-1'-enyl acyl PLs (plamalogens)



X: CHOLINE
ETHANOLAMINE
SERINE
INOSITOL

3) The diacyl PLs, which have ester linkages at both sn-1 and sn-2 positions (Structure D).

The PC component is composed of 81% diacyl, 10% alkyl-acyl and 9% of the plasmalogen forms, while the PE is composed of 36% diacyl, 4% of alkyl-acyl and 60% of the plasmalogen classes (Mueller et al., 1983). Mahadevappa and Holub (1982), and Nordoey and Lund (1968) measured a plasmalogen PE percentage of 32, whereas Vishnubhatla et al. (1987) reported a range of 34-68% for the same PE molecular class. Mueller's group (1983) demonstrated that AA was found on the sn-2 position of all the PLs. AA, which is an w-6 unsaturated 20 carbon chain (Structure E), comprises 44%, 23% and 25% of the alkyl-acyl, diacyl and plasmalogen forms of PC, respectively, whereas in PE, it constitutes 20%, 60% and 68% of the respective molecular classes (Mueller et al., 1983; Vishnubhatla, 1986).

1.1.2. Glycerophospholipid Metabolism

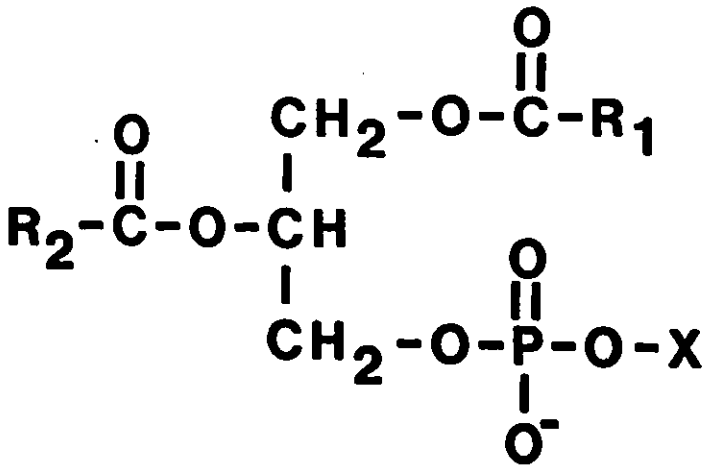
Three pathways for the biosynthesis of GPL have been described in platelets: The de novo synthesis, the remodeling pathway and the transmethylation pathway.

1) The de novo synthesis (or Kennedy) pathway (Kennedy, 1962). Evidence for this pathway is corroborated by the fact that radioactive glycerol is incorporated by washed human platelets into all of the major GPLs (Lewis and Majerus, 1969). The glycerol is converted to glycerol-3-phosphate, which is acylated to form PA, the precursor of all PLs (FIGURE 1). There are two routes for the conversion of PA into PLs:

a) Dephosphorylation of PA to diacylglycerol (DAG) followed by attachment of the phosphorylated head groups, choline and ethanolamine, forming PC and PE, respectively.

Structure D

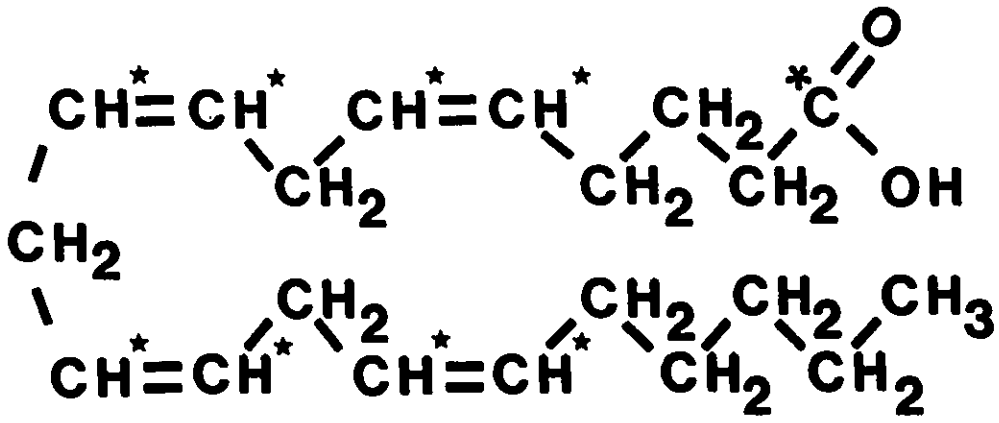
Diacyl PLs



X: CHOLINE
ETHANOLAMINE
SERINE
INOSITOL

Structure E

Arachidonic acid
$[^{14}C]^*$ or $[^{3}H]^*$



$(20:4^{\Delta 5,8,11,14})$

b) Conversion of PA to phosphatidyl glycerol (PG) by the reaction of cytidine diphosphate diglycerol with glycerophosphate or the reaction of cytidine diphosphate diglyceride with inositol to give PI. PG is subsequently converted to CL by the reaction with cytidine diphosphate diacylglycerol.

c) PS is formed by an enzymatic exchange of the ethanolamine head group of PE with serine. PS also undergoes a decarboxylation reaction to give PE.

All the essential enzymes required for the de novo synthesis of GPLs have been shown to be present in human platelets (Call and Williams, 1973; Call and Rubert, 1975).

2) The remodeling pathway for PL synthesis or Lands pathway (Lands, 1960) (FIGURE 1). This pathway has two steps: a deacylation reaction catalyzed by phospholipase A_2 (PLA_2) followed by reacylation of the lyso-PL with a fatty acyl group derived from fatty acyl coenzyme A (CoA) (Elsbach et al., 1971).

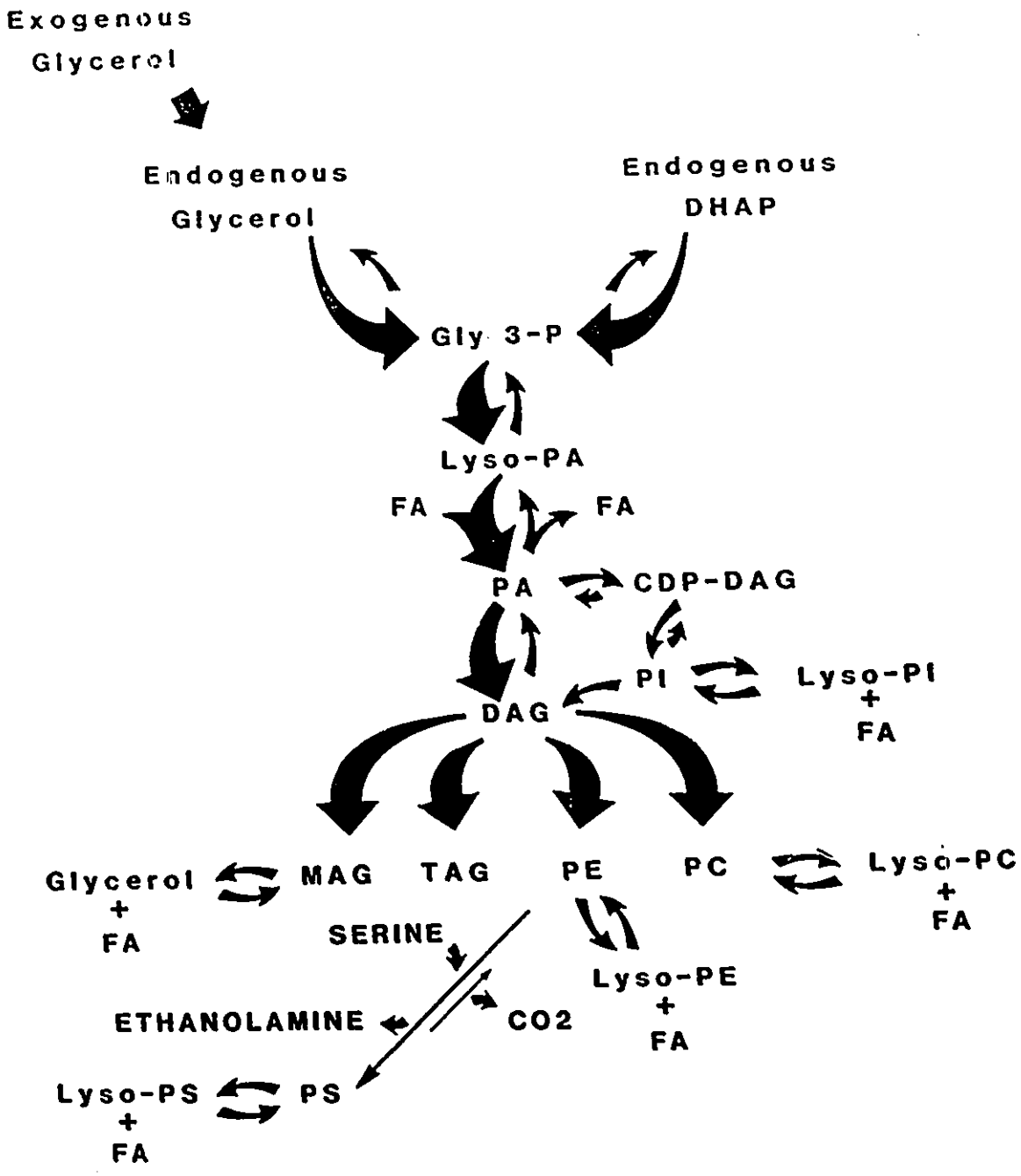
3) The transmethylation pathway. This pathway has been shown to be responsible for the conversion of PE to PC, by stepwise transfer of methyl group using S-adenosyl methionine as methyl donor. The existence of this pathway in human platelets has been demonstrated by Cordasco et al. (1981). However, it has been estimated that the activity of this pathway for the biosynthesis of PC is minor relative to the de novo and remodeling pathways (Shattil et al., 1981).

1.1.3. Arachidonate and Glycerol Uptake and Incorporation into Platelets

In vivo, platelets are exposed continuously to the different albumin-bound fatty acids (FA/Alb) contained in the plasma. It has been shown that human platelets can take up FAs (Cohen, 1968). Bills et al. (1977) and Chambaz et al. (1979) have shown that AA can be taken up by platelets. A mechanism by which FA are incorporated into platelets has been proposed by Spector (1987). This

FIGURE 1: De novo GPL Synthesis

FIGURE 1


Exogenous
Glycerol
Endogenous
Glycerol
Endogenous
DHAP
Gly 3-P
Lyso-PA
FA
FA
PA
CDP-DAG
PI
Lyso-PI
+
FA
DAG
Glycerol
+
FA
MAG
TAG
PE
PC
Lyso-PC
+
FA
SERINE
Lyso-PE
+
FA
ETHANOLAMINE
CO2
Lyso-PS
+
FA
PS

model (FIGURE 2) suggests that the binding of FA/albumin to the platelet membrane is a reversible process, so that FAs are exchangeable and can rapidly dissociate from the platelet membrane. According to this model, there is no indication that the capacity of the platelet to bind FA can be saturated. Schick et al. (1984), demonstrated that AA taken up in vitro does not equilibrate with all of the AA pools in platelets. Spector et al. (1970), showed that more than 95% of the uptake that remains as free FA can be released back to the extracellular fluid if albumin is present. Yardimici et al. (1980), concluded that transport into the platelet occurred by diffusion. The extent to which FAs accumulate in unesterified form is determined primarily by three factors:

a) The molar ratio of FA/albumin which regulates the dissociation of FA-albumin complex (Spector, 1975).

b) The rate of metabolism of the FA.

c) The binding capacity of the platelet membrane for the FA.

Wilson et al. (1982), observed two rates of uptake of FAs into washed human platelets occurring by two distinct enzymatic processes: one is a non-specific, long-chain, acyl, CoA synthetase and the other is an acyl CoA synthetase which is specific for AA.

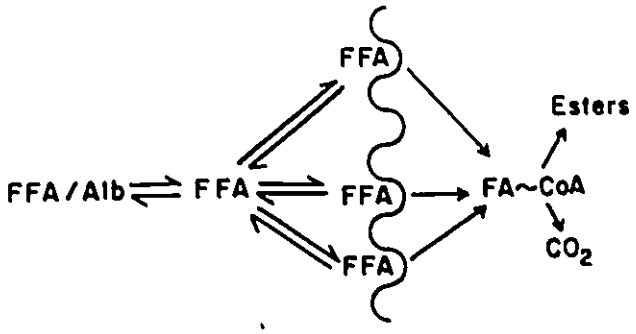
The AA-acyl CoA synthetase is responsible for the rapid esterification of AA into platelet PLs. This specific enzyme probably represents the physiological mechanism for platelet AA uptake (Neufeld et al., 1984). Neufeld et al. (1983), concluded that platelet esterification of AA is relatively independent of albumin concentration. Since AA serves as a precursor for various mediators of cell functions, such an uptake system can serve two important roles:

a) Even though the plasma level of free FA is 1 nM with only about 1% of this is AA, the AA-acyl CoA synthetase can efficiently scavenge AA from

FIGURE 2: Model for Free Fatty Acid Utilization by Platelets

The abbreviations used are: Alb for plasma albumin and FFA for free fatty acid. The wavy line signifies the platelet membrane (taken from Spector, 1987).

FIGURE 2



the plasma (Dole et al., 1959).

b) It can keep free AA levels within unstimulated platelets so low that cyclooxygenase and lipoxygenase have negligible reaction rates, inhibiting prostaglandin production (Neufeld et al., 1983).

The rapid incorporation of AA into PLs may require PLA_2 activity to provide a lyso-PL acceptor for the AA (see deacylation-reacylation pathway of Lands, FIGURE 1). After the incorporation of the AA, PL renovation could occur via two different ATP-independent-AA-specific transacylation enzymes, one being a CoA-dependent transacylase, while the other is a CoA-independent transacylase (Trotter et al., 1982; Kameyama et al., 1983; Kramer et al., 1984b). The CoA-dependent enzyme is known to transfer the AA from one PL to another without passing through the free FA intermediate. It seems that this enzyme, preferentially uses PC as the donor of the arachidonyl group, and transfers it to lyso-PLs in the following order of preference: lyso-PS> lyso-PE> lyso-PI. In contrast the CoA-independent enzyme transfers AA predominantly to the plasmalogen form of lyso-PE (Kramer et al., 1984).

Another mechanism for the renovation of the PLs in the platelets involves the exchange of PLs between platelets and plasma lipoproteins. This exchange has been demonstrated in vitro between platelet PLs and lipoproteins enriched with radiolabelled AA-PC (Bereziat et al., 1978; Plantavid et al., 1982).

1.2. Storage of Human Platelets

1.2.1. Effects of Storage on Platelets

Blood platelets are routinely isolated from whole blood, concentrated, and stored in plasma for use in transfusion therapy. Such platelet concentrates (PU) can be stored for five days and still be therapeutically effective in

thrombocytopenic patients (Adams et al., 1986).

During storage of PUs, a progressive decline in platelet viability indices, such as morphology scores (Holme et al., 1978), hypotonic shock response (Rock and Figueredo, 1976; Holme et al., 1978; Rock et al., 1981; Wong and Rock, 1982), serotonin uptake (Adams et al., 1982), aggregation and release reactions to single stimuli (Rock and Figueredo, 1976; Holme et al., 1978; Rock et al., 1981; Wong and Rock, 1982; DiMinno et al., 1983), have been consistently observed.

In general, platelets which are stored at 22°C for more than 24 hours transform from normal discoid cells to spherically shaped cells (White and Kritvit, 1967; McGill, 1978). Such metamorphosis appears irreversible if the pH falls below 6.0 (White and Kritvit, 1967; McGill, 1978). Stored platelets do not respond normally to single stimuli, such as thrombin (THR), adenosine diphosphate (ADP) or collagen (COLL), after 24 hours of storage (Moroff, 1981). However, this impaired aggregation response can be corrected when a second stimulus is used simultaneously (Moroff, 1981; White, 1981; Fratantoni, 1981; Adams et al., 1986). This ability of stored platelets to aggregate fully, when combinations of stimuli are used, implies that the membrane receptors are intact and that the cellular machinery responsible for aggregation is functional. Perhaps it is the transmembrane signal or the intracellular second messenger which is dysfunctional in stored platelets (Adams, 1982). Membrane phospholipid metabolism, calcium mobilization, transmethylation reactions, cyclic adenosine monophosphate (cAMP), and protein phosphorylation are all interconnected intraplatelet control systems. These systems must be explored further before normal platelet physiology or the defects in stored platelets can be understood (Adams, 1982).

Vishnubhatla (1986) studied the PL composition of resting human platelets stored for 5 days in autologous plasma, and concluded that there is no significant

change in total PLs; the amounts of PC, PE, PI and PS remain constant and the AA composition of those PL subclasses is not affected by the long-term storage period. Molecular class analysis revealed that there is no significant change in the plasmalogen and diacyl forms of total lipids (TLs), PC and PE. However, there was a statistically significant decrease in the alkyl-acyl forms of PC after three days of storage but the significance of this long-term change remains obscure.

1.2.2. Storage of Platelets in an Artificial Medium

It is known that some conditions such as temperature or change in pH can irreversibly inactivate platelets (Zucker and Grant, 1978). The specific role of plasma in supporting platelets during storage has not yet been defined. Indeed, there are several reasons why plasma might be deleterious rather than beneficial:

The collection of blood stimulates cellular secretion which results in plasma being contaminated with proteolytic and glycolytic enzymes. The activity of these enzymes could erode the integrity of the platelet membrane (George, 1976; Bolin et al., 1981), a phenomenon known to occur during storage (Rock et al., 1981).

In the limited plasma environment of platelet concentrate (60 mL), there is relatively little buffering capacity to prevent the decrease of pH below 6.0, a level known to be lethal for stored platelets (Murphy and Gardner, 1975).

Plasma is generally not required by patients receiving platelet therapy and would be used more efficaciously if directed to blood fractionation (Adams et al.,

1986). A report of the ability of non-plasma medium (Plasmalyte A/citrate/dextrose, designated PCD) to support platelet storage has been presented by Adams (1986). It was demonstrated that platelets stored in PCD will respond to pairs of stimulating agents equivalently to platelets stored in plasma. It was shown that plasma is not required to maintain in vitro platelet function during storage of platelet concentrate (Rock et al., 1985).

In fact, in vivo platelet recovery studies have been done and Adams et al. (1986) demonstrated that there is no statistical difference in the percentage of platelet recovery or survival, in normal recipients, between the platelets stored for 5 days in plasma versus PCD. Other in vitro tests of platelet function have demonstrated no differences between platelets stored in plasma or PCD (Adams et al., 1987). The effective storage of platelets in non-plasma medium was also supported by the studies of Holme et al. (1984).

1.3. Rationale and Aims of the Present Work

The role of membrane PLs in maintaining cellular integrity has been well established. Although it has been shown by many investigators (White and Krivit, 1967; Baldini, 1968; Murphy and Gardner, 1971; White et al., 1978) that significant deterioration of platelet function takes place during storage, very little information has been available about the effects of storage on platelet PL metabolism (Imai et al., 1984).

The human platelet is a particularly good model to study PL metabolism since:

a) Platelets can be easily obtained fresh from human donors, in large quantities.

b) The therapeutic necessity of platelet transfusion has promoted the development of advanced methods for preserving the cells in vitro in their native state.

c) Platelets PLs are important mediators of physiological function both as platelet agonists and vasoactive agents.

d) Platelets readily modify their lipids upon stimulation with agonistic agents.

It is very important to consider the fact that platelet lipid composition and response appear to differ between different species (Meyers et al., 1980; Leach and Thorburn, 1982; Agwu et al., 1983; Nishizawa et al., 1983). The results obtained from the present study are thus most applicable to human platelets.

After platelets became available for transfusion, numerous studies have been carried out in order to optimize storage protocol which would allow efficient utilization of platelets (White et al., 1978). For many years, whole blood was stored in glass bottles. After the development of plastic bags about 20 years ago, blood could be fractionated into its components. At this time, platelets were stored as platelet concentrate units (PUs) for 3 days in di(-2-ethylhexyl)phthalate polyvinyl chloride (DEHP-PVC) bags. The permissible interval for storage of PUs was increased from 3 to 5 days by the office of Biologic Research and Review in 1982. This extension was made possible by the development of the new plastic, polyolefin that improved preservation of pH in PUs because of its increased gas permeability. This allowed greater amounts of oxygen to enter the bag and greater amounts of carbon dioxide to escape. The maintenance of neutral pH improved viability and extended the platelet storage period up to 5 days for therapeutic use (Murphy et al., 1982). There is no doubt that the storage container is critical in platelet metabolism and the results from the research done with platelets stored in polyolefin bags are certainly different from results

obtained from cells stored in PVC bags.

The storage of human platelets in PCD is not used as a standard procedure in blood banking. However, this medium constitutes a more controlled environment which is ideal for biochemical studies since its composition is constant whereas plasma composition can vary from one individual to another.

The purpose of this research was to study the kinetics of GPL metabolism in resting human platelets stored for five days in a non-plasma medium. In particular, the routes used to achieve this goal were:

a) Determination of cellular uptake and incorporation into the glycerophospholipids (GPLs) of AA and glycerol during storage of the platelets in plasma-free medium. In this report, the term "uptake " refers to diffusion or transport of a substance into the cell, whereas the term "incorporation" refers to the covalent binding of a substance to one or more cellular component(s).

b) Analysis of GPL metabolism of stored platelets, in terms of de novo synthesis and remodelling pathway.

c) Correlation of the PL changes with decrease of the agonist-platelet-response that occurs with time.

The uptake and incorporation into GPLs of AA and glycerol have been well documented by Imai et al. (1982) who studied human platelets in plasma and by Neufeld and Majerus (1983) who looked at the human platelets in different types of phosphate buffers. The distribution of AA into GPLs of resting and stimulated platelets has been looked at by Cohen and Derksen (1969); Marcus et al. (1969); Bills et al. (1976); Chambaz et al. (1979); Rittenhouse-Simmons and Deykin (1981); Guichardant and Lagarde (1983); Kramer and Deykin (1983); Schick et al. (1984); Aznar et al. (1985); Weaver and Holub (1985); Wilhem Weiner and

Sprecher (1985); Baker and Chang (1986); Takamura et al. (1987), who used different experimental conditions. Only Imai et al. (1982) and Lewis and Majerus (1969) were interested in looking at the details of the de novo synthesis in human platelet.

The present study is unique since it is the first one intended to study the long-term incorporation pattern of AA and glycerol in human platelets resuspended in PCD. The results obtained from this research should help to answer some of the questions about the metabolic behavior of PLs during the in vitro storage of mammalian excitable cells. This knowledge will allow the formulation of more specific hypotheses concerning of the role of platelet PL metabolism in the "storage defects" known to result in transfusion technology.

2. MATERIALS AND METHODS

2.1. Materials and Reagents

Non-plasma medium was prepared by mixing 0.5 mL of concentrated sodium citrate (46.7% trisodium citrate, Haemonetics Corporation, Braintree, Mass), 0.5 mL of dextrose for injection (50% in water, sterile and nonpyrogenic), and 59 mL of Plasma-Lyte A (90 mM NaCl, 23 mM sodium gluconate, 27 mM sodium acetate, 5 mM of KCl, 3 mM of $MgCl_2$, 294 mOsm, pH 7.4; Travenol Canada Inc, Mississauga, Ontario). The standard non-plasma medium was designated PCD.

All solvents were HPLC grade, distilled in glass (Accusolv, Anachemia Canada Inc., Montreal, Quebec). Fatty acid and phospholipid standards were purchased from Sigma Chemical Company (St Louis, MO). [1-^{14}C]Arachidonic acid was from Amersham (Searle, Boston, MA); [5,6,8,9,11,12,14,15-^{3}H(N)]AA and [^{14}C(U)]glycerol were purchased from New England Nuclear Research products (Albany, Boston, MA). Radioactivity was counted in polypropylene scintillation vials (Fisher Scientific, Ottawa, Ontario) containing Universal cocktail (ICN Biomedica Canada, Montreal, Quebec).

2.2. Methods

2.2.1. Platelet Preparation in Non-plasma Medium

Whole blood from normal healthy donors was collected into citrate-phosphate-dextrose-adenine (CPDA-1) anticoagulant using established procedures of the Canadian Red Cross (Blood Transfusion Service, Ottawa Centre, Ontario). The fresh blood was centrifuged (RC-3B, Sorvall, Newton, CT) at 1000 x g for 7 minutes at 22^{o}C. Using aseptic techniques, the platelet rich plasma (PRP) from 2-7 units was pooled and divided into 6 new polyolefin transfer packs (Fenwal

PL732, 300 mL capacity) to give approximately 72 x 10^9 platelets per bag. The platelet concentration was determined by electronic particle counting (Coulter Inc., Hialeah, FLA.), the platelet count given by the coulter counter has a coefficient of variation of 4.2% (Bull et al., 1965). All 6 units were centrifuged at 3,350 x g for 6 minutes in a Beckman Model J-6M centrifuge, and most of the plasma was removed (7-9 mL remaining). Knowing that the albumin concentration in normal plasma is 40 g/L (Scientific Tables, 1962), the albumin concentration in PCD is calculated to be 6 g/L. The concentrated platelets were left at room temperature undisturbed for one hour, after which PCD was added. The final volume of each PU was 60 mL. The PUs were stored at 22°C on a vertically rotating shaker at 6 rpm (Helmer Labs Inc, St Paul). The platelets were tested for in vitro function on day 0, and after 5 days of storage. The pH of the PUs was monitored throughout the storage using a Metrohm 632 pH-meter (Brinckmann, Montreal, Quebec).

2.2.2. Platelet Labelling

The freshly resuspended PUs (60 mL) were labelled with trace amounts of either [1-^{14}C]AA, (33.6 nmol) or [^{14}C(U)]glycerol, (29.2 nmol). The specific activity of the radiolabelled compounds was 59.6 mCi/mmol and 165.8 mCi/mmol, respectively. Double label experiments were conducted using [5,6,8,9,11,12,14,15-^{3}H(N)]AA, (33.6 nmol) and [^{14}C(U)]glycerol (29.2 nmol). The specific activity of the [^{3}H]AA was 240 mCi/mmol.

Subsamples were withdrawn from the radiolabelled PUs on day 0 (blood collection day) up to day 5. The radioactivity was measured (using a Beckman LS7500 liquid scintillation system, Beckman Instruments Inc., Irvine, CA.) on each subsampling day. Several polyunsaturated FAs have been shown to influence

platelet functions markedly. The best known of all, which is AA, induces platelet aggregation. Several investigators have observed that addition of AA to platelets (0.025-0.1 mM) results in platelet activation (Smith et al., 1974; Linder et al., 1979; Frantatoni and Poindexter, 1981; Linder and Goodman, 1982; Aharony et al., 1982). High concentrations of AA (0.15-0.35 mM), have been shown to cause inhibition of platelet function (Linder and Goodman, 1982; Cattaneo et al., 1982), and even higher concentrations (> 0.4 mM) cause cell lysis (Frantatoni and Poindexter, 1981). Since the amount of tracer AA used to label the platelets was 560 nM (0.56×10^{-3} mM), we can exclude the possibility of platelet activation or damage created by the tracer.

2.2.3. Platelet Function Testing

Platelet aggregation was measured as follows: PUs were diluted to 3×10^8 platelets per mL with autologous plasma. The stimulating agents were added to the platelet suspensions separately or in pairs. The following aggregating agents were used:

Adenosine diphosphate (ADP) (Sigma), dissolved in normal saline to 10^{-4} mol/L and frozen.

Bovine tendon collagen (COLL) (Sigma), prepared by the method of Cazenave et al. (1973).

Thrombin (THR) (Parke-Davis Canada Inc., Scarborough, Ont.), diluted in normal saline to 2 U/mL.

The final concentrations of the aggregating agents used were:

AGGREGATING AGENT	FINAL CONCENTRATION
ADP	10^{-5} M
COLL	5.0 μg/mL
THR	0.1 U/mL

The extent of aggregation was measured turbidimetrically (Born, 1962). Aggregation, which was defined as the maximum change in optical density after stimulation, was monitored for 4 minutes on an aggregometer (Payton Instruments, Toronto, Ont.).

2.2.4. Cellular Radiolabel Uptake

To follow the uptake of radioactivity into the platelets, 0.5 mL of the radiolabelled PU was subsampled, at regular time intervals, counted and centrifuged at 15,000 x g (Eppendorf centrifuge 5412, West Germany); the supernatant (platelet-free PCD) was then counted. The difference between the total and the platelet-free PCD radioactivity counts, was taken as a measure of the cellular uptake of radioactivity with storage time.

2.2.5. Platelet Washing Procedure

The PU subsamples were washed according to the method of Mustard et al. (1972), in order to remove the un-incorporated radiolabelled material and the non-platelet cells. The platelet suspension was first acidified to pH 6.3 with acid-citrate- dextrose solution USP (ACD) Formula A (Fenwal Laboratories, Malton, Ontario) and centrifuged in a Sorvall RT 6000 (Du Pont Company, Wilmington, DE) at 1000 x g for 12 minutes at 22°C. After the removal of the supernatant, the cells were resuspended in the same volume of a modified Tyrode's wash buffer

(137 mM NaCl, 2.7 mM KCl, 12 mM $NaHCO_3$, 0.4mM $NaH_2PO_4 \cdot H_2O$, 25 mM HEPES, 0.1% glucose, 0.35% FA-free bovine albumin, pH 7.35) containing 25 U/mL of porcine intestinal mucosa heparin sodium salt (Organon Canada LTD., Toronto, Ontario) and 20 μL/mL of apyrase (prepared from potatoes by the method of Molnar & Lorand (1961)), pre-warmed at 37°C. It is important to note that special care was taken to avoid resuspension of RBCs and WBCs. The resuspended platelets were transferred to another tube and kept at 37°C in a shaking water bath for 15 minutes, then centrifuged and washed as above with the same buffer, containing only 10 μL/mL of apyrase but without heparin. After a second 15 minute period at 37°C. the washed cells were resuspended in 8 mL of modified Tyrode's buffer containing only 1 μL/mL of apyrase. Washing efficiency was determined by platelet counts before and after the washing procedure.

2.2.6. Total Cellular Lipid Extraction

The washed platelets had their total cellular lipids extracted via a modified method of Bligh and Dyer (1959), (Kates, 1986). The cells (approximately $72x10^9$ platelets in 8 mL) were transfered into 30 mL of CH_3OH:$CHCl_3$ [2:1, v/v], mixed and kept at room temperature for 30 minutes. This suspension was filtered through a Buchner funnel layered with Whatman #1 filter paper coated with glass wool. After filtration the Buchner funnel was rinsed with 38 mL of CH_3OH:$CHCl_3$:modified Tyrode's buffer [20:10:8, v/v/v]. $CHCl_3$ and H_2O (20 mL each) were added to the filtrate and washings (one phase system) to make it into a two phase system composed of $CHCl_3$:CH_3OH:H_2O [1:1:0.9, v/v/v]. The lower $CHCl_3$ layer was separated from the upper aqueous-methanol layer [9:10]. The latter was washed twice with 5 mL of $CHCl_3$. The combined $CHCl_3$ layers were concentrated using a rotary evaporator, with the addition of 10% benzene to aid-

in the removal of traces of water. The residual total lipids were dissolved in 0.5 mL of $CHCl_3$:CH_3OH [2:1, v/v], flushed with N_2 and stored at -20°C until used.

2.2.7. Determination of Total Mass of Lipids in Intact Platelets

The total mass of lipid extracted from one unlabelled PU was determined in triplicate as follows: platelets ($72x10^9$ cells) were acidified to pH 6.3 with ACD Formula A and centrifuged at 1,000 x g for 12 minutes at 22°C as above. The pellet was resuspended in a total volume of 8 mL of modified Tyrode's buffer. The lipids were then extracted as above. The $CHCl_3$ layer was evaporated in a pre-weighed 50 mL pear-shape flask, with about 10% benzene and dried overnight in a 140°C vacuum oven. The residual lipids (approximately 45 mg) were allowed to cool at room temperature in a dessicator and finally weighed on an analytical balance. The results were expressed as μg lipids/10^9 platelets.

2.2.8. Fractionation of Total Cellular Lipids

The NLs and PL subclasses were separated by HPLC liquid chromatography according to a modified method of Patton et al. (1982), on an HPLC Varian Model 5000 Liquid Chromatograph (Varian Canada Inc., Ottawa, Ontario) equipped with a radiodetector (Technical Marketing Associates Limited, Ottawa, Ontario) connected to a servo graphic recorder (Pharmacia Fine Chemicals, Piscataway, NJ). Half of the total cellular lipid extracts resuspended in 50 μL of $CHCl_3$:CH_3OH [2:1, v/v], were injected onto a (9.4 mm x 25 cm) Partisil 10 Magnum 9 Preparative HPLC Column (Whatman Chemical Separation Inc., Clifton, NJ). To facilitate the PLs separation, the elution procedure of Patton et al. (1982) was modified as follows: For the first 115 minutes, the mobile phase composed of hexane/2-propanol/25mM potassium phosphate buffer (pH 7.0)/ CH_3OH/ CH_3COOH [367:490:62:100:0.6, v/v],-

(Patton et al., 1982), running at a flow rate of 1 mL/minute, allowed the elution of the NLs, PE, PI and PS. To allow a more rapid elution of PC, the solvent system was substituted (for the next 25 minutes) by $CH_3CN/CH_3OH/H_2O$ [75:21:14], v/v/v], (Jungalwala et al., 1976), at a flow rate of 3.0 mL/minute.

The eluates of separated lipids were collected at 2.5 minute intervals, into 10 x 75 mm borosilicate glass disposable tubes containing 5 x 10^{-5} M butylated hydroxy toluene (BHT) (Aldrich Chem. Co. Ltd., Milwaukee, Wisconsin), to prevent autoxidation of the lipids (Guichardant and Lagarde, 1983), using a FC-80K Fractionator (Gilson Medical Electronics Inc., Middleton, Wisconsin). Aliquots of fractions were recounted in the scintillation counter, pooled according to the elution profiles of each known phospholipid subclass, evaporated with 10% benzene on the rotary evaporator, transfered into a 1 mL vial and stored in $CHCl_3$:CH_3OH [2:1, v/v], at -20°C as above until used. The different PLs were identified by comparison with HPLC retention times (R.T.) of unlabelled and labelled PL standards. The unlabelled PLs standards were detected with an UV-100 ultraviolet detector (Varian Canada Inc.), operated at a wavelength of 205 nm, whereas the labelled PL standards were detected with a radiodetector as previously described. The identity and the purity of each separated PL was confirmed by its mobility on thin-layer chromatography (TLC), as described below. The percentage distribution of radioactivity into each subclass was determined and the incorporation of radioactivity into PLs plotted as a function of storage time.

2.2.9. Thin-layer Chromatography

Analytical TLC was carried out with the total cellular lipid extracts, purified PLs and NLs. The mobilities (R_f values) of those samples were compared to appropriate standards. TLC was performed on 20 x 20 cm silica gel G plates-

(Sigma) with 250 μm layer thickness, 2-25 μm mean particle size and 60 Å mean pore diameter. The solvent systems used for TLC were as follows:

TYPE OF LIPID	SOLVENT SYSTEMS
Neutral lipids:	Petroleum ether: diethyl ether: CH_3COOH [90: 10: 1, v/v/v], (Skipski and Barclay, 1969).
Phospholipids:	Acidic system: $CHCl_3$: CH_3OH: CH_3COOH: H_2O [50:25:4:2, v/v/v/v], (Skipski and Barclay, 1969).

Following development of each TLC plate, the spots were detected by two methods: Iodine vapour as general stain for lipids (Sims and Larose, 1962) and/or the molybdate reagent for phosphate groups (Vaskovsky and Kostetsey, 1968). The radioactive spots were detected by autoradiographic techniques (Mangold, 1969).

2.2.10. Lipid-phosphate Determination

The phosphorus content of total cellular lipid extracts and separated PLs was determined colorimetrically by the modified method of Bartlett (Kates, 1986). Since the separated PE, PI, and PS were eluted from the HPLC column with a phosphate containing mobile phase, special care was taken to avoid any contamination of the samples with phosphate. These PLs were treated as follows:

The samples were evaporated to dryness under nitrogen with 10% benzene, and resuspended in a known volume of $CHCl_3$. A known aliquot of this solution was carefully removed for the assay, leaving behind the inorganic phosphate bound to the glass. As a control, a known volume of the buffered system (15mL) containing 11.6 mg phosphorus was assayed as above and only traces of

the phosphate could be detected. The results were expressed as μg phosphorus/ 10^9 platelets.

2.2.11. Design of Experiments and Statistical Analysis

Most of the platelet samples reported in this study were from different pools. The n values (number of analyses) used in statistical analysis refer to the total number of repeated analyses done. Three types of labelling experiments were conducted:

1) A single labelling experiment (using AA) was done twice.
2) A single labelling experiment (using glycerol) was also done in duplicate.
3) A double labelled experiment (using simultaneously AA and glycerol) was done in duplicate.

The reported results for AA labelling represent the means of experiments 1 and 3, whereas the reported results for glycerol labelling represent the means of experiments 2 and 3 described above. The results are expressed as means ± standard error (SE) for $n \geq 4$.

The SE was calculated as follows:

$$SE = \frac{\text{Standard deviation (SD)}}{\sqrt{n}}$$

where SD is the positive square root of the variance (measurement of the dispersion of a distribution).

For $n \leq 3$, the results were expressed as mean $\pm$ 95% confidence interval. The 95% confidence interval was calculated as follows:

$$95\% \text{ confidence interval} = \frac{SD}{\sqrt{n}} \times T$$

where T is the t statistic value for n analyses at 95% confidence intervals from the Student's-t distribution (Hogg and Tanis, 1977).

3. RESULTS

3.1. Preparation and Storage of Platelets in Artificial Medium

The resuspension of fresh platelets in PCD and the storage of those cells in PL 732 bags for a period of 5 days is a standardized method in our laboratories. The platelet count of each PU was initially adjusted to an average value of $1.16 \times 10^6 \pm 0.07 \times 10^6$ platelets/μL. This platelet count did not change significantly ($p < 0.025$) on day 5 since it was up to $1.19 \times 10^6 \pm 0.07 \times 10^6$ platelets/μL, indicating minimum cell lysis during storage.

The pH of the medium did not change significantly during the storage ($p<0.025$), varying from 6.96 ± 0.04 on day 0 to 7.06 ± 0.04 on day 5. pH maintenance throughout the storage period is an indication of good storage conditions and of absence of bacterial contaminants. The constant mean pH value of 7.0, was ideal for platelet storage, since White and Kritvit (1967); McGill (1978) and Watts et al. (1983) have shown that pH values below 6.0 and above 8.0 cause irreversible platelet damage.

The platelet function testing shown in TABLE 1 demonstrated that there is a significant decrease of the aggregation response after 5 days of storage when the cells are stimulated with a single agonist, whereas no significant changes were observed in the responses of cells stimulated with the double agonists ADP + COLL and COLL + THR, ($p<0.025$). The fact that ADP and THR was the only combination unable to restore the aggregation response on day 5, is probably because the response given by THR is always more difficult to interpret since clot formation occurs rather than the small platelet aggregates normally obtained. The loss of aggregation response to single agonist after 5 days of storage and the recovery of their activity in presence of two agonists is an indication that

TABLE I: Platelet Function Testing During Storage

Stimulating Agent	Aggregation (% transmission) Storage Time	
	Day 0	Day 5
ADP	32.3 ± 2.1	6.7 ± 7.6
COLL	69.7 ± 17.8	14.7 ± 9.4
THR	28.9 ± 9.9	2.0*
ADP + COLL	71.0 ± 19.3	79.7 ± 20.6
ADP + THR	49.0 ± 4.3	15.0*
COLL + THR	56.0 ± 17.2	41.0 ± 38.7

The final concentrations of the aggregating agents used were 10^{-5} M of ADP, 5.0 ug/mL of COLL, and 0.1 U/mL of THR.

The values are the mean ± 95% confidence interval of separate pools of PU.

* No variations between values were recorded.

platelet function was preserved during storage. This normal behavior observed in platelets stored for more than one day, has been demonstrated by many investigators (Moroff, 1981; White, 1981; Fratantoni, 1981; DiMinno et al., 1982; Adams et al., 1986). It has been shown by Adams et al. (1986) that 5 day stored platelets function normally in vivo, when transfused into thrombocytopenic patients.

3.2. Platelet Labelling

The total amount of radioactivity introduced into the bags did not change significantly ($p<0.025$), during the 5 day storage period (TABLE 2). After the introduction of the AA tracer into the PU, an uptake equilibrium state was reached after 10 minutes (FIGURE 3). The maximum uptake of AA by the resting human platelets was 70.6 ± 10.2% of the total amount of tracer present in the bags. This uptake value was not significantly different ($p<0.025$) from the value reached on day 5 of the storage period (77 ± 4.5%).

However glycerol uptake by the platelets showed a different pattern; the uptake equilibrium state was reached after 6 hours at a maximum uptake of 22.3 ± 5.4% of the total amount of tracer introduced into the bags (FIGURE 4). As demonstrated for the AA uptake, the glycerol uptake did not significantly change after 5 days remaining at the level of 21.3 ± 1.7%.

3.3. Platelet Washing Efficiency

The platelet washing procedure used, consists in washing out most of the un-incorporated radioactive materials that are left in the PCD and to eliminate as much as possible any other non-platelet cells that are always present in the PUs. The washing efficiency calculated on the basis of platelet counts, was 58.0 ± 2.3%.

TABLE 2: Retention of Radioactivity in PUs During Storage

Radioactive Tracer	Concentration of Radioactivity in PU (μCi/PU)	
	Storage Time	
	Day 0	Day 5
[^{14}C]AA	2.47 ± 0.34	2.42 ± 0.31
[^{3}H]AA	3.23 ± 0.21	3.15*
[^{14}C]glycerol	4.50 ± 1.08	4.92 ± 1.44

The values are the mean ± SE of separate pools of PU.

* No variation between values.

FIGURE 3: Rate of Radiolabelled AA Uptake into Human Platelets.

After addition of the radioactive AA to the cell suspension, samples were taken at regular time intervals, platelets were centrifuged and the radioactivity was counted as described in Materials & Methods. The difference between the total and the platelet-free PCD radioactivity counts, was taken as a measure of the cellular uptake of AA and was plotted as percentage of total counts versus time.

The results are the mean $\pm$ 95% confidence interval, of a single PU.

FIGURE 3

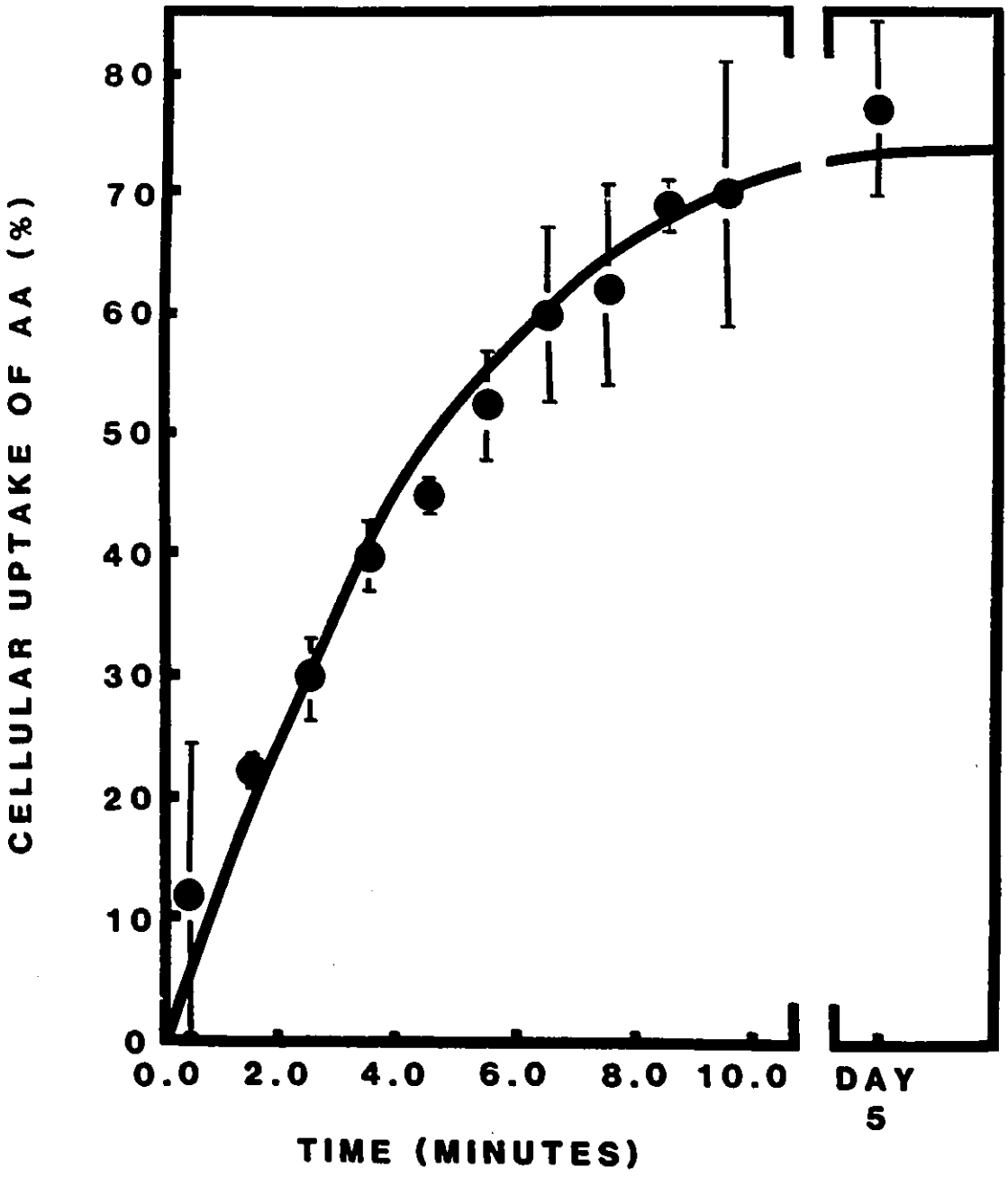
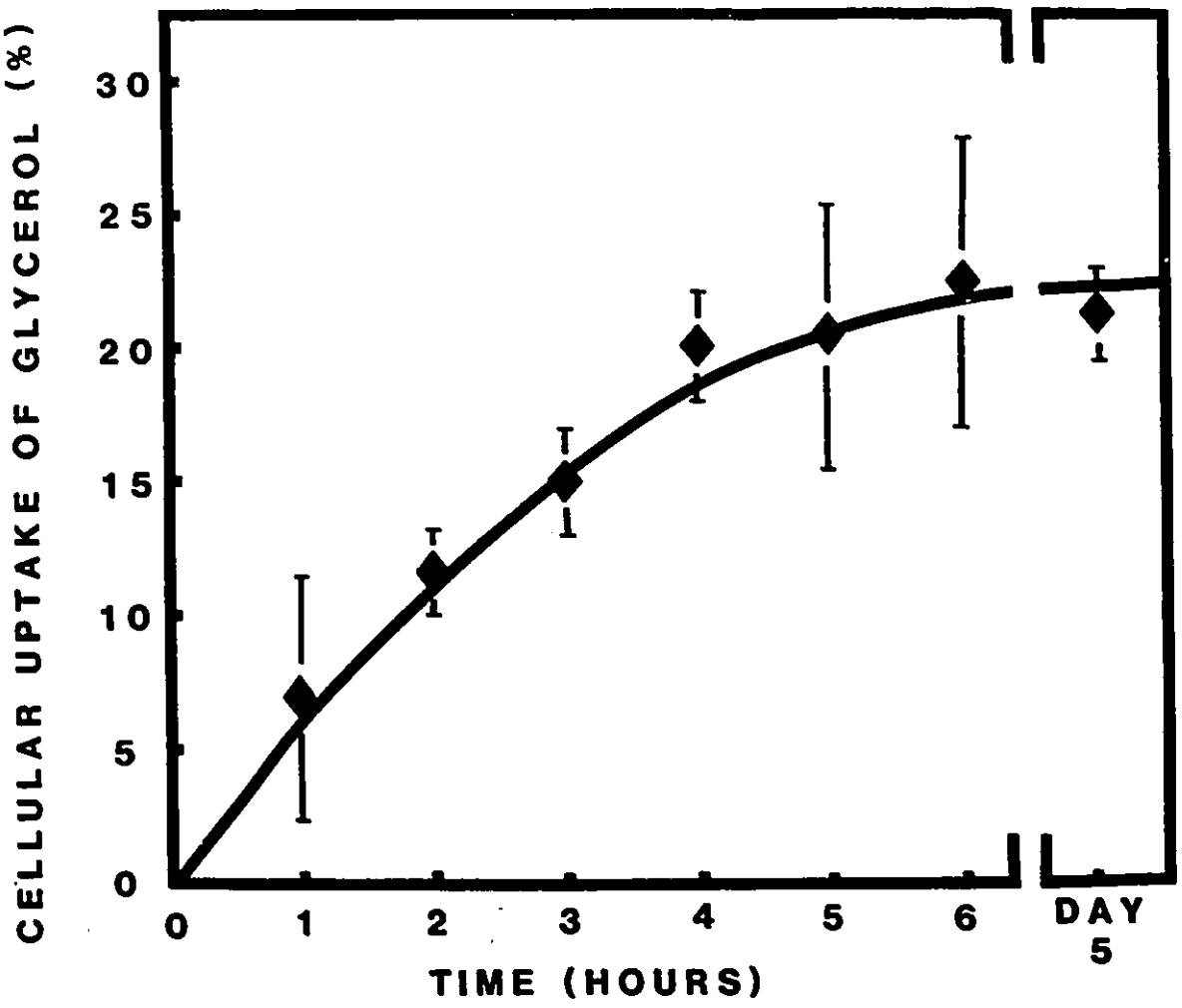

CELLULAR UPTAKE OF AA (%)
80
70
60
50
40
30
20
10
0
0.0
2.0
4.0
6.0
8.0
10.0
DAY 5
TIME (MINUTES)

FIGURE 4: Rate of Radiolabelled Glycerol Uptake into Human Platelets.

After addition of the radioactive glycerol to the cell suspension, samples were taken at 1 hour time intervals, platelets were centrifuged and the radioactivity was counted as described in Materials & Methods. The difference between the total and the platelet-free PCD radioactivity counts, was taken as a measure of the cellular uptake of glycerol and was plotted as percentage of total counts versus time.

The results are the mean ± 95% confidence interval, of a single PU.

FIGURE 4


CELLULAR UPTAKE OF GLYCEROL (%)
30
25
20
15
10
5
0
0
1
2
3
4
5
6
DAY 5
TIME (HOURS)

To evaluate the purity of the washed platelet suspensions, a cell differential test was conducted by counting the non-platelet cells on a washed platelets smear stained with Wright stain using light microscopy. The cell differential test revealed the absence of neutrophils but the presence of 1 WBC per 1600 platelets.

3.4. Lipid Extraction of Platelets

Extraction of intact platelets by the modified Bligh and Dyer (1959) procedure (Kates, 1986) gave a total lipid content of 642 ± 117 $\mu g/10^9$ platelets. Extraction of AA-radiolabelled cells resulted in 98.7 ± 0.1%, recovery of radioactive lipids, whereas the extraction of glycerol-radiolabelled platelets gave a recovery of only 85.1 ± 1.1% of the glycerol taken up by the platelets.

3.5. Fractionation of Total Cellular Lipids

Recovery of total [^{14}C]-labelled lipids after HPLC fractionation (see methods section 2.2.8.) was 101.41 ± 0.53%. The HPLC tracings of both [^{3}H]AA and [^{14}C]glycerol are shown in FIGURE 5. HPLC peaks were identified by comparison of their R.T. with those of corresponding commercially obtained PL standards that are listed in TABLE 3. Peak fractions were also subjected to TLC and autoradiographed on analytical TLC plates developed with either solvent system for NLs (FIGURE 6) or for PLs (FIGURE 7).

The NL peak was chromatographed on TLC and autoradiographed (FIGURE 6). The purified glycerol-containing NL fraction was mainly composed of TAG whereas, very little DAG was measured (TABLE 4). The small amount of radioactivity detected at the origin of the NL autoradiogram could have been glycerol or MAG, or both. The purified AA-labelled NL fraction was mainly composed of TAG. Small amounts of free AA and MAG were found, whereas only

FIGURE 5: HPLC Tracing of Separated PLs

A total lipid extract of 5 day old human platelets, labelled with both [^{3}H]AA and [^{14}C]glycerol was chromatographed by HPLC as described in Materials & Methods. The radiolabelled GPL were detected via a radiodetector. NL, PE, PI, and PS were separated within 115 minutes with solvent system hexane/2-propanol/25mM potassium phosphate, pH 7.0/CH_3Cl/CH_3COOH [367:490:62:100:0.6, v/v/v/v/v] solvent system at a flow rate of 1.0 mL/minute, whereas PC was eluted within the next 25 minutes with a CH_3CN/CH_3OH/H_2O solvent system at a flow rate of 3.0 mL/minute.

The solid line shows the ^{3}H-AA tracer whereas the dotted line represents the ^{14}C-glycerol incorporated in the GPL.

FIGURE 5

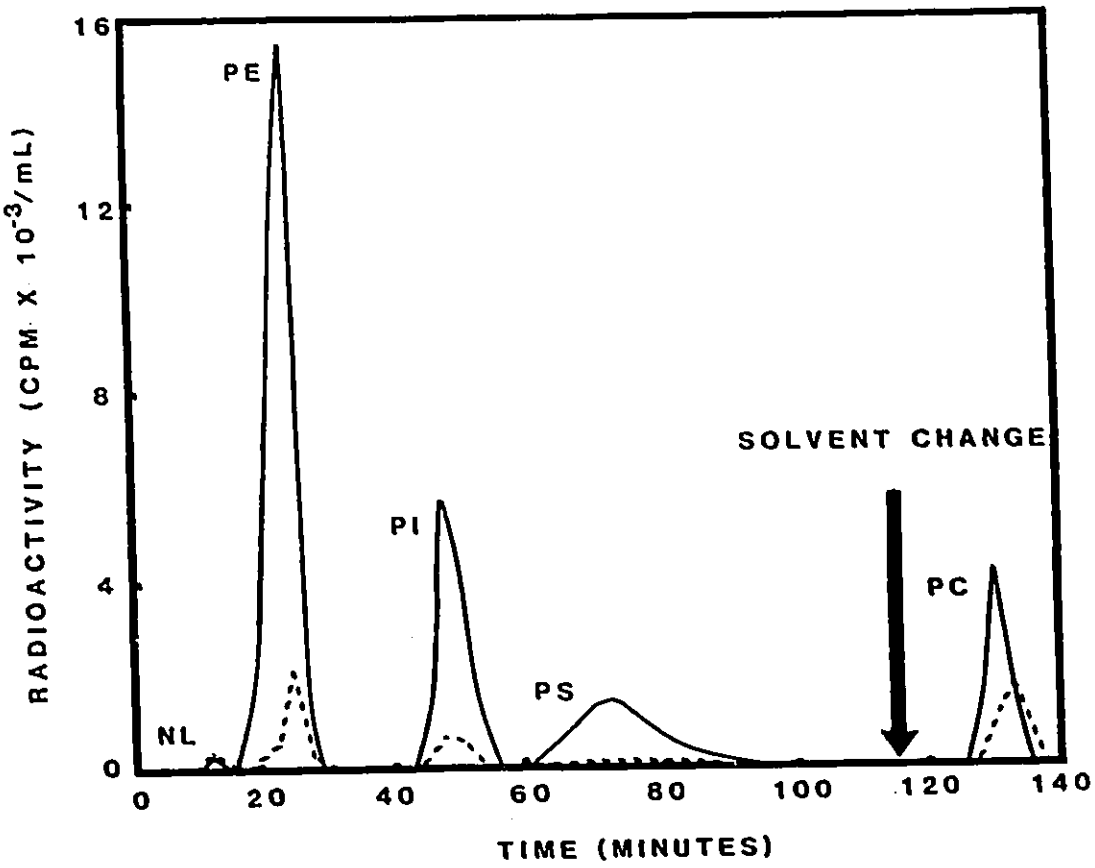

16
12
8
4
0
RADIOACTIVITY (CPM X 10^{-3}/mL)
PE
PI
PS
NL
PC
SOLVENT CHANGE
0
20
40
60
80
100
120
140
TIME (MINUTES)

TABLE 3: Separation of PLs by HPLC

PLs	SOLVENT SYSTEM*	FLOW RATE (mL/min)	R.T. RANGE (MINUTES) standards	labelled platelet GPLs
NL+	A	1.0	12.5-15.0	13.0-14.0
PE	A	1.0	22.5-25.0	22.5-24.0
PA	A	1.0	27.5-30.0	-
PI	A	1.0	45.0-48.0	46.0-47.5
LPE	A	1.0	57.0	-
PS	A	1.0	70.0-72.5	69.0-73.0
CL	A	1.0	80.5	-
PC	B	3.0	130.0-135.0x	132.0-134.5
SM	B	3.0	137.0-139.0x	-
LPC	B	3.0	147.0-152.0x	-

* Solvent system A is hexane/2-propanol/25mM potassium phosphate buffer (pH 7.0)/CH_3OH/CH_3COOH [367:490:62:100:0.6, v/v/v/v/v].
Solvent system B is CH_3CN/CH_3OH/H_2O [75:21:14, v/v/v].

\+ NLs analyzed were TAG, DAG, MAG, cholesteryl-FAs and FA methyl esters.

x R.T. range after the mobile phase change from A to B.

FIGURE 6: Autoradiogram of Chromatogram with HPLC Separated NLs from Double Labelled Platelets with [^{3}H]AA and [^{14}C]glycerol after 5 day storage.

The silica gel plate was run in a neutral lipid solvent system composed of petroleum ether: diethyl ether: CH_3COOH [90: 10: 1, v/v/v]. The spots were detected by iodine vapour.

FIGURE 6

NL8

FIGURE 7: Autoradiogram of Chromatogram with HPLC Separated GPLs from Double Labelled Platelets with [^{3}H]AA and [^{14}C]glycerol after 5 Day Storage.

The silica gel plate was run in an acidic solvent system containing $CHCl_3$: CH_3OH: CH_3COOH: H_2O [50: 25: 4: 2, v/v/v/v/]. The spots were detected via iodine vapour and/or molybdate reagent.

FIGURE 7

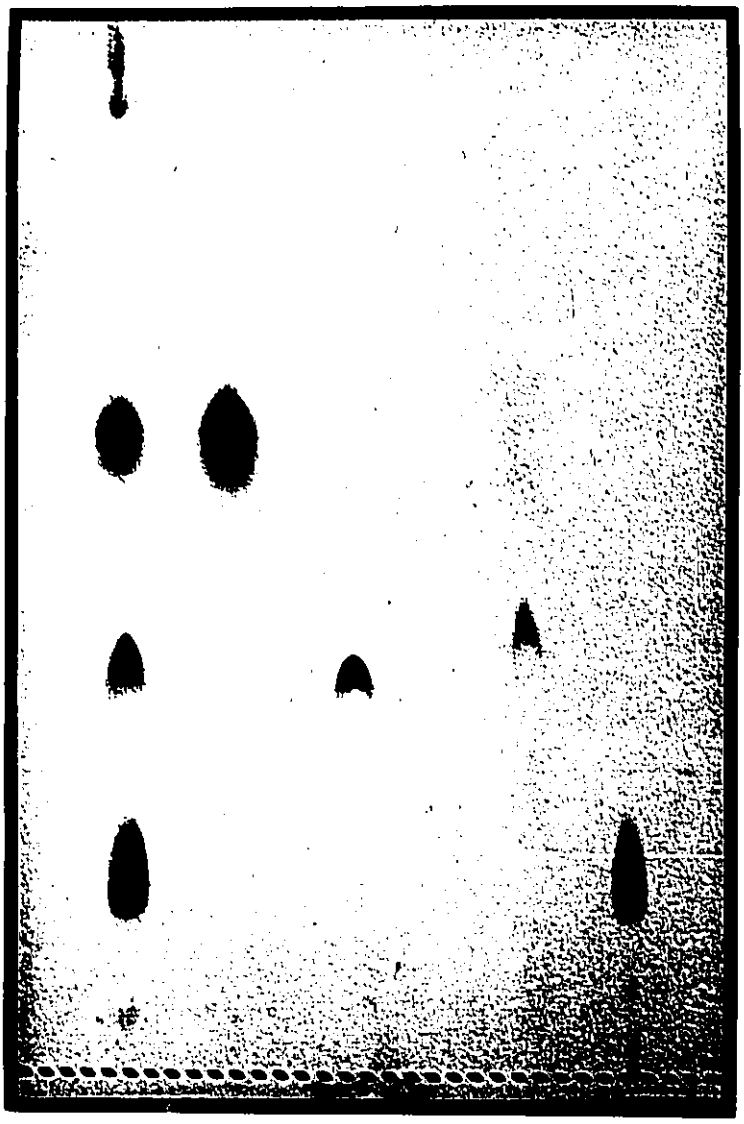


TABLE 4: Distribution of Radioactivity among NL Components*

NLs (HPLC fraction)	R_f	Radioactivity (%)	
		[^{3}H]AA	[^{14}C]glycerol
MAG + glycerol	origin	16	6
DG	0.10	7	6
AA	0.21	20	1
TG	0.37	45	81
Unidentified component	0.48	4	5
AA-sterol	0.92	8	1

* Chromatographic analysis of the NL-TLC (FIGURE 6).

Standards used for identification of each spots, are listed here with their relative R_f values in the solvent system composed of petroleum ether: diethyl ether: CH_3COOH [90:10:1,v/v/v]: Glycerol (origin), MAG (origin), DAG (0.09), TAG (0.4), AA (0.2), arachidonyl-sterol (0.9).

traces of DAG and AA-sterol were measured (TABLE 4). The total cellular lipid extract (TL) and the separated GPLs were chromatographed on TLC and the autoradiogram demonstrated that good separation of each radioactive component of the TL had been achieved by HPLC. Each GPL fraction is 98.4 ± 0.4% pure (FIGURE 7). The distribution of radioactivity among the lipids separated either by TLC (TABLE 5) or HPLC (FIGURES 8-12) are in good agreement.

All the radioactive-AA counts were found in NL, PE, PI, PS and PC (FIGURE 5), and no radiolabelled AA was detected in lyso-PC, lyso-PE, CL, and SM. Very little free radiolabelled AA was found in the NL fraction, most of it being in PC, PE, PI and PS. All the radioactive glycerol counts were recovered in NL, PE, PI and PC, but only traces of [^{14}C]glycerol were incorporated in PS. Low amount of radiolabelled glycerol was found in the NL fraction. No radioactivity was detected in lyso-PC, lyso-PE, CL and SM. The percent of the total radioactivity incorporated with time, into each GPL and NL is plotted in FIGURES 8-12, and are discussed below.

3.5.1. Effect of Long-term Storage of Platelets on Incorporation Pattern of AA

The percentage of radiolabelled AA incorporated in NL, PI and PS did not significantly change ($p>0.025$) during the 5 day storage period (FIGURES 8, 10 and 11, respectively). There was a (28%) increase in AA incorporation in the PE fraction after 5 days of storage (FIGURE 9). This incorporation pattern was linear with time and was statistically significant ($p>0.025$). The opposite phenomenon was observed with the incorporation pattern of the AA tracer in PC (FIGURE 12); there was a significant (28%) decrease in the AA incorporation ($p<0.025$) which was also linear with increasing storage time.

TABLE 5: Distribution of Radioactivity among GPLs*

LIPIDS	R_f	Radioactivity (%)	
		[^{3}H]AA	[^{14}C]glycerol
TOTAL LIPIDS			
PC	0.19	26	57
PI + PS	0.38	30	12+
PE	0.59	41	24
NL	front	2	6

* Chromatographic analysis of the GPL-TLC (FIGURE 7).
\+ Contains only trace amounts of glycerol-labelled PS.

Standards used for the identification of each spots, are listed here with their relative R_f values in the solvent system composed of $CHCl_3$: CH_3OH: CH_3COOH: H_2O [50:25:4:2, v/v/v/v]: PC (0.18), PI (0.37), PS (0.41) and PE (0.59).

FIGURE 8: Incorporation of Radiolabelled AA and Glycerol into NLs during Storage.

The total lipid extracts from the radiolabelled platelets subsampled at different stages of storage, were separated using HPLC. The NL peak (which was the first peak eluted from the column), was counted as described in Materials & Methods. The counts in the NL peaks as a % of the total recovered from the column were plotted as percent of cellular uptake in NL versus time of storage.
Each value plotted represents the mean ± 95% confidence interval of separate pools of PU. Very small error bars could not be represented on the figure.

FIGURE 8

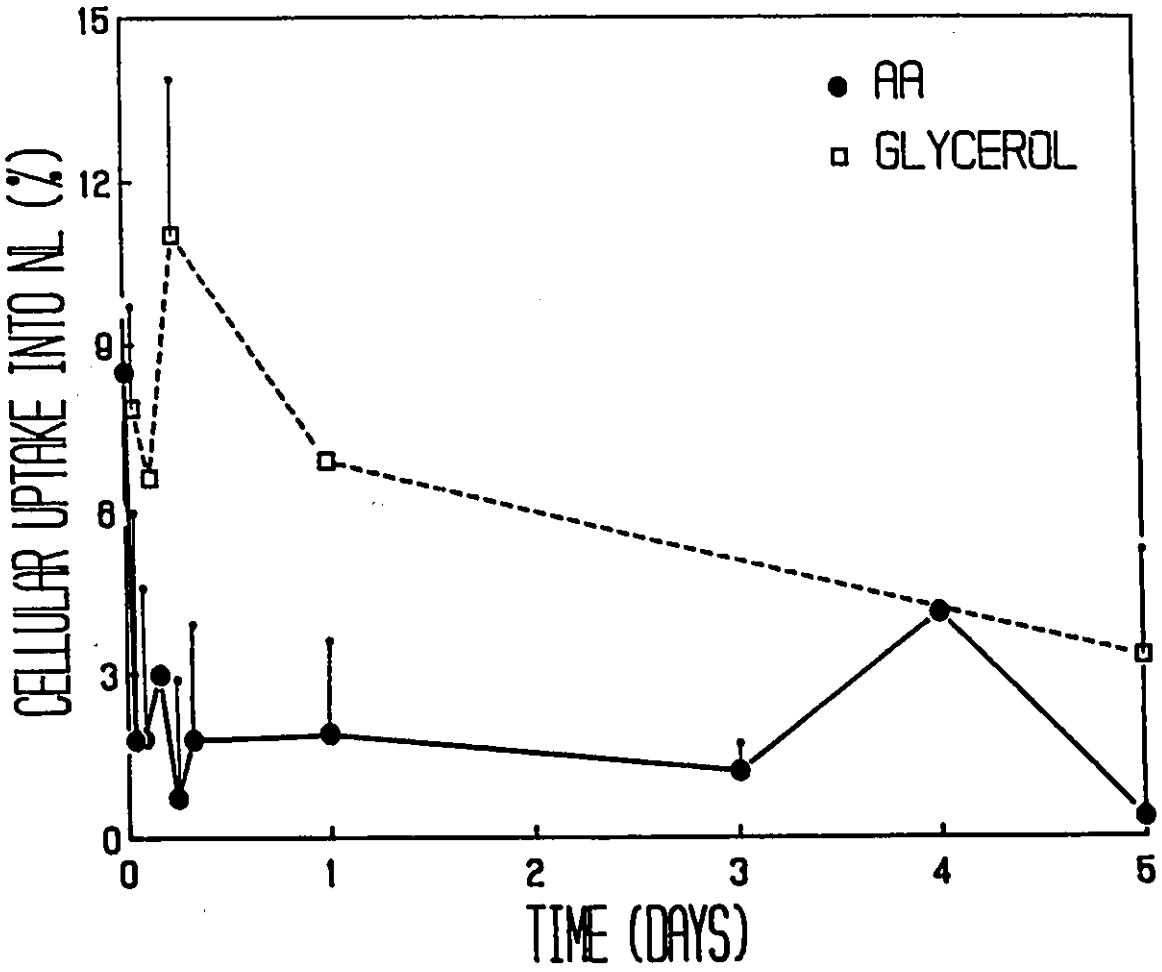

15
12
9
6
3
0
0
1
2
3
4
5
CELLULAR UPTAKE INTO NL (%)
TIME (DAYS)
AA
GLYCEROL

FIGURE 9: Incorporation of Radiolabelled AA and Glycerol into PE During Storage.

The total lipid extracts from the radiolabelled platelets subsampled at different stages of storage, were separated using HPLC. The PE peak (which was the second peak eluted from the column), was counted as described in Materials & Methods. The counts in the PE peaks as a % of the total recovered from the column were plotted as percent of cellular uptake in PE versus time of storage.
Each value plotted represents the mean ± 95% confidence interval of separate pools of PU. Very small error bars could not be visualized on the figure.

FIGURE 9

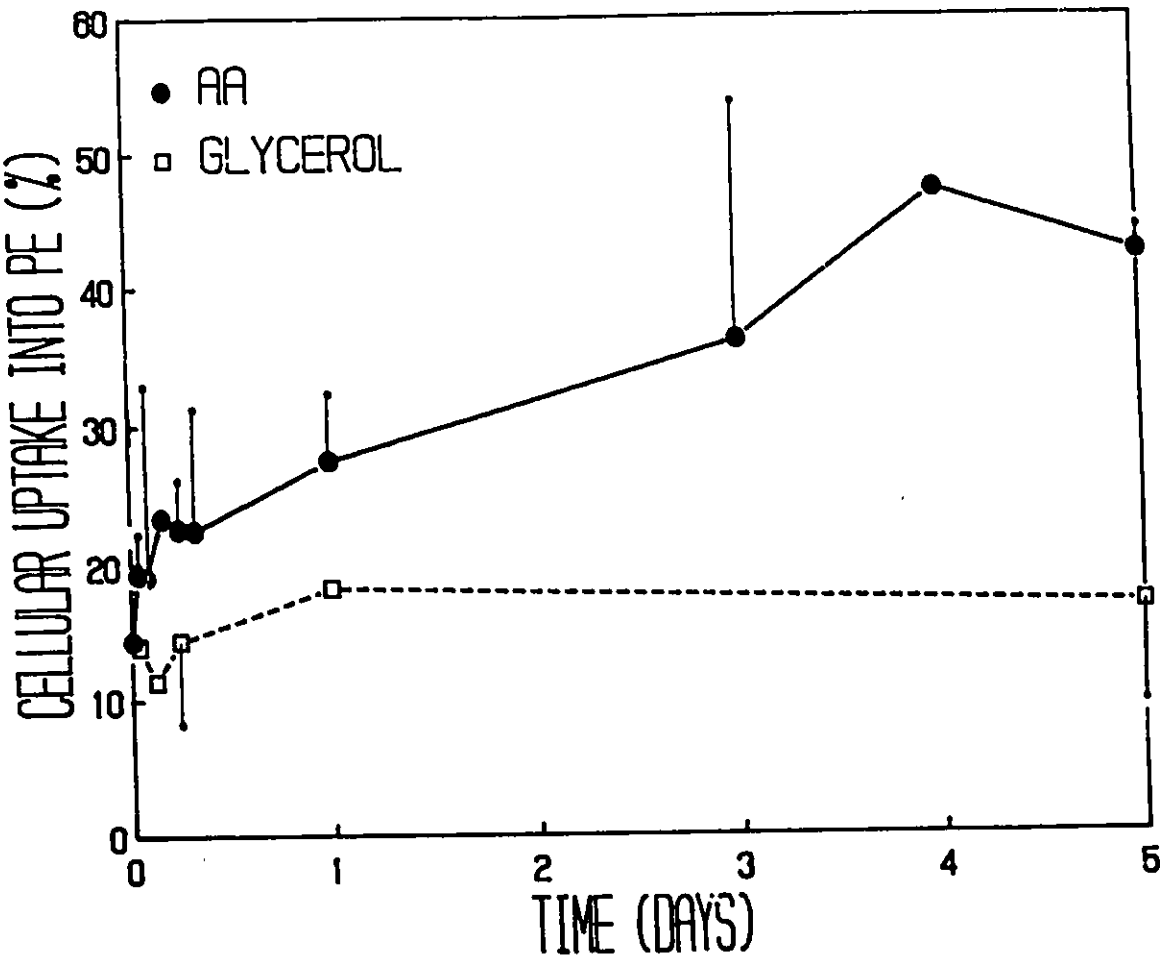

60
50
40
30
20
10
0
AA
GLYCEROL
CELLULAR UPTAKE INTO PE (%)
0
1
2
3
4
5
TIME (DAYS)

FIGURE 10: Incorporation of Radiolabelled AA and Glycerol into PI During Storage.

The total lipid extracts from the radiolabelled platelets subsampled at different stages of storage, were separated using HPLC. The PI peak (which was the third peak eluted from the column), was counted as described in Materials & Methods. The counts in the PI peaks as a % of the total recovered from the column were plotted as percent of cellular uptake in PI versus time of storage.
Each value plotted represents the mean ± 95% confidence interval of separate pools of PU. Very small error bars could not be visualized on the figure.

FIGURE 10

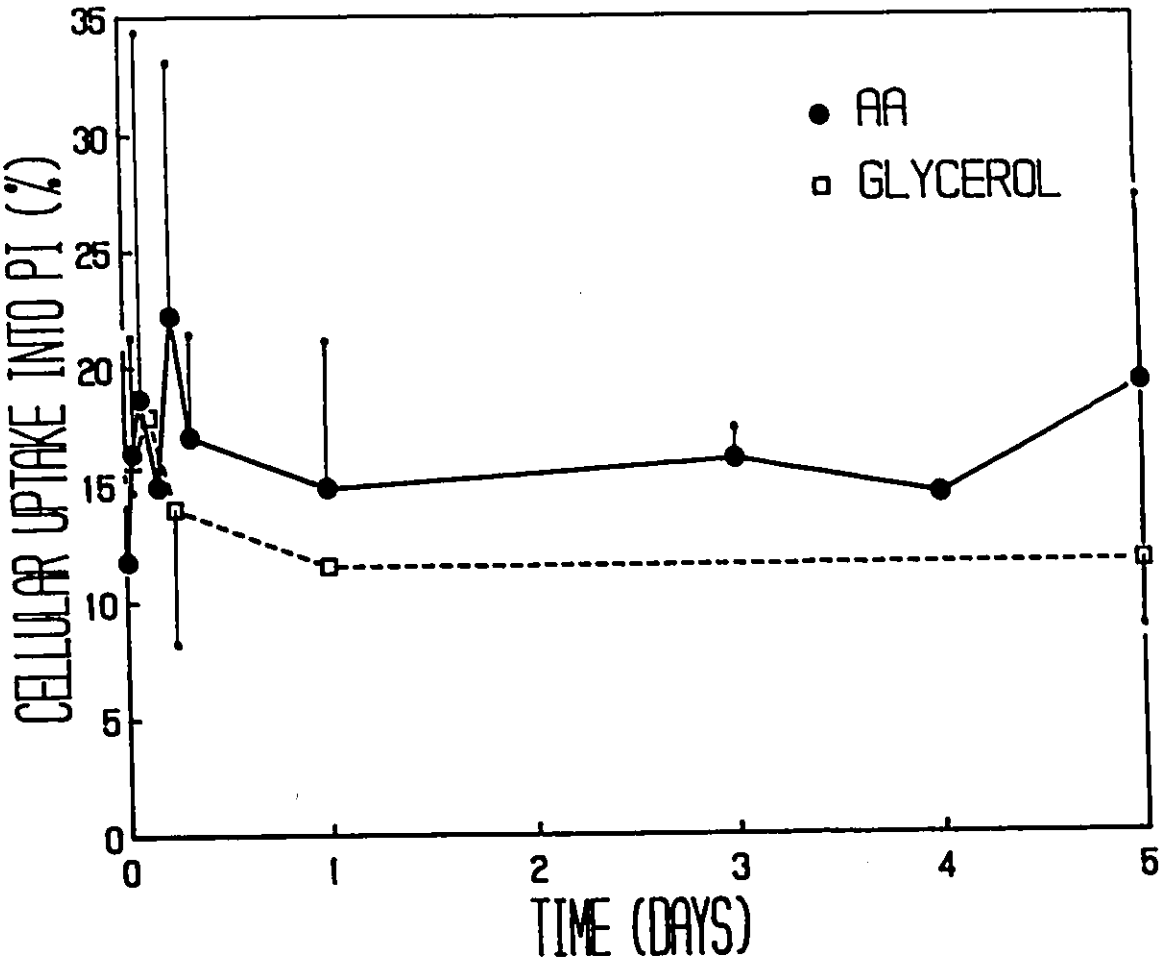

CELLULAR UPTAKE INTO PI (%)
35
30
25
20
15
10
5
0
0
1
2
3
4
5
TIME (DAYS)
AA
GLYCEROL

FIGURE 11: Incorporation of Radiolabelled AA and Glycerol into PS During Storage.

The total lipid extracts from the radiolabelled platelets subsampled at different stages of storage, were separated using HPLC. The PS peak (which was the fourth peak eluted from the column), was counted as described in Materials & Methods. The counts in the PS peaks as a % of the total recovered from the column were plotted as percent of cellular uptake in PS versus time of storage.
Each value plotted represents the mean ± 95% confidence interval of separate pools of PU. Very small error bars could not be visualized on the figure.

FIGURE 11

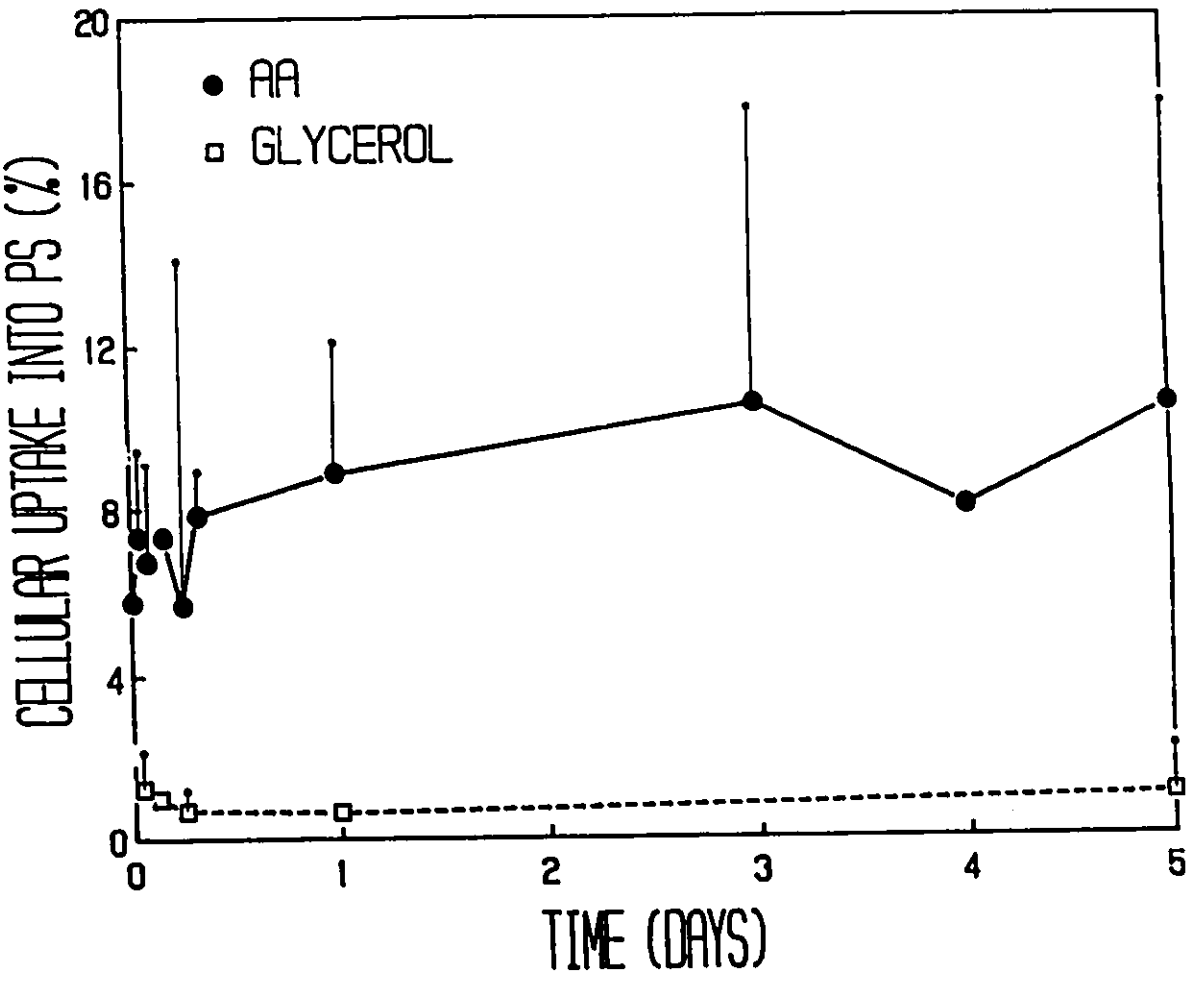
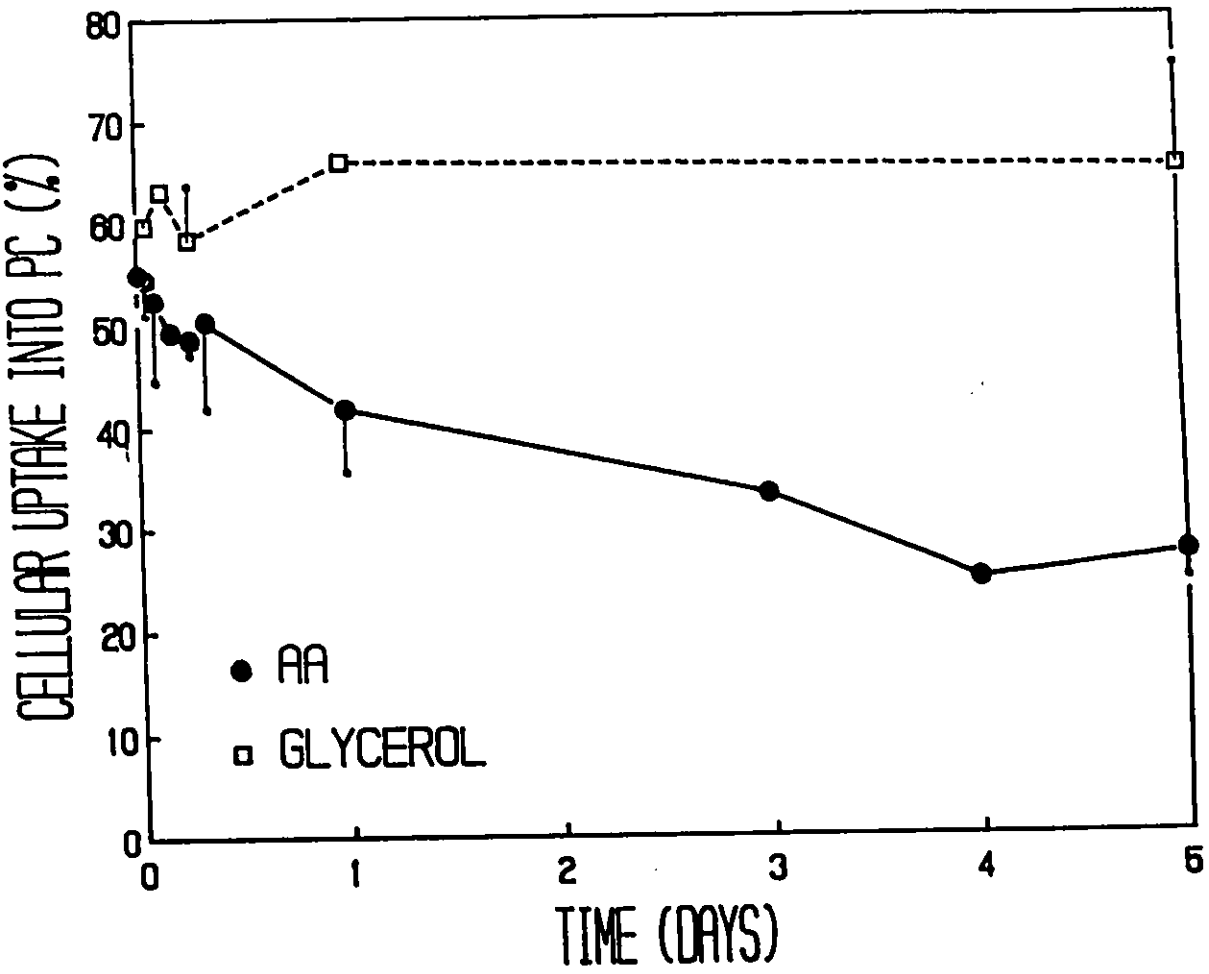

20
16
12
8
4
0
AA
GLYCEROL
CELLULAR UPTAKE INTO PS (%)
0
1
2
3
4
5
TIME (DAYS)

FIGURE 12: Incorporation of Radiolabelled AA and Glycerol into PC During Storage.

The total lipid extracts from the radiolabelled platelets subsampled at different stages of storage, were separated using HPLC. The PC peak (which was the fifth peak eluted from the column), was counted as described in Materials & Methods. The counts in the PC peaks as a % of the total recovered from the column were plotted as percent of cellular uptake in PC versus time of storage.
Each value plotted represents the mean ± 95% confidence interval of separate pools of PU. Very small error bars could not be visualized on the figure.

FIGURE 12


CELLULAR UPTAKE INTO PC (%)
80
70
60
50
40
30
20
10
0
0
1
2
3
4
5
TIME (DAYS)
AA
GLYCEROL

3.5.2. Effect of Long-term Storage of Platelets on Incorporation Pattern of Glycerol

The percentage of radiolabelled glycerol present in NL, PE, PI, PS, and PC did not significantly change ($p<0.025$) during the 5 day storage period (FIGURES 8-12, respectively).

3.6. Glycerophospholipid Content and Composition of Human Platelets

The GPL content of human platelets was determined by phosphate analysis as nmole/10^9 platelets and as mole% of PE, PI, PS and PC (TABLE 6). Since the HPLC-separated GPLs were identified using a radiodetector and because none of the radioactive labels were incorporated into SM, this last one was not collected, therefore it was not included in this study. TABLE 6 illustrates that human platelet GPLs are composed mostly of PC and PE, with a PC/PE mole ratio of 1.4. PS and PI are minor conponents of GPL. These results are found to be within the range reported in the literature (Marcus et al., 1969; Cohen and Derksen, 1969; Broekman et al., 1980; Prescott and Majerus, 1981; Skeaff and Holub, 1985). However it is very important to keep in mind that the actual percent of GPLs (in mole%) presented in this study, excludes SM which is found in significant amounts (>15%) in human platelets (Broekman et al., 1980).

3.7. Effect of Storage Media on Aracidonate Incorporation in Glycerophospholipids

To determine whether the incorporation pattern of AA into GPL of platelets stored in PCD is comparable to the literature data, a short term study was conducted. Platelet-rich plasma (6 mL) was placed in two tubes, one sample

TABLE 6: The GPL Content and Composition of Human Platelets

	GPL content of human platelets* (nmole/10^9 platelets)	(mole%)+
PE	79.0 ± 2.5	34.7 ± 1.1
PI	10.7 ± 3.3	4.7 ± 1.4
PS	26.9 ± 2.5	11.8 ± 1.1
PC	111.2 ± 8.3	48.8 ± 3.6

* Based on phosphate-determination by the modified method of Bartlett (Kates, 1986), as described in Materials & Methods.
The values are the mean ± SEM of separate pools of PU.

\+ SM was excluded for the % mole calculation

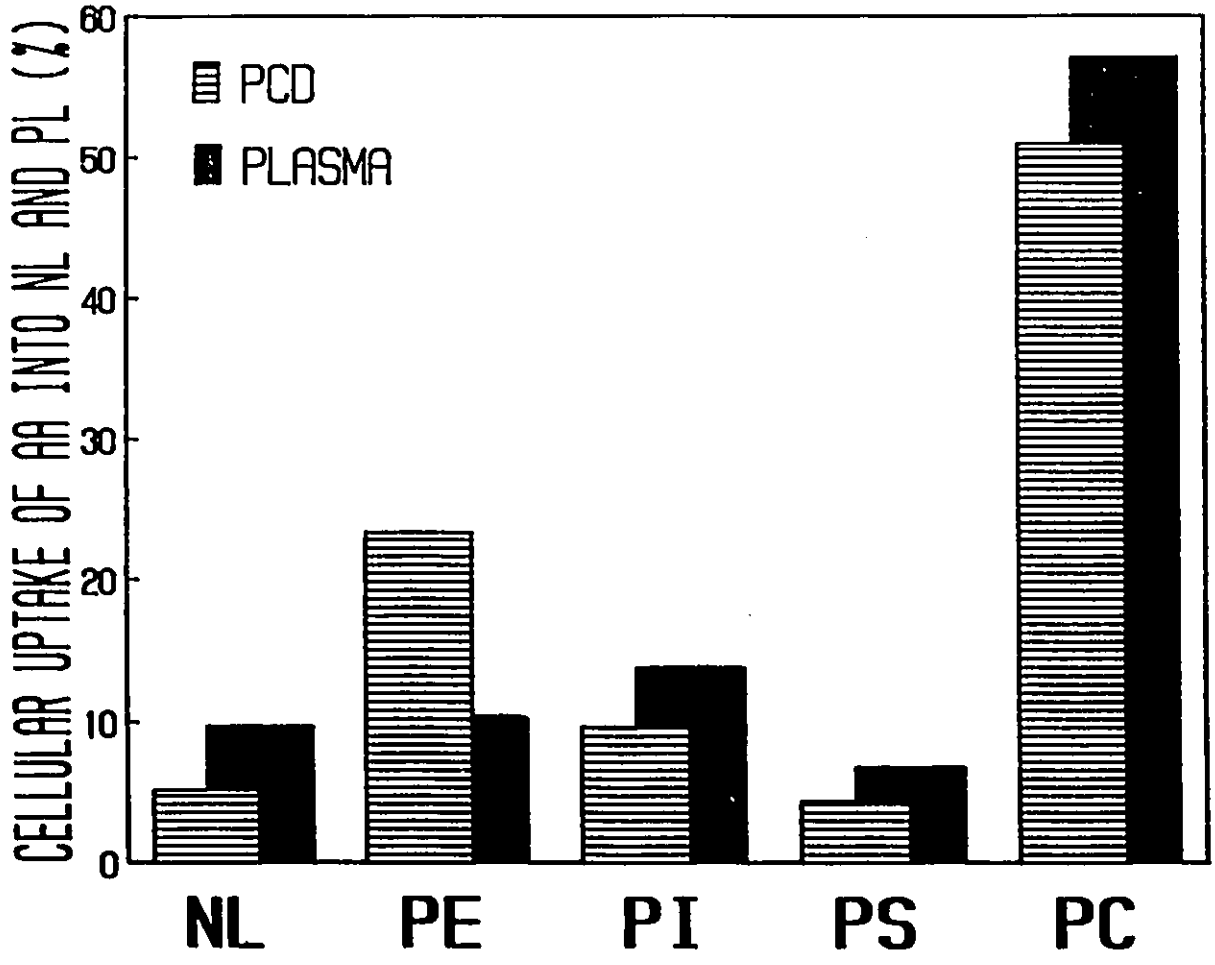
had most of the plasma replaced by PCD, whereas the other was kept intact. Both cell suspensions were incubated at 37^{o}C in a shaking water bath with 2 uCi of [^{14}C]AA for a period of 1.5 hour. After the incubation, the cells were washed, extracted and the lipids were separated on the HPLC as previously described in Materials and Methods. The amount of radiolabelled AA incorporated into GPLs obtained for both storage media was compared to the results of Schacky et al. (1985), who used the same labelling protocol.

The incorporation pattern of AA into the PCD stored platelets and plasma stored cells (FIGURE 13) was not significantly different ($p>0.025$) from the 1-2 hour data points of the AA incorporation into PL obtained from the storage experiments (FIGURES 8-12).

FIGURE 13: Distribution of [^{14}C]AA (% of total) into Platelet Lipid Classes Stored for 1.5 hour in Two Different Media: PCD and Autologous Plasma.

The platelets from the same unit were resuspended either in plasma or PCD. Radiolabelled AA was added to both suspensions. After an 1.5 hour incubation, the cells were washed, extracted and separated on the HPLC as described in Materials & Methods.

FIGURE 13


CELLULAR UPTAKE OF AA INTO NL AND PL (%)
60
50
40
30
20
10
0
PCD
PLASMA
NL
PE
PI
PS
PC

4. DISCUSSION

4.1. Uptake and Incorporation of Radioactive Arachidonate into Fresh Human Platelets

Platelets contain a FA synthetase system (Deykin and Dresser, 1968) and thus are able to make palmitate for subsequent acylation into GPLs (Majerus et al., 1969), but lack Δ^6-desaturase activity necessary for the synthesis of AA from linoleate (Needleman et al., 1982). Therefore, human platelets must take up preformed AA from an extracellular source. Long-chain FAs have been shown to diffuse readily in and out of platelet membrane (Spector et al., 1970). After AA diffusion inside the cell membrane, it is very rapidly esterified into GPLs (Wilson et al., 1982).

The maximum uptake of AA into platelets (75%) was reached after 10 minutes and no variation was recorded after 5 days incubation at 22°C in PCD, which contains 6 g/L albumin (FIGURE 3). These results are consistent with the findings of Neufeld and Majerus (1983) who measured 60-75% AA uptake independently of albumin concentration ranging from 25-200 μg/mL. Imai et al. (1982) demonstrated that the uptake of AA into platelets resuspended in plasma (which contain 40 g/L albumin, Scientific Tables, 1962), was only 20%. The low uptake value for AA, observed by Imai et al., (1982) is consistent with the conclusions of Hashimoto et al. (1985) concerning the fact that AA added to platelet suspensions containing albumin, binds partly to the albumin and the remainder enters the cells. If the suspension medium contains no albumin, most of the AA added is taken up by the platelets. Based on the observations of Neufeld and Majerus (1983), Imai et al. (1982), Hashimoto et al. (1985) and ours, we can conclude that the AA uptake in human platelets is inversely proportional to the albumin concentration of the external medium.

The radioactive AA taken up by platelets was mainly recovered in GPLs, however a small fraction of the tracer (2-5% of the total taken up) was also detected in the NLs (FIGURE 8). The AA measured in the NL fraction was mainly found esterified to TAG (45%, TABLE 4). Only 0.36% of the AA remained free, which is in agreement with the findings of Kramer and Deykin (1983). The remaining AA in the NLs was distributed among MAG, DAG, cholesteryl-AA and an unidentified compound (TABLE 4). On the basis of its R_f value, this unidentified compound might either be arachidonyl methyl ester, alkyl DAG or alk-enyl DAG, or a mixture of these (Kates, 1986). Further analysis is required to determine the identity of this component.

The high incorporation into GPLs (95-98%) of the AA taken up by platelets (FIGURE 9-12) is in agreement with the results reported by Imai et al. (1982) and Guichardant and Lagarde (1983). The initial rate of AA incorporation into the GPLs of fresh intact human platelets incubated in PCD (at 22°C) was 28 pmole of AA/minute/10^9 platelets. This rate of AA incorporation, which was determined as the initial slope of the curve of the amount of AA incorporated into all GPLs per 10^9 platelets as a function of time (FIGURE 14), was consistent with the value reported by Neufeld et al. (1983), for platelets incubated at 37°C. The rate of AA incorporation into GPLs, estimated in the present study, could be more accurately determined with a short-term kinetic study i.e. 1-2 minutes time intervals rather than 10 minutes as in FIGURE 14.

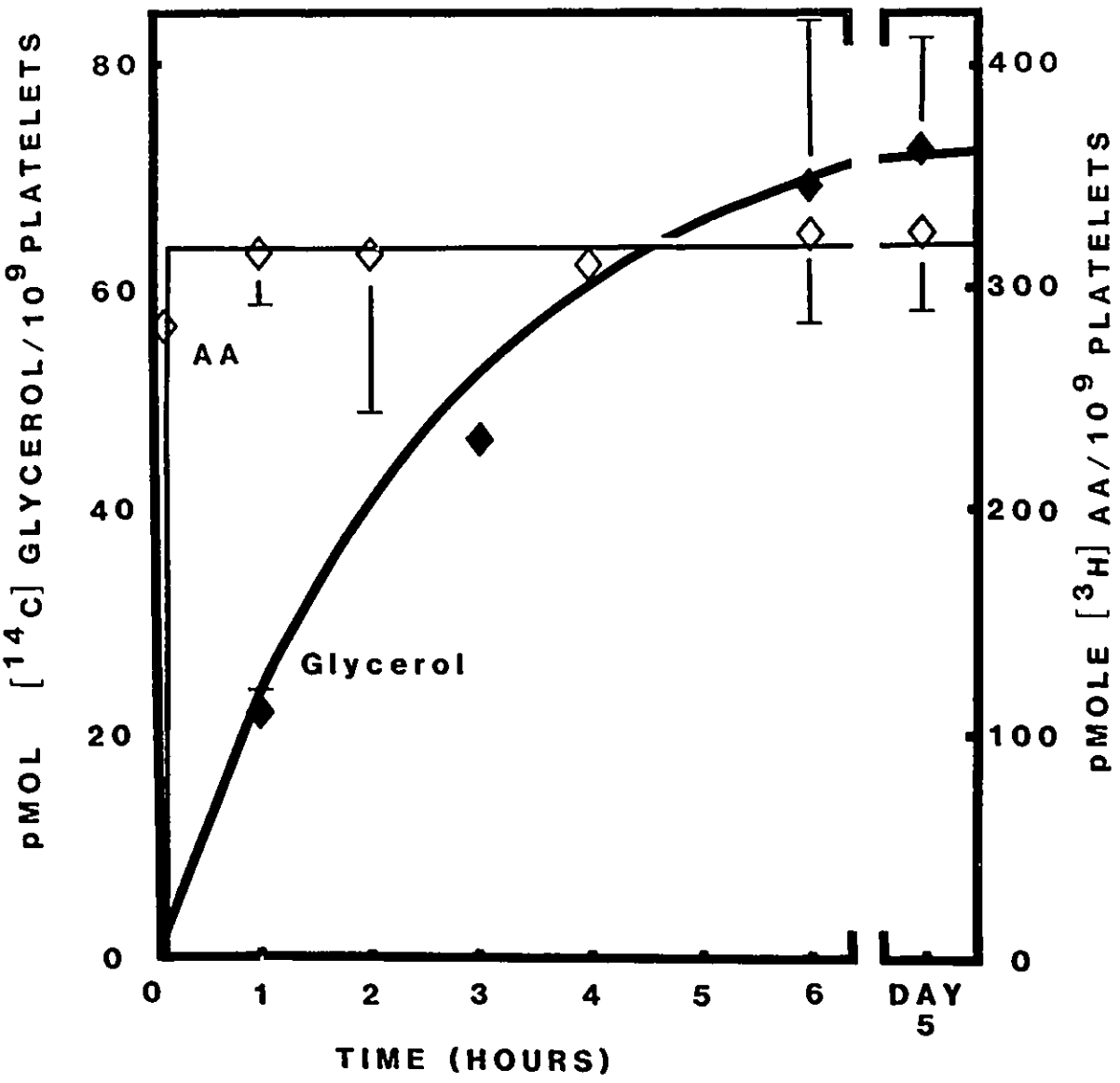
The distribution of AA among GPLs after a short-term incubation (1-2 hours, FIGURES 8-12), agreed with the findings of Marcus et al. (1969); Bills et al. (1976); Guichardant and Lagarde (1983); Kramer and Deykin (1983); Weaver and Holub (1985); Takamura et al. (1987), despite the fact that different conditions were used by each group, such as, cell and radiolabel concentrations,

FIGURE 14: Incorporation of Radiolabelled AA and Glycerol into Platelet GPLs as a Function of Time.

The pmoles of glycerol and AA incorporated into GPLs per 10^9 platelets was calculated from the sum of the total counts of [^{14}C]glycerol or [^{3}H]AA incorporated into PC, PE, PI,and PS and, plotted as a function of time.

Each value plotted represents the mean $\pm$ 95% confidence interval of separate pools of PU. Very small error bars could not be visualized on the figure.

FIGURE 14


pMOL [14C] GLYCEROL/10^9 PLATELETS
pMOLE [3H] AA/10^9 PLATELETS
AA
Glycerol
TIME (HOURS)
DAY 5
0
20
40
60
80
100
200
300
400
1
2
3
4
5
6

incubation time, temperature, and medium composition (plasma, phosphate buffer with or without albumin). All of these studies showed essentially that the PC and PE fractions account for 3/4 of the total radiolabelled AA-GPLs in platelets. All reports agreed that no radiolabelled AA is found in lyso GPLs, CL and SM (Bills et al., 1976; Imai et al., 1982; Kramer and Deykin, 1983; Guichardant and Lagarde, 1983; Weaver and Holub, 1985; Takamura et al., 1987). The absence of AA in lyso-GPLs could be due to the fact that no AA is present in the sn-1-position (Blackwell et al., 1978; Mueller et al., 1983; Chilton et al., 1987).

The 5-6 fold higher specific activity of PI compared to PC, PE and PS (FIGURE 15), suggest a much higher turnover rate for PI relative to that of PC, PE or PS. These results agree with the specific activity data reported for fresh platelets (Chambaz et al., 1979; Rittenhouse-Simmons and Deykin, 1981; Holub, 1984; Wilhelm Weiner and Sprecher, 1985; Baker and Chang, 1986).

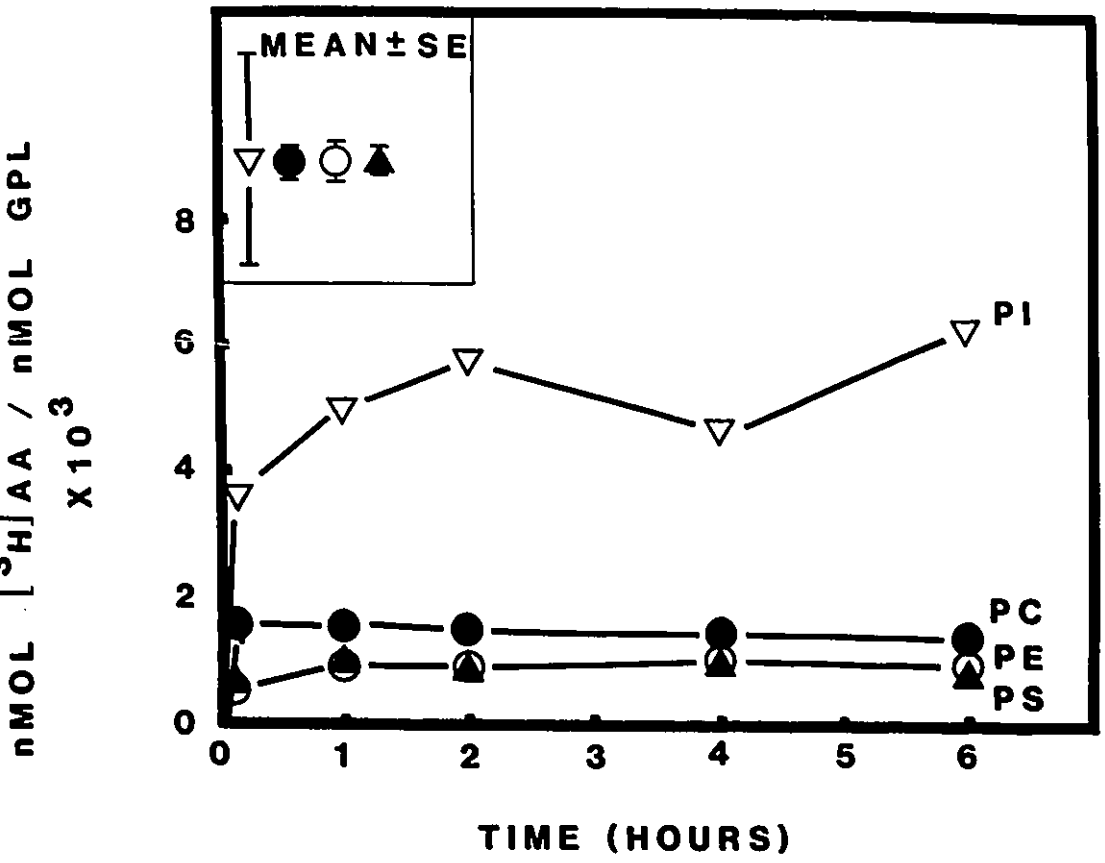
After its incorporation into human platelets, AA is esterified very rapidly into PI, and less quickly into PC, PE and PS, without AA oxygenation via the lipoxygenase or cyclooxygenase pathways (Rittenhouse-Simmons and Deykin, 1981). Fatty acids are known to be inserted into PLs by the de novo pathway or by the deacylation-reacylation pathway. Both of these pathways involve fatty acyl CoA transferase activity. Saturated and monounsaturated FA are known to be preferred for the de novo pathway (Okuma et al., 1973) whereas the polyunsaturated FAs are incorporated by the deacylation-reacylation pathway. It has been well documented that arachidonoyl-CoA is the prefered FA donor for the various 1-acyl-lyso PL acceptors (Lands and Crawford, 1976; Holub and Kuksis, 1978). McKean and co-workers (1982) demonstrated that human platelets contain a lysoPC acyltransferase with a preference for unsaturated acyl CoA, but no absolute specificity of AA-CoA. AA remodelling can also take place via two

FIGURE 15: Changes in Specific Activity of Radiolabelled AA Containing-GPLs with Time.

The nmole [^{3}H]AA/nmole GPL was calculated from the total counts of AA incorporated into each GPL over the respective amount of PC, PE, PI or PS (nmole), found in human platelets (TABLE 6). The specific activity of each GPL was plotted as a function of time.

Each value plotted represents the mean ± 95% confidence interval of separate pools of PU. Very small error bars could not be visualized on the figure.

FIGURE 15


MEAN ± SE
PI
PC
PE
PS
nMOL [3H]AA / nMOL GPL X 10^3
TIME (HOURS)
0
2
4
6
8
0
1
2
3
4
5
6

arachidonoyl-transacylases, one being CoA dependent and the other CoA independent (Trotter et al.,1982; Kameyama et al., 1983; Kramer et al., 1984).

The platelet GPLs PC, PE, PI and PS are reported to contain, respectively, 25%, 48%, 13% and 14% of the total cellular AA (Marcus et al., 1969; Cohen and Derksen, 1969; Broekman et al., 1976; Chap et al., 1982; Mahadevappa and Holub, 1983; Skeaff and Holub, 1985). Our results demonstrated that after 1-2 hours incubation, 54% of the AA tracer is incorporated into PC and 19% into PE (FIGURES 9 and 12), which does not reflect the normal AA pool sizes of these GPLs. This discrepancy between platelet endogenous-AA content in GPLs and radioactive AA incorporation within those GPLs, has been recognized for a long time (Cohen and Derksen, 1969; Bills et al., 1977; Schick et al., 1984; Wilhem Weiner and Sprecher, 1985; Aznar et al., 1985; Purdon et al., 1987). Therefore, the results obtained from the short-term incorporation of AA support the conclusions made by Schick et al. (1984) and Wilhelm Weiner and Sprecher (1985) about the fact that AA incorporated in vitro does not equilibrate initially with all the AA pools in GPLs.

4.2. Uptake and Incorporation of Radioactive Glycerol into Fresh Human Platelets

Glycerol uptake by platelets is a prerequisite for the de novo synthesis of GPLs. This study found a maximum uptake of glycerol of 20% into platelets incubated 6 hours in PCD at 22°C, with no subsequent variation recorded after 5 days of storage (FIGURE 4). These results do not agree with the study of Imai et al. (1982) who demonstrated that 40% of glycerol label was taken up into human platelets resuspended in plasma, after 1 hour incubation at 37°C. Two arguments could possibly explain the discrepancy between the results of the

present study and Imai et al. (1982):

a) The radiolabelled glycerol concentration of the external medium: Imai et al.(1982) incubated the platelets with 100 times higher glycerol concentration than used in the present study (3,000 nmol / $72x10^9$ versus 29.2 nmol / $72x10^9$ platelets). The high glycerol concentration used by Imai's group (1982), probably favours the initial diffusion of glycerol into the platelet cytosol.

b) The incubation temperature: The higher temperature (which was used by Imai et al., 1982), could possibly be considered as a factor favouring a higher rate of glycerol uptake.

This study demonstrated that 15% of the platelet-glycerol taken up, remained in the cytosol whereas the remainer (85%) was membrane-incorporated, 7% of which being incorporated into the NLs (FIGURE 8) and 78% into the GPLs (FIGURE 9-12). These finding are in agreement with those of Imai et al. (1982) and Lewis and Majerus (1969) who found 80% and 75%, respectively, of the total glycerol taken up was incorporated into GPLs. Of the radioactive glycerol in the NL fraction, 80% was accounted for by TAG (TABLE 4) which is consistent with the findings of Imai et al. (1982) who also reported that the NL fraction was mainly TAG. The remaining activity was distributed equally among glycerol and/or MAG, DAG and the unidentified component discussed above (section 4.1.).

Since no mechanism showing the exchange of glycerol backbone between GPLs has been demonstrated in blood cells, the incorporation of glycerol is assumed to reflect de novo synthesis. The rate of GPL de novo synthesis was determined and reported as the amount of glycerol incorporated per 10^9 platelets as a function of time (FIGURE 14). The rate of glycerol incorporation at 22°C in PCD was 0.28 pmol of glycerol / minute / 10^9 platelets. Neufeld and Majerus

(1983) demonstrated an initial rate range of 80-300 pmol / minute / 10^9 platelets, for cells incubated at 37°C. The difference in temperature between the study of Neufeld and Majerus (1983) and the present one (37°C versus 22°C) is suspected to be the main reason for the 300 fold slower GPLs de novo rate of incorporation reported here.

Based on the results generated from the incorporation of radioactive glycerol into GPLs, we can conclude that human platelets are capable of de novo synthesis of PI, PC and PE however, PS synthesis is very limited. It is not known if this limited PS synthesis is due to low enzymatic activity or simply due to the positional distribution of PS. These results are in agreement with the findings of Lewis and Majerus (1969) except for PA which was reported to be actively synthesized. However, more recently, it has been well documented that PA constitutes a minor component of resting platelet membranes (Holub, 1984). Imai et al. (1982) reported the presence of radiolabelled glycerol into lyso-GPLs of human platelets. This is in good agreement with the fact that the authors introduced 100 times more radiolabelled glycerol in the external medium than what was used in the present study and, being at 37°C, the cellular uptake was found to be 40% rather than 20% (reported here) resulting in higher GPL specific activities.

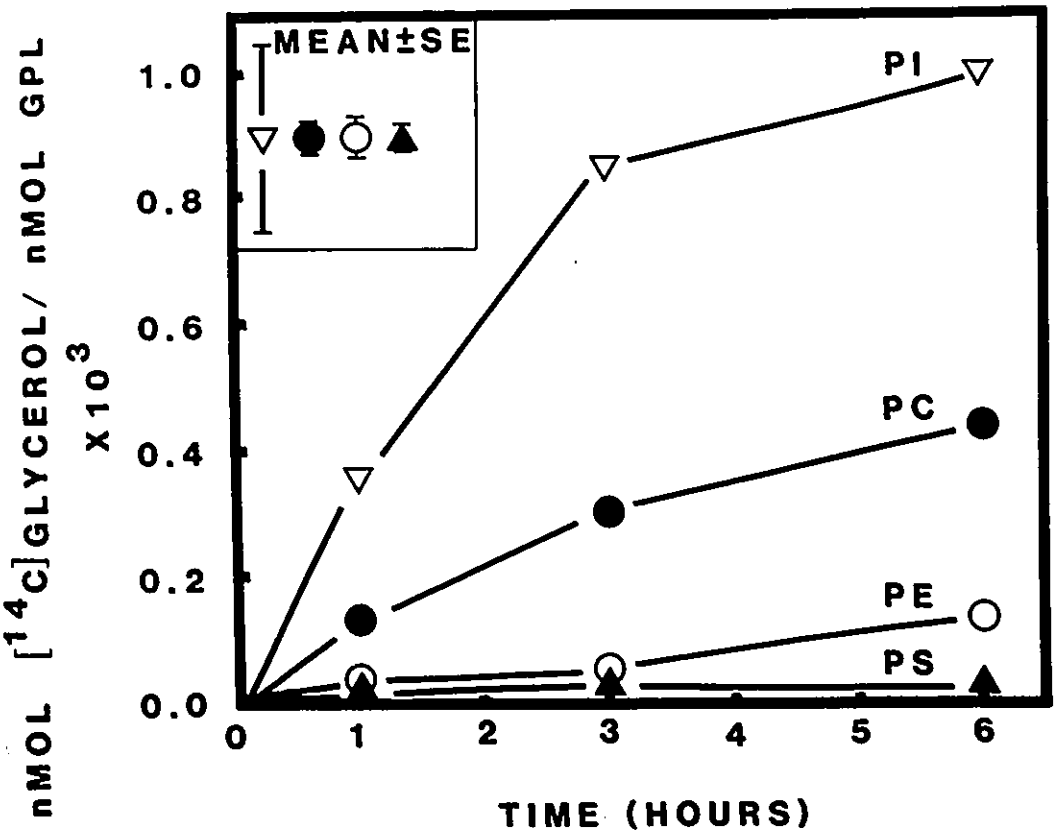
The specific activities of the GPLs presented in FIGURE 16, demonstrated that de novo synthesis of PI occurs 2.5 times faster than PC and 7 times faster than PE. As reported above, the specific activity of PS is almost negligable. These specific activity differences are probably due to the cumulative effects of the turnover rate and the positional distribution of each GPL. The results presented in this study are consistent with those reported in the literature (Lewis and Majerus, 1969; Imai et al., 1982).

FIGURE 16: Changes in Specific Activity of Radiolabelled Glycerol Containing-GPLs with Time.

The nmole [^{14}C]glycerol/nmole GPL was calculated from the total counts of glycerol incorporated into each GPL, over the respective amount of PC, PE, PI or PS (nmole), found in human platelets (TABLE 6). The specific activity of each GPL was plotted as a function of time.

Each value plotted represents the mean ± 95% confidence interval of separate pools of PU. Very small error bars could not be visualized on the figure.

FIGURE 16


MEAN±SE
PI
PC
PE
PS
1.0
0.8
0.6
0.4
0.2
0.0
0
1
2
3
4
5
6
nMOL [14C]GLYCEROL/ nMOL GPL X10^3
TIME (HOURS)

After its diffusion into the platelet cytosol, glycerol is phosphorylated to glycerol-3-phosphate which is the precursor of lyso PA, one of the first steps involved in the de novo synthesis of GPLs (FIGURE 1).

In summary, the present study of short-term uptake and incorporation of radiolabelled AA and glycerol in human platelets incubated in PCD at 22°C, demonstrated:

a) The AA and glycerol cellular uptake.

b) The incorporation of AA into GPLs.

c) The de novo synthesis of GPLs.

d) The de novo synthesis of GPLs was demonstrated to be 100 times slower than AA incorporation into GPLs.

The results generated from these studies, served as a basis for long-term PL metabolism studies during platelet storage, which were subsequently carried out.

4.3. Effects of Storage on Human Platelets

The validity of the results generated from this long-term storage is supported by the cellular viability measurements done throughout the storage period (Results, section 3.1.) that were in good agreement with the behavior of healthy 5 day stored human platelets.

PL 732 plastic bags, which were used to store platelets, have the property of being gas permeable. The significant decrease in radioactivity counts observed during storage would suggest that oxidation of AA to CO_2 took place, followed by subsequent liberation of the radioactive gas into the atmosphere. The present results demonstrated that the total AA radioactive counts added to the platelet suspension, did not significantly change throughout the 5 day storage period

(TABLE 2), therefore we can conclude that the AA was not oxidized to CO_2 during storage. Free FA oxidation has been shown to take place with palmitate and oleate (Spector et al., 1970; Donabedian and Nemerson, 1971) however, AA oxidation to CO_2 in platelet, has never been reported in the literature. The absence of this specific reaction could be rationalized by the fact that: 1) AA is very rapidly incorporated into GPLs (Neufeld et al., 1984). 2) The remodelling pathway of AA does not proceed via the free form of the acid (Trotter et al., 1982; Kameyama et al., 1983; Kramer et al., 1984b). 3) There is a very low level of endogenous free arachidonate in resting cells (Neufeld et al., 1983).

Vishnubhatla (1986) concluded that platelet phospholipid composition with respect to AA content, does not undergo change with storage in plasma up to 5 days. However, the use of labelled AA tracer in this study, permitted the observation of a time-dependent simultaneous reciprocal correlation of the AA-incorporation pattern between PC and PE (FIGURE 17a). This significant linear event suggests that AA migrates from PC into PE in resting platelets. This remodelling of AA was maintained at a slow-rate during the entire storage period. As discussed earlier (Results, section 4.1.), human platelet PC has been reported to contain 25% of the total cellular AA, whereas PE contains 48% of AA (Marcus et al., 1969; Cohen and Derksen, 1969; Broekman et al., 1976; Chap et al., 1982; Mahadevappa and Holub, 1983; Sheaff and Holub, 1985). After 1-2 hours of incubation, the AA tracer incorporation is 54% into PC and 19% into PE. However, after 5 days of storage, only 26% of the tracer was left in PC, whereas 42% was incorporated into PE (FIGURE 17a). The sum of the AA contents in PC and PE on day zero equalled the sum on day 5, suggesting that the AA was transfered from one GPL to the other. The distribution of AA radiolabel on day 5 was much more representative of the endogenous distribution of the AA in

FIGURE 17: Distribution of Radiolabelled AA and Glycerol into PE and PC During Storage.

The graphs represent: a) The incorporation of AA into PE and PC (taken from FIGURE 9 and FIGURE 12) and b) The incorporation of glycerol into PE and PC (taken from FIGURE 9 and FIGURE 12).

Each value represents the mean ± 95% confidence interval of separate pools of platelets. Very small error bars could not be visualized on those figures. The opened symbols represent PE whereas the closed one represent PC.

FIGURE 17a

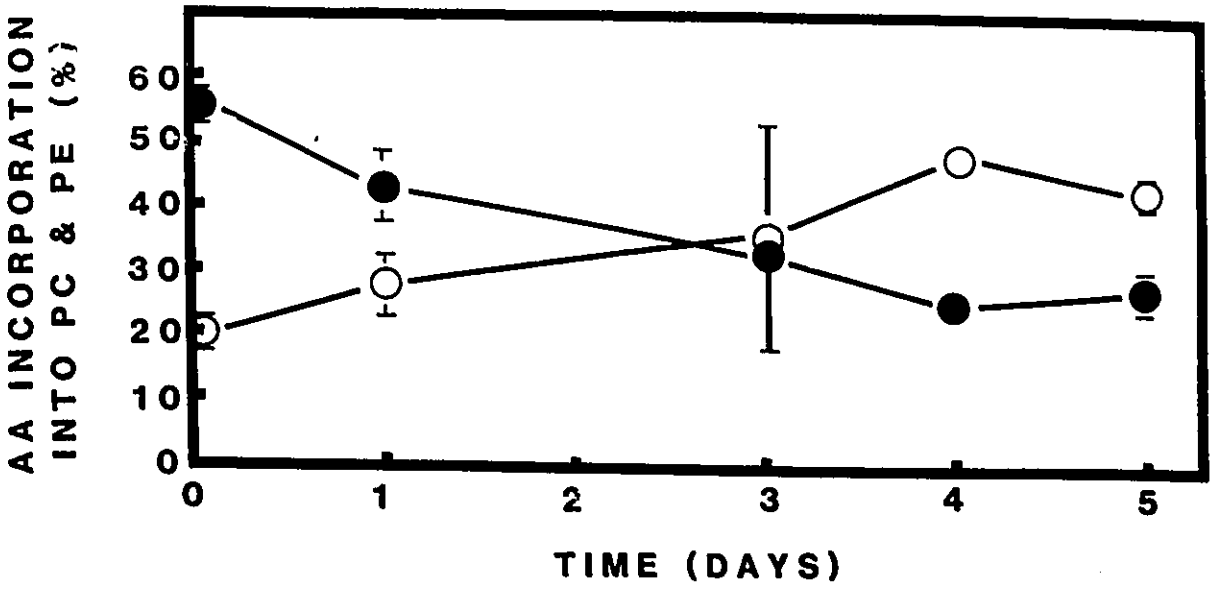
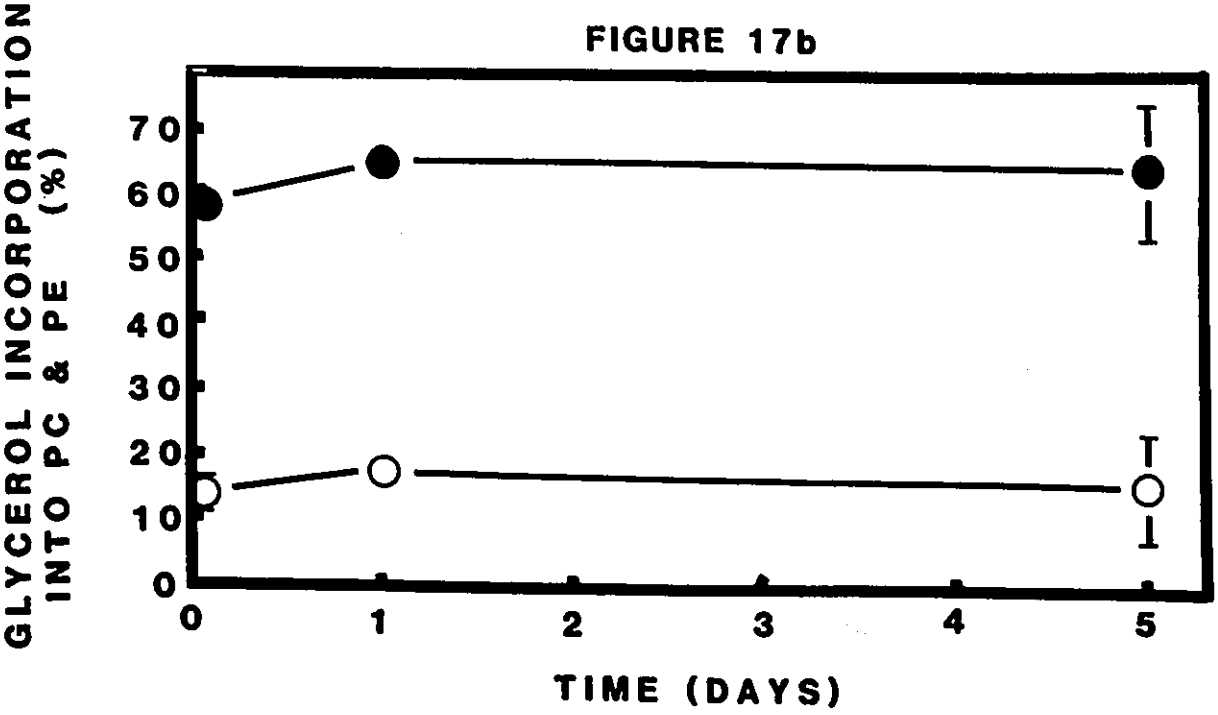


FIGURE 17b



GPLs, than the short-term incorporation results. These results of long-term distribution of AA, permit a conclusion that 5 days or more are required for equilibration of AA-endogenous GPL pools in human platelets stored at 22°C in PCD. While the data do not exclude transfer through an intermadiate lipid class, direct transfer of AA from PC to PE is the most likely mechanism. There are 4 possible mechanisms which could be involved in this remodelling pattern: 1) Free AA (released from PC by PLA_2) could be reincorporated into lyso-PE by acyl-CoA-dependent acyltransferase (Hill and Lands, 1970). 2) Alternatively, AA could be transfered by direct transacylation. Further evidence for the deacylation-reacylation pathway of AA from PC into PE was given by Kramer and Deykin (1983), who reported the presence of arachidonoyl transacylase activity in human platelets. That enzyme has been shown to transfer AA (CoA-independently) from PC prelabelled crude platelet membrane preparations to exogenous lyso plasmalogen PE. A similar pathway has been demonstrated in canine heart (Reddy and Schmid, 1985), in rabbit macrophages (Sugiura and Waku, 1985), and in rat macrophages as well (Robinson et al., 1985). 3) Howard et al. (1986) found in bovine endothelial cells, the presence of a CoA-dependent transacylase that preferentially transfers AA from diacyl PC to alkyl lyso-PE. 4) The polar head exchange of choline with ethanolamine. The AA labelling data did not distinguish which mechanism was responsible for the long-term AA migration. This remodelling of AA took place at a much slower rate than the initial incorporation of the tracer, since it was demonstrated that 10 minutes were required to initially label the GPLs (FIGURES 3, 9-12) whereas more than 5 days were required for equilibration with the AA pool of PE, PI, PS and PC (FIGURES 9-12). This long-term remodelling might simply be due to the positional distribution of each GPL in human platelets, which is poorly known.

In contrast to the AA incorporation data, there was no significant effect of storage on the glycerol incorporation pattern (FIGURE 17b). These results ruled out the possible mechanism for the AA migration from PC into PE involving the exchange of the polar head groups, suggesting that this remodelling may occur via the deacylation-reacylation pathway (CoA-dependent /CoA-independent) and/or via the acyltransferase pathway (as described above, FIGURE 1).

The long-term remodelling of AA from PC into PE, is in agreement with other studies demonstrating the same pathway in rat platelets (Colard et al., 1984), rat testes (Blank et al., 1985), rabbit alveolar macrophages (Sugiura et al., 1984) and also in bovine aortic endothelial cells (Howard et al., 1986).
Sugiura et al. (1984) concluded that the mobilization pathway is an AA-specific event in rabbit alveolar macrophages.

This long-term PL remodelling of AA is PLA_2-dependent since sufficient amounts of lyso PE must be available as AA-acceptor. Labow et al. (1986) demonstrated that PLA_2 activity is not affected by these storage conditions.

4.4. Limitations of the Study and Future Aspects of this Research

Wilhelm Weiner and Sprecher (1985) demonstrated that human platelets can chain elongate the arachidonyl-CoA and subsequently incorporate the 22-carbon acid into PLs. This 22-carbon acid, named docosatetraenoic acid, has been shown to label all the glycerol-containing lipids. The technique used in the present study does not allowed us to comment on this possible reaction. However, gas-liquid chromatography of the incorporated AA would probably clarify this point.

The storage conditions used in this study (see Methods) are essential for the maintenance of platelet integrity. The storage temperature ($22^{o}C$), is far below the physiological temperature, however, Colard et al. (1984) demonstrated

that rat platelets do not aggregate after 18 hour incubation at 37°C. It is probably beneficial to maintain the cells for 5 days at 22°C, since their metabolism is slowed down, details of the AA remodelling pathway could be studied.

Blood transfusion centers store their platelets in plasma, the use of PCD in this study was simply to provide a better controlled environment. The short-term study, carried out to evaluate the distribution of [^{14}C]AA into platelet lipids after storage in either PCD or plasma (FIGURE 13), demonstrated that the non-plasma medium used to store the cells plays a similar role as plasma since no significant differences were observed in the AA labelling pattern of PLs (FIGURE 13). However, it should be kept in mind that this short-term evaluation of storage media might not reflect possible changes over a long-term storage.

Cell differential tests on washed human platelets (Results, section 3.3.) revealed that slightly more than 0.05% of the cells extracted were WBCs, which is almost negligible as contaminant possibly distorting the AA-labelling pattern of PLs. This contamination factor could be ignored for the study of de novo synthesis since it has been demonstrated that mature WBCs from human blood are unable to synthesize PLs because they lack acetyl-CoA carboxylase, the first enzyme in this pathway (Majerus and Lastra, 1967).

The present study was not designed to identify the AA-labelling pattern of each GPL molecular class. However, short-term incubation (5 minutes to 2 hours, at 37°C) of different cell types (such as rat testes and platelets and, also human platelets) with AA, reported that PE and PC diacyl classes had the highest specific activity, whereas alkylacyl and plasmalogen forms of PE and PC were very poorly labelled (Colard et al., 1984; Blank et al., 1985; Purdon et al., 1987). The remodelling pathway reported here in human platelets could possibly explain

the high AA concentration present in plasmalogen PE, reported by Mueller et al. (1983) and Vishnubhatla (1986).

Colard and co-workers (1984) were the first to report the equilibrium labelling of rat resting platelets with AA. After a 30 minute incubation with AA, the platelets were washed and reincubated for 18 hours at 37°C. This long-term incubation was sufficient to show significant movement of AA from diacyl PC into alkylacyl PE and PC, and plasmalogen PE. The authors demonstrated that the transfer of label was linear up to 6 hours, then remained constant. Sugiura et al. (1984) showed the AA mobilization from diacyl PC to alkylacyl PC and plasmalogen PE in rabbit alveolar macrophages incubated for 2 hours at 37°C. Incubation of rat testes with AA for 24 hours at 37°C permitted Blank et al. (1985) to conclude that diacyl PC and PE serve as a source of AA which is ultimately transferred to ethanolamine plasmalogens. Howard et al. (1986) demonstrated that the transfer of AA from diacyl PC to both diacyl and plasmalogen PE, takes place in bovine endothelial cells after more than 8 hour incubation at 37°C. Finally Chilton et al. (1987), who only studied PC remodelling, demonstrated that a 2 hour incubation at 37°C of washed prelabelled-AA-human neutrophils is sufficient to show the movement of AA from diacyl PC to alkylacyl PC. Chilton et al. (1987) supported the studies of Sugiura and Waku (1985) who used rabbit macrophage microsomes as their model.

Based on the above literature review, it is known that in many cells, AA is first incorporated into the diacyl forms of PE and PC, then is transported into alkylacyl PC and PE, and also into plasmalogen PE. The present reported the movement of AA from PC into PE in human resting platelets. This pathway does not involve polar head exchange between choline and ethanolamine, but possibly involve arachidonoyl transacylase (CoA-dependent/CoA-independent) and/or the

transacylase activities. However, to elucidate the exact details of this poorly understood pathway existing in many cells, more work is required to study the kinetics of AA labelling of PE and PC molecular classes. The model used in this study is ideal for kinetic studies since the AA remodelling takes place at a very slow rate (5 days as opposed to a few hours in other systems).

In the area of platelet research, stimulation is still poorly understood. One of the enigmas is the source of AA liberated during stimulation, which is the precursor of the prostaglandins and thromboxane (Marcus, 1978; Holub, 1984). Platelet stimulation studies are often contradictory and very hard to interpret since different conditions are used. All the literature related to AA incorporation followed by stimulation of human platelets, implies short-term incorporation which does not reflect equilibrated labelling of AA for PE and PC-GPLs. Mahadevappa and Holub (1983) were aware of the situation since they cautioned against over-interpretation of results derived from prelabelling with AA, because it can lead to highly heterogeneous labelling of different pools. The validity of all the short-term AA incorporation studies done with stimulated platelets is questionable since any decrease of radioactive AA within a specific molecular class of PC or PE, after stimulation, could simply be the result of an accelerated remodelling pathway, rather than donation of AA for thromboxane and prostaglandin synthesis. The only way to study the AA release during stimulation is with an equilibrated-AA- labelled human platelet model (involving long-term labelling). Before analysis of complex stimulation pathways, it is crucial to know in much more detail, the AA remodelling sequential events in resting human platelets .

CONCLUSIONS

The kinetic studies of GPL metabolism in resting platelets stored for five days at 22°C, in a non-plasma medium demonstrated:

1) Equilibration of AA with all the GPL pools is a long-term process that involves the shuttle of AA from PC into PE. The present study is the first one to provide convincing evidence for the existence of that pathway in human platelets. The actual mechanism for this remodelling is unknown however, we demonstrated that it does not involve polar head exchange between choline and ethanolamine, suggesting that it could possibly be the result of arachidonoyl transacylase activities (CoA-dependent /CoA-independent) and/or the acyltransferase pathway.
2) The human platelet contains all the enzymes required for the de novo synthesis. However, the formation of PS from PE is very limited.
3) AA is rapidly taken up by human platelets where it is immediately incorporated into GPLs with a high specific activity for PI. AA is not B-oxidized to CO_2 and is not converted into prostaglandins or thromboxane, in the resting cells.
4) The initial rate of AA incorporation (at 22°C) in human platelets is 100 times faster than the de novo synthesis.

In summary, the present study allowed us to discover a new pathway for the AA remodelling in GPLs, that until now was unknown in human platelets. This long term shuttle of AA from PC into PE constitutes an excellent explanation for the high AA content of plasmalogen PE. Although, we showed PL remodelling during storage, it could not be correlated with any decreases of agonist-platelet-response that occurs with storage time.

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APPENDICES

A) RATE OF RADIOLABELLED AA UPTAKE INTO HUMAN PLATELETS (FIGURE 3)

TIME (minutes)	CELLULAR UPTAKE OF AA (%) (mean ± 95% confidence interval)
0.5	12.4 ± 12.0
1.5	21.8 ± 1.2
2.5	29.7 ± 3.3
3.5	39.3 ± 3.7
4.5	45.0 ± 1.4
5.5	52.2 ± 4.9
6.5	59.6 ± 7.8
7.5	62.3 ± 8.5
8.5	68.9 ± 2.1
9.5	70.6 ± 10.2
TIME (days)	
5	77.4 ± 4.5

n=2

B) RATE OF RADIOLABELLED GLYCEROL UPTAKE INTO HUMAN PLATELETS (FIGURE 4)

TIME (hours)	CELLULAR UPTAKE OF GLYCEROL (%) (mean ± 95% confidence interval)
1	6.9 ± 4.7
2	11.5 ± 1.4
3	14.8 ± 1.8
4	20.0 ± 1.5
5	20.5 ± 5.1
6	22.3 ± 5.4
TIME (days)	
5	21.3 ± 5.4

n=2

C) INCORPORATION OF RADIOLABELLED AA AND GLYCEROL INTO NLs DURING STORAGE (FIGURE 8)

TIME	AA INCORPORATION INTO NLs (%) (mean ± 95% confidence interval)
10 minutes	8.5*
1 hour	1.8 ± 4.2
2 hours	1.8 ± 2.7
4 hours	3.0*
6 hours	0.7 ± 2.2
8 hours	1.8 ± 2.1
1 day	1.9 ± 1.7
3 days	1.2 ± 2.1
4 days	4.1*
5 days	0.3 ± 0.1

TIME	GLYCEROL INCORPORATION INTO NLs (%) (mean ± 95% confidence interval)
1 hour	7.9 ± 1.8
3 hours	6.6*
6 hours	11.0 ± 2.9
1 day	6.9*
5 days	3.3 ± 1.9

n=3
* n=1

D) INCORPORATION OF RADIOLABELLED AA AND GLYCEROL INTO PE DURING STORAGE (FIGURE 9).

TIME	AA INCORPORATION INTO PE (%) (mean ± 95% confidence interval)
10 minutes	14.2*
1 hour	19.2 ± 3.0
2 hours	19.0 ± 13.8
4 hours	23.3*
6 hours	22.5 ± 3.5
8 hours	22.4 ± 8.8
1 day	27.5 ± 4.7
3 days	36.1 ± 17.6
4 days	47.1*
5 days	42.4 ± 1.8

TIME	GLYCEROL INCORPORATION INTO PE (%) (mean ± 95% confidence interval)
1 hour	13.9 ± 0.1
3 hours	11.3*
6 hours	14.3 ± 6.1
1 day	18.1*
5 days	16.9 ± 7.4

n=3
* n=1

E) INCORPORATION OF RADIOLABELLED AA AND GLYCEROL INTO PI DURING STORAGE (FIGURE 10).

TIME	AA INCORPORATION INTO PI (%) (mean ± 95% confidence interval)
10 minutes	11.7*
1 hour	16.3 ± 5.0
2 hours	18.6 ± 15.7
4 hours	14.9*
6 hours	22.1 ± 10.9
8 hours	17.0 ± 4.3
1 day	14.8 ± 6.3
3 days	16.0 ± 1.3
4 days	14.5*
5 days	19.1 ± 8.1

TIME	GLYCEROL INCORPORATION INTO PI (%) (mean ± 95% confidence interval)
1 hour	16.0 ± 1.3
3 hours	17.9*
6 hours	13.9 ± 5.8
1 day	11.5*
5 days	11.6 ± 2.8

n=3
* n=1

F) INCORPORATION OF RADIOLABELLED AA AND GLYCEROL INTO PS DURING STORAGE (FIGURE 11).

TIME	AA INCORPORATION INTO PS (%) (mean ± 95% confidence interval)
10 minutes	5.7*
1 hour	7.3 ± 2.1
2 hours	6.7 ± 2.4
4 hours	7.3*
6 hours	5.6 ± 8.4
8 hours	7.8 ± 1.0
1 day	8.4 ± 3.2
3 days	10.5 ± 7.2
4 days	8.0*
5 days	10.4 ± 7.4

TIME	GLYCEROL INCORPORATION INTO PS (%) (mean ± 95% confidence interval)
1 hour	1.2 ± 0.9
3 hours	1.0*
6 hours	0.7 ± 0.5
1 day	0.6*
5 days	1.0 ± 1.1

n=3
* n=1

G) INCORPORATION OF RADIOLABELLED AA AND GLYCEROL INTO PC DURING STORAGE (FIGURE 12)

TIME	AA INCORPORATION INTO PC (%) (mean ± 95% confidence interval)
10 minutes	54.9*
1 hour	54.4 ± 3.3
2 hours	52.3 ± 7.7
4 hours	49.3*
6 hours	48.5 ± 1.4
8 hours	50.3 ± 8.4
1 day	41.7 ± 6.2
3 days	33.1 ± 0.4
4 days	24.8*
5 days	27.2 ± 2.6

TIME	GLYCEROL INCORPORATION INTO PC (%) (mean ± 95% confidence interval)
1 hour	59.8 ± 0.2
3 hours	63.2*
6 hours	58.3 ± 5.3
1 day	66.0*
5 days	65.0 ± 10.0

n=3
* n=1

H) INCORPORATION OF RADIOLABELLED AA AND GLYCEROL INTO PLATELET GPLs AS A FUNCTION OF TIME (FIGURE 14)

TIME	AA INCORPORATION INTO GPLs (pmol [^{3}H]AA/10^9 platelets)
10 minutes	282.5*
1 hour	316.6 ± 22.3
2 hours	316.0 ± 72.0
4 hours	309.5*
6 hours	324.5 ± 46.0
5 days	325.0 ± 36.0

TIME	GLYCEROL INCORPORATION INTO GPLs (pmol [^{14}C]glycerol/10^9 platelets)
1 hour	21.7 ± 2.2
3 hours	46.2*
6 hours	69.2 ± 17.0
5 days	72.6 ± 9.8

n=3
* n=1

I) CHANGES IN SPECIFIC ACTIVITY OF RADIOLABELLED AA CONTAINING-GPLs WITH TIME (FIGURE 15).

TIME	SPECIFIC ACTIVITY OF GPLs (mean ± 95% confidence interval) x 10^3
	(nmol [^{3}H]AA/nmol PI)
10 minutes	3.6*
1 hour	5.0 ± 0.5
2 hours	5.7 ± 3.8
4 hours	4.5*
6 hours	6.7 ± 2.3
	(nmol [^{3}H]AA/nmol PC)
10 minutes	1.6*
1 hour	1.6 ± 0.1
2 hours	1.5 ± 0.2
4 hours	1.4*
6 hours	1.4 ± 0.04
	(nmol [^{3}H]AA/nmol PE)
10 minutes	0.6*
1 hour	0.8 ± 0.1
2 hours	0.8 ± 0.6
4 hours	1.0*
6 hours	0.9 ± 0.07
	(nmol [^{3}H]AA/nmol PS)
10 minutes	0.7*
1 hour	0.9 ± 0.3
2 hours	0.8 ± 0.3
4 hours	0.9*
6 hours	0.7 ± 0.1

n=3
* n=1

J) CHANGES IN SPECIFIC ACTIVITY OF RADIOLABELLED GLYCEROL CONTAINING-GPLs WITH TIME (FIGURE 16).

TIME (hours)	SPECIFIC ACTIVITY OF GPLs (mean ± 95% confidence interval) x 10^3
	(nmol [^{14}C]glycerol/nmol PI)
1	0.36 ± 0.03
3	0.85*
6	1.00 ± 0.4
	(nmol [^{14}C]glycerol/nmol PC)
1	0.13 ± 0.01
3	0.30*
6	0.40 ± 0.04
	(nmol [^{14}C]glycerol/nmol PE)
1	0.04 ± 0.01
3	0.06*
6	0.14 ± 0.06
	(nmol [^{14}C]glycerol/nmol PS)
1	0.01 ± 0.01
3	0.02*
6	0.02 ± 0.01

n=3
* n=1

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