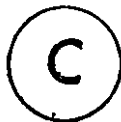


METABOLISM OF PHOSPHOLIPIDS IN PLATELETS IN RESPONSE TO STIMULI

By



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ABSTRACT

Platelet aggregation plays an important role in both the formation of a hemostatic plug and in the generation of a thrombus. The mechanism of platelet aggregation has not been fully elucidated. It is conceivable that changes in the synthesis, breakdown or rate of turnover of some of the constituents of the platelet membrane may be important in the mechanisms of aggregation. Recent experimental evidence in other mammalian tissues has implicated the closed circle conversion between MPI and PA as part of the membrane receptor-effector interaction process, and a possible role of TPI-DPI interconversion in the regulation of membrane permeability toward Na^+ and K^+ . Platelets have been recognized to be a useful model for these studies because of its abundance in phosphoinositides and easiness of obtaining pure platelet suspensions. Previous studies showed that addition of ADP to ^{32}P -labeled washed platelets caused an increase in radioactivity in PA in 2 sec, DPI in 30 sec and MPI in 2-3 min. TPI-DPI interconversion was suspected but consistent changes in ^{32}P -radioactivity in TPI was not detected. Similar changes were observed in platelets in response to thrombin stimulation. In none of the studies in other tissues all the metabolic pathways for inositol lipids were studied simultaneously. The aims of the present experiments were to investigate TPI-DPI interconversion and MPI metabolism during platelet aggregation and the release reaction caused by ADP, ionophore A23,187 and thrombin. Since these stimuli are considered to act on platelets by different mechanisms, their effects on the platelet phosphoinositide metabolism were compared. Furthermore, experiments were carried out with unstimulated platelets

labeled with $^{32}\text{PO}_4$ to find out if there were differences in the patterns of $^{32}\text{PO}_4$ incorporation into the major platelet phospholipids (PC, PE, PS) under in vitro and in vivo conditions, as claimed by former investigators.

Using improved methods for separation of phosphoinositides, it was found that the pattern of ^{32}P -incorporation into the major phospholipids in platelet suspension was similar to that of in vivo platelets when the incubation was carried out for many hours. Therefore, the phosphate moiety in all phospholipids turn over; but the phosphate in the major phospholipids turn over much more slowly than that in the phosphoinositides.

During ADP-induced platelet aggregation, hydrolysis of TPI to DPI was measurable at 60 sec, with a loss of $^{32}\text{PO}_4$, ^{14}C -arachidonic acid and ^3H -inositol from TPI. This hydrolysis was abolished by AMP, an inhibitor of ADP-induced aggregation. Resynthesis of TPI occurred during platelet deaggregation, with the $^{32}\text{PO}_4$ radioactivity of this compound being restored to the control level. With A23,187-induced aggregation and release, hydrolysis of TPI did not occur. While there was a significant amount of MPI converted into PA, the majority of MPI appeared to be hydrolysed to fatty acid and lyso MPI. Using platelets labelled with ^{14}C -arachidonic acid, ^3H -glycerol, ^3H -inositol, $^{32}\text{PO}_4$ and phosphorus assay, it was found that thrombin caused a decrease in TPI ($-5.64 \pm 1.55\%$, $P < 0.005$) as early as 9 sec when platelet shape change was maximal, ^{32}P -content was also decreased. Resynthesis of TPI was measurable at 60 sec. Most of the MPI was metabolised via the 1,2-diacylglycerol pathway. The amount of PA was increased. A small amount of the MPI was converted to polyphosphoinositides or lyso MPI and free fatty acid.

These experiments have shown a close relationship between changes in phosphoinositide metabolism and platelet aggregation and the release reaction.

These principal changes are: (1) increased TPI - DPI interconversion; (2) increased conversion of MPI to 1,2-diacylglycerol PA and MPI; (3) with the release reaction conversion of MPI to lyso ME and free fatty acids.

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LIST OF ABBREVIATIONS

PA	phosphatidic acid
MPI	monophosphoinositide
DPI	diphosphoinositide
TPI	triphosphoinositide
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PS	phosphatidylserine
DG	diacylglycerol
TG	triacylglycerol
PGE ₁	prostaglandin E ₁

CHAPTER ONE

INTRODUCTION

PART I. Platelets - General Review

Platelets are discoid annucleated cell-like entities in the blood circulation. They are formed from the megakaryocytes in the bone marrow (Behnke, 1969). The use of ^{32}P -diisopropylfluorophosphate (Leeksa and Cohen, 1956; Mustard et al., 1966) and $^{51}\text{CrO}_4$ technique (Gardner, 1966) has shown that human platelets have a life span of about ten days and a half life approximately 3.7 days. The normal turnover rate of platelets is about 3.5×10^7 /ml/day in man (Harker and Finch, 1969).

The normal platelets are about 2 μM in diameter. The most striking feature of platelet structure as revealed by electromicrograph is the presence of electron dense storage granules and the less dense lysosomal granules. The dense granules mainly contain 5-hydroxytryptamine (Pletscher and DaPrada, 1968), adenine nucleotides ADP and ATP (Pletscher et al., 1969), pyrophosphate (Silcox et al., 1973), calcium and magnesium (Berneis et al., 1969). Platelet antiplasmin made available during the release reaction has also been considered as a component of these granules (Joist et al., 1976). Lysosomal granules contain potassium (Lages et al., 1975), chemotactic factor (Weksler and Coupal, 1973), cationic protein (Nachman et al., 1970), fibrinogen (Day et al., 1975) and many hydrolytic enzymes including β -galactosidase, β -glucuronidase, α -arabinosidase, N-acetyl- β -D-glucosaminidase and acid phosphatase (Holmsen et al., 1969; Day and Holmsen, 1969).

In addition, platelets contain mitochondria which enable platelets to carry out aerobic respiration. Electron micrographs have revealed a surface-connected canalicular system possibly derived from the in-folding of megakaryocyte membranes. There is also a dense tubular system of unknown origin that could possibly be the site for reversible calcium storage in similarity to the sarcoplasmic reticulum in muscle cells.

Platelets play a major role in hemostasis and thrombosis. The subject has been extensively reviewed by a number of authors (Marcus and Zucker, 1965; Mustard and Packham, 1970; Brinkhous et al., 1971; Holmsen, 1972; Mustard, 1976). Platelets are actively involved in almost all responses of blood to injury (Mustard and Packham, 1970, 1971). For instance, they respond to the injury of endothelium of blood vessels (Jørgensen, 1971; Moore, 1974; Mustard and Packham, 1975), and to immunological and inflammatory reactions (Pfeuller and Luscher, 1972; Zucker, 1974). They also interact with various artificial surfaces (Salzman, 1971 a,b,c; Berger and Salzman, 1974; Nossel, 1975) and phagocytose foreign particles in the blood circulation (Mustard and Packham, 1968).

Platelets exhibit a wide variety of cellular reactions. This versatility, combined with the ready accessibility of platelets as a homogeneous population of intact cells, makes them one of the most investigated tissues for a large spectrum of cellular functions in the last ten years.

1. Membrane Properties of Platelets

Shape change, aggregation or the release reaction of platelets in response to aggregating stimuli manifest the dynamic interactions between stimuli and the membrane surface components. An understanding of the mechanisms of these functional changes is dependent on a thorough knowledge of the organization of the platelet plasma membrane and its associated structures. Although this has not been feasible at the present time, many reported platelet reactions toward stimuli can be appreciated in the light of the Fluid Mosaic Model of cell membrane proposed by Singer and Nicolson (1972) a few years ago.

In essence, the model states that the matrix of cell membranes is mainly composed of phospholipid and cholesterol. The lipid molecules are organized into a fluid bilayer configuration, with their polar head groups facing the aqueous phase, and the fatty acyl chains sequestered from the aqueous phase and stabilized by an apolar environment. The fluidity of the membrane depends on the presence of cholesterol and temperature which influences the packing and the stiffness of the fatty acid residues. Depending on the temperature, lipid molecules of the same species may flock together to form islands (crystalline phase) which trap or exclude protein molecules.

Protein molecules are mainly globular. They may be associated with the lipid bilayer loosely as soluble particles or they may span the matrix to form integral proteins that cannot be readily separated from the membrane, yet are accessible to chemical and enzymatic

modification at either membrane surface (Bretscher, 1971 a,b,c; Morrison et al., 1974; Cabantchik et al., 1975). These protein molecules are structurally amphipathic or asymmetric with regards to their hydrophilic and hydrophobic portions of their structure (Segrest et al., 1973; Strittmatter et al., 1972).

There are carbohydrate residues complexed with some of the membrane protein or lipid molecules. Such residues are located only on the outer membrane surface. The feature of this model is that the membrane components are capable of lateral mobility. Lipid molecules inter-mix freely in the horizontal plane but they flip-flop very slowly between the bilayers. This is important in the preservation of asymmetry in the membrane. Chemical labelling techniques (Bretscher, 1972) and enzymatic hydrolysis of phospholipids (Verkleij et al., 1973; Waah et al., 1973) have demonstrated that in the human erythrocyte membrane, phosphatidylethanolamine and phosphatidylserine are predominant in the inner half of the bilayer while sphingomyelin and phosphatidylcholine in the outer half.

Protein molecules also undergo lateral movement which is restrained by several mechanisms. Thus integral proteins do not tumble from one side of the membrane to the other at an appreciable rate. Furthermore, the display of surface receptors in some cells is non-random (Nicolson and Yanagimachi, 1974). Platelet membranes consist of 80% phospholipids and 20% cholesterol of the total lipids (Marcus et al., 1969). They are formed from the membrane fragments of

megakaryocytes. The organization of platelet membrane components is therefore likely similar to that of other mammalian cells. Using a chemical probe trinitrobenzene sulfonate, Shick and associates (1976) found that most of the platelet phosphatidylethanolamine and all phosphatidylserine are located in the inner leaflet of the bilayer. In addition, the glycoproteins on platelet surface are rich in sialic acid which may play a role in the survival of platelets in the circulation (Greenberg et al., 1976).

2. Membrane restraints on lateral mobility in platelets and in other cells

(i) Planar association of membrane components - glycoproteins

The fluid mosaic model suggests that grouping of membrane proteins at specific locations to form cell junctions, or the interwinding of the carbohydrate residues between glycoproteins or glycolipid molecules, can impede lateral mobility of membrane components. The latter could be of great importance in platelet function.

There have been approximately twenty types of proteins found in platelet membrane (Nurden and Caen, 1976; Jamieson and Smith, 1976; Mills and Macfarlane, 1976). The presence of surface glycocalyx has been demonstrated with special staining techniques such as ruthenium red. The three major types of glycoproteins separated by polyacrylamide gel electrophoresis have been classified as type I, II and III (Nachman and Ferris, 1972; Phillips, 1972). Type II glycoprotein was further

resolved into two components designed as IIa and IIb (Jenkins et al.; 1976). However, none of the glycoprotein has been identified as receptor for any specific stimulus of platelets.

Owing to the large number of side chains and combinations among monosaccharides (about ten of them are common in membrane structures), glycoproteins confer antigenicity to cells. For example, glycophorin in red cell membrane bears receptors for the blood groups MN, ABO and influenza viruses (Marchesi and Andrews, 1971), or component III bears receptors for concanavalin A (Findlay, 1974). These structures have been implicated in cell-cell recognition and adhesion. Furthermore, glycoprotein aggregating factors have been isolated from the suspending medium of retinal cells and cerebral lobe cells (Balsamo and Lilien, 1975). The specificity of the terminal sugar in the carbohydrate chain is critical for this cellular antigenicity since SV 40 transformed 3T3 cells adhere to galactose derivatised sephadex beads and form nuclei. These cells will not respond to sephadex beads coated with glucose or N-acetyl-D-glucosamine (Chipowsky et al., 1973). The enzyme-substrate complex formation during the transfer of a galactose unit from platelet membrane surface components to the incomplete galactosyl hydroxylysine groups of collagen by the galactosyl transferase has been proposed as a mechanism for platelet adhesion to collagen (Jamieson, 1974). The enzyme was claimed to be present on the membrane surface (Jamieson et al., 1971). However, this hypothesis has been challenged by the findings that platelets

adhere to collagen lacking galactosyllysine groups (Jaffe and Deykin, 1974), and also to the collagen treated with periodate (Puett et al., 1973). Cazenave et al. (1975) reported that UDP-galactose is not an efficient inhibitor of platelet-collagen adhesion. A possible role of glycoproteins in platelet membrane comes from the study of platelets in patients with thrombasthenia (Glanzmann's disease), von Willebrand's disease, and Bernard-Soulier syndrome (Tschopp et al., 1976; Nurden and Caen, 1976). Platelets from patients with the Bernard-Soulier syndrome which have reduced content of glycoprotein I (Nurden and Caen, 1975) have been demonstrated to adhere less effectively to subendothelium (Weiss et al., 1974). By contrast, platelets from thrombasthenic patients which lack glycoprotein II (Nurden and Caen, 1974) show normal interaction with subendothelium; i.e. normal adhesion, although there is no subsequent aggregation of these platelets.

(ii) Protein-lipid membrane domains

Integral protein molecules may be associated with each other via their hydrophobic portions embedded in the hydrophobic region of the lipid bilayer, thus hindering their own movement. Cell membrane contains a considerable amount of cholesterol (20-40%). The presence of this lipid, divalent cations such as calcium and long chain fatty acid residues determine the fluidity of cell membrane and the formation of a mixture of fluid and solid domains at physiological

temperatures (Bretscher, 1973). It has been found that high concentration of cholesterol and sphingolipids which have long chain acyl residues and low content of polyunsaturated fatty acids render myelin membrane more rigid and ordered than synaptosomal plasma membrane or erythrocyte membranes (Boggs and Moscarello, 1978). In the fluid phase cholesterol reduces lateral molecular separation and bilayer fluidity, while in solid phase cholesterol increases lateral molecular spacing thereby making the bilayer more fluid (Demel et al., 1967; Marsh, 1974; Marsh and Smith, 1973). Temperature influences the stiffness of acyl residues in the bilayer. This modification in lipid phase fluidity could determine more favorable or less favorable protein-lipid interactions which in turn would result in selective sequestration or exclusion of proteins to specific domains. If proteins were sequestered into solid domains, their lateral mobility would be limited to that of the domain. And if excluded from extensive solid domains, their display would be confined to those areas of fluid lipid (Nicolson, 1976).


In addition to the above long range interaction between lipids and proteins, there is direct interaction between these two compounds contributing to the restraint of membrane mobility. Electrostatic interaction between the basic protein and the charged head groups of lipid molecules has been demonstrated in myelin membrane (Boggs and Moscarello, 1978). This interaction leads to some portion of the peripheral proteins penetrating into the hydrocarbon

region of the bilayer, thus disrupting the lipid packing. With lipid vesicles, this type of interaction causes an increase in vesicle permeability to glucose (Gould, 1972) and sodium (Papanadjopoulos et al., 1975), as well as a decrease in lipid phase-transition temperature.

On the other hand, hydrophobic proteins that span across the membrane are ^{non-}covalently attached to the fatty acyl residues in the hydrophobic region, thus immobilizing a layer of lipid molecules. This interaction excludes cholesterol from its boundary layer possibly because the steroid is too rigid to conform to the irregular surface of the protein molecule (Boggs and Moscarello, 1978). Hydrophobic proteins seem to interact electrostatically with acidic lipids as well, perhaps not to the same extent as basic proteins do. Although related evidence has not been directly demonstrated in platelets, the effect of temperatures on the ability of platelets to aggregate is well established. Platelets aggregate optimally at 37°C and much less effectively at 4°C.

(iii) Peripheral membrane restraint

The peripheral membrane proteins lie predominantly or completely outside the integral membrane zone formed by the lipid bilayer (Nicolson, 1976), or they may exist as α -helices on membrane surface with their N-terminals partially dipping into the hydrocarbon region of the bilayer (Boggs and Moscarello, 1978). These peripheral proteins are stabilized by interacting with integral membrane components, by ionic or hydrogen bonds and other interactions. The best example



was shown in human red cells in which the spectrin molecules form a peripheral lattice across large portions of the inner membrane (Nicolson and Singer, 1971). Arrangement such as this may be present in platelets. A membrane protein (Mol. wt. 190,000) without proteolytic activity and being released into the medium from thrombin-stimulated platelets or phytohemagglutinin-treated platelets was reported by Baenziger et al. (1971). The protein is released into the medium from platelets in a form that is indistinguishable from that present in the untreated platelets (Baenziger et al., 1972). This protein therefore may solely be of structural importance to platelets. Peripheral proteins of this nature may link specific components or an array of different components and impede their lateral mobility.

(iv) Membrane-associated (cytoskeletal) restraints

The three basic types of cytoskeletal components which are at times associated with cell membranes are the microfilaments, thick filaments (myosin-like) and microtubules. All three have been identified in platelets. They differ from peripheral membrane components in being transient in nature, in their requirement of ATP for maintenance and in their sensitivity to drugs which disrupt their organization.

Microfilaments contain actin, ATP and divalent cations, calcium or magnesium. They exist as double helical filamentous structures. They form arrow heads with meromyosin in vitro in the

presence of KCl and $MgCl_2$ (Bettex-Galland et al., 1972). The thick filaments (meromyosin) contains ATPase. In platelets; they consist of 2 heavy chains (M.W. 200,000) and 2 pairs of light chains (M.W. 20,000 and 17,000 respectively) (Adelstein et al., 1975; Daniel and Adelstein, 1976). The actomyosin system (formerly called thrombosthenin) makes up approximately 15% of platelet protein. It is not visible in resting platelets and becomes apparent in swollen platelets or platelet aggregates (Bettex-Galland and Luscher, 1965). The microfilaments are adherent to the inner platelet membrane surface and are considered to play a role in shape change and pseudopod formation of platelets. Cytochalasin B which disrupts microfilament organization and blocks contractile activity in many cells (Wrenn and Wessells, 1970; Spudich, 1973) maintains platelets in disc shape (White, 1971). Microfilaments may be involved in platelet secretion activities. Activation of the contractile system is thought to be brought about by the increase in intracellular calcium ion concentration (Massini and Luscher, 1976). The calcium ions may be released from the dense tubular system which anatomically resembles the sarcoplasmic reticulum in muscle cells, or calcium ions may influx through the plasma membrane during stimulation of platelets.

Microtubules are polymeric complexes of the protein tubulin. Tubulin dimers assemble to form the microtubular structure. The assembly and disassembly of microtubules are sensitive to temperature, Ca^{2+} concentration, pH, pressure and certain alkaloids (Nicolson, 1976). Low temperatures and high Ca^{2+} concentrations favor depolymerization of microtubules to tubulin

subunits (Olmsted and Borisy, 1973; Weisenberg, 1972; Allison, 1973).

This could partially explain the disappearance of microtubules during the platelet release reaction when intracellular calcium ion concentration is raised.

Although microtubules are not contractile proteins, they are essential to the trans-membrane control mechanism of many cellular activities as these may be the structures that join peripheral components to cytoplasmic components. Thus, microtubule transformations are implicated in cellular secretion and pseudopod formation or processes involved in cell-adhesion to substrate (Nicolson, 1976). Many of these cellular activities can be disrupted by the presence of colchicine. Low concentration of this alkaloid, however, does not modify response of platelets to thrombin (Detwiler et al., 1975).

3. Response of platelets to aggregating stimuli

The essential feature of platelet function is their response to various stimuli, for instance, ADP, collagen, thrombin and ionophore A23,187. Despite the diversified nature of these stimuli, the response of the platelet consists of the same sequence of events: rapid shape change, from a circular disc to a spiny sphere. Depending upon the nature and the concentration of the stimulus, shape change of platelets may be followed by aggregation and/or release of the contents of the storage granules. The uniformity of this response led Luscher and Massini (1975) to suggest that all

primary effects on the platelet surface either lead to the same alteration of membrane structures, or always activate the same 'second messenger' system which could be cyclic AMP or movement of Ca^{2+} ions or both. The ability of platelets to aggregate and release granule contents enable them to participate in hemostasis, or formation of thrombi and many other biological functions.

(i) Platelet response to ADP

In the early sixties, Gaarder et al. (1961) identified ADP from broken red cells as the substance that induces platelets to aggregate. Later, Haslam (1964) postulated release of ADP from platelets as the mechanism of thrombin-induced platelet aggregation. Since platelets contain higher concentrations of ADP than most other mammalian tissues, the revelation of the mechanism of ADP-induced platelet aggregation becomes very important in the prevention of thromboembolic diseases. Investigation into this topic has been advanced considerably by the development of photometric monitoring of platelet aggregation (Born, 1962; O'Brien, 1962), preparation of responsive washed platelets with enzyme apyrase (Ardlie et al., 1971) and various methods for isolation and separation of membrane proteins by electrophoresis.

ADP is present in at least two compartments in platelets. ADP in the dense granules is referred to as the releasable pool which is metabolically inactive and is extruded from the platelets during the

release reaction (Holmsen et al., 1969; Ireland, 1967). ADP in the cytoplasm or membrane fractions and mitochondria represents the metabolic pool which turns over rapidly as indicated by a rapid incorporation of precursors (^{32}P -orthophosphoric acid, ^{14}C -adenine or ^{14}C -adenosine) into this nucleotide (Holmsen et al., 1969). The nucleotides in the releasable pool are not as inert as it was believed, however, because Reimers and colleagues (1975) observed incorporation of ^{32}P -orthophosphoric acid and ^{14}C -adenosine into the granule nucleotides upon prolonged incubation of platelets with the isotopes. They postulated that the isotopes enter the granules from the cytoplasmic pool as ATP.

The response of platelets to ADP is very much like other excitable cells. Depending on the experimental conditions and species of platelets, ADP may cause only platelet shape change, shape change and aggregation, or shape change, aggregation and the release reaction. ADP stimulated washed platelets without the release reaction normally deaggregate spontaneously following maximum aggregation. Shortly after deaggregation they exhibit a period of refractoriness during which further addition of ADP does not elicit new response. Platelets will aggregate again if ADP is added to the suspension after the refractory period. In general, low concentration of ADP (e.g. 10^{-8} M) cause only platelet shape change which does not require the presence of exogenous Ca^{2+} . High concentration of ADP induce platelet aggregation of human platelets suspended in a medium with no added calcium. ADP added to human platelets in citrated platelet-rich plasma causes aggregation and the release reaction, but no release reaction

takes place with human platelets in hirudin platelet-rich plasma or when washed platelets are suspended in a calcium containing medium (Mustard, 1975). Platelets of rabbits or pigs require exogenous Ca^{2+} for aggregation to occur, while human platelets require no external Ca^{2+} (Mustard et al., 1975). ADP-induced platelet aggregation needs fibrinogen (Mustard and Packham, 1970). Responsiveness of washed platelets is best maintained in the presence of enzymes such as apyrase that degrade ADP (E.C. 3.6.1.5) (Ardlie et al., 1971). The pH of suspending medium influences platelet response to ADP; the most desirable value is between pH 7-8 (McLean and Veloso, 1967).

There are several theories on ADP-induced platelet aggregation, each with inconclusive experimental evidence. Gaarder and Laland (1964) suggested that ADP attaches to the platelet surface. The negative charges of the ADP molecules on separate platelets are bridged by the divalent calcium cation. This hypothesis fails to account for other effects of ADP or for aggregation induced by other agents.

ADP could also act as a competitive inhibitor of ATPases present on the external surface of the platelet membrane, whose activity is assumed to be necessary for maintaining platelets in the nonadhesive state. Different types of ATPase has been proposed to serve as target for ADP (Salzman et al., 1966; Mason and Saba, 1969; Moake et al., 1970).

Salzman and associates (1966) and Chamber et al. (1967) proposed that the ecto-ATPase on platelet surface that is inhibited by ADP is

similar, if not identical, to thrombosthenin (actomyosin) of platelets. The proposal was questioned on the ground that (1) an external source of substrate for ecto-ATPase is unlikely; (2) there is very little conversion of ^{14}C -ATP by washed rabbit platelets (Packham et al., 1969); (3) the hypothesis does not account for deaggregation when concentration of ADP is more than adequate to induce aggregation (Mustard and Packham, 1970).

Mason and Saba (1969) and Moake and associates (1970) proposed that the Na^+ , K^+ -ATPase was a target for ADP. However, Mills and Macfarlane (1976) maintained that ADP has not been shown to inhibit the Na^+ , K^+ -ATPase in intact platelets and ouabain, a known inhibitor of the enzyme, does not cause platelet aggregation. Therefore, if ADP acts by regulating a membrane enzyme it must do so through a specific regulating site in the membrane rather than by simple product inhibition of the enzyme.

Since ADP-platelet interaction indicates a remarkable degree of structural specificity, for instance, small changes in the purine ring other than in the 2-position of ADP cause marked reduction of activity (Gaarder et al., 1961), many investigators believe in the presence of ADP receptors on platelet surface. Nachman and Ferris (1974) investigated the nature of ADP-receptor and came up with 10^5 binding sites per platelet. It was observed that divalent cation was needed and binding was inhibited by sulphhydryl reagents and 2-chloro adenosine, to a lesser extent by ATP and AMP, and not at all by PGE_1 . Isolated membrane vesicles bind ADP. The process is

reversible and temperature dependent. Furthermore, exposure of the isolated membrane to trypsin, chymotrypsin or pronase inhibited ^{14}C -ADP uptake. A soluble protein prepared from frozen-thawed washed platelets appears to possess similar characteristics as the ADP receptor of intact platelets. But it has not been possible to relate these findings to any previously described membrane enzyme systems (Nachman, 1975).

A recent theory brings forward the possibility that ADP acts as a phosphate acceptor and that phosphorylation of the exogenous ADP could be mediated by the enzyme nucleotide diphosphate kinase (NDPK, E.C. 2-7.4.6) (Mustard et al., 1975). The essence of this hypothesis is that NDPK phosphorylates external ADP by drawing ATP from the inside of the plasma membrane, thus decreasing the amount of ATP available to adenylate cyclase and the thrombosthenin-associated ATPase. The activities of both of these enzymes are presumably important in maintaining the disc shape of platelets (Salzman et al., 1966; Zieve and Greenough, 1969). In agreement with the concept of transmembrane phosphorylation, ^{14}C -ADP added to washed rabbit platelets is rapidly phosphorylated to ^{14}C -ATP in the medium and this conversion is enhanced by adding unlabelled ATP to the suspension (Guccione et al., 1971). Furthermore, ^{32}P -ATP is formed in the medium after addition of ADP to the ^{32}P -labelled platelets (Mustard et al., 1975). The enzyme, if it is NDPK, is highly specific. It phosphorylates ^{14}C -CDP and ^{14}C -GDP less rapidly and its inhibitors AMP and PGE_1

also inhibits platelet aggregation. Adenosine monophosphate possibly exerts its action by competitive inhibition, while PGE_1 possibly acts by stimulating synthesis of cAMP, thus diverting the substrate ATP away from NDPK.

(ii) Thrombin and the platelet release reaction

Thrombin is one of the most powerful activators of platelets in hemostasis. Local formation of minute amounts of thrombin by the extrinsic pathway as in endothelial injury might be important in hemostasis (Nemerson and Pitlick, 1972). Minute amounts of thrombin might also induce platelet aggregation and the release reaction and could be involved in generation of thrombi.

Thrombin is a proteolytic enzyme of the active serine class (E.C. 3.4.21.5) (Magnusson, 1971). It normally hydrolyses the peptide bond between the arginyl and lysyl residues in polypeptides and to a certain extent behaves like trypsin. Its activity is irreversibly inhibited by agents such as DFP, PMSF and TLCK (Mills and Macfarlane, 1976). Thrombin also has esterolytic activity against substrates such as TAME, BAME (Cole et al., 1967). The enzyme catalyses the splitting of fibrinopeptide A and B from fibrinogen. The fibrin thus formed polymerize and platelets readily adhere to this polymerizing fibrin

(Niewiarowski et al., 1972); but not to fibrinogen or fully polymerized fibrin in which the thrombin has been neutralized (Hovig et al., 1968). Besides fibrinogen, thrombin hydrolyses a number of proteins isolated from platelets, including fibrinogen (Ganguly, 1972), platelet factor XIII (Kisselbach and Wagner, 1966), the contractile protein thrombosthenin M (Cohen et al., 1969), but none of these has been shown to be hydrolysed in intact platelets during aggregation and release (Majerus et al., 1976).

At low concentration, thrombin only induces platelet shape change. At high concentrations, it causes aggregation and the release reaction. The release reaction occurs with extrusion of contents from both of the amine granules and the α -granules. The substances released by thrombin from the amine granules include 5-hydroxytryptamine, ADP, ATP, calcium, pyrophosphate and antiplasmin. The compounds released from the α -granules include acid hydrolases, potassium, fibrinogen, permeability factor and chemotactic factor, bactericidal factor and platelet factor 4 is uncertain (Holmsen, 1973).

Thrombin is capable of causing many metabolic changes in platelets. It induces a burst of oxygen consumption (Hussain and Newcombe, 1964) which was attributed to a rapid oxygenation of endogenously released arachidonic acid by thrombin (Pickett and Cohen, 1976). There is a reduction of metabolic ATP content due to hydrolysis by ATPase or conversion of ATP to IMP and hypoxanthine (Holmsen, 1975). Thrombin also causes a rapid generation of

endoperoxides and prostaglandin derivatives (Hamberg et al., 1974), phosphorylation of membrane proteins and hydrolysis of glycoproteins (Phillips and Agin, 1974). Platelet metabolism is accelerated as demonstrated by increased uptake of glucose and increased lactate production.

Platelets degranulated with thrombin and recovered properly retain their sensitivity to ADP, ionophore A23,187 (Kinlough-Rathbone et al., 1975). The platelets which remained after repeated thrombin treatment and loss of their granule contents had a survival time in the circulation that was not significantly different from that of unstimulated platelets (Reimers, thesis, 1976).

(iii) Mechanism of thrombin-induced platelet aggregation and the release reaction

The mechanism of the thrombin-induced platelet release reaction is not clear. It is likely that the excitation-secretion coupling process resembles that in other secretory cells including mast cells, chromaffin cells of the adrenal medulla and synaptic nerve endings (Stormorken, 1969). The procedure possibly involves depolarization of the plasma membrane which triggers the release of Ca^{2+} ion from inside of the cell or influx of Ca^{2+} from the extracellular medium. In an unknown manner, this calcium causes the fusion between granular and the plasma membranes, leading to extrusion of the granule contents. The granule membrane is retained

by the platelets. In nerve endings, the membrane is reutilized in packaging of neurotransmitters.

The release reaction of the amine granules and of the α granules apparently is governed by a separate mechanism (Holmsen, 1975). The evidence being that higher thrombin concentration is required to trigger the release reaction from α -granules, and material from amine granules is released more rapidly than from α -granules (Holmsen and Day, 1970). Furthermore, aspirin which inhibits thromboxane A_2 -mediated release reaction of amine granules does not inhibit the release reaction of α -granules (Mustard and Packham, 1970).

Several mechanisms have been suggested for thrombin-induced platelet aggregation. The earliest theory was that the aggregation was mediated by the ADP released from storage granules (Grette, 1962; Haslam, 1964). Synergism between thrombin and ADP was observed by Packham and colleagues (1973). They showed that addition of low concentrations of thrombin which causes only shape change and ADP which causes moderate aggregation to washed platelets would result in extensive aggregation and the release reaction.

Another theory concerns the generation of endoperoxides and thromboxane A_2 during thrombin-induced platelet aggregation. These prostaglandin precursors arise from the oxidation of arachidonic acid by platelet cyclooxygenase and the arachidonic acid is cleaved from the membrane phospholipids (Hamberg et al., 1973, 1974, 1975;

Flower and Blackwell, 1976). Thromboxane A_2 is an extremely potent platelet aggregating agent. This pathway may only play a minor role in the mechanism of thrombin-induced platelet functional changes as indomethacin, a non-steroidal anti-inflammatory drug and a potent cyclooxygenase inhibitor, does not abolish platelet aggregation or the release reaction caused by thrombin (Kinlough-Rathbone et al., 1977), nor does it abolish the synergistic effect of low concentrations of thrombin (shape change only) and ADP (Kinlough-Rathbone et al., 1977).

It was observed that thrombin-induced platelet aggregation is not inhibited by creatinine phosphate/creatinine phosphokinase (CP/CPK) which converts released ADP to ATP (Kinlough-Rathbone et al., 1977). Thus it appears that thrombin induces platelet shape change, aggregation and release by at least three pathways: released ADP, the formation of prostaglandin endoperoxides and thromboxane A_2 , and at least one other pathway which is independent of these two pathways (Packham et al., 1977; Kinlough-Rathbone et al., 1977). These three pathways may act synergistically with each other to enhance aggregation and the release reaction.

(iv) Thrombin receptors of platelets

From the earlier description of the properties of thrombin, it is obvious that there exists a specific receptor-thrombin interaction as the initiating step for all the observed

platelet response to thrombin. Currently, not much is known about the nature of this receptor.

Two schools of opinion prevail regarding the mechanism of thrombin-receptor interaction. One theory proposed that the proteolytic activity of thrombin is the basis for its action on platelets. The proposed membrane substrates for thrombin include fibrinogen (Ardlie et al., 1970); thrombostenin proteins or glycoproteins; each without substantial evidence. The role of thrombostenin as substrate for thrombin remains controversial. Some investigators (Booyse et al., 1972; Cohen et al., 1969) proposed myosin component as the substrate; while other investigators (Adelstein et al., 1972; Muszbek, 1976; Weber and Olsson, 1974) regard actin as the substrate.

On exposure of platelets to thrombin, a glycoprotein (Mol. Wt. 118,000) was earlier reported to have lost from platelets (Phillips and Agin, 1974). The timing of observation (30 min) and the concentration of thrombin (10 u/ml) was too high to be regarded as bearing a meaningful relationship to the physiological effect of thrombin on platelets (Majerus et al., 1976). Recently, Phillips and Agin (1977) treated platelet surface glycoproteins by galactose oxidase with subsequent ³H-sodium borohydride reduction. They found that the amount of platelet glycoprotein V (Mol. Wt. 89,000) was greatly reduced, and a glycopeptide hydrolytic product (apparently Mol. Wt. 70,000) was released into the soluble fraction.

Additional evidence of the proteolytic action of thrombin on platelets is that trypsin treatment of platelets (under conditions without platelet aggregation and release reaction) reduces the binding capacity for thrombin and renders platelets unresponsive to the stimuli (Ganguly, 1977). Yet neuraminidase treatment of platelets enhances their response to thrombin, presumably the removal of sialic acid residues helps to expose the receptors.

Another theory proposed that thrombin would be acting like a hormone. Its binding to the receptor without bond cleavage may cause a distortion of platelet membrane, resulting in functional changes of platelets.

Kinetic studies have shown that the prospective receptor is substrate specific. These are two populations of receptors, several hundreds per platelet of the low affinity-binding ones and approximately 50,000 per platelet of the high affinity-binding receptors (Majerus et al., 1976; Detwiler et al., 1975; Workman et al., 1977). Binding of thrombin to the receptors involves no turnover of thrombin as will be expected of a hydrolytic enzyme (Majerus et al., 1976; Detwiler et al., 1975). Negative binding cooperativity similar to the one between the binding of glucagon or insulin to their receptors was suggested by Majerus et al. (1976) but not by other investigators. Chemical deactivation with diisopropyl fluorophosphate or alteration of the serine or histidine residues in the active site of thrombin does not influence thrombin binding to platelets. Acetylation of thrombin with the enzyme

retaining esterase activity, or chemical modification of a single tryptophan residue at or near the binding site of thrombin, diminishes the high affinity binding capacity considerably with some loss of platelet release reaction. It was thus concluded that the high-affinity and low-affinity binding sites on the platelets are far apart. The high affinity binding site is believed to be involved in the initiation of the release reaction and aggregation (Workman et al., 1977).

4. Role of calcium and cyclic nucleotides in platelet function

Although little is known about how the message of stimulus-receptor interaction on cell surfaces is transmitted for various platelet responses, it is likely that some of the 'second messengers' known to function in cellular regulatory processes in other tissues are involved. The principal candidates of the 'second messenger' are calcium ions and cyclic AMP. Their actions in cell functions may be independent or interrelated.

The calcium ions mediating the contractile or secretory activities of many tissues are dependent on an external source. For instance, the release of neurotransmitter from the presynaptic nerve endings (Hutter and Kostial, 1954), catecholamines from chromaffin granules (Douglas and Rubin, 1961) and potassium from parotid slices (Selinger et al., 1974). In skeletal and smooth muscle

cells, Ca^{2+} is released into cytoplasm from the sarcoplasmic reticulum. In cardiac muscle, Ca^{2+} required for the contractile activity comes from both outside the cells, the transverse tubular system and the sarcoplasmic reticulum (Guyton, 1976). The requirement and sources of Ca^{2+} for platelet function is very complex. Aggregation of rabbit platelets requires the presence of exogenous Ca^{2+} while exogenous calcium is not necessary for human platelet aggregation. With human platelet shape change and the release reaction can occur in the absence of external Ca^{2+} (Kinlough-Rathbone et al., 1975; Feinman and Detwiler, 1975), while with rabbit platelet external calcium is necessary for the release reaction. The intracellular binding sites of Ca^{2+} in platelets have not been defined. The so-called dense tubular system may be one of the sources since it bears anatomical resemblance to the sarcoplasmic reticulum of skeletal muscle (White, 1972b). Another possible source of Ca^{2+} not mentioned in the literature on platelet research is the phospholipids. The acidic phospholipids were shown to bind much more calcium than the proteins do in the cell membrane (Anghileri, 1972). In contrast, the dense granules in human platelets, although rich in calcium are not considered to be a source of Ca^{2+} in the Ca^{2+} -mediated platelet function since the contents of the dense granules are thought to be directly extruded to the external environment during the release reaction (Murer and Holme, 1970).

The mechanism of Ca^{2+} as a mediator of platelet response to aggregating stimuli is not known. Presumably, it involves the release of Ca^{2+} from the inner surface of membrane binding sites upon interaction of stimulus and membrane receptors. This calcium then induces further release of calcium from the dense tubular system

or an increased membrane permeability for exogenous calcium.

The rise of cytoplasmic Ca^{2+} concentration activates a number of Ca^{2+} -dependent biochemical reactions. These include the activation of actomyosin ATPase, depolymerization of microtubules, activation of glycogenolysis and phospholipase A_2 and increased phosphorylation of specific proteins (Haslam et al., 1978). Activation of phospholipase A_2 would increase the release of free arachidonic acid from phospholipids in the membrane. The arachidonic acid is converted to endoperoxides and thromboxane A_2 which stimulate further release of Ca^{2+} from the dense tubular system. Calcium also activates guanylate cyclase and increase the production of cyclic GMP (Haslam et al., 1978).

Cyclic AMP is formed from ATP by the enzyme adenylate cyclase and cAMP is degraded to AMP by phosphodiesterase. Exogenous cAMP and its more cell-penetrating analogue dibutyryl cAMP inhibit both aggregation and the release reaction of platelets (Mills and Smith, 1971). The common inhibitory agonists prostaglandin E_1 , adenosine and α -adrenergic agonists and the common phosphodiesterase inhibitors such as caffeine have been shown to exert their effects by increasing platelet cyclic AMP concentrations (Mills and Macfarlane, 1974; Tateson et al., 1977). Inhibition of adenylate cyclase with deoxyadenosine therefore abolishes the inhibitory effect of PGE_1 on platelet aggregation induced by ADP (Haslam et al., 1978). Furthermore, combined use of PGE_1 and cyclic AMP phosphodiesterase inhibitors appear to be more effective in

inhibiting aggregation induced by ADP or collagen than the use of PGE₁ alone.

It has been suggested that a decrease in the concentration of cAMP below that normally present in circulating platelets may favor platelet aggregation (Salzman and Levine, 1971). There is however, no convincing evidence that aggregation is linked to a decrease in cAMP and that cAMP can be lowered under basal conditions (Haslam, 1975) although aggregating agents such as ADP or adrenaline can lower cAMP if its basal level has been previously elevated (Haslam, 1973).

The detailed mechanisms by which cAMP exerts its effect are not known. Measurements of changes in the phosphorylation of proteins in intact platelets labeled with ³²P-orthophosphate have shown that PGE₁ selectively increases the phosphorylation of a 24,000 dalton polypeptide present in a microsomal fraction that can actively take up Ca²⁺ ions (Haslam et al., 1978). Booyse and associates (1976) demonstrated in platelets a cAMP-dependent protein kinase and cAMP-independent kinase which can phosphorylate several membrane proteins. In addition, Kaser-Glanzmann and coworkers (1977) showed that cAMP can increase the ATP-dependent uptake of Ca²⁺ ions by a microsomal fraction. It is also known that cAMP increases the uptake of calcium into the sarcoplasmic reticulum of cardiac and smooth muscle cells (Morkin and LaKaia, 1974). It is thus possible that many of the effects of increased cAMP levels on aspects of platelet function may result from the rapid reuptake of Ca²⁺ ions released into the

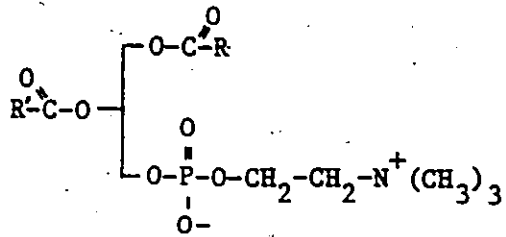
platelet cytosol by aggregating agents, with consequent inhibition of Ca^{2+} -dependent reactions (Haslam et al., 1978).

The role of cyclic GMP in mammalian tissues remains largely unknown. Studies on the effects of ADP, collagen, arachidonic acid and other agents have shown that increases in platelet cGMP correlate with the extent of platelet aggregation and not with the release of 5-HT. Furthermore, EDTA which blocks platelet aggregation and change in cGMP induced by collagen does not inhibit the release reaction. Haslam and associates (1978) therefore suggest that contact between membranes of aggregating platelets is necessary for the physiological activation of guanylate cyclase in intact platelets and that increases in platelet cGMP may be an effect rather than a potential cause of aggregation. In addition, a muscle relaxant SNP which elevates cGMP level in smooth muscle (Schultz et al., 1977) is found to inhibit platelet aggregation and increase the concentration of cGMP, suggesting a role as a feedback inhibitor of this cyclic nucleotide (Haslam et al., 1978).

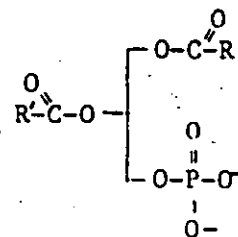
PART II. Phospholipids

Phospholipids are ubiquitous and essential components of cell membranes. The amount of phospholipids in biological membranes is approximately 40-80% of the dried weight of total lipid. The proportions of phospholipids are relatively high in mammalian cell membrane preparations but vary appreciably from one type of membrane to another and also vary among the types of tissues. It was suggested previously that biosynthesis of phospholipids is localized in the microsomal fraction and the products are then shuffled to different locations within the cell. Recently, however, there is evidence suggesting compartmentalization of phospholipid biosynthesis (Soto et al., 1977).

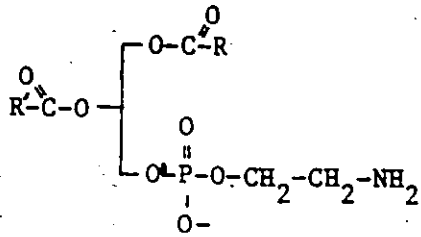
Phospholipids have in common that they contain a phosphate group and at least one fatty acid molecule and one alcohol. Depending on the nature of the alcohol they can be grouped as glycerol phospholipids and sphingophospholipids. This thesis project is confined to glycerol phospholipids and therefore only the metabolism of this group of phospholipids would be discussed. The most widely studied members of this group include phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), monophosphoinositide (MPI), diphosphoinositide (DPI), triphosphoinositide (TPI), phosphatidic acid (PA) and diphosphatidylglycerol (DPG). In platelets, PC is the most abundant, followed by PE, SPH and PS. The rest of phospholipids make up less than 10% of total lipid phosphorus. Depending on the nature of the substituent polar head groups and the fatty acids, there are



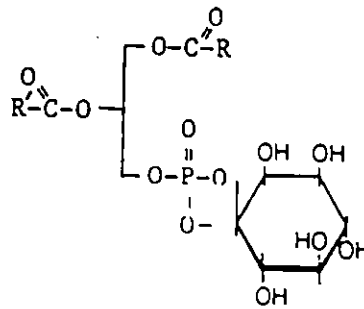
PHOSPHATIDYLCHOLINE



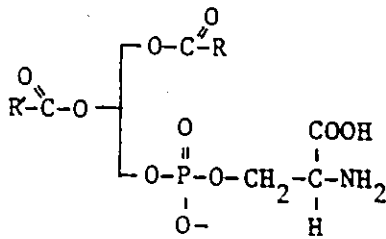
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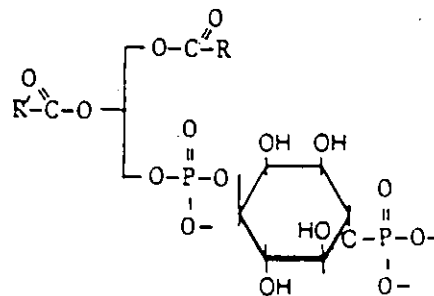
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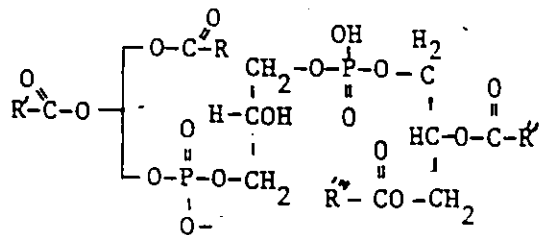
MONOPHOSPHOINOSITIDE



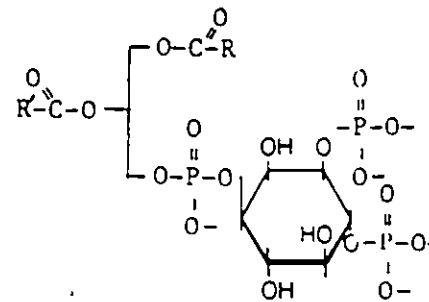
PHOSPHATIDYLSERINE



DIPHOSPHOINOSITIDE



DIPHOSPHATIDYLGLYCEROL



TRIPHOSPHOINOSITIDE

Figure 1. Structures of phospholipids.

almost infinite varieties of phospholipids. For example, milk fat alone contains about 95 different phosphatidylcholine species (Gurr and James, 1971). The structures of diacylglycerol phospholipids are shown in Figure 1. It can be seen from these structures that phospholipids are differently charged. All of them bear negative charges except PC which is electrically neutral. PA and the phosphoinositides are extremely acidic lipids because of their free phosphate moieties and the phosphoinositides are very polar molecules owing to the hydroxyl groups on the inositol residue. The acidity and polarity of phospholipids influence their interaction with proteins and divalent cations in the membrane.

1. Biosynthesis

The de novo synthesis of phospholipids begins with esterification of 3-Sn-glycerol phosphate by activated long chain fatty acids to form 1,2-diacyl-3-Sn-glycerol phosphate (phosphatidic acid) (Kornberg and Pricer, 1953). This is the parent molecule of all the glycerol phospholipid family and as such it does not ordinarily accumulate in tissues by carefully regulated process. The coenzyme A derivatives of fatty acid arise either by de novo synthesis from acetate within the cell or by the action of microsomal and mitochondrial enzymes which couple free fatty acid with coenzyme A in an ATP-requiring reaction (Lippel et al., 1970). 3-Sn-Glycerol phosphate is supplied by glycolysis of glucose which in turn is made abundant by the breakdown of

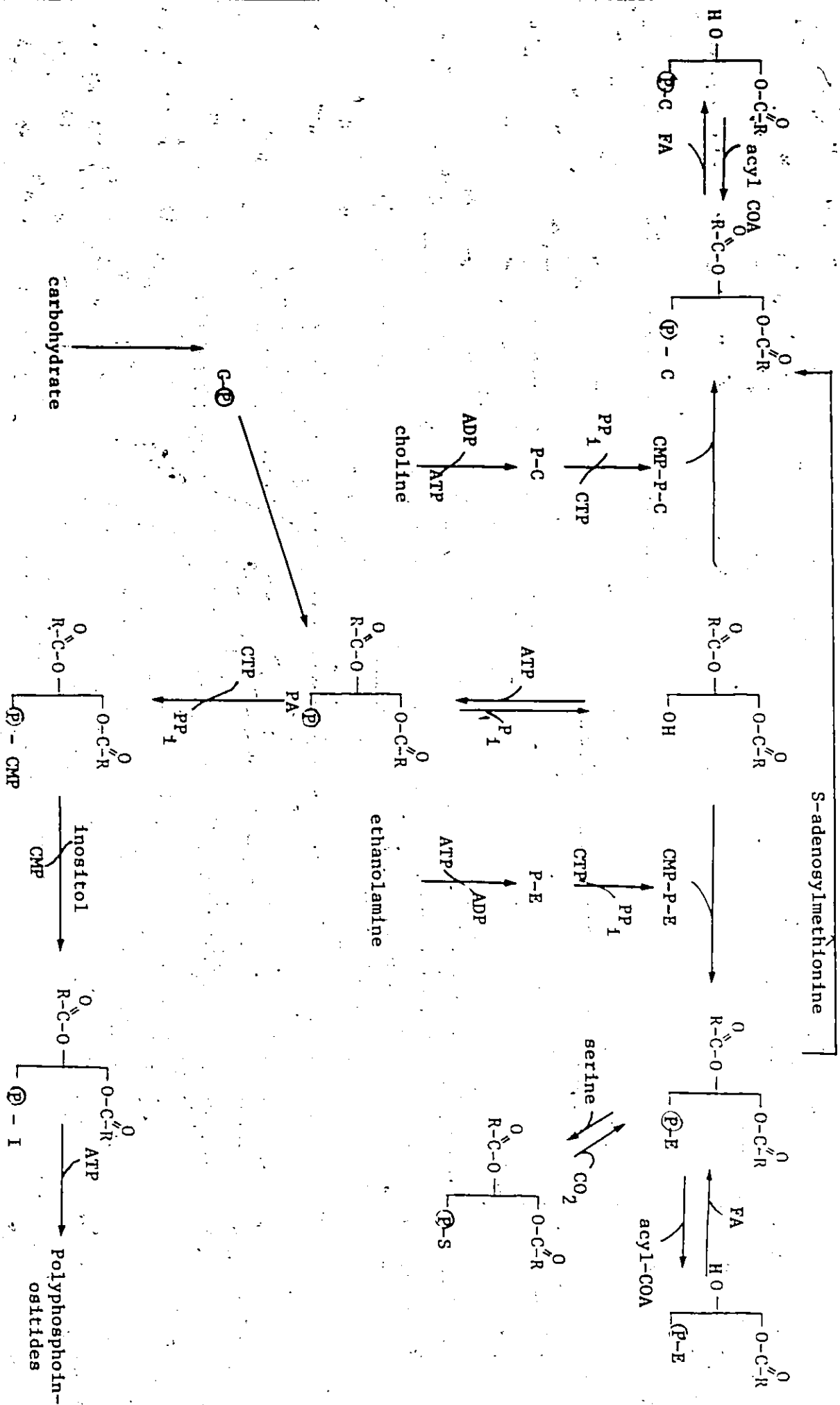


Figure 2. Biosynthesis pathways of phospholipids. P = phosphate; C = choline; E = ethanolamine; I = inositol;

FA = fatty acid; S = serine; G-P = glycerol-3-phosphate; P-C, P-E = phosphoryl choline, ethanolamine;

carbohydrates. PA can also be synthesized by phosphorylation of monoacylglycerol to lyso-PA, followed by esterification with fatty acyl CoA. Alternatively, monoacylglycerol is first esterified and then phosphorylated. Another pathway for PA synthesis involves esterification of dihydroxyacetone phosphate with fatty acyl-CoA. All these three pathways, however, play only minor roles in the maintenance of PA quantity in the cell (Thompson; 1973):

Presently known pathways for the biosynthesis of various glycerophospholipids are shown in Figure 2. Cytidine-containing coenzyme play a general role in these reaction patterns. The coenzyme is involved in the de novo generation of the monophosphate diester linkage and such a requirement has been unequivocally demonstrated in the biosynthesis of PE, PC, MPI, phosphatidylglycerol and sphingomyelin. The first evidence for the enzymatic formation of cytidine diphosphate diacylglycerol was reported by Agranoff and colleagues (1958) who found conversion of labeled cytidine monophosphate into a chloroform-soluble product (CDP-diacylglycerol) in the presence of a particulate enzyme from kidney. Later experiments by Paulus and Kennedy (1958) showed that CDP-diacylglycerol reacts with inositol to yield MPI. Phosphorylation of MPI yields DPI and further TPI. By using ^{32}P -labeled 3-Sn-glycerol phosphate, they also demonstrated that the phosphate moiety of this compound remains with the glycerol backbone throughout the formation of PA to CDP-diacylglycerol and subsequently appears in MPI. By a similar pathway, phosphatidylglycerol

is formed from CDP-diacylglycerol which is the precursor of diposphatidylglycerol. The latter is a major phospholipid species of the inner mitochondrial membrane and bacterial membrane lipids. Formation of PS in bacteria is also mediated by CDP-diacylglycerol. PS can then undergo decarboxylation to form PE which is further methylated to form PC. In animal tissues however, PE synthesis begins with phosphorylation of ethanolamine by the action of ethanolamine kinase, with ATP being the phosphate donor. The phosphoethanolamine reacts with cytidine triphosphate to yield cytidine diphosphoethanolamine. The reaction is catalysed by the phosphoethanolamine cytidyltransferase. In the last step the cytidine monophosphate moiety of CDP-ethanolamine is cleaved and the phosphoethanolamine is transferred to 1,2-diacylglycerol to form PE (Kennedy, 1956). Biosynthesis of PC in mammalian tissues follows two pathways. A pathway similar to that of PE synthesis is demonstrated in the nervous system (Rossiter et al., 1957), kidney and other tissues. In this pathway choline is activated instead of ethanolamine. Kornberg and Pricer (1952) demonstrated that phosphorylcholine labeled with both $^{32}\text{PO}_4$ and ^{14}C on treatment with an enzyme preparation would yield PC in which $^{32}\text{PO}_4$ and ^{14}C remain in the same proportion as the phosphorylcholine precursor. This indicates that phosphorylcholine molecule is incorporated into PC as an entity. The alternate pathway for PC synthesis is by stepwise methylation of PE, S-adenosylmethionine being the methyl donor

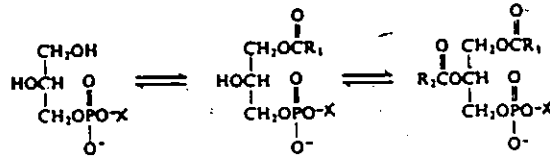
(Bremer and Greenberg, 1961). This pathway is important only in the liver and contributes a considerable amount of plasma PC to the circulation.

Platelets have the enzyme system for de novo synthesis of fatty acids and chain elongation. They are also capable of de novo synthesis of phospholipids. Deykin and Desser (1968) observed active incorporation of ^{14}C -1-acetate into the free fatty acids and PC of platelets in both buffer and plasma systems. There was also incorporation of ^{14}C -1-palmitic acid into phospholipids. The activity of acetyl-coenzyme A carboxylase which converts acetyl-CoA and CO_2 to malonyl-CoA was high in human platelet extracts (Majerus et al., 1969). Lewis and Majerus (1969) observed incorporation of radioactive glycerol into platelet phospholipids, indicating de novo synthesis. Platelets possess phosphatidate phosphatase which catalyses conversion of PA to 1,2-diacylglycerol for the synthesis of PC and PE (Call and Williams, 1973). They also have enzymes CTP: diacylglycerophosphate-cytidyl transferase (Call and Williams, 1970) and CDP-diacylglycerol-myoinositol phosphatidyl transferase (Lucas et al., 1970) to mediate the synthesis of MPI. Recently, Kaulen and Gross (1976) reported activity of specific kinases that phosphorylate MPI to DPI and DPI to TPI in human platelets.

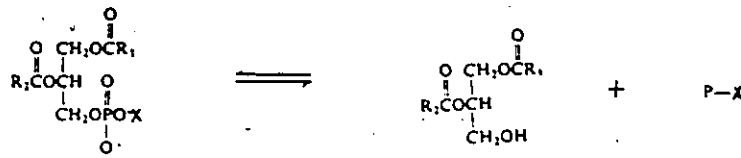
2. Metabolism of major phospholipids

The life span of phospholipids in the cell is known to be

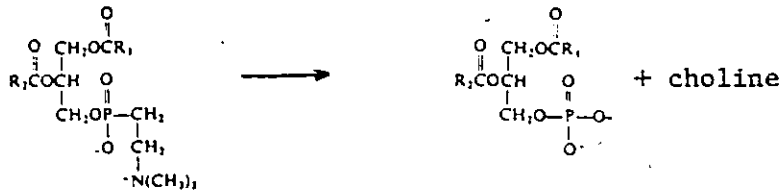
ACYLATION - DEACYLATION



PHOSPHOLIPASE C TYPE REACTION



PHOSPHOLIPASE D TYPE REACTION



BASE EXCHANGE

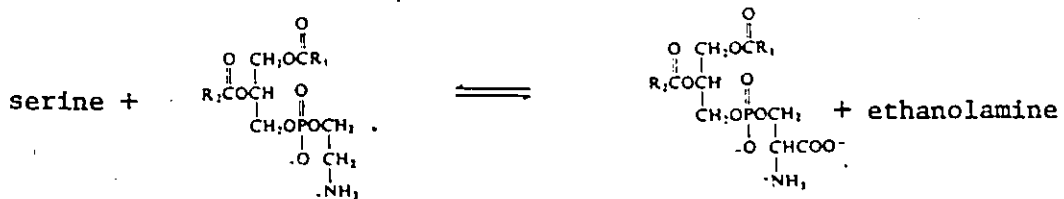


Figure 3(a). Major metabolic pathways of phospholipids.

shorter than most other structural components. Isotopic studies have shown that the half-lives of various phospholipids of adult rat brain range from 12.5 days in MPI to 40 days in sphingomyelin (Thompson, 1973). Unlike the metabolic turnover of proteins which is an all-or-nothing process, phospholipid molecules can have part of their structural components renewed, leaving the other part intact. The types of reactions include acylation and deacylation, formation and cleavage of the glycerol-phosphate ester linkage, formation and cleavage of the phosphodiesterase linkage between the phosphate and the base, as well as modification of the base. The specificity and subcellular distribution of the enzymes involved in these reactions are extremely complex. In general, there are various types of phospholipases to cleave the molecular linkages and transferase for bond formations.

Deacylation of intact phospholipids at the 1- or the 2-position yields free fatty acid and lysophospholipid (Figure 3(a)). The reaction is carried out by phospholipase A₁ and A₂ respectively and phospholipase B attacks at both positions. These enzymes are ubiquitous. Some have been prepared from various snake venoms, pancreatic juice (De Haas et al., 1968), arterial tissue (Eisenberg et al., 1968), heart muscle (Weglicki et al., 1971) and liver (Waitz and van Deenen, 1967). Most of the phospholipase A₂ enzymes are Ca²⁺-dependent and enzymes prepared from different species exhibit preferential hydrolysis of substrates. For example, the snake venom preparations deacylate lecithin readily

while pancreatic phospholipase A₂ rapidly attacks very acidic phospholipids such as PA, MPI and cardiolipin, and hydrolyses lecithin slowly regardless of chain length or degree of saturation of the fatty acid (De Haas et al., 1968). In spleen slices and platelets labeled with ¹⁴C-arachidonic acid and stimulated with appropriate agents, there was formation of free arachidonic acid and radioactive prostaglandin derivatives or intermediates, indicating the activation of phospholipase A₂ in the tissues (Flower and Blackwell, 1976; Bills et al., 1976).

Acylation of lysophospholipids to form diacylphospholipids requires the acyl-CoA-phospholipid acyltransferases. These enzymes are highly selective in the type of fatty acids they transfer and there is a marked specificity for esterifying saturated fatty acids at the 1-hydroxyl position and unsaturated fatty acids at the 2-hydroxyl position (Reitz et al., 1968; Van Den Bosch et al., 1968). For instance, the specificity of the microsomal liver enzyme in transferring the test acyl-CoA to 1-acyllyso phosphatidylcholine follows the order of oleate > linoleate > palmitate > stearate. Contrary to this, the same enzyme preparation transfers stearate or palmitate to the C₁-position of 2-acyllyso-phosphatidylcholine more efficiently than oleate or linoleate (Van Den Bosch et al., 1968). Thus the selectivity of the enzyme depends strictly upon the position of the hydroxyl group to be acylated and not being influenced by the nature of the acid attached to the other position

of the lysophosphatidate, resulting in predominantly unsaturated fatty acids in the C₂-position and saturated fatty acids at the C₁-position of mammalian phospholipids.

Exchange of fatty acyl groups also occurs between two lyso phospholipid molecules to yield diacylphospholipid and glycerophosphate derivative, or between a diacylphospholipid and a cholesterol molecule to form cholesterol ester and lyso-phospholipid. The latter reaction is an important source for cholesterol ester in the plasma (Glomset, 1968). Platelets are capable of direct acylation of plasma lysophosphatidylcholine with free fatty acid and take in this product to replenish their diacylphosphatidylcholine "compartment" (Elsbach et al., 1971). This process is not affected by ADP or thrombin. However, in the presence of polystyrene particles, platelets ingest them and demonstrate increased formation of glycerophosphorylcholine and phosphatidylcholine from lysophosphatidylcholine.

Cleavage of the phosphate-glycerol linkages of phospholipids yields diacylglycerol and a phosphate base, phosphorylcholine in the case of phosphatidylcholine. The reactions are catalysed by phospholipase C. Under proper conditions, the same enzyme preparation attacks phosphatidylcholine, phosphatidylethanolamine and sphingomyelin. Most data concerning phospholipase C comes from the studies of enzymes of bacterial origin. Activities of these enzymes are not well studied in animal tissues as interpretation are often complicated by the concurrent action of other phospholipases in the systems under

examination. Phospholipase C preparations obtained from liver cell lysosomes (Fowler, 1969) and brain (Gatt, 1970) show no dependence on Ca^{2+} for full activity. The liver preparation is much more active toward sphingomyelin than PC. On the other hand, phospholipase C occurring in intestinal mucosa and other animal tissues, attacking only phosphatidylinositol and di- and triphosphoinositides is dependent upon the presence of Ca^{2+} for activity (Atherton and Hawthorne, 1968). Formation of the phosphate-glycerol linkages occur in the synthesis of PE and PC as mentioned previously.

Platelets contain no detectable endogenous phospholipase C activity and treatment of platelets with phospholipase C from *Bacillus cereus* leads to a loss of 45% of total phospholipids without triggering the release reaction (Otnaess and Holm, 1976). These phospholipase C-treated platelets respond to ADP or thrombin. On the other hand, phospholipase C of *Clostridium perfringens* or *Clostridium welchii* induces aggregation and the release reaction of platelets (Otnaess and Holm, 1976; Chap and Douste-Blazy, 1974).

Cleavage of the phosphate-base linkages yields phosphatidic acid and a base. The enzymes catalysing these reactions are the phospholipases D. However, attempts to isolate phospholipases D in animal cells have been met with little success although they are present in abundance in higher plants. The base exchange processes among PC, PE and PS belong to this type of reactions which account for a considerable amount of intracellular lipid modification. The

base exchange activity is particularly pronounced in the absence of de novo phospholipid synthesis (Williams and Bygrave, 1970).

Modification of the base in phospholipids is exemplified by the decarboxylation of PS to form PE (in bacteria), the methylation of PE to PC or the phosphorylation of MPI to become polyphosphoinositides. The latter reactions will be discussed in the next paragraph.

3. Metabolism of phosphatidic acid and phosphoinositides

Phosphatidic acid and the phosphoinositides comprise less than 12% of total phospholipids in the cell membrane. Since PA is an intermediate in the synthesis of all other glycerophospholipids, it does not accumulate in the tissue and is usually less than 1% of the total lipid phosphorus. Monophosphoinositide (MPI) makes up 3-10% of lipid phosphorus (Hawthorne and Kemp, 1964). Diphosphoinositide (DPI) and triphosphoinositide (TPI) are each approximately 10-25% of the amount of MPI (Ditlmer and Douglass, 1969). These minor phospholipids distinguish themselves from other phospholipids in being extremely acidic and have high affinity for divalent cations (Kerr et al., 1964; Eichberg and Dawson, 1965; Hendrickson and Reinertsen, 1971). In addition, the monoesterified phosphate groups on the polyphosphoinositides (DPI and TPI) interact strongly with proteins (Dawson, 1965) and a variety of amphiphilic cations such as alkylamines, local anesthetics and phenothiazine tranquilizers (Seeman, 1972). The inositol phospholipids are also unlike other phospholipids in

that they are very rich in arachidonic acid and stearic acid which are esterified to the C₂ and C₁-position respectively (Baker and Thompson, 1972). Phosphatidic acid on the other hand is rich in palmitic and oleic acid with little arachidonate.

Studies on the metabolism of PA and the phosphoinositides began with the observation by Hokin and Hokin (1955) that ³²P and ³²P-orthophosphate was incorporated more rapidly into PA and the phosphoinositides than into other phospholipids, particularly when the tissues were stimulated with acetylcholine. Since their observations, numerous experiments have been conducted with an ever expanding list of stimuli and of responsive tissues to find out the biological roles of these phospholipids.

When ³²P-orthophosphate is injected into animals or when tissues are incubated with this isotope, MPI, DPI and TPI are rapidly labeled with ³²PO₄ and in a few hours, the specific radioactivity of these inositol lipids exhibits an order of TPI > DPI > MPI (Dawson, 1969; Sheltawy and Dawson, 1969; Yagihara and Hawthorne, 1972). The specific radioactivity of PA is also very high, whereas the specific radioactivity of major phospholipids remains low. These changes have been observed in various tissues including the heart, liver (Ts'ao and Cornatzer, 1967), red cells (Hokin and Hokin, 1964), kidney (Anrade and Huggins, 1964) and brain slices (Humphrey et al., 1969).

The monoesterified phosphates of DPI and TPI in brain slices

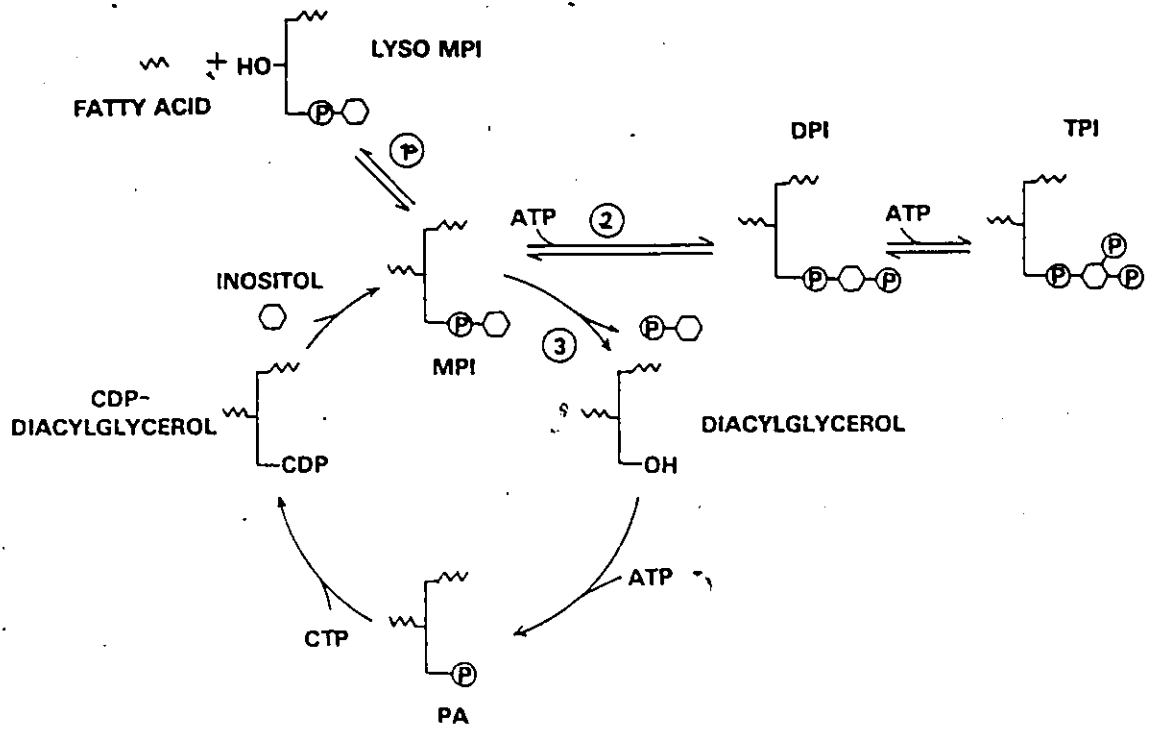


Fig. 3b. Metabolic pathways of phosphatidic acid and phosphoinositides.

reached a higher specific radioactivity than the diesterified phosphate on incubation with ^{32}P -orthophosphate (Ellis and Hawthorne, 1962; Saunders and Bellou, 1966). This was further proven by experiments in which the monoesterified phosphates were hydrolysed with alkaline phosphatase from red cells previously incubated with (γ - ^{32}P) ATP for an hour. The results show that over 90% of the radioactivity incorporated into DPI and TPI reside in the monoesterified phosphates (Hokin and Hokin, 1964). It is thus clear that enzymes involved in the turnover of monoesterified phosphates of the polyphosphoinositides have very high activity.

It was later shown that TPI phosphomonoesterase which hydrolyses the phosphate groups from TPI has an activity almost as high as the acetylcholine esterase in brain tissues (Kai and Hawthorne, 1969).

The high rate of labelling of the phosphate group of PA is likely a result of the combined action of phosphatidate phosphatase and diacylglycerol kinase. The activity of both of these enzyme is very high in red cell and brain tissue (Hokin and Hokin, 1963; Lapetina and Hawthorne, 1971; Schact and Agranoff, 1974).

Incorporation of $^{32}\text{PO}_4$ into MPI in unstimulated tissues is due to a cyclical conversion as shown in Figure 3(b). Since ^3H -glycerol is also incorporated into MPI (Hokin, L.E., 1959), some degree of de novo synthesis is indicated. In the cyclical event, MPI is degraded by MPI phosphodiesterase to form 1,2-diacylglycerol. The latter is phosphorylated by (γ - ^{32}P) ATP to form PA and the ^{32}P -

labelling remains with the intermediates, and subsequently appears in the newly synthesized MPI molecule. During this conversion, the diacylglycerol backbone is conserved (Hokin-Neaverson, 1974):

Current interest in PA and phosphoinositide metabolism of tissues in response to stimuli focuses on 2 types of reactions, namely the degradation of MPI and the interconversion between DPI and TPI.

It has been observed in many tissues treated with acetylcholine, salivary glands in response to adrenaline, anterior pituitary exposed to corticotrophic-releasing factor or pancreozymin-treated pancreas an increased rate of $^{32}\text{PO}_4$ incorporation into MPI and often also into PA. The changes occur within a few minutes after the application of a stimulus and persists for as long as the stimulus is applied. The only common link between the isotope incorporation among the various tissues in response to different stimuli is that rapid incorporation of $^{32}\text{PO}_4$ into PA and MPI occurs only when the stimuli exert their effects on their target cells through interaction with receptor sites on cell surface, rather than by entering and directly affecting intracellular processes in a manner analogous to steroid hormones (Michell, 1975). Thus this change of metabolism in PA and MPI exhibits some kind of specificity which is further illustrated by the observation that the only affected lipids are PA and the phosphoinositides. Initially it was not shown whether the increased labelling was due to enhanced synthesis of MPI de novo or to an

increased rate of turnover of a cellular pool of MPI of essentially unchanged size. Soon, however, the Hokins (1958) demonstrated that in cerebral cortex the enhanced incorporation of ^{32}P -orthophosphate and of labelled inositol into MPI was not accompanied by increased incorporation of labelled glycerol, indicating that the phosphoryl-inositol portion of the molecule was being renewed as a response to stimulation but that the diacylglycerol backbone of the molecule was reutilized. Measurements of the MPI content of stimulated tissues confirmed the impression that the process under study did not involve an increase in synthesis of MPI de novo. Studies on many other tissues including lymphocytes (Fisher and Mueller, 1971); pancreas (Miller and Dawson, 1972) and polymorphonuclear leukocytes (Tou and Stjernholm, 1974) also conform to the same pattern. Preliminary evidence for a phosphodiesteratic cleavage of MPI in acetylcholine-stimulated brain homogenate and a closed-cycle turnover of MPI mediated by the formation of PA was first provided by Durell and Garland (1969). Recently, a decrease in the amount of MPI was measured in stimulated lymphocytes (Allan and Michell, 1974) and in acetylcholine-treated rat pancreas (Hokin-Nemerson, 1974). Concurrently, a several fold increase in the amount of 1,2-diacylglycerol, a product of phosphodiesteratic cleavage of phospholipids, was reported in red cells treated with ionophore A23,187 and acetylcholine-stimulated rat pancreas (Allan et al., 1976; Banschbach et al., 1974). The concept of the closed-circle metabolism

of MPI is considerably strengthened by the findings of a selective decrease in stearic acid and arachidonate from MPI and a stoichiometric increase in these fatty acids in PA of the acetylcholine stimulated rat pancreas as well as a reversal of these changes when an acetylcholine antagonist was added to the medium (Geison et al., 1976).

In summary, therefore, a closed cycle of reactions seems to be operating, at least in the majority of affected tissues. This cycle of reactions shares two reactions with the synthesis of MPI de novo, but the cleavage of the head group from MPI and the diacylglycerol kinase-catalysed synthesis of PA are metabolic conversions which appear to participate only in this cycle reaction sequence. The increase in ^{32}P -labelling in MPI in stimulated tissues is therefore secondary to the phosphodiesteratic cleavage of MPI and $^{32}\text{PO}_4$ is incorporated into the newly synthesized MPI molecules during the phosphorylation of 1,2-diacylglycerol to PA. Some of the enzymes involved in these cyclical reactions such as 1,2-diacylglycerol kinase (Kaulen and Gross, 1976), CTP: phosphatidate, CMP transferase (Call and Williams, 1970) and cytidine diphosphate diacylglycerol: myoinositol, phosphatidyl transferase (Lucas et al., 1970) have been shown in platelets in the membrane-bound fractions. Increased incorporation of ^{32}P -orthophosphate into PA and phosphoinositides have been observed in platelets following ADP or thrombin stimulation (Lloyd et al., 1973; Kaulen and Gross, 1976). However, the mechanisms

behind these changes in PA and MPI were not defined in these experiments.

Another pathway of degradation of MPI is mediated by phospholipase A₂ with the formation of free fatty acid and lyso MPI (Figure 3b). Formation of lyso MPI was first detected in pigeon pancreas in response to acetyl- or carbamylcholine (Keenan and Hokin, 1962). Investigation into this area is of interest because the arachidonic acid cleaved from MPI or other phospholipids in stimulated tissues may become the precursor for prostaglandin synthesis. It has been established that some intermediates of the prostaglandin pathway such as the endoperoxides and the thromboxane A₂ are potent platelet aggregating agents (Hamberg et al., 1975). Loss of ¹⁴C-arachidonic acid from MPI of thrombin-stimulated platelets was measured by several investigators (Bills et al., 1976; Rittenhouse-Simmons et al., 1976).

It is also possible that MPI is phosphorylated to form the polyphosphoinositides in tissues in response to stimuli. However, such occurrence has never been reported in literature.

The proposals of involvement of DPI and TPI interconversion in the generation and propagation of action potentials in stimulated neurons began with the observation of rapid turnover of DPI and TPI in nerve trunks (Sheltaw and Dawson, 1969; White and Larrabee, 1973). The key features of all of the proposals have been that TPI has very high affinity for Ca²⁺ and this affinity is lost by 60% when TPI is

converted to DPI (Hendrickson and Reinertsen, 1971). The interconversion of inositol lipids might therefore change the quantity of Ca^{2+} bound to specific sites on nerve membrane. It was considered that these changes might be synonymous with the modulation of membrane-bound Ca^{2+} levels which electrophysiological experiments indicate are involved in the opening and closing of Na^+ and K^+ "gates" and thus in the generation and/or propagation of action potentials.

The loss of ^{32}P -labelling in TPI was first reported by Birnberger et al. (1971) in lobster nerves after long incubations and brief electrical stimulation. White and Larrabee (1973) reported a specific decrease in the labelling of TPI in rat vagus nerve after electrical stimulation for 3 hours. Before their observations, information on the changes in TPI in tissues in response to stimulation was either lacking or controversial. For examples, despite many attempts in the past to alter the labelling of DPI and TPI by the addition of acetylcholine to media bathing brain slices (Palmer and Rossiter, 1965; Hokin, 1969, 1970), sympathetic or vagal ganglia (Hokin, 1965), synaptosomes (Yagihara and Hawthorne, 1972) no significant changes have been found. By contrast Schacht and Agranoff (1972) observed a decrease in the labelling of polyphosphoinositides with ^{32}P -orthophosphate in guinea pig brain cortex subfractions incubated with acetylcholine. But these decreases in labelling were regarded as non-specific by these authors as they did not respond

to the effect of an acetylcholine antagonist. Kaulen and Gross (1976) reported an increase in ^{32}P -labelling in human platelets following thrombin stimulation, but Lloyd et al. (1973) could not find consistent changes in TPI in platelets aggregated by ADP. In the last year, hydrolysis of TPI was confirmed by several investigators. A decrease in TPI content concurrent to an increase in the permeability toward K^+ was measured in crab nerve fibers depolarized by acetylcholine (Tret'jak et al., 1977). Abdel-Latif et al. (1977) reported a decrease in the content as well as ^{32}P -labelling in TPI of rabbit iris muscle treated with acetylcholine and defined this TPI effect being the consequence of the interaction between acetylcholine and the muscarinic receptors and not the nicotinic receptors of the smooth muscle. The same authors (Abdel-Latif et al., 1978) observed similar changes in TPI in sympathetically denervated rabbit iris muscle and indicated that such changes are mediated through α -adrenergic receptors and not through β -receptors.

The distribution of the enzymes involved in DPI and TPI interconversion in the cells is worthy of attention. Unlike most other phospholipid metabolism enzymes, TPI monophosphate hydrolase and DPI kinase are primarily soluble enzymes, although in some tissues these enzymes are also membrane-bound (Kai et al., 1966; Cooper et al., 1976). The distribution of these enzymes strongly suggests that these inositol phospholipids mainly reside on the inner surface of cell membrane. This proposal is further supported by the observation

that phosphorylation of the phosphoinositides is insignificant when external (γ - ^{32}P) ATP serves as a precursor to the sealed erythrocyte ghosts (Redman, 1972).

4. Biological significance of phospholipids

Phospholipids are essential for the structure of all biological membranes. The presence of lipophilic and hydrophilic groups make these molecules highly suitable for interfaces. The hydrocarbon chains present an apolar barrier by lipid-lipid interaction and in addition they interact with the hydrophobic region of membrane proteins. The polar head groups allow electrostatic interaction between lipids and proteins, binding of cations and formation of ternary complexes. The role of phospholipid bilayer in the simplest sense, is therefore to protect the cell interior. This idea is based upon the observation that artificial phospholipid bilayers are impermeable to ions and to other water-soluble compounds (Papahadjopoulos, D., 1973). The phospholipid molecules in some biological membranes are so closely packed that intact erythrocytes are not susceptible to pure phospholipase A_2 attack regardless of the source of the enzymes while phospholipids of erythrocyte ghosts or resealed ghosts previously swollen and lysed are readily hydrolysed. (Zwaal et al., 1973). Similarly, mycoplasma hominis, an organism that lacks a cell wall, is not ingested and killed by intact granulocytes and its phospholipids are not broken down unless

the packing of these molecules is altered by treatment with detergent (Elsbach, 1972). The integrity of the protective phospholipid bilayer in cell membranes is assured by the active turnover of the phospholipid molecules. This is particularly crucial to the well-being of the rapidly circulating blood elements such as platelets and lymphocytes.

The primary role of phospholipids, appears to be, however, not so much their being structural components as they are in membrane function. These include their roles in the selective control of the movements of ions and various solutes, packaging and translocation of macromolecules, or grouping and orientation of vectorially-directed enzyme systems in the membrane. These functions of phospholipids are influenced by the composition and the membrane distribution of phospholipids as well as the protein-phospholipid interaction. These factors in turn determine the fluidity and the mobility of proteins in the membrane.

(i) Effect of phospholipid composition on membrane function

The composition of phospholipids may influence membrane permeability in several ways. For instance, by the closeness of molecular packing and by activation of the enzymes participating in membrane transport. Essentially, substitution of long chain or saturated fatty acids with short chain or unsaturated fatty acids in phospholipids causes expansion of phospholipid membrane (Van Deenen et al., 1962). Modification of the polar head group with

unchanged acyl glycerol back bone of phospholipids may similarly alter the assembly of phospholipids and affect the osmotic fragility of biological membranes. The above situations are illustrated by the following examples. *Mycoplasma laidlawii* can incorporate into their membrane phospholipids large amounts of saturated fatty acids (up to 80%) (Razin et al., 1966). Depending on the particular fatty acid accumulated and the growth temperature, this organism may change from filamentous to rounded swollen structures which cease to grow and lyse. The great variation of acyl substituents in phospholipids is also important in the maintenance of membrane fluidity over a wide range of temperatures. The fluid state of membranes makes morphological changes such as pseudopod formation possible in phagocytosing cells. In general, each type of acyl chain has its own phase transition temperature below which the fatty acid molecule exhibits stiffness, and unsaturated fatty acids have lower transition temperatures than the saturated ones.

The effect of modification of the polar head group of phospholipids on membrane permeability is observed in gram-positive bacteria such as *staphylococcus aureus* (Heast et al., 1972). The major phospholipids in these bacteria are phosphatidylglycerol and lysylphosphatidylglycerol. The two lipids have the same fatty acid composition. Phosphatidylglycerol, however, has a negatively charged head group, is more bulky and prevents close packing

of the acyl chains. Membrane composed mainly of phosphatidylglycerol exhibits cation selectivity. Lysylphosphatidylglycerol however, is positively charged and anion selective. It was found that valinomycin is highly active as a monovalent cation carrier through the bacterial membrane enriched with phosphatidylglycerol. In contrast, the ionophore appears to be immobilized with a lysylphosphatidylglycerol-rich bilayer in the same kind of bacteria.

The relationship between membrane permeability and phospholipid composition is further complicated by the complex interaction between phospholipids and membrane proteins. It has been observed that incorporation of proteins into artificial phospholipid bilayers greatly increases the permeability of the latter. During the reconstitution of the sodium pump (Tanaka and Sakamoto, 1969) and calcium pump (Warren and Metcalfe, 1978) it was found that phospholipids are required for the activity of $(\text{Na}^+ - \text{K}^+) - \text{ATPase}$ and $\text{Ca}^{2+} - \text{ATPase}$. Warren and Metcalfe (1978) reported that unsaturated phospholipids and a mixture of two phospholipids are the best for Ca^{2+} influx and retention. When dipalmitoylphosphatidylcholine is tested, uptake of Ca^{2+} is not efficient as measured by $\text{Ca}^{2+}/\text{ATP}$ ratio. The interpretation is that the saturated acyl chains are too rigid in the membrane. For phospholipids with different head groups, zwitterionic phospholipids appear to support a higher activity of the enzyme than phospholipids with a net negative charge. Dioleoylphosphatidic acid which has two negative charges support even lower activity of the $\text{Ca}^{2+} - \text{ATPase}$.

Interconversion between TPI and DPI as previously discussed is another example that alteration of phospholipid composition may change the permeability of membrane toward monovalent cations. Hydrolysis of TPI with concurrent release of bound Ca^{2+} by the appropriate enzymes may modify the charge of the sodium channels in the membrane (Hendrickson and Reinertsen, 1971). It has been observed in erythrocyte ghosts that the amount of Ca^{2+} bound to the membrane is in proportion to the amount of polyphosphoinositides present in the membrane (Buckley and Hawthorne, 1972).

(ii) Effect of phospholipid distribution on membrane function

Asymmetry of plasma membrane composition has always been suspected. Yet evidence for asymmetric distribution of phospholipids in cell membranes has only been revealed recently. It is now established that in eukaryotic cells the choline-containing phospholipids are preferentially localized in the outer leaflet of the bilayer (70 and 80% for PC and sphingomyelin respectively) while MPI, PS and PA are concentrated in the inner leaflet (Verkleij et al., 1973; Renooj et al., 1976; Emmelot and Van Hoven, 1975; Kahlenberg et al., 1974). The distribution of PE is also asymmetric, with approximately 70% in the inner leaflet (Bretscher, 1972; Haest and Deuticke, 1976). Similar distribution of phospholipids has been found in human platelets (Shick, 1976). The major phospholipids of the endoplasmic reticulum have a distribution opposite to that in the plasma membrane (Depierre and Dallner, 1975). The

asymmetry of phospholipid distribution in membranes may be controlled by some unknown inner proteins (Tanaka and Ohnishi, 1976). In addition, the flip-flop rates of phospholipid molecules between the inner and outer leaflet are rather slow. It was estimated in the erythrocyte membranes that the half-time for flip-flop is a matter of days at 37°C (Rothman and Dawidowicz, 1975). The asymmetric distribution of phospholipids in cell membranes results in a difference of microviscosity in the inner and outer leaflets, with the outer leaflet apparently more rigid than the inner one (Tanaka and Ohnishi, 1976). Although more rapid, phospholipid molecules in the outer leaflet participate in the exchange reactions with phospholipid molecules in the environment. In contrast, phospholipid molecules in the inner leaflet do not undergo similar exchange reactions (Rothman and Dawidowicz, 1975).

The behaviour of phospholipids in the membrane is also influenced by the presence of cholesterol as mentioned previously. The amount of cholesterol in the bilayer (presumably the outer leaflet) can be increased to such an extent that the killing power of a malignant cell is markedly inhibited (Inbar and Shinitzky, 1974).

Another biological significance of asymmetric distribution of membrane phospholipids is their influence on the orientation of membrane-bound enzymes. For instance, enzymes such as 5'-nucleotidase, Ca²⁺-ATPase, cholinesterase have their catalytic sites facing the external surface while enzymes adenylate cyclase, MPI

kinase, Na^+ , K^+ -ATPase and glyceraldehyde-3-phosphate dehydrogenase have catalytic sites at the cytoplasmic surface (Trams, 1977).

In addition to the roles mentioned above, phospholipids and their metabolic products have highly diversified properties. For instance lysophospholipids which are products of the action of phospholipases A on diacylphospholipids are powerful lytic agents. Addition of lysocompounds promptly causes lysis of mammalian cells. Formation of lysophospholipids in the cell membrane during stimulation of secretory cells has been proposed to be a mechanism leading to fusion of granules to the plasma membranes (Lucy, 1970). Free arachidonic acid cleaved from phospholipids by phospholipase A_2 may become the precursor of prostaglandins. The latter have a broad spectrum of biological influences on cellular functions and have been implicated in various inflammatory processes. In platelets, free arachidonic acid may be converted to endoperoxides and thromboxane A_2 which are potent platelet aggregating agents (Hamberg et al., 1975). 1,2-Diacylglycerol, a product of phospholipase C action, accumulate in red cells treated with ionophore A23,187. The formation of this lipid is considered to be related to erythrocyte transformation from disc shape to spherocyte (Allan et al., 1975). Diphosphoinositide may be involved in arsenate transport in yeast (Carbon, 1970). Secretion of proteins by yeast and of lipoproteins by liver depend upon adequate supply of inositol (Matile, 1966; Yagi and Lotaki, 1969).

Furthermore, 1,2-cyclic phosphate inositol formed from phosphodiesteratic cleavage of phosphoinositides was proposed to be a cyclic AMP-like mediator of cellular response to stimuli (Michell, 1975).

PART III. Aims of Study

Lewis and Majerus (1969) incubated washed human platelets with isotopically labelled glycerol and demonstrated that platelets synthesized the major phospholipid classes de novo. Bills and coworkers (1976) observed that approximately 75% of the ^{14}C -arachidonic acid incorporated into platelet phospholipids were found in PC, PE and PS following 1 h incubation of platelets with the isotope. However, when washed platelets were incubated with ^{32}P -orthophosphate, almost all the radioactivity in phospholipids was located in PA and the phosphoinositides and none of the major phospholipids was significantly labelled (Cohen et al., 1971; Lloyd et al., 1972). In contrast, all major phospholipids of platelets were labelled if ^{32}P -orthophosphate was infused intravenously into animals or therapeutically given to humans (Cohen et al., 1971; Lloyd et al., 1972). Cohen (Cohen et al., 1971) and Marcus (1972) could not account for the differences in the in vitro and in vivo observations and suggested different pathways of $^{32}\text{PO}_4$ -incorporation under these two conditions. In view of the frequency with which washed platelets are used for various biochemical investigations, it is pertinent to determine whether membrane phospholipid metabolism in washed platelets behave differently from platelets in the circulation.

When platelets labelled with ^{32}P -orthophosphate were stimulated by stimuli such as ADP, thrombin or collagen, increased ^{32}P content in PA and DPI was detectable seconds after the addition

of the stimuli. Increase in ^{32}P -labelling in MPI occurred in 2 to 3 min. Increase in ^{32}P -labelling in TPI was measured with thrombin stimulated platelets (Kaulen and Gross, 1976) while Lloyd and coworkers (1973) could not find consistent changes in ^{32}P -labelling in TPI of platelets in response to ADP. In addition, Kaulen and Gross (1976) could not detect changes in ^{14}C -glycerol in PA and MPI 2 min after thrombin treatment of platelets and reported insignificant incorporation of this isotope into the polyphosphoinositide. On the other hand, loss of ^{14}C -arachidonic acid from MPI was reported in platelets treated with thrombin or ionophore A23,187 (Bills et al., 1976; Rittenhouse-Simmons et al., 1977). In view of the importance of MPI as a source for the precursor of prostaglandin synthesis and as a source for diacylglycerol which is implicated in fusion and shape change of erythrocytes (Allan et al., 1976), it is of interest to clarify the above conflicting observations and to map out a pathway for MPI metabolism in platelets in response to stimuli. Furthermore, interconversion of DPI and TPI has always been of theoretical interest in their possible involvement in the regulation of membrane permeability in excitable cells. Experimental evidence for the hypothesis, however, has been scarce. Since platelets contain a high concentration of these polyphosphoinositides and pure preparations of platelets are readily available, it is of interest to investigate whether interconversion of TPI and DPI occurs during platelet aggregation and to determine if the changes are primarily

involved in aggregation or secondary to this process.

The existing evidence has shown that ADP, thrombin and the divalent cation ionophore A23,187 cause platelet aggregation by different pathways. Although the subsequent manifestations of platelet response are the same, that is, platelets undergo shape change, aggregation and/or release reaction. Thrombin and ADP appear to cause platelet aggregation via interaction with the appropriate receptors on the platelet surface, while A23,187 apparently activates the aggregation machinery by directly transporting calcium into platelets or by mobilizing calcium from the intracellular binding sites (Nachman, 1975; Majerus et al., 1976; Feinman and Detwiler, 1974). It is therefore reasonable to expect that the metabolism of PA and phosphoinositides which are among the earliest detectable biochemical changes among mammalian tissues in response to stimuli would demonstrate differences in platelets treated with ADP, thrombin or the ionophore.

Immediate aims of the study were to:

- (1) Determine whether incorporation of ^{32}P -orthophosphate into major phospholipids occurs in unstimulated washed platelets.
- (2) Investigate whether hydrolysis of TPI occurs in platelets within the first minute of ADP stimulation, and whether interconversion between DPI and TPI occurs during platelet deaggregation and whether these changes can be abolished by an inhibitor of ADP-induced platelet aggregation.

- (3) Determine whether there are differences in PA and phosphoinositide metabolism in platelets in response to ADP and ionophore A23,187 within the first minute of stimulation.
- (4) Study the different pathways of MPI metabolism and change in DPI and TPI in thrombin-stimulated platelets. These changes were compared with those occurring in response to ADP and the ionophore A23,187.

CHAPTER TWO

MATERIALS AND METHODS

A. MATERIALS

1. Phospholipids used as Reference Standards for Thin Layer Chromatography

All phospholipid standards were purchased from Supelco, Inc., Pennsylvania, or from Sedary Research Laboratories Inc., London, Ontario. These standards were high purity lipids, containing negligible trace contaminants as revealed by thin layer chromatography. Phospholipids were stored at -20°C either as the powder or as a solution in benzene, or methanol-chloroform (2:1 v/v). Precautions were taken so that no silica gel particles were introduced into the lipid samples and the containers were flushed with nitrogen gas before storage.

The phospholipid standards include:

- (a) MPI (pig liver)
- (b) PG (phosphatidylglycerol, bacterial)
- (c) PA (egg lecithin)
- (d) PS (bovine brain)
- (e) PE (pig liver)
- (f) PC (bovine brain)
- (g) SPH (bovine brain)
- (h) LPE (lysophosphatidyl ethanolamine, pig liver)
- (i) DPI and TPI. These were a gift from Dr. K.M.W. Keough, Department of Biochemistry, University of Toronto, Ontario, Canada.
- (j) LPC (lysophosphatidylcholine, bovine brain)

2. Neutral lipid

1,2-Diacylglycerides (pig liver lecithin, Sedary Research Laboratories Inc., Ontario).

3. Radioactive Chemicals

(i) ^{32}P -orthophosphate ($^{32}\text{PO}_4$) free of carrier PO_4 was purchased as a solution in 0.1 N HCl from New England Nuclear, Boston, Massachusetts. It was diluted by a factor of 10 with distilled water before use. HCl was used for storage of $^{32}\text{PO}_4$ because it has been shown that this inhibits the polymerization of PO_4 to pyrophosphate and other polyphosphates (Zelenay and Pasternak, 1966).

(ii) myo-2- ^3H (N)-inositol (specific activity 17.4 Ci/mmol) was obtained as a solution in 60% (v/v) ethanol from New England Nuclear, Boston, Massachusetts.

(iii) 2- ^3H (N)-glycerol (specific activity 6.35 mCi/mmol) was obtained as an aqueous solution from New England Nuclear, Boston, Massachusetts.

(iv) 1- ^{14}C -arachidonic acid (specific activity 61 mCi/mmmole) was obtained as a solution in benzene from New England Nuclear, Boston, Massachusetts.

4. Potato apyrase (E.C. 3.6.1.5). This was prepared from potatoes by the method of Molnar and Lorand (1961) modified by dialysing the final material against 0.9% NaCl (Mustard et al., 1972). The solution was stored at -20°C .

5. Solutions used for wash . suspending platelets.

(i) CF Tyrodes. This Tyrodes buffer contained 0.1% glucose, 0.35 g/100 ml albumin (fraction V, Pentex, Miles Laboratories, Kankakee, Illinois), 2×10^{-3} M Mg^{2+} and no Ca^{2+} . EGTA (0.2×10^{-3} M) was added to this medium.

(ii) Tyrodes Albumin Solution. The suspending medium was Tyrodes buffer, pH 7.35, containing 0.35 g/100 ml albumin, 0.1% glucose, 2×10^{-3} M Ca^{2+} and 1×10^{-3} M Mg^{2+} . As suggested by Ardlie (Ardlie et al., 1971), small amounts of potato apyrase were added to the suspending medium, such that 1.8 nmole ATP/min/ μ g protein and 300 nmole ADP/min/ μ g protein were degraded (Reimers, 1977).

For rabbit platelets, the osmolarity of the washing and suspending solutions was 280 milliosmoles per litre. The same washing and suspending solutions were used for human platelets.

6. Solutions added as Test Substances to Platelet Suspensions

(i) Modified Tyrodes solution. This was Tyrodes buffer modified to contain no Ca^{2+} or Mg^{2+} ions. Most of the test substances were dissolved in this solution and this solution was also used as a control.

(ii) ADP and AMP purchased as the sodium salts from Sigma Company, St. Louis, Missouri, were dissolved in modified Tyrodes solution and buffered to pH 7.35.

(iii) Thrombin. Crude bovine thrombin purchased from Parke-Davis, Detroit, Michigan was dissolved in modified Tyrodes solution.

(iv) CaCl₂ was 0.2 M in distilled water.

(v) Ethyleneglycoltetraacetic acid (EGTA) purchased from Koch-Light Laboratories Limited, Colnbrook, Bucks, England was prepared as a 0.1M solution in deionized distilled water and then diluted to 5×10^{-2} M with modified Tyrodes buffer.

(vi) Fibrinogen was lyophilized human fibrinogen (Grade L) from Kabi Stockholm, Sweden. This was prepared as a 4% solution and treated with diisopropylfluorophosphate (DFP) for 60 minutes at 37°C. The final concentration of DFP was 10^{-3} M.

(vii) Ionophore A23,187 (M.W. 523) was a gift from Eli Lilly Company, Indianapolis, Indiana. A23,187 was dissolved in dimethylsulfoxide (DMSO).

7. ATP was purchased as sodium salt from Sigma Company, St. Louis, Missouri and was dissolved in modified Tyrodes buffer before used as a marker in paper electrophoresis of nucleotides.

8. Compounds used in the preparation of thrombin-degranulated platelets.

(i) PGE₁ (prostaglandin E₁) was a gift from Upjohn Company, Kalamazoo, Michigan. It was prepared as a stock solution (2.9×10^{-3} M) in 95% (v/v) ethanol (0.1 ml/mg) and 0.02% Na₂CO₃ solution (0.9 ml/mg). This was stored at -20°C. Dilutions of the stock solution were made with modified Tyrodes solution.

(ii) Pig plasmin (4.17 NE/mg, Batch 74-21) was from Novo Fabrik, Copenhagen, Denmark.

(iii) TAMe (p-tosyl-L-arginine methylester (HCl) was obtained from Mann Research Labs., Inc., New York 6, New York.

(iv) Hepes (N-2-hydroxy-ethyl-piperazine-N'-2-ethane-sulfonic acid) was from Sigma Chemical Company, St. Louis, Missouri.

(v) Heparin was from Connaught Laboratories, Toronto, Ontario, Canada.

(vi) Soya bean trypsin inhibitor (SBTI) was obtained from Sigma Chemical Company, St. Louis, Missouri.

9. Organic solvents. These were reagent grade and were distilled before use.

10. Silica gel H was purchased from E. Merck, Darmstadt, Germany, and silica gel G (with binder) was distributed by Brinkman Instrument Limited, Rexdale, Ontario, Canada.

B. METHODS

Experiment A. In vitro and in vivo Incorporation of ^{32}P -
orthophosphate into Platelet Phospholipids.

1. Preparation of Platelet Suspensions

Rabbit or human platelet suspensions were prepared by the method of Ardlie et al. (1970) and Mustard et al. (1972) with some modifications. Blood was collected into acid citrate dextrose (6 vols. of blood to 1 vol. of anticoagulant) (Aster and Jandl, 1964). The blood was centrifuged at room temperature for 15 minutes at 190 x g. Platelet-rich plasma (PRP) was obtained and centrifuged for 15 minutes at 190 x g to remove red cells. The platelet-rich plasma (PRP) was then centrifuged at room temperature for 15 minutes at 2500 x g. The platelets obtained from PRP were washed at room temperature in a calcium-free and phosphate-free modified Tyrode solution (pH 6.2) containing 0.35% albumin and 0.01% EGTA (ethylene glycol bis (β -aminoethylether)-N-N¹-tetraacetic acid). After centrifugation at 1200 x g for 10 minutes, the platelets were resuspended in a phosphate-free modified Tyrode solution containing 0.35% albumin (pH 7.35) and apyrase (10 μ l/ml). (The enzyme apyrase degrades adenosine diphosphate (ADP) and adenosine triphosphate (ATP) that may possibly leak from platelets to adenosine monophosphate (AMP), thereby preventing the platelets from becoming refractory to aggregating stimuli. The activity of apyrase was such that it caused the hydrolysis of 18 nmole/ATP/min/ μ g protein and 300 nmole ADP/min/ μ g of protein (Reimers, 1977).

The platelet suspension was incubated for 60 min at 37°C with 50-60 μCi of carrier-free $^{32}\text{PO}_4$ per ml. Aliquots of the platelet suspension were subjected to lipid extraction at 15 min, 30 min, and 60 min. Tyrode-albumin solution containing unlabelled phosphate (8.3×10^{-3} M) was added to the suspension at the end of the labelling period. The suspension was then centrifuged at 1200 x g for 10 min. The platelets were washed once more in a calcium-free modified Tyrode solution containing 0.35% albumin and resuspended in Tyrode-albumin solution containing apyrase. The platelet count of the final suspension was adjusted to be between 0.8×10^6 and $1.2 \times 10^6/\text{mm}^3$ for rabbit platelets and between 3×10^5 and $6 \times 10^5/\text{mm}^3$ for human platelets. The platelet suspensions were kept at 37°C throughout the experiment. Every six hours, the platelets were transferred to fresh medium to eliminate the lactic acid produced by the platelets and to renew the glucose supply for the platelets. Throughout the experimental period, the pH of the suspension was maintained at 7.35 with a 5% CO_2 -air mixture in the atmosphere above the platelet suspension. Aliquots of the platelet suspension were subjected to lipid extraction at hourly intervals. In order to rule out the possibility of ^{32}P -incorporation as a consequence of bacterial growth over these prolonged incubation hours, in some experiments all the used materials were sterilized by heat and the solutions sterilized by filtration through a membrane filter (Millipore pore size 0.22 μ) and the bacterial growth was monitored by bacterial culture of the platelet suspension on horse blood agar petri dishes.

2. Animal Studies

Rabbit platelets obtained from six rabbits were labelled with $^{32}\text{P}\text{O}_4$ in vitro as described above. Approximately 8.8×10^9 of the labelled platelets in 2.5 ml of platelet suspending medium was injected intravenously into each of seven rabbits whose total number of platelets had been estimated on the basis of the peripheral blood platelet count and on the assumption that the blood volume is about 58 ml per kg (Reimers et al., 1973). The dilution factor was calculated for each animal, which was the fraction of the number of infused platelets over the sum of platelets of the recipient rabbit and the infused platelets. The labelled platelets accounted for about 10-15% of the total number of platelets in the host rabbit. Using the dilution factor, the amount of phosphorus in the individual phospholipids (obtained by phosphorus assay, see below) of the infused platelets can be computed. The carotid arteries of recipient rabbits were cannulated at different times and platelet suspensions were prepared from their blood and subjected to lipid extraction. Assuming that the ^{32}P -labelled platelets were completely mixed with those of the host rabbit in the circulation, that the labelled platelets and the recipient rabbit's own platelets were removed from the circulation at the same rate, and that the average survival time of rabbit platelets is 4 days (Reimers, H.-J., Personal Communication), we estimated the fraction of ^{32}P -labelled platelets lost from the circulation during the experimental period and determined that 1% of the infused platelets was lost per hour.

A rabbit ~~exsanguinated~~ 34 hours after platelet injection would thus have 66% of the infused platelets left. This correction factor together with the dilution factor were applied to the quantitative analysis of the platelet phospholipids. Although it is realized that a number of assumptions have had to be made in making these calculations, the values obtained do allow examination of the changes among the different phospholipids.

3. Lipid extraction

Lipids were extracted from the platelets by a modification of the method of Bligh and Dyer (1959). For the extraction of DPI and TPI, the extracting solvent consisted of chloroform, methanol, 10 N HCl and butylated hydroxytoluene in a ratio of 125:250:20:0.9 (v/v/v/v). Butylated hydroxytoluene was used as an antioxidant. The extracting solvent (12 ml) was added to 2.4 ml of platelet suspension and thoroughly mixed for 30 sec. The one phase mixture was immediately separated into 2 phases by addition of 3.75 ml CHCl_3 and 3.75 ml of H_2O . The mixture was mixed for 1 minute and centrifuged for 3 minutes at 1000 r.p.m. in a Soval II Centrifuge. The CHCl_3 lower phase was transferred to another test tube. Each aliquot of the platelet suspension was extracted four times and the chloroform extract of lipids was made up to 5 ml. For the extraction of PC, PE, PS, SPH, MPI and PA, a neutral solvent was used. The solvent consists of CHCl_3 : CH_3OH :hydroxybutylated toluene (125:250:0.9 v/v/v). The solvent (9 ml) was added to 2.4 ml of platelet suspension and mixed

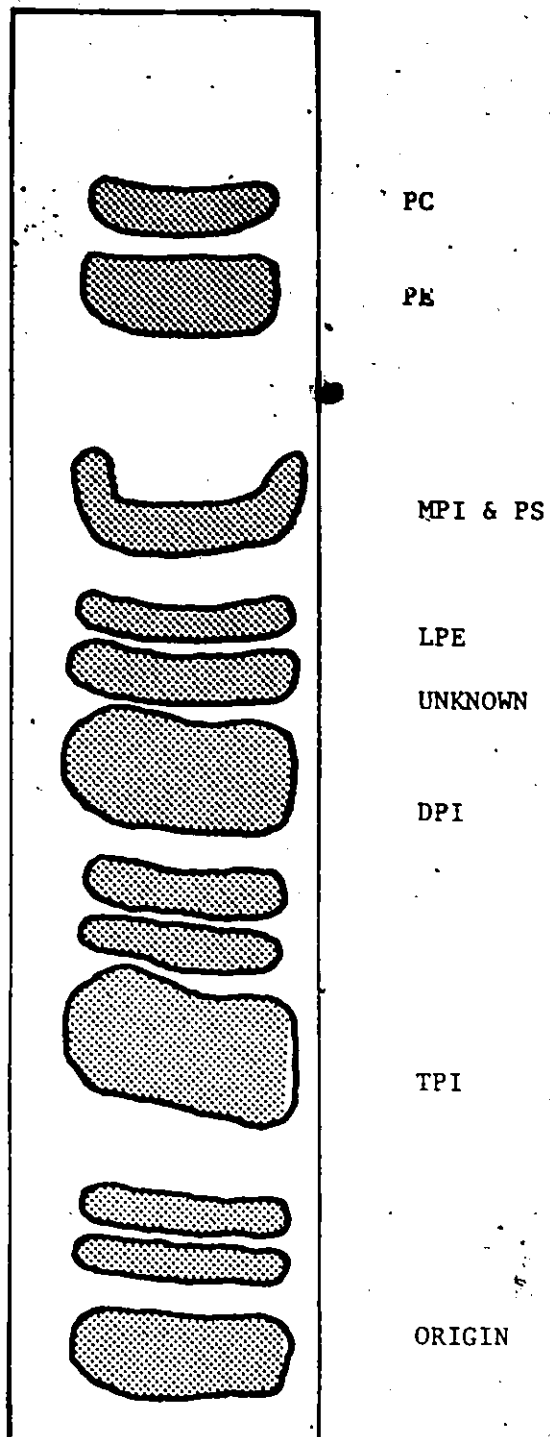


Figure 4. Thin layer chromatography on 1% potassium oxalate silica plate using a mixture of chloroform, methanol, 28% ammonia and water (25:25:4:5.25 v/v/v/v) as solvent. TPI and DPI of platelets labelled with $^{32}\text{PO}_4$ were separated from other phospholipids as revealed by the X-ray film.

for 1 minute. The mixture was kept in darkness at room temperature for two hours and was mixed for 30 sec every half an hour. In order to separate the one phase mixture into 2 phases, 3 ml of chloroform and 3 ml of 2 M KCl solution were added and mixed. The mixture was centrifuged and the CHCl_3 phase was transferred to another test tube. The aqueous phase was further extracted with 2 ml of CHCl_3 three times. The CHCl_3 extract was evaporated in nitrogen to dryness and redissolved in CHCl_3 immediately. The final volume of CHCl_3 extract was made up to 5 ml in a volumetric flask.

4. Thin layer chromatography

Method 1 is a modification (Lloyd et al., 1972) of the methods of Gonzalez-Sastre and Folch-Pi (1968) and is a one dimensional thin layer chromatography technique done on 1% potassium oxalate silica H plates using a mixture of chloroform, methanol, 28% ammonia and water (25:25:4:5.25 v/v/v/v) as solvent. The humidity was between 40 - 50%. After the solvent front reach the top, the TLC plate was further developed for another 20 min. This method was used to separate triphosphoinositide (TPI) and diphosphoinositide (DPI) from other lipids (Figure 4).

Method 2 is a two dimensional thin layer chromatography method using magnesium acetate plates (0.5 gm of magnesium acetate and 27 gm silica H in 71 ml water). This is a modification of the method described by Rouser et al. 1970). The plates were activated at

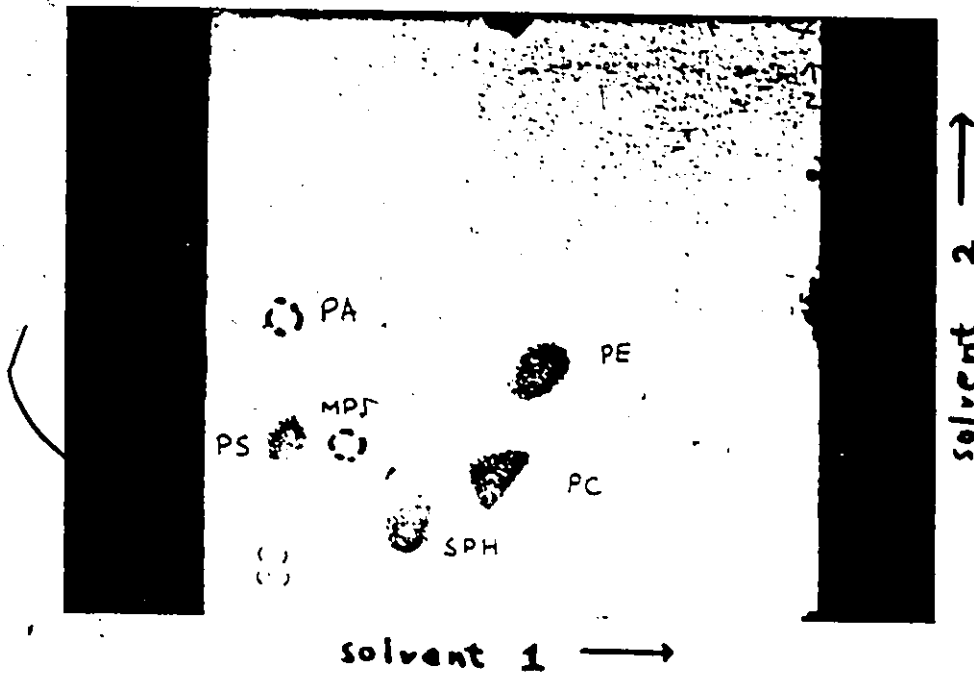


Figure 5(a). Two dimensional thin layer chromatography using magnesium acetate plate. First solvent was chloroform, methanol and 28% ammonia (44:35:5 v/v/v). Second solvent was a mixture of chloroform, methanol, acetone, acetic acid and water (195:97.5:78:65:19.5 v/v/v/v/v).

120°C for 20 minutes before use. The TLC plates containing the lipid sample were first run in a solvent of chloroform, methanol and 28% ammonia (44:35:5 v/v/v). When the solvent reached the top, the plate was allowed to develop another half hour. The plate was then dried in a current of nitrogen and rerun in an acidic solvent consisting of chloroform, methanol, acetone, acetic acid and water (195:97.5:78:65:19.5 v/v/v) in a direction perpendicular to the first one. Humidity was regulated between 40-50%. By this method, phosphatidic acid (PA), phosphatidylinositol (MPI), phosphatidylserine (PS), phosphatidylcholine (PC), sphingomyelin (SPH), lysophosphatidylcholine (LPC), lysophosphatidylethanolamine and phosphatidylethanolamine were separated from one another (Figures 5(a) and 5(b)). The separated phospholipids were scraped directly into vials and their radioactivity determined by liquid scintillation counting. Individual phospholipids were identified by running authentic standards, using Dittmer-Lester staining (Kates, 1972), ninhydrin staining (Kates, 1972), Dragendorff staining (Kates, 1972), iodine staining and autoradiography.

5. Liquid scintillation counting

Individual phospholipids were scraped from the TLC plates and transferred into scintillation vials. The scintillation fluid contained 2 ml absolute alcohol and 10 ml of scintillator solution composed of 5 g of 2,5-diphenyloxazole and 0.3 g of 2,2-l-phenylenebis-(5-phenyloxazole) in 1 litre of toluene. Radioactivity (cpm) of individual phospholipids was corrected for decay that had occurred

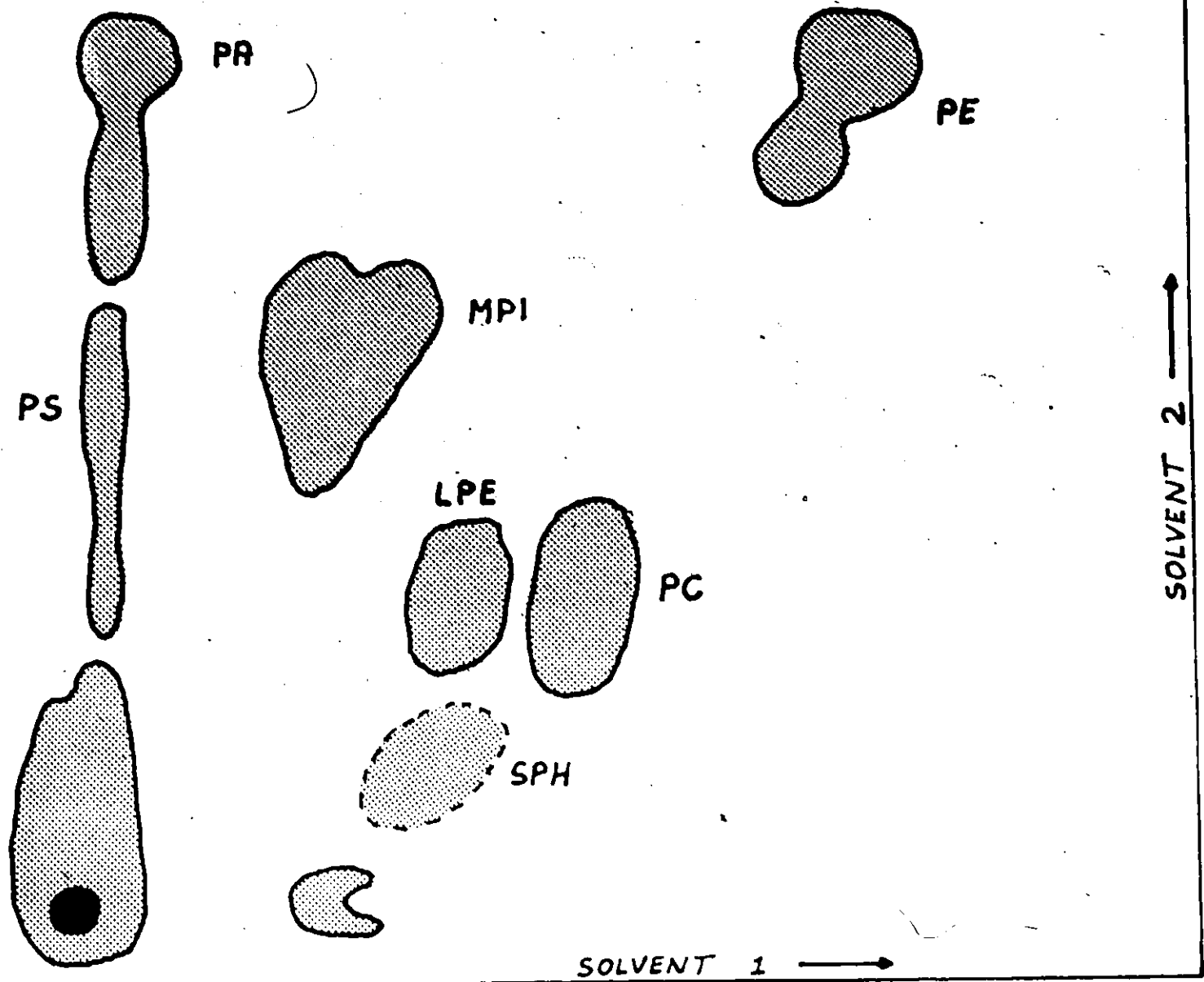


Figure 5(b). X-ray film of the two dimensional chromatography method using magnesium acetate plate. The solvents were the same as in Figure 5(a). The ^{32}P radioactivity in sphingomyelin was too little to be visible on the film.

between the day of ^{32}P -incorporation and the day of counting the samples.

6. Phosphorus assay

Phospholipids separated by thin layer chromatography were scraped into test tubes free of phosphate. Phospholipids used for phosphorus assays were obtained from samples containing three times as many platelets as other samples used for the study of ^{32}P -radioactivity. Phosphorus was assayed by the method of Rouser et al. (1970). PA, MPI, DPI and TPI were assayed by the "small spot method"; PC and other major phospholipids were assayed by the "big spot method" (Rouser et al., 1970).

7. Recovery of phospholipids

Phospholipid standards PC, PE, PS, PA and MPI obtained from Supleco (Pennsylvania, U.S.A.) were checked for purity by thin layer chromatography before being used for the recovery study. A known amount of phospholipid was spotted on TLC plates and scraped into test tubes for phosphorus assay. Equal amounts of phospholipid solution mixed with platelet suspending medium were subjected to the Bligh and Dyer extraction procedure, followed by thin layer chromatographic separation. The spot was then scraped and assayed for phosphorus. The experiment was carried out in triplicate. The percentages recovered were MPI - 63 ± 3 ; PC - 89 ± 5 ; PE - 80 ± 6 ; PS - 84 ± 5 ; PA - 61 ± 3 . The recovery study of DPI and TPI carried

out by Lloyd (1972 thesis) of this laboratory using an acidified Bligh and Dyer solution showed that the percentage recovery for DPI and TPI was 93 ± 6 and 76 ± 8 respectively. This method is the most effective method available for extraction of polyphosphoinositides (Lang et al., 1977). The recovery study of DPI and TPI was also carried out by preparing ^{32}P -labelled DPI and TPI from the platelets. A known amount of ^{32}P -labeling of DPI or TPI was spotted on TLC plates and scraped for radioactivity counting in liquid scintillation vials. An equal amount of DPI or TPI was added to unlabelled platelet suspension and subjected to lipid extraction. The presence of platelets was to minimize the loss of ^{32}P -labeled DPI or TPI since the quantity of these labeled lipids was very small. The lipids were then separated by TLC method 1. and the radioactivity was counted. The percentage recovery of TPI or DPI was DPI - 76 ± 6 ; TPI - 70 ± 8 . Using the ^{32}P -labelling method, the percentage recovery of PA or MPI was MPI - 62 ± 4 ; PA - 65 ± 7 . The relatively low recovery of DPI and TPI by the second method could be due to some breakdown of these lipids during preparation of the radioactive material. The increased recovery of PE and PA compared to that of Lloyd (Lloyd, thesis, 1972) could be due to the use of a neutral extracting solvent in these studies. It has been found that the use of an acidic extracting solvent caused extensive breakdown of PE to lyso PE. Phosphatidylethanolamine and phosphatidic acid in mammalian cell membranes contain considerable amounts of plasmalogen (White, 1973) species and they are sensitive to acid degradation.

Experiment B. Changes of Isotopic Labeling in Phosphatidic Acid
And Phosphoinositides of Rabbit Platelets
in Response to ADP.

1. Preparation of platelet suspension.

Platelet suspension labelled with $^{32}\text{PO}_4$ were prepared in the same way as in Experiment A. The final suspension contained 1.5×10^6 platelets/ mm^3 . Some platelet suspensions were labelled with 1- ^{14}C -arachidonic acid (0.4 $\mu\text{Ci/ml}$), 2- ^3H -inositol (20 $\mu\text{Ci/ml}$) or 2- ^3H -glycerol (20 $\mu\text{Ci/ml}$). Uptake of these isotopes into platelets in one hour was 10-20% for ^{14}C -arachidonic acid and glycerol, less than 1% for ^3H -inositol and approximately 10% for ^{32}P -orthophosphate. Methods were similar to the preparation of ^{32}P -labelled platelets except that the washing solutions contain unlabelled phosphate.

2. Incorporation of $^{32}\text{PO}_4$ into TPI of platelets.

During the one hour incubation with ^{32}P -orthophosphate, aliquots of platelet suspension were subjected to lipid extraction at 15 min, 30 min and 60 min. The remaining platelets were washed and resuspended in Tyrode-albumin solution (pH 7.35, 37°C) containing unlabeled phosphate (8.3 mM). Aliquots of this suspension were subjected to lipid extraction at various times up to 6 h after the addition of $^{32}\text{PO}_4$. After the phospholipids were separated by thin layer chromatography, the phosphorus content in TPI was assayed by the small spot method of Rouser et al. (1970). The reactivity in the TPI samples was counted and the specific radioactivities were calculated.

3. Incorporation of $^{32}\text{PO}_4$ into adenosine triphosphate of platelets

$^{32}\text{PO}_4$ incorporation into ATP by the platelets was examined.

At 1, 3, 5 and 11 h aliquots of platelet suspension (0.2 ml) were mixed with perchloric acid (70%, 0.3 ml, 0°C , 10 min). Potassium citrate solution (27%, 0.2 ml) and methyl orange (1 drop) were added. The ^{32}P -labeled adenine nucleotides were separated from each other and from inorganic phosphate by low voltage paper chromatography for 16 h at 4°C , with citrate buffer (0.12 M, pH 4.78) (Reimers et al., 1975). The separated compounds were then counted for radioactivity. Aliquots of 0.5 ml platelet suspension were also taken at these time intervals for the assay of platelet ATP content by the luciferin-luciferase method (Holmsen et al., 1972). The specific radioactivity of ATP at these time intervals was calculated. These calculations were based on the total ATP content of the platelets since it has been shown elsewhere (Reimers et al., 1975) that the metabolic and storage pools of ATP slowly equilibrate over long incubation periods.

4(a). Aggregation studies

Concentrations of all chemical agents are expressed as final concentrations throughout this thesis except when specified. Samples (2.3 ml) of the platelet suspension were placed in siliconized cuvettes and stirred in Payton aggregometers (Payton Co., Rexdale, Ontario) for 1 min at 37°C . One control sample and one ADP sample were run simultaneously in aggregometers. One hundred microliters of ADP dissolved in Tyrode's solution was added to the platelets suspension. Tyrode solution was added to the control sample. The concentrations of ADP given

in the tables and figures are the final concentrations in the platelet suspension. The samples were subjected to lipid extraction at the same time by emptying the contents of the cuvettes directly into 8 ml portions of the extracting solvent (see "Extraction of Phospholipids"). Each cuvette was rinsed twice with 2 ml portions of the extracting solvent. Lipid extraction was carried out at 7, 20, 45 or 60 sec and 2.5, 10, 20 or 30 min after the addition of ADP or Tyrode solution. The extent of platelet aggregation was determined by recording the changes in light transmission through the stirred suspension. Samples of 20 or 30 min were stirred in the aggregometers for the first 10 min and the last 3 min.

During the short time interval studies, three to seven pairs of samples (control and stimulated platelets) at each time (7, 20, 45 and 60 sec) were prepared in each experiment. The experiment was repeated at least three times on different days so that a total of 9-20 pairs of samples at each time interval was obtained.

In some experiments ADP was added to give a final concentration of 4 μ M. Three minutes or 30 min afterwards, an equal amount of ADP was added. The effect of a second addition of ADP on TPI labelling was studied. The platelet suspensions were subjected to lipid extraction 1.5 min after the second addition of ADP. Control experiments were done in which modified Tyrode solution replaced the ADP solution. The 30 min samples were stirred in the aggregometer for the first 10 min and the last 3 min. In each experiment, four sets of samples for the

3 min studies and four sets of samples for the 30 min studies were prepared. Each experiment was repeated at least three times.

4(b). Inhibition of ADP-induced platelet aggregation by AMP

To examine whether the changes in PA and phosphoinositide metabolism is related to platelet aggregation, AMP which is a specific inhibitor of ADP effects was added to ^{32}P -labelled platelet suspension 1 min before addition of ADP. Four studies with stimuli Tyrode, Tyrode; Tyrode, ADP; AMP, Tyrode; AMP, ADP were run simultaneously in the aggregometers. The platelets were subjected to lipid extraction 1½ min after the addition of the second agent. The experiment was repeated 3 times, each containing 5-7 pairs of samples. Concentration of ADP was 0.9 μM . Concentration of AMP was 0.7 mM.

5. Lipid extraction and thin layer chromatography

See Experiment A.

6. Liquid scintillation counting

The scintillation fluid used was the same as in Experiment A. The separated phospholipids were scraped into scintillation vials. Extra silica gel was added when necessary so that control and experimental samples contained the same amount of silica to minimize differences in quenching.

7. Statistical analysis

The statistical analysis of the experimental data was based on

using the proportionate (percentage) changes of ^{32}P -labelling in individual phospholipids as compared to the control sample rather than actual differences. Logarithms of the original data (counts per minute) were taken and the differences of the corresponding pairs of logged data were then analysed, using analysis of variance procedures. Since in some experiments the magnitude of the variation within an experiment or a group increased with the mean level of the percentage changes, the problem of changes in the magnitude of the variation was avoided by taking the logarithms of the original data. The overall mean and standard error for the percentage changes were estimated taking into consideration the possible systemic variation from experiment to experiment. The accuracy of the mean was indicated by the 95% confidence interval for the true value.

Experiment C. Comparison of Changes in Isotopically Labelled Phosphatidic Acid and the Phosphoinositides of Platelet in Response to Ionophore A23,187 and ADP.

1. Preparation of platelet suspension and thrombin-degranulated platelets

Platelet suspensions labelled with $^{32}\text{PO}_4$ was prepared in the same way as in Experiment A. Platelets labelled with $1\text{-}^{14}\text{C}$ -arachidonic acid was prepared similarly except that the platelets were first washed

In Tyrode solution containing phosphate. $1\text{-}^{14}\text{C}$ -arachidonic acid (0.4 $\mu\text{Ci/ml}$) was added to the second washing solution of Tyrode albumin and incubated with the platelets for 1 h.

In experiments requiring thrombin degranulated platelets, the $^{32}\text{PO}_4$ -labelled platelet suspensions were divided into two equal parts and kept at 37°C . One part was treated with thrombin (0.45 units/ml) to degranulate the platelets. The concentrations of all chemicals referred to the final concentration. Tyrode was added to the other part as control and was later treated in the same way as the thrombin degranulated platelets. After 1 min, aliquots of suspensions were measured for the amount of nucleotide released at 259 nm as an indicator of the release reaction.

At this time, plasmin (0.025%) was added to both the control and thrombin treated platelets. PGE_1 ($10\mu\text{M}$) was added at the same time. PGE_1 restored the disc shape of the platelets and prevented plasmin from causing the release reaction (Niewiarowski et al., 1973). Plasmin digested the fibrin formed during the treatment of platelets with thrombin. Platelets were incubated with PGE_1 and plasmin for 30 min. TAME (1 mM) and soya bean trypsin inhibitor (0.025%) were then added. The platelets were centrifuged at $1,200 \times g$ and resuspended in fresh, calcium-free Tyrode albumin containing heparin (50 units/ml) and "heparin cofactor" (100 μl of rabbit serum/ml suspension) to neutralise any thrombin left in the suspending medium. The suspensions were centrifuged at $1,200 \times g$ and the platelets were resuspended in Tyrode albumin solution containing apyrase and 5 mM HEPES

to maintain a pH of 7.3. The platelet count was adjusted to $10^6/\text{mm}^3$, Non-degranulated washed rabbit platelets labelled with ^{14}C -arachidonic acid ($0.4 \mu\text{Ci}/\text{ml}$) were prepared as in Experiment B.

2. Aggregation studies

Duplicate samples of 1.5 ml of thrombin-degranulated platelets and duplicate samples of 1.5 ml of non-thrombin treated platelets were placed in a cuvette in the aggregometer at the same time. Ten microliter of A23,187 dissolved in DMSO (6×10^{-7} M final) was added to one thrombin-degranulated sample and to one non-thrombin degranulated sample. Tyrode solution was added to the remaining two samples as control. The platelet suspensions were subjected to lipid analysis 10 sec and at 60 sec after the addition of A23,187 or Tyrode.

To study the effect of A23,187 on $1\text{-}^{14}\text{C}$ -arachidonic acid labelled phospholipids of non-thrombin degranulated platelets in the presence or absence of calcium, calcium chloride solution (6.6×10^{-3} M) or Tyrode was added to 1.5 ml of platelet suspension. A23,187 (6.7×10^{-7} M final) was added 30 sec later. The suspension was subjected to lipid extraction 60 sec after addition of the ionophore.

A time study of non-thrombin treated platelets labelled with $1\text{-}^{14}\text{C}$ -arachidonic acid was carried out similarly. A23,187 (6.7×10^{-7} M) was added to 1.5 ml of platelet suspension placed in the aggregometer. At the same time, Tyrode was added to the control sample. The platelet suspensions were subjected to lipid extraction at 10, 30 and 60 sec. Five pairs of samples were prepared for each

time interval. The experiment was repeated twice.

In some experiments, platelet suspensions were treated with A23,187 at two concentrations (0.6 μM and 1.2 μM) for 3 min and then subjected to phosphorus analysis.

3. Extraction of phospholipids and analysis

See Experiment B.

Experiment D. Early Degradation of Monophosphoinositide and Triphosphoinositide of Platelets in Response to Thrombin Stimulation.

1. Labeling platelets with isotopes

Rabbit platelets labeled with $^{32}\text{P}\text{O}_4$ was prepared in the same way as in Experiment A. Platelets labeled with radioactive glycerol, arachidonic acid or inositol were prepared with the method as in Experiment C. The concentrations of isotopes added to the incubation medium were 2- ^3H -glycerol (20 $\mu\text{Ci/ml}$), 1- ^{14}C -arachidonic acid (0.4 $\mu\text{Ci/ml}$) or 2- ^3H -inositol (20 $\mu\text{Ci/ml}$).

To obtain some idea of the distributions of various isotopes in platelet lipids at the time of aggregation studies, platelet suspensions labelled with the individual isotopes were subjected to lipid extraction 3 h after the initial addition of the isotopes to the platelet suspension.

2. Aggregation studies

Aliquots of 1.5 ml platelet suspension (platelet count $1.5 \times 10^6/\text{mm}^3$) were placed in the aggregometer simultaneously. After 1 min of stirring, 0.1 ml of bovine thrombin (0.33 units/ml final) was added to the sample and Tyrode was added to the control. The platelet suspensions were subjected to lipid extraction at 9, 30, and 60 sec after the addition of thrombin, unless stated otherwise. At 9 sec, platelets attained maximum shape change.

In some experiments, low concentrations of thrombin (0.01 U/ml) was used. This concentration of thrombin caused only platelet shape change. The platelet suspensions were subjected to lipid extraction 9 sec or 5 min after addition of thrombin.

3. Extraction and Separation of Lipids

See Experiment A.

Free fatty acids, diacylglycerol and triacylglycerol were separated by one-dimensional TLC with silica G plates. The solvent was chloroform, acetone and methanol (96:4:1 v/v/v).

4. Phosphorus Assay

See Experiment A.

In an attempt to measure the differences in the quantities of PA, MPI, DPI and TPI between the thrombin stimulated samples and the control samples 9 sec or 1 min after the addition of thrombin,

large amounts of platelets were required. Usually, the platelets obtained from the blood of 8 to 11 rabbits were pooled. Aliquots of 2.3 ml platelet suspensions (platelet count $2.2 \times 10^6/\text{mm}^3$) were used for the aggregation studies. Higher concentration of thrombin (0.41 unit/ml) was required to bring about the same extent of aggregation as measured by the aggregometer tracing. After the phospholipids were extracted and separated by TLC, three spots were combined to make one sample for phosphorus assay. Thus the amount of phospholipid in each sample was obtained from approximately 1.6×10^{10} platelets, or approximately the amount of platelets from the blood obtained from one rabbit. The experiments were repeated three times for the 9 sec and the 60 sec studies each consisting of 4 or 5 pairs of samples. The quantities of phospholipids were corrected for recovery for the calculation of specific radioactivity. The percentage recovery for PA, MPI, DPI and TPI used for calculating of specific radioactivities were taken as 65, 62, 76 and 70% respectively.

C. COMMENTS ON METHODS

1. Extraction of Phospholipids from Platelets

The greatest difficulties encountered in the experiments with phosphatidic acid and phosphoinositides are their scarcity in cell membranes and their highly acidic nature that renders them to bind strongly to proteins, divalent cations and even glasswares. Divalent cation-bound polyphosphoinositides are insoluble in the normal chloroform and methanol extracting solvents used for the extraction of many other lipids. Furthermore, polyphosphoinositides are unstable. Postmortem TPI degradation was invariably reported in many tissues (Dawson and Eichberg, 1965). Thus in the early part of this project, effort was devoted to the improvement of extraction methods, recovery and the separation of these lipids by thin layer chromatography. Several investigators had quantitated phospholipids in platelets. Their results as well as those from the present experiments, regarding the percentage distribution of the individual phospholipids, phosphatidic acid and of the phosphoinositides are presented in Table 1 and 2.

Several methods were used for the extraction of DPI and TPI. One of them was based on the Folch method (Folch et al., 1957); modified by Dittmer and Douglas (1969) and further by Lloyd and Associates (1972). The method involved lyophilizing the platelet samples, sonication of the residue in $\text{CHCl}_3:\text{CH}_3\text{OH}$ (2:1), and further resuspending the residue in a similar solvent containing hydrochloric

TABLE I. COMPARISON OF THE PERCENTAGE DISTRIBUTION OF PHOSPHORUS AMONG FIVE PHOSPHOLIPIDS
IN THE PRESENT STUDY WITH OTHER PUBLISHED VALUES FOR PLATELETS.

SPECIES	METHOD OF EXTRACTION	NO.	SPH	PC	PS % (mean ± S.E.M.)	MPI	PE	REFERENCES
HUMAN	MODIFICATION OF FOLCH METHOD (1957)	7	17.4 ± 0.9	39.4 ± 1.5	10.4 ± 0.7	5.1 ± 0.6	27.7 ± 1.4	MARCUS et al., 1969
HUMAN	BLIGH AND DYER (1959)	4	18.7 ± 0.3	40.5 ± 0.7	9.0 ± 0.4	3.8 ± 0.1	28.0 ± 0.6	COHEN & DERKSON 1969
RABBIT	FOLCH et al., (1957)	4	23.3 ± 1.0	32.8 ± 1.3	11.4 ± 0.5	2.5 ± 0.5	30.0 ± 1.9	ANDREOLI, 1969
RABBIT	LLOYD et al., (1972)	4	24.4 ± 1.1	32.0 ± 2.8	11.8 ± 0.6	6.5 ± 0.8	25.3 ± 0.7	LLOYD et al., 1972
RABBIT	PRESENT STUDY	3	19.0 ± 2.3	37.1 ± 1.7	10.5 ± 0.6	4.5 ± 0.3	28.6 ± 1.0	PRESENT STUDY

In the present study, the percentages of phospholipids represent the mean values from three experiments of phosphorus assay, each containing three to four samples, and have been corrected for recovery.

Abbreviations : SPH = sphingomyelin, PC = phosphatidylcholine, PS = phosphatidylserine, PE = phosphatidylethanolamine, MPI = monophosphoinositide.

TABLE II. AMOUNTS OF PA, MPI, DPI AND TPI IN WASHED PLATELETS FROM RABBIT

PHOSPHOLIPID	n MOLES/10 ⁹ PLATELETS*	PERCENTAGE OF TOTAL PHOSPHOLIPIDS**	
		THIS STUDY (RABBIT PLATELETS)	LLOYD et al., (1972) (RABBIT PLATELETS) COHEN et al., (1971) (HUMAN PLATELETS)
PA	0.72 ± 0.18	0.29 ± 0.07	1.45 ± 0.19
MPI	10.87 ± 0.20	4.40 ± 0.27	5.68 ± 0.72
DPI	1.51 ± 0.11	0.71 ± 0.02	0.75 ± 0.05
TPI	1.48 ± 0.10	0.65 ± 0.02	0.90 ± 0.14

* Calculated as n moles of phospholipid per 10⁹ platelets allowing for two PO₄ groups in DPI and three in TPI.

The values have been corrected for recovery. Three experiments were done, each containing four samples. The results (mean ± S.E.M.) presented in this table were from one of the three experiments.

** The total phospholipid quantity was calculated by summing up the amounts (n moles) of phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, sphingomyelin, PA, MPI, DPI and TPI. The values for PA, MPI, DPI and TPI are expressed as a percentage of this total (see also TABLE I) and represent the mean ± S.E.M. from three experiments. Each experiment contained three to four samples.

acid. Partitioning of the lipids was carried out with a solvent containing chloroform, methanol, hydrochloric acid and sodium chloride. Acetone was omitted in the modified method as this solvent caused breakdown of TPI. Lyophilization of platelet samples is rather time consuming. The number of samples that can be prepared per experiment is limited and the reproducibility for these minor phospholipids is low.

The Bligh and Dyer method (1959) involves suspending the platelets in a one phase mixture of chloroform, methanol, and water in a ratio of 1:2:0.8 (v/v/v). After a mixing period of about 2 h, more chloroform and water is added to change the volumetric ratio to 1:1:0.9. The mixture becomes two phases. The lipids stay in the chloroform phase (lower layer). This method was used in the extraction of the major phospholipids, PA and MPI in the present project. It was found that lysophospholipids, particularly lyso MPI remain in the aqueous phase. The Bligh and Dyer method is simple in operation and time saving compared to the Folch method. By using the 50 ml round bottom centrifuge tubes, up to eighty to one hundred samples were prepared routinely in the present experiments. Cohen and Associates (Cohen et al., 1971) reported the use of EDTA-KCl solution for the water component of the Bligh and Dyer procedure. Presumably EDTA chelates the divalent cations and frees triphosphoinositide from cell membrane while KCl increases the polarity of the water phase and drives the polar lipids into the chloroform phase. We tried this

5

method but without much success for some unknown reason. Lloyd and associates (1972) modified the Bligh and Dyer method (1959) and the method by Palmer and Rossiter (1965) by including 0.5 N HCl (final concentration) in the extracting solvent. This solvent produces a clear solution on mixing with platelet suspension. This concentration of acid is apparently necessary for a thorough solubilization of the membrane proteins as the use of 0.2 N HCl did not dissolve the platelets completely. The amount of DPI and TPI recovered with 0.2 N HCl was lower and not as reproducible. Lloyd's method (1972) is convenient as platelet samples can be poured directly into the round bottom centrifuge tubes containing the acidified solvent and the platelet reactions are stopped almost instantly. Large quantity of samples can be prepared. The effectiveness of this method (use of acidified Bligh and Dyer solvent) was confirmed recently by Lang et al. (1977). But the acid component destroys plasmalogen phospholipids. Phosphatidylethanolamine and phosphatidic acid are particularly vulnerable. Large amounts of lyso-PE is produced in the acid extraction procedure. This could account for the lower yield of PE in Lloyd's experiments (thesis 1972). Thus neutral solvent of the Bligh and Dyer method was used for the extraction of lipids other than DPI and TPI. It is not known how much DPI and TPI was destroyed by this acid solvent. Since DPI and TPI are produced by phosphorylation of MPI and MPI contains little plasmalogenic species, it is reasonable to believe that the acid extraction procedure may not affect the yield of these polyphosphoinositides to any considerable extent. The

possible destruction of these phospholipids by acid was minimized by partitioning the lipids into the chloroform and aqueous phases immediately following mixing the one phase mixture.

2. Separation of phospholipids by thin layer chromatography

The use of chloroform and methanol mixture for lipid extraction from tissues and the development of thin layer chromatography methods using silica gel for separation of lipids have been the revolutionary steps in phospholipid research. Yet no one method of TLC reported in literatures is capable of separating all the major phospholipids, PA and the phosphoinositides. Poor separation of MPI from PS has been the major difficulty in the investigations on the metabolism of these lipids. Of a large number of two dimensional TLC methods tried in the present projects, the method reported by Rouser and Associates (1972) using magnesium acetate salt with silica gel H for TLC plate preparation appears to give the cleanest separation among PC, PE, PS, SpH, MPI, PA and lyso PE. DPI and TPI remain close to the origin. However, clean separation of phospholipids by TLC depends on many factors, even if the same solvent is used. Humidity is one of the most influential factors, not only humidity in the air, but also humidity in the solvents and in the plates. Temperature also affects the outcome to a great deal. For these reasons and also others, the solvents used by Rouser (1970) was modified for the present experiments. It was also found that overrunning the plate in the ammonia solvent for half an hour enhanced separation between PS and MPI.

Chromatographic separation of TPI and DPI was based on the method of Gonzales-Sastre and Folch-Pi (1968). Overrunning the plates for 15 min enhanced separation of DPI from adjacent lipids. The separation, however, was exceedingly sensitive to humidity and temperature. Separation was most successful in the winter time and troublesome in the summer when humidity and room temperatures were high. It was fortunate that in the last part of the project a humidity control room became available so that accurate determinations of the phosphorus amount of DPI and TPI were possible as a result of clean TLC separation of these lipids.

CHAPTER THREE

Experiment A

In Vitro and In Vivo Incorporation of ^{32}P -Orthophosphate
into Platelet Phospholipids

1. Introduction

The essential feature of platelet function is their responsiveness to aggregating stimuli which cause them to change shape, aggregate or to release their granule contents. This responsiveness demonstrates the pliancy of platelet membrane, which is determined to a large extent by the composition and fluid bilayer configuration of phospholipids. While in circulation, platelets are subjected to the wear and tear action of the dynamics of blood flow. Platelets respond to this continuous stress by having an active metabolism, thus to maintain the integrity of the membrane. Platelets have an efficient enzyme system that enable them to incorporate labeled acetate into phospholipids, ceramide, free fatty acids, triglyceride and cholesterol (Deykin and Dessler, 1968; Andreoli, 1970; Okuma et al., 1971a and 1971b). The acetate is incorporated into fatty acids by both de novo synthesis (Hennes et al., 1966) and by chain elongation. Platelets also synthesise glycerophospholipids de novo for they can incorporate labeled glycerol into the glycerol moiety of phospholipids (Lewis and Majerus, 1969). In addition, they incorporate $^{32}\text{PO}_4$ and labelled fatty acids (Firkin and Williams, 1961; Deykin and Dessler, 1968). The fatty acids may be utilized as substrates for energy production (Donabedian and Nemerson, 1971).

Until apyrase was used in washed platelet suspensions (Ardlie et al., 1971) investigation of phospholipid metabolism in platelets was carried out by infusing the radioactive isotopes into the blood circulation of animals or by incubating platelets in platelet-

rich plasma with the isotope for brief periods. The enzyme apyrase degrades ADP leaked from platelets thereby maintaining the responsiveness and disc shape of platelets (Ardlie et al., 1971) and allowing experiments using washed platelets to last for many hours. A number of investigators reported incorporation of $^{32}\text{PO}_4$ into phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine of platelets harvested from animals infused with $\text{H}_3^{32}\text{PO}_4$ (Lloyd et al., 1972; Cohen et al., 1971), but they were not able to find ^{32}P -labelling in these phospholipids when platelets were labelled in vitro with $^{32}\text{PO}_4$. The only phospholipids that have been reported to be labeled in vitro with $^{32}\text{PO}_4$ are phosphatidic acid and the phosphoinositides. Cohen et al. (1971) and Marcus (1972) could not explain this difference between in vivo and in vitro labelling. Marcus (1972) suggested that the in vivo labelling of the major phospholipids of platelets probably occurs during the formation of megakaryocytes in the bone marrow. In view of the popularity of using washed platelets for various biochemical and functional studies which now may be carried out over prolonged periods, it is pertinent to find out if there is indeed a difference between in vitro and in vivo behaviour of $^{32}\text{PO}_4$ incorporation into the membrane phospholipids of platelets, or whether this difference simply reflects the difference in the turnover rates of the various phospholipids.

The following experiments were done to examine these possibilities. The incorporation of $^{32}\text{PO}_4$ into the major phospholipids of platelets was compared with the incorporation of $^{32}\text{PO}_4$ into the phosphoinositides in vivo and during prolonged incubation in vitro.

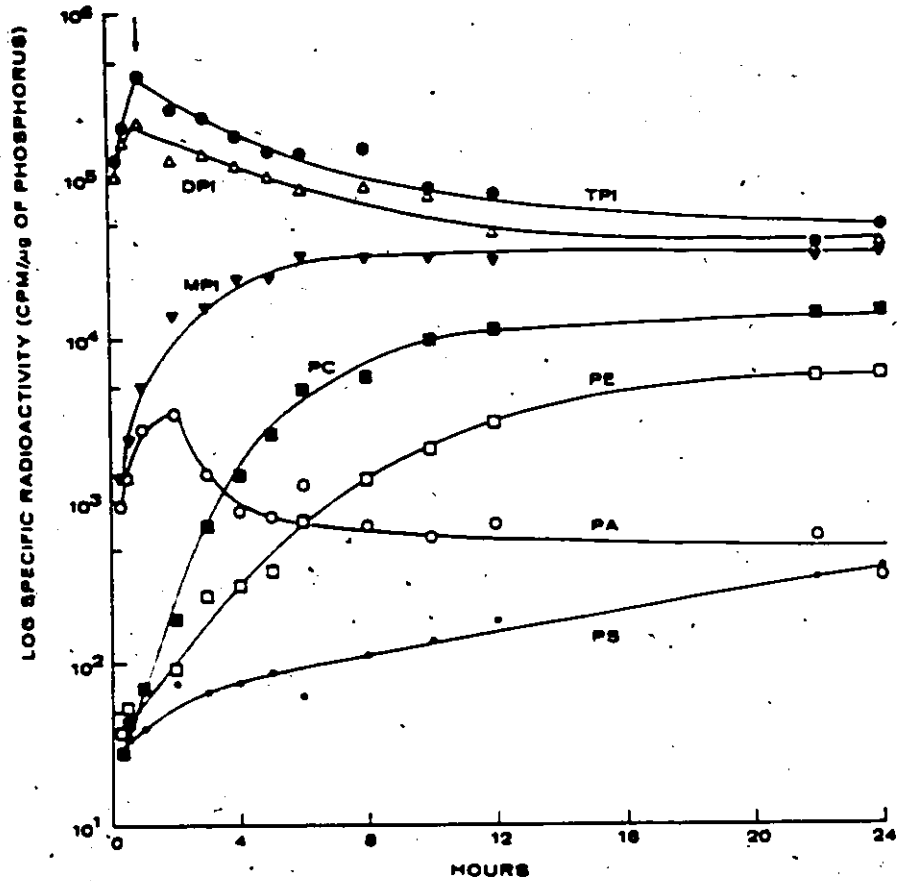


Figure 6. Incorporation of $^{32}\text{PO}_4$ into phospholipids of rabbit platelets expressed as the logarithm of specific radioactivity vs. time. Platelets were labelled for 1 h and washed once before being suspended in a medium containing unlabelled PO_4 . The values shown at 1 h are the values before the platelets were washed at the time indicated by the arrow. Subsequent values (after the arrow) were obtained after the platelets had been resuspended in a medium containing unlabelled PO_4 . The results are from one of three experiments that give similar results. Each value represents the mean of 3 samples.

2. Results

Changes in Specific Radioactivity of Phospholipids

(a) In vitro

Washed rabbit platelets were incubated with $^{32}\text{PO}_4$ for one hour at 37°C . At the end of the labelling period approximately 24% of the added $^{32}\text{PO}_4$ had been incorporated into the platelets, and 4-5% of the incorporated radioactivity was found in the phospholipid fraction. TPI, DPI and PA had a greater specific radioactivity than PC, PE or PS (Figure 6). TPI had the highest specific radioactivity. When the platelets were washed and resuspended in Tyrode-albumin solution containing unlabelled phosphate, the specific radioactivity of TPI and DPI declined progressively over 24 hours. The pattern of incorporation of $^{32}\text{PO}_4$ into MPI was different from that into the poly-phosphoinositides in that the specific radioactivity of MPI increased and reached a plateau 7 hours after the labelling of the platelets with $^{32}\text{PO}_4$. The specific radioactivity of the phosphate in the major phospholipids (PC, PE and PS) increased gradually over the 24 hour incubation period. After one hour of incubation, the specific radioactivity of TPI was 80 times that of MPI. However, after 24 hours of incubation, the difference between the specific radioactivity of TPI and MPI was small. The specific radioactivity of PC at one hour was negligible, and at 24 hours it was about half that of MPI. The specific radioactivity of PE increased more slowly than that of PC. Although the increases in specific radioactivity in PS was not as great as that in PC or PE, it continued to increase over the 24 hour period.

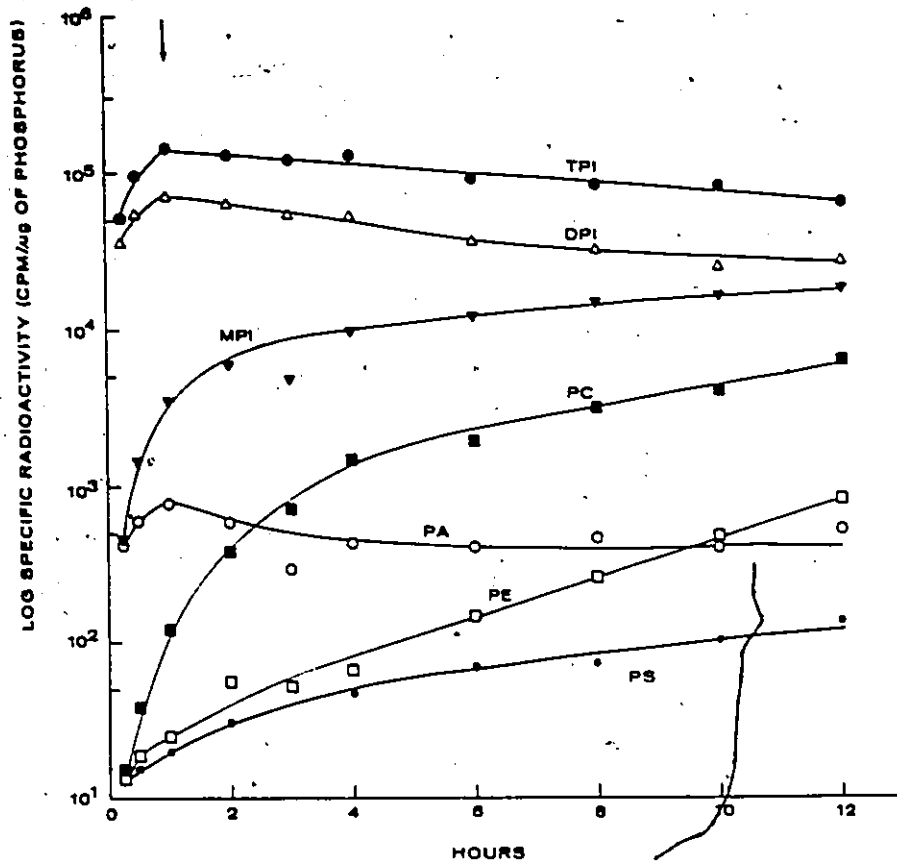


Figure 7. Incorporation of $^{32}\text{P}\text{O}_4$ into human platelets. Experimental conditions were the same as specified for Figure 6. The results are from one of two experiments that gave similar results. Each value represents the mean of 3 samples.

A similar pattern of changes of specific radioactivity was observed with washed human platelets (Figure 7).

(b) In vivo

Rabbit platelets were labelled for 1 hour by incubation with $^{32}\text{PO}_4$. They were then washed, resuspended and infused into rabbits. Blood samples were taken at intervals up to 35 hours. When platelets were collected 4 hours after their infusion (6 hours after the beginning of labelling), the specific radioactivities of the phosphorus of the phosphoinositides were much higher than those of the major phospholipids (Figure 8). The pattern of change in the specific radioactivities of all the phospholipids was similar to that found in the in vitro experiments. That is, the specific radioactivities of the phosphorus of TPI and DPI decreased after the labelling period, whereas the specific radioactivities of PC and PE increased. In contrast to the in vitro experiments, the specific radioactivity of the phosphorus of MPI appeared to decline by 16 hours. By 35 hours, the specific radioactivities of the phosphorus of TPI, DPI and MPI and PC were similar. At this time, the specific radioactivity of the phosphorus of PE appeared to be about 1/3 that of MPI (Figure 8).

Changes in the Total Radioactivity of Individual Phospholipids
During Prolonged Incubation in Vitro

Since the amounts of the phosphoinositides and the major phospholipids in platelets are different, the specific radioactivities

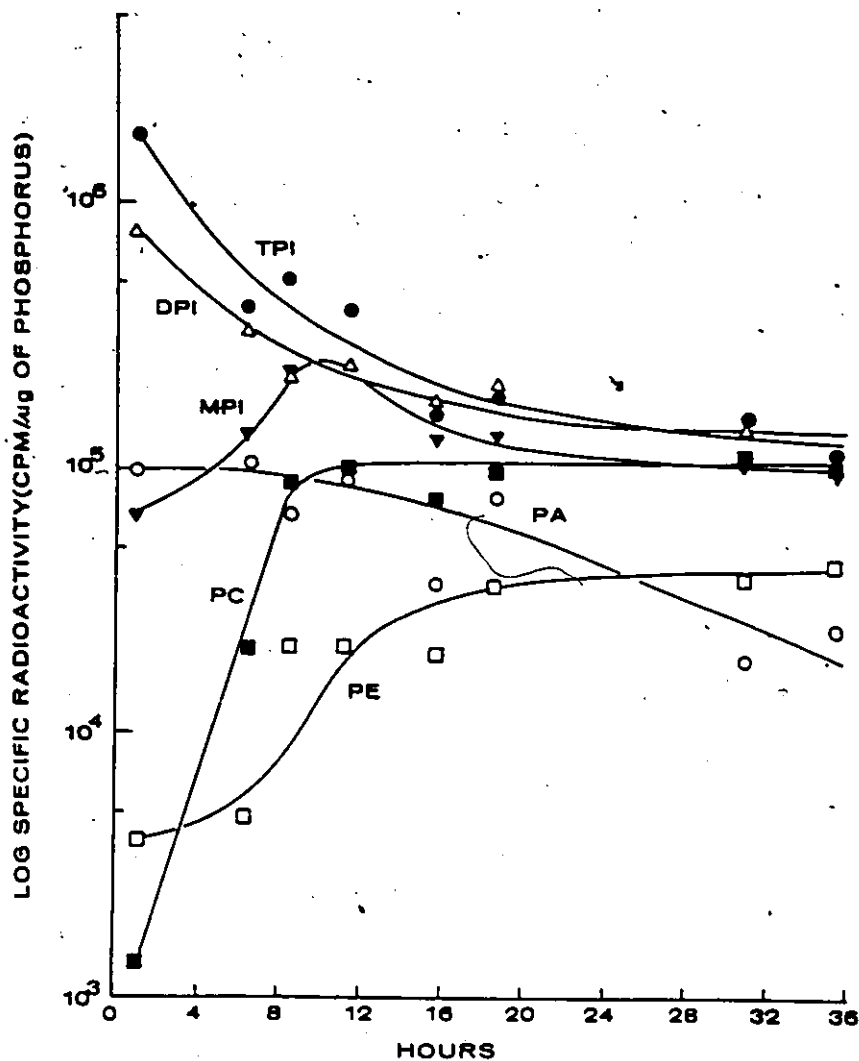


Figure 8. $^{32}\text{PO}_4$ incorporation into phospholipids of rabbit platelets labelled in vitro with $^{32}\text{PO}_4$ and infused intravenously into 7 rabbits. The first set of samples was prepared from the platelet suspension 1 h after addition of $^{32}\text{PO}_4$. The subsequent samples were taken after the ^{32}P -labelled platelets had been infused into rabbits; the samples were prepared from blood harvested at the times indicated. The values have been corrected as described in the "Methods" section. Incorporation of $^{32}\text{PO}_4$ into PS was not studied in this experiment. Each value represents a separate animal.

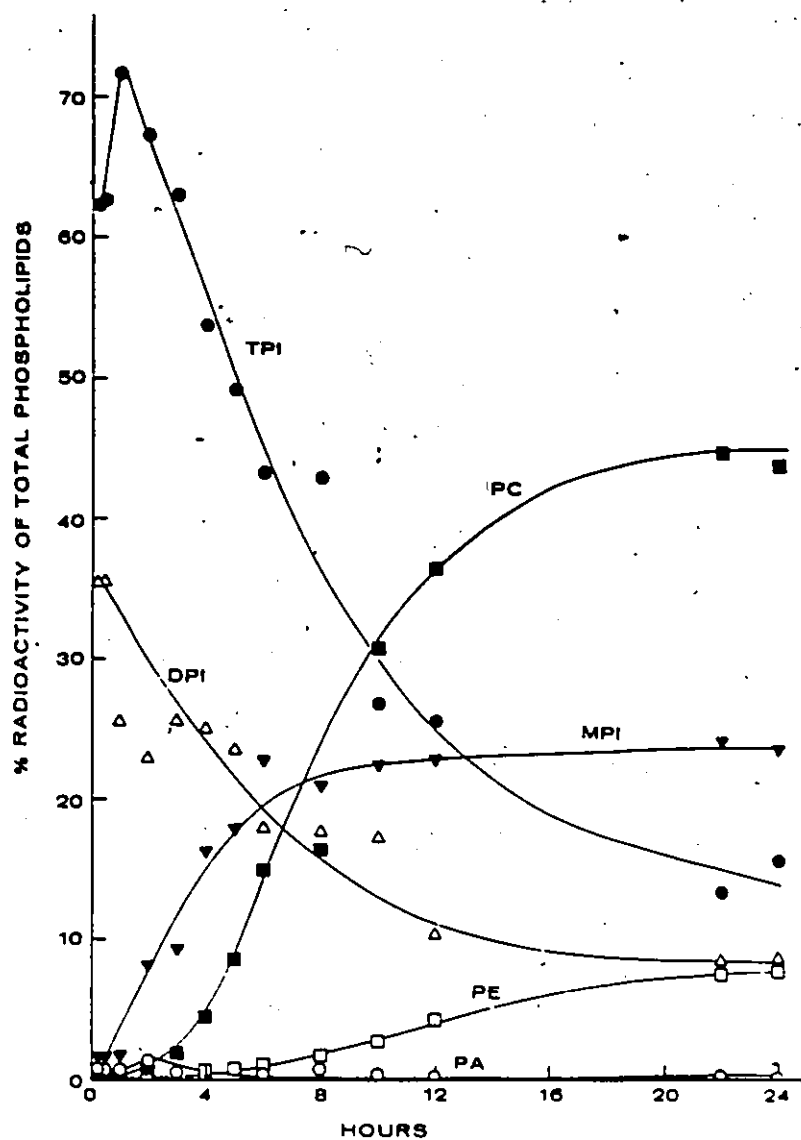


Figure 9. In vitro incorporation of $^{32}\text{PO}_4$ into individual phospholipids of rabbit platelets expressed as the percentage of total radioactivity of all the phospholipids vs. time. The data are averages of three experiments. Standard deviations are not shown to avoid complexity in the figures. The sum of the radioactivity in TPI, DPI, MPI, PA, PC, PE and PS at each time interval was taken as 100%. The percentage of radioactivity in PS was too low to be shown.

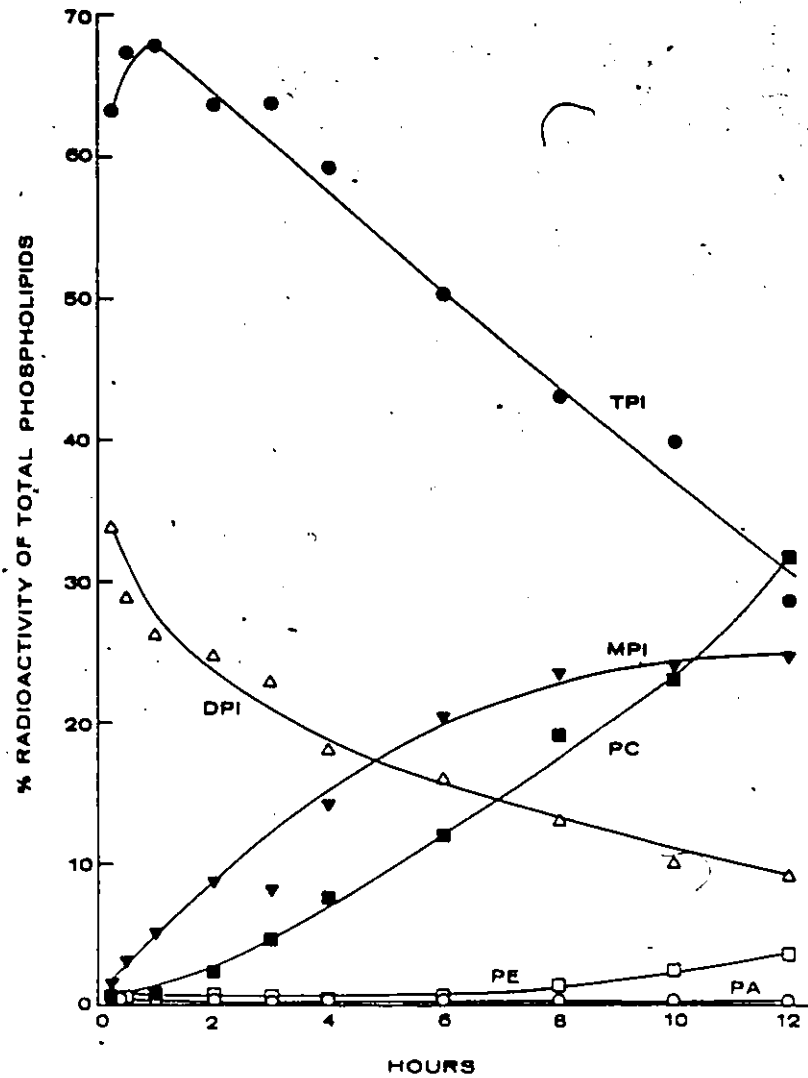


Figure 10. In vitro incorporation of $^{32}\text{PO}_4$ into individual phospholipids of human platelets expressed as the percentage of the total radioactivity in all the phospholipids vs. time (see caption of Figure 9).

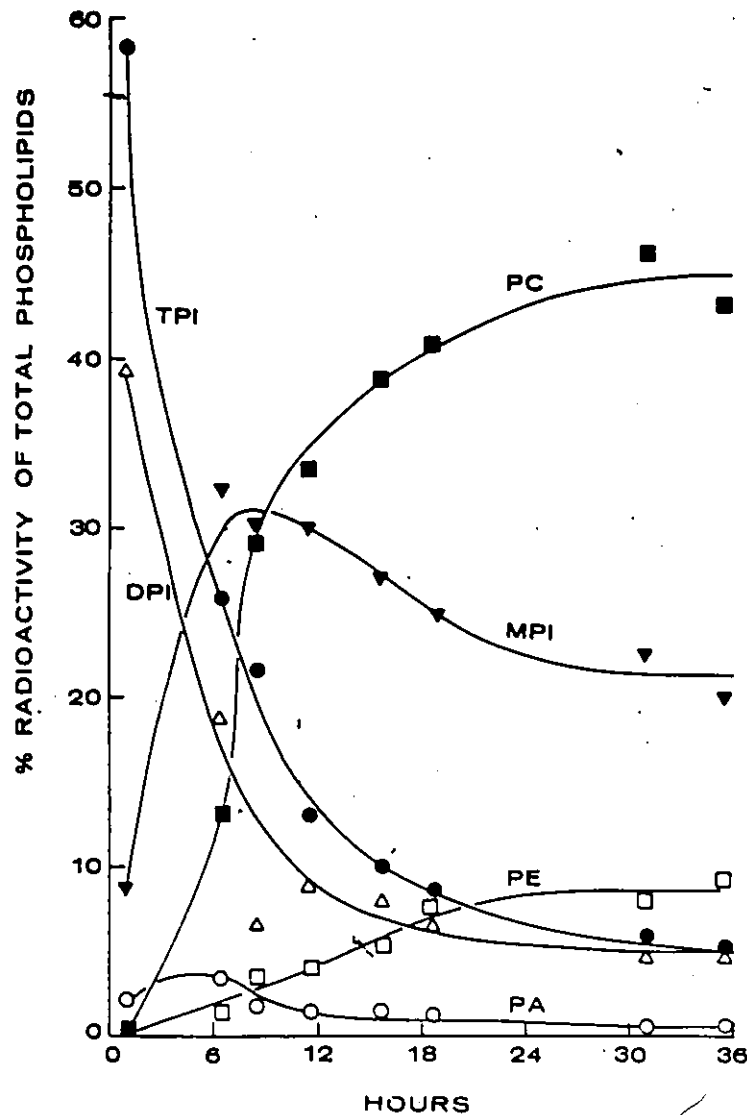


Figure 11. $^{32}\text{PO}_4$ incorporation into phospholipids of rabbit platelets expressed as percentage of total radioactivity of all the phospholipids which included MPI, DPI, TPI, PA, PC, PE, SPH and LPC. Each point represents the percentage of the radioactivity in the phospholipids from one rabbit. The procedure is described in the legend of Figure 8. (Radioactivity of total phospholipids in a single sample at 1 h was approximately 1.2×10^6 cpm).

of their phosphorus do not reflect the actual amounts of $^{32}\text{PO}_4$ incorporated into the major phospholipids. Therefore, we plotted the amounts of $^{32}\text{PO}_4$ incorporated into the individual phospholipids of rabbit platelets (Figure 9) and human platelets (Figure 10), expressed as percentages of the total radioactivity in the phospholipids at each time. With rabbit platelets, PC contained less than 1% of the total radioactivity in the phospholipids 1 hour after the addition of $^{32}\text{PO}_4$ but had more than 40% after 24 hours of incubation. The radioactivity in PE increased from about 1% of the total radioactivity in the phospholipids 1 hour after the addition of $^{32}\text{PO}_4$ to about 8% at 24 hours. Over the same period of time, PS showed little change (less than 1% in 24 hours). In contrast, TPI which had 72% of the radioactivity at 1 hour, had only 15% of the phospholipid radioactivity at 24 hours. DPI radioactivity also fell extensively.

Figure 10 shows similar results obtained in 3 experiments in which human platelets were used.

When rabbit platelets labelled with $^{32}\text{PO}_4$ in vitro were infused into rabbits, more than 40% of the total radioactivity in phospholipids was in PC after 35 hours, whereas less than 10% of the total radioactivity was in DPI and TPI (Figure 11).

3. Discussion

The experimental results illustrate the rates of incorporation of $^{32}\text{PO}_4$ into various platelet phospholipids over prolonged incubation

in terms of changes in the specific radioactivity and of the percentage distribution of $^{32}\text{PO}_4$ in the lipids. Although no attempts have been made to study the dynamics of $^{32}\text{PO}_4$ incorporation, several salient points have emerged from these studies.

Polyphosphoinositides (DPI and TPI) have exceedingly high rates of phosphate metabolism (Figures 1-3). These phosphoinositides constitute less than 2% of platelet phospholipids, yet possess over 90% of total phospholipid $^{32}\text{PO}_4$ one hour following incubation of platelets with $^{32}\text{PO}_4$. The phosphate metabolism of these lipids is very sensitive to the changes in the cellular phosphate concentration. This is illustrated by the rapid fall in their specific radioactivities and $^{32}\text{PO}_4$ percentages once unlabelled phosphate was added to the platelets labelled with $^{32}\text{PO}_4$ in a phosphate-free medium. Furthermore, although MPI is the immediate precursor of DPI and TPI, the rapid phosphate incorporation into these lipids has little to do with de novo synthesis from MPI. If de novo synthesis takes place, specific radioactivity of MPI would be higher than that of DPI and TPI and the specific radioactivity curve of MPI would be declining and cross the peaks of those of DPI and TPI once $^{32}\text{PO}_4$ was withdrawn from the medium. This has not happened and the results therefore indirectly demonstrate the presence of a very active DPI or TPI phosphomonoesterase in the platelets and that most of the $^{32}\text{PO}_4$ incorporated into DPI and TPI resides in the monoesterified phosphate moieties. This has been demonstrated in red cell ghosts labelled with (γ - ^{32}P)ATP (Hokin and Hokin,

1964). TPI phosphomonoesterase has been shown in various mammalian tissues (Salway et al., 1967; Sheltaw et al., 1972). It is primarily a soluble enzyme. The extremely rapid turnover of the monoesterified phosphates of DPI and TPI implies the ready accessibility of the polar head groups of these lipids to the phosphomonoesterase, thus adding support to the concept that these phospholipids are predominantly located in the inner cell membrane (Verkleij et al., 1973).

Rapid incorporation of $^{32}\text{PO}_4$ also occurs with MPI. Yet unlike the polyphosphoinositides, dilution of the $^{32}\text{PO}_4$ pool in platelets with unlabelled phosphate had ~~no~~ immediate effect on the incorporation course of $^{32}\text{PO}_4$ into MPI. Since incorporation of $^{32}\text{PO}_4$ into MPI has to be mediated by the phosphorylation of diacylglycerol with $(\gamma\text{-}^{32}\text{P})\text{ATP}$ to form PA, which is later converted to MPI, it is expected that the specific radioactivity of PA to be higher than that of MPI. The consistently lower specific radioactivity of PA than MPI in these experiments could be a consequence of compartmentalization of PA metabolism in platelets. It is possible that only a small pool of PA is metabolically active to serve as a precursor of MPI. The specific radioactivity of this PA could thus be much higher than the observed value. In addition, MPI could be derived from DPI. Rapid incorporation of $^{32}\text{PO}_4$ into phosphatidic acid and phosphoinositides under resting conditions and an increased $^{32}\text{PO}_4$ incorporation upon stimulation has been observed in brain (Dawson, 1954), nerve tissue (White et al., 1974), pancreas (Hokin and Hokin, 1955), red cells (Hokin and Hokin, 1964;

Karnovsky and Wallach, 1961; Fisher and Mueller, 1971), kidney (Andrade and Higgins, 1964), pineal gland (Eichberg et al., 1973) and platelets (Cohen et al., 1971). This active turnover of inositol phospholipids support the possible role in specialized cell functions such as synaptic transmission in nerves (Larrabee and Leicht, 1965) and to a more general role of transport across cell membranes and secretion from cells (Hokin, 1969).

Unlike the previous studies in which $^{32}\text{PO}_4$ incorporation into major phospholipids of platelets has been claimed to occur only in vivo (Lloyd et al., 1972; Cohen et al., 1971; Marcus, 1972), our studies on $^{32}\text{PO}_4$ incorporation into platelet phospholipids demonstrate that washed platelets incorporate $^{32}\text{PO}_4$ in vitro into PC, PE and PS when long incubation times are used. The results of the in vitro incubation studies were confirmed by the in vivo experiments, since platelets infused into rabbits immediately after labelling gave similar results. Like PA and inositol phospholipids, the specific radioactivities of these lipids eventually leveled off fifteen to twenty hours following the pulse labelling, indicating that the metabolism of $^{32}\text{PO}_4$ in platelets had reached a steady state. The differences in the values of specific radioactivities of the individual phospholipids at this time possibly reflect the divergence of substrate affinity and substrate availability in the phosphate metabolizing enzymes. This is in agreement with the observation of asymmetric distributions of phospholipids in cell membranes (Verkleij et al., 1973) and the variation in the susceptibility

to hydrolytic enzymes of phospholipids with different fatty acid composition or different head groups (Zwaal et al., 1973). Much of the information regarding the dynamic interplay between cellular enzymes and membrane phospholipids, however, remains unknown. Firkin and Williams (1961) reported radioactivity in autoradiograms in positions representing PC, PS and PE of platelet phospholipids labelled with $^{32}\text{PO}_4$ for 6 hours in vitro, they did not comment on these findings, and later investigators do not appear to have been aware of them. Prolonged incubation in vitro has also demonstrated incorporation of $^{32}\text{PO}_4$ into the major phospholipids in pineal glands (Eichberg et al., 1973) and SV 40 transformed mouse cells (Marggraf et al., 1972). Marinetti and coworkers (1957) have also noticed a difference in ^{32}P -labelling of liver tissues under in vivo and in vitro conditions.

In vitro studies with ^{14}C -acetate and ^{14}C -glycerol have demonstrated ready labelling of the major phospholipids of platelets (Deykin, 1973; Lewis and Majerus, 1969). As in the present $^{32}\text{PO}_4$ experiments, PC incorporates the largest amount of radioactivity among the major phospholipids. The ^{14}C -acetate incorporation probably represents the synthesis of new fatty acids which are incorporated into the phospholipids. The amount of $^{32}\text{PO}_4$ incorporated within 1 hour into PC in the present investigation is comparable to the amount of ^{14}C -1,3 -glycerol radioactivity incorporated into PC as found by Lewis and Majerus (1969). Since ^{14}C -1,3 -glycerol can be incorporated into phospholipids only by de novo synthesis, it is likely that $^{32}\text{PO}_4$

incorporated into PC in vitro in our experiments also represents de novo synthesis of the molecule.

In contrast to previous suggestions (Cohén et al., 1971; Marcus, 1972), these experiments demonstrate that platelets do not lose their capacity to turn over the phosphates in the major phospholipids after they have separated from the megakaryocytes. These experiments further demonstrate that MPI has a higher rate of phosphate turnover than the other major phospholipids. All this evidence is in keeping with the observations that the phosphates in the inositol phospholipids have a very high rate of turnover in comparison to the phosphate of PC, PE and PS. The turnover of the phosphate groups in the inositol phospholipids is probably an important aspect of membrane function and the rapid increase in turnover when platelets are stimulated by ADP or thrombin indicates that they are involved in the platelet response to these agents (Lloyd et al., 1972; Lloyd and Mustard, 1974).

CHAPTER FOUR

Experiment B

Changes of Isotopic Labellings of Phosphatidic Acid and Phosphoinositides
of Rabbit Platelets in Response to ADP

1. Introduction

Myo-inositol is present in both plant and animal tissues and in eukaryocytes is incorporated principally into phosphoinositides (Hawthorne and Kemp, 1964). These inositol lipids comprise less than 10% of the total phospholipids in cell membranes and appear to be involved in cell functions such as transmission of action potentials along nerves (Hawthorne and Kai, 1970; Dawson, 1969), membrane transport of ions (Hawthorne and Kai, 1970) and secretion of proteins (Hokin, 1969).

Much of the work on phosphoinositides has been done with brain tissue (Hawthorne and Kai, 1970), nerves (White and Larrabee, 1973) and synaptosomes (Schacht and Agranoff, 1972). Enhanced turnover of the phosphoryl moiety of monophosphoinositide (MPI) has been observed in response to a variety of stimuli such as acetylcholine (Redman and Hokin, 1964) and electrical stimulation (White and Larabee, 1973). Micheli (1975) has recently pointed out that platelets may be useful for studying the metabolism of phosphoinositides. Lloyd and associates (1973a) reported that ADP-induced platelet shape change was associated with increased incorporation of $^{32}\text{PO}_4$ into phosphatidic acid (PA). This change was observed as early as 2 sec after the addition to ADP to a suspension of washed rabbit platelets. Increased $^{32}\text{PO}_4$ incorporation into diphosphoinositide (DPI) was observed at 30 sec (Lloyd et al., 1973 a,b). Similar changes in MPI were not detected until 2-3 min after the addition of ADP, when platelet aggregation was already well underway (Lloyd et al., 1973). In the initial studies of changes in

³²P-labelling of triphosphoinositide (TPI) following the addition of ADP, consistent changes in TPI were not demonstrated (Lloyd et al., 1973a; 1973b).

The possibility that TPI hydrolysis mediates changes in membrane permeability in nerve excitation was suggested by Hawthorne and Kai (1970). Hendrickson and Reinertsen (1971) proposed that since TPI has a higher affinity for calcium than does DPI, interconversion between DPI and TPI might modify calcium binding in membranes leading to changes in sodium and potassium permeability. Hydrolysis of TPI has only recently been observed in acetylcholine-stimulated crab nerve (Tret'jak et al., 1977) and rabbit iris smooth muscle (Abdel-Latif et al., 1977). Significant loss of TPI was observed at least 10 min after additional acetylcholine.

Interaction of ADP with its receptors on the platelet surface and the subsequent changes in platelet shape indicate alteration in the membrane permeability of platelets. The lack of changes in the ³²P-labelling in major membrane phospholipids (e.g. phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine) during ADP-induced platelet aggregation (Lloyd et al., 1973b) emphasizes the importance of investigating the effect of ADP on the possible change of phosphoinositides particularly TPI in the first 60 sec of platelet response to this stimulus.

The specific objectives of the present studies were to examine whether changes of radioactive isotope contents in TPI occurred during

TABLE III. INCORPORATION OF ³²P-ORTHOPHOSPHATE INTO PHOSPHOLIPIDS.

<u>Phospholipid</u>	<u>Specific Radioactivity* cpm/ug phosphorus</u>
TPI	735,000
DPI	410,000
PA	100,000
MPI	32,000

*The values represent the average specific radioactivities from 4 samples in a typical experiment of phospholipids in the rabbit platelets after 1 h labelling.

ADP-induced platelet aggregation using washed rabbit platelets labelled with ^{32}P -orthophosphate, ^{14}C -arachidonic acid, 2- ^{14}C -glycerol and myo- ^3H -inositol. In addition, we investigated whether these changes were influenced by adenosine monophosphate, an inhibitor of ADP-induced platelet aggregation. After ADP-induced aggregation, platelets deaggregate and restore their disc shape. The present experiments also studied the change of ^{32}P -labelling in PA and the inositol phospholipids during this recovery period.

2. Results

Incorporation of $^{32}\text{PO}_4$ into triphosphoinositide and ATP

Incubation of washed rabbit platelets for 1 hour with $^{32}\text{PO}_4$ -orthophosphate resulted in the rapid labelling of TPI, DPI and, to a lesser extent, PA and MPI (Table III). Among these lipids the phosphorus in TPI had the greatest specific radioactivity. This declined after the platelets had been resuspended in a medium containing unlabelled phosphate (Figure 12). Therefore, in all experiments, the experimental samples and the corresponding control samples were run simultaneously. The specific radioactivity of ATP also declined after the removal of external $^{32}\text{PO}_4$, but remained several hundred fold higher than that of TPI (Figure 13).

Aggregation studies within 60 sec

Addition of $31\ \mu\text{M}$ ADP to the suspension of washed platelets labeled with ^{32}P -orthophosphate caused the platelets to change shape and to aggregate (Figure 14). ADP does not cause release reaction of

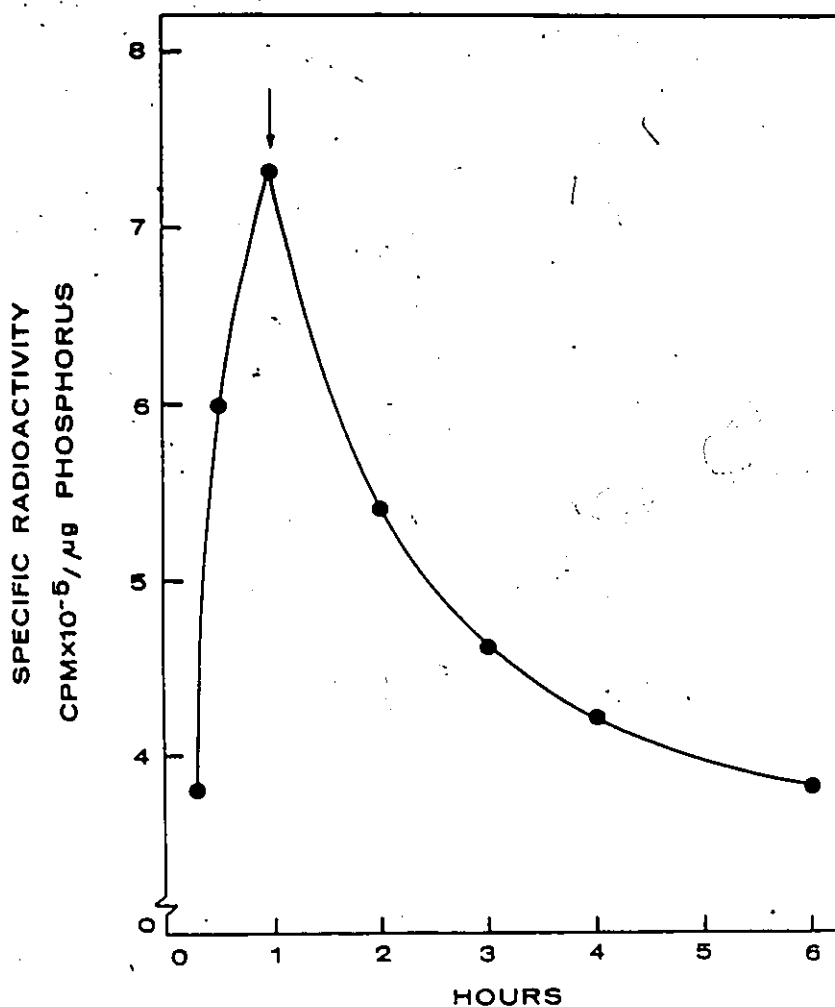


Figure 12. Changes in specific radioactivity of the phosphorus in TPI of rabbit platelets during the one hour labelling with $^{32}\text{PO}_4$ and the rapid decline in specific radioactivity of phosphorus in this lipid after the platelets had been resuspended in a medium containing unlabelled phosphate at the time indicated by the arrow. The data represent the average of 3 samples.

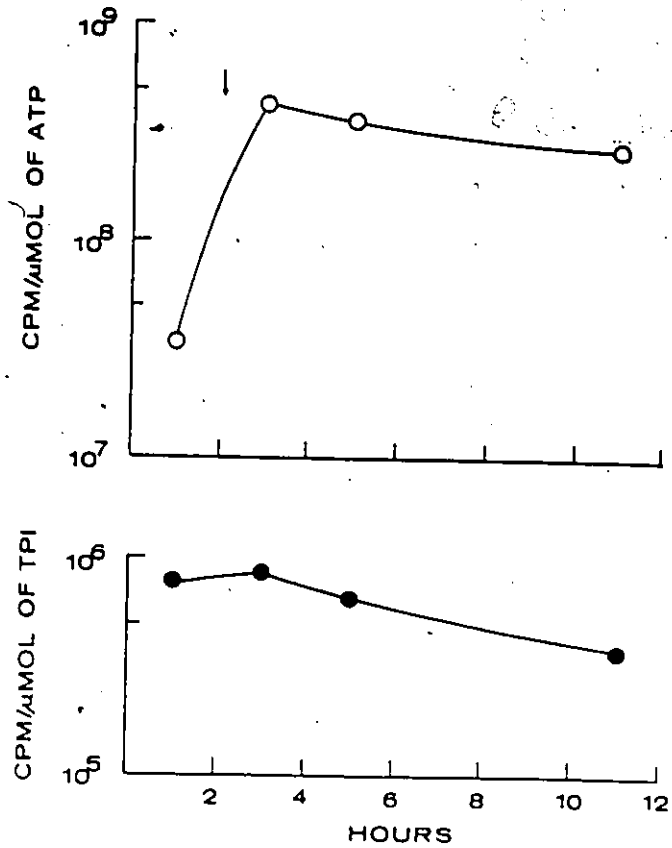


Figure 13. Incorporation of $^{32}\text{PO}_4$ into ATP and TPI of rabbit platelets expressed as specific radioactivity (logarithm) vs. time. Platelets were washed and resuspended in medium containing unlabelled phosphate after 2 h incubation with $^{32}\text{PO}_4$ as indicated by the arrow. The data represent the mean of 3 samples.

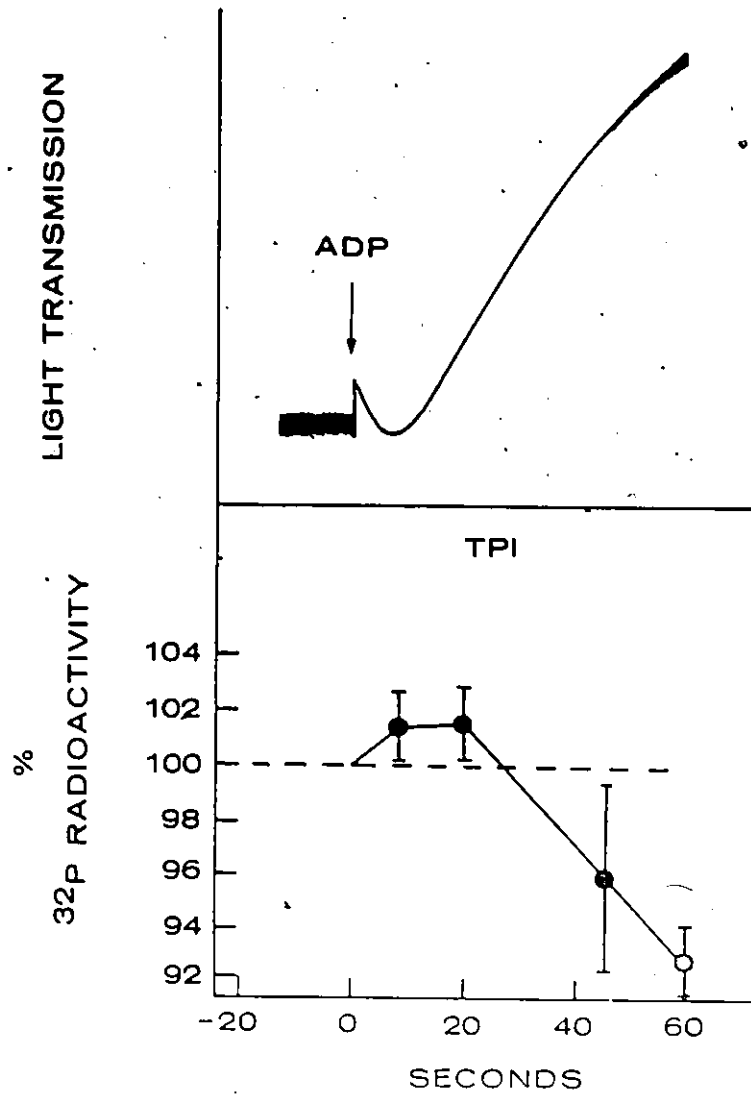


Figure 14.

Figure 14. Changes in light transmission during ADP-induced platelet shape change and aggregation (top) and changes in ^{32}P -content in TPI of platelets during ADP-induced platelet aggregation (bottom). The final concentration of ADP was $31\ \mu\text{M}$. The amount of $^{32}\text{PO}_4$ in TPI in the control samples was taken as 100% (the dotted horizontal line) and the amount of $^{32}\text{PO}_4$ in TPI in the stimulated platelets was expressed as a percentage of this control value. The data were calculated as mean \pm S.E. of 9-20 pairs of samples from at least three experiments at each time interval. A 5-7% decrease in ^{32}P -content in TPI represented 2000-7000 cpm in different experiments. The ^{32}P -content in TPI at 60 s was significantly less than for the controls ($p < 0.01$).

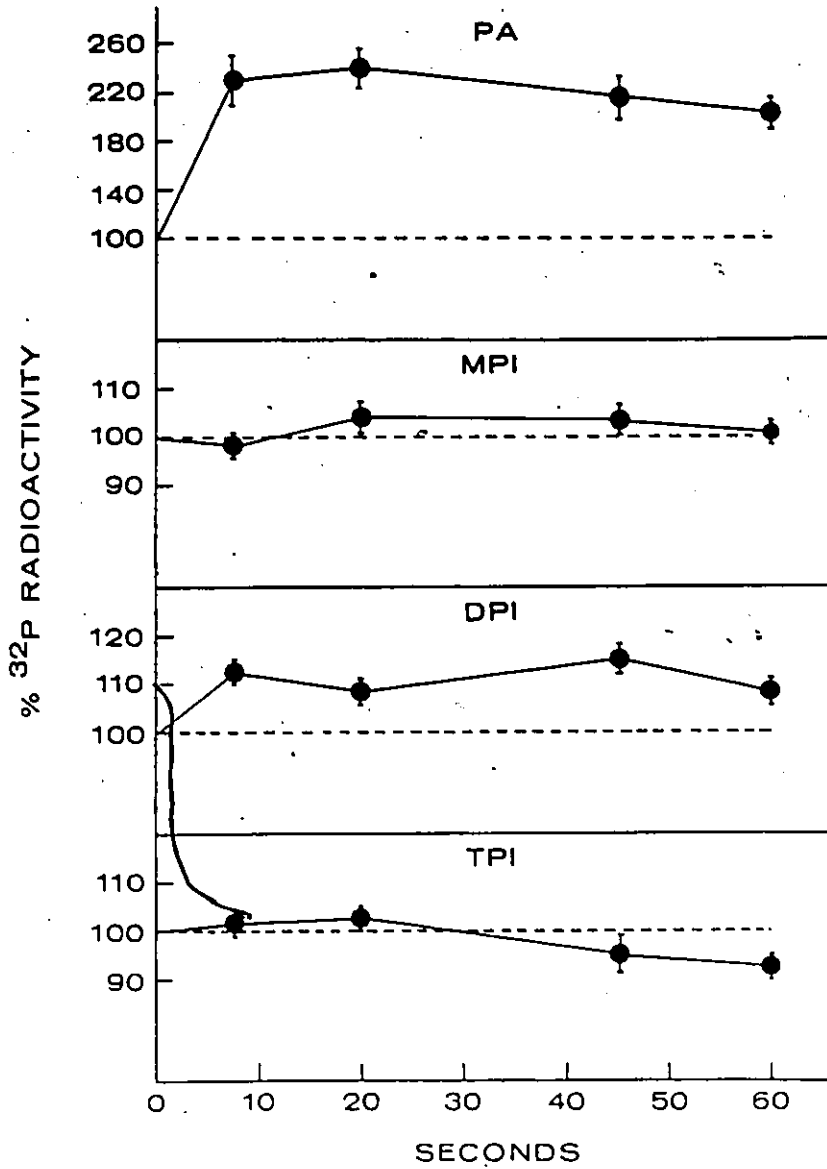


Figure 15.

Figure 15. Changes in ^{32}P -content in PA, MPI, DPI AND TPI during ADP-induced platelet aggregation. The final concentration of ADP was $31\ \mu\text{M}$. Data are presented in the same way as in Figure 14. In a representative experiment, the average amounts of ^{32}P -labelling in TPI, DPI, MPI and PA of the control samples were 41891, 35859, 15389 and 2990 cpm, respectively. Significance of the difference (P values) between untreated platelets and platelets treated with ADP:-

	<u>7 sec</u>	<u>20 sec</u>	<u>45 sec</u>	<u>60 sec</u>
PA	<0.001	<0.001	<0.001	<0.001
MPI	NS	<0.05	NS	NS
DPI	<0.001	<0.05	<0.001	<0.001
TPI	NS	NS	NS	<0.01

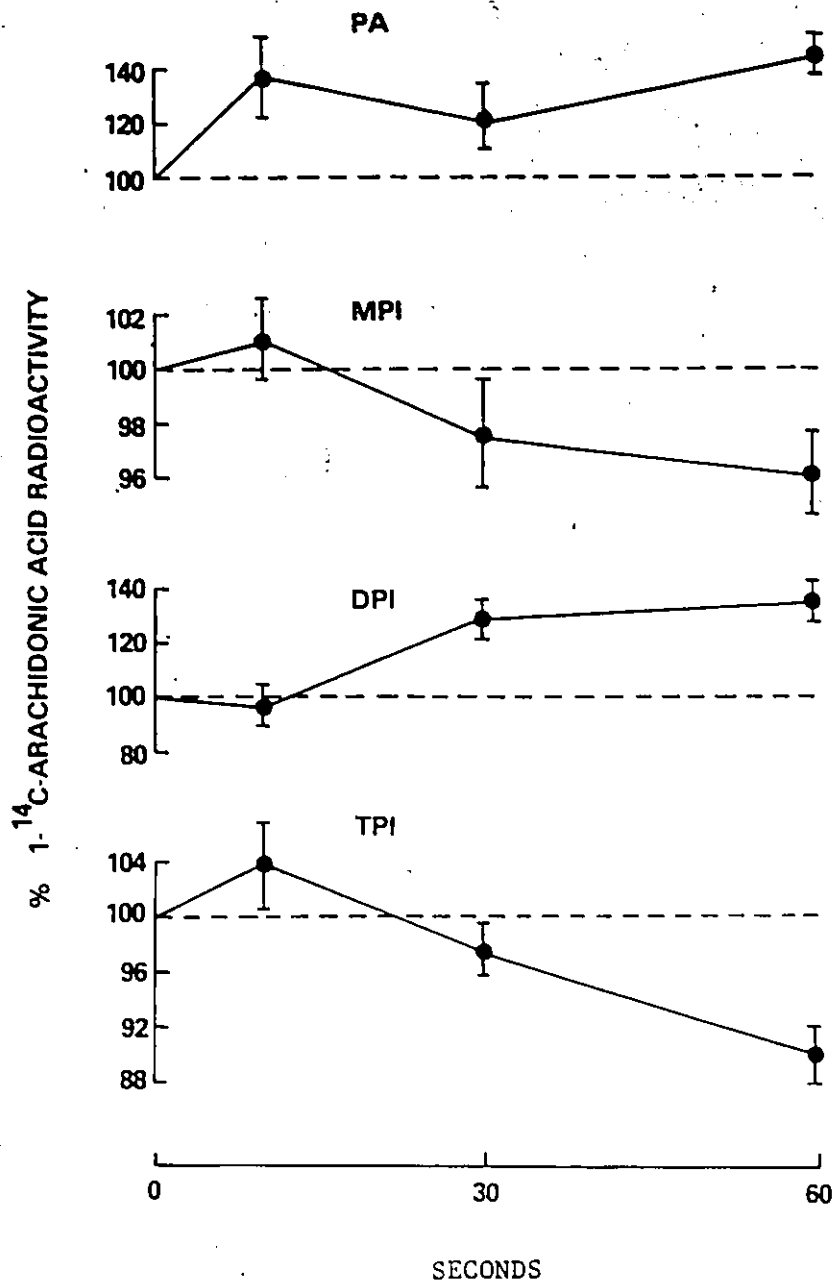


Figure 16.

Figure 16. Changes in 1-¹⁴C-arachidonic acid in PA, DPI, MPI and TPI during ADP-induced platelet aggregation. The final concentration of ADP was 31 μ M. Data are presented in the same way as in Figure 14. Data at each time interval were calculated as mean \pm S.E.M. of 10 pairs of samples from two experiments that yield similar results. In a representative experiment, the average amount of 1-¹⁴C-arachidonic acid in PA, MPI, DPI and TPI of the control samples were 192, 11712, 1555 and 1736 dpm, respectively. Significance of the difference (P values) between untreated platelets and platelets treated with ADP:-

	<u>10 sec</u>	<u>30 sec</u>	<u>60 sec</u>
PA	<0.01	<0.01	<0.005
MPI	NS	NS	<0.02
DPI	NS	<0.001	<0.001
TPI	NS	NS	<0.001

NS = not significant

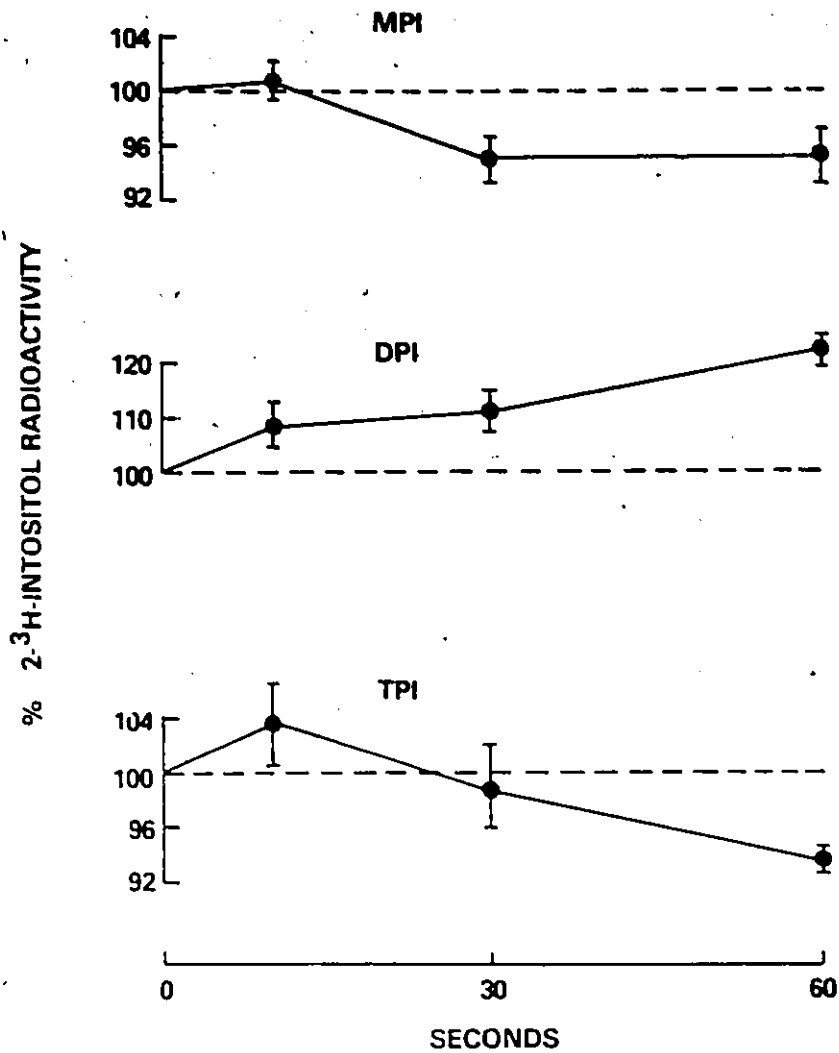


Figure 17.

Figure 17. Changes in 2-³H-inositol labelling in MPI, DPI and TPI in platelets in response to ADP. The concentration of ADP was 31 μ M. Data are presented in the same way as in Figure 14. Data at each time interval were calculated as mean \pm S.E.M. of 10 samples from two experiments. In a representative experiment, the average amounts of radioactivity in MPI, DPI and TPI of the control samples were 50291, 6986 and 7626 dpm respectively. Significance of the difference (P values) between untreated platelets and platelets treated with ADP:-

	<u>10 sec</u>	<u>30 sec</u>	<u>60 sec</u>
MPI	NS	<0.025	<0.05
DPI	<0.05	<0.01	<0.001
TPI	NS	NS	<0.001

NS = not significant

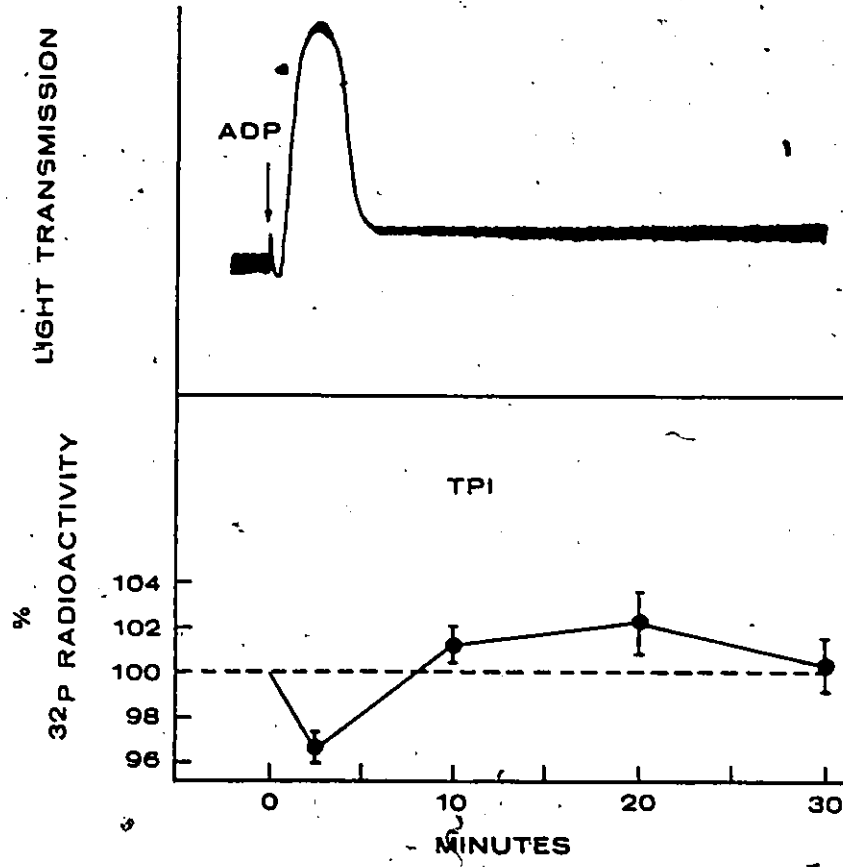


Figure 18.

Figure 18. The aggregometer tracing of platelet aggregation and deaggregation is illustrated in the upper diagram. The lower diagram shows changes in the ^{32}P -content in TPI during platelet aggregation and deaggregation after the addition of ADP (10 μM). Data are presented in the same way as in Figure 14. The data were calculated as mean \pm S.E.M. of 10-20 pairs of samples from at least three experiments at each time interval. The mean ^{32}P -content in TPI at 2.5 min was significantly less than for the control ($P < 0.05$). At 10, 20 and 30 min the ^{32}P -contents in TPI were not significantly different from the controls.

washed rabbit platelets (Packham et al., 1971). The mean values of ^{32}P -labelling (cpm) of TPI 7 and 20 sec after the addition of ADP were not significantly different from the mean values for the control platelets. However, 60 sec after the addition of ADP, the amount of $^{32}\text{PO}_4$ in the TPI was significantly decreased in platelets treated with ADP compared to the control platelets (Figure 14).

In contrast to TPI, the labelling of the DPI increased significantly as early as 7 sec after the addition of ADP (Figure 15). This was the earliest time after the addition of ADP that we attempted to extract the lipids. The labelling of DPI remained elevated up to 60 sec. There were no consistent changes in the ^{32}P -labelling of MPI within 1 min of the addition of ADP. The labelling in PA increased to more than twice the value of the control. Changes in the ^{14}C -arachidonic acid labelling in PA and phosphoinositides 60 sec following the addition of ADP are shown in Figure 16. There was a moderate increase in radioactivity in PA and DPI and a small but significant decrease in MPI and TPI by 60 sec. Similar changes were observed in the 2- ^3H -inositol labelling of MPI, DPI and TPI (Figure 17). In platelets labelled with ^{14}C -glycerol, there was a $48 \pm 1.2\%$ increase in radioactivity in PA and a $4 \pm 0.1\%$ decrease in MPI 60 sec following ADP stimulation, with a significance of $P < 0.001$ and $P < 0.05$ respectively.

Aggregation studies between 2.5 min to 30 min

The change in labelling of TPI after prolonged incubation with ADP is shown in Figure 18. There was a significant decrease in the

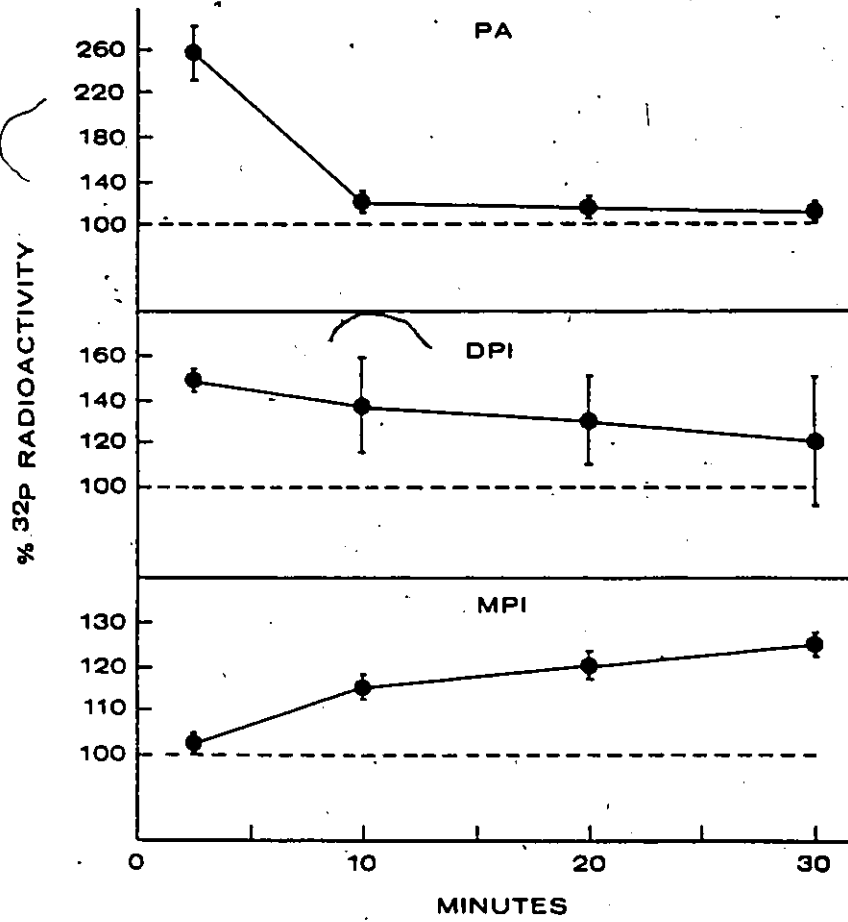


Figure 19.

Figure 19. Changes in the ^{32}P -content in PA, DPI and MPI during platelet aggregation and deaggregation after addition of ADP: The concentration of ADP was $10\ \mu\text{M}$. Data are presented in the same way as in Figure 14. Data were calculated as mean \pm S.E.M. of 10-20 pairs of samples from at least three experiments. The probabilities for difference (P values) in mean $^{32}\text{PO}_4$ radioactivity between the control and the stimulated platelets for PA, MPI and DPI are as follows:-

	<u>2.5 min</u>	<u>10 min</u>	<u>20 min</u>	<u>30 min</u>
PA	<0.001	<0.05	<0.05	NS
MPI	<0.05	<0.001	<0.001	<0.001
DPI	<0.001	NS	NS	NS

NS = not significant

labelling of TPI 2.5 min after the addition of ADP ($10\mu\text{M}$) a time at which the platelets were completely aggregated. However, at 10 min after exposure to ADP the platelets had deaggregated and the labelling of TPI was no longer significantly different from that of the control platelets. The ^{32}P -content in DPI and PA, which had increased during platelet aggregation, decreased in association with platelet deaggregation (Figure 19). The ^{32}P -content of PA approached that of the control sample 10 min after the addition of ADP. By 10 min the platelets had deaggregated, and by 30 min they had returned to their disc shape. At 10, 20 and 30 min the DPI values were not significantly different from the control values. In platelets labelled with ^{14}C -glycerol, the net changes of radioactivity in PA and MPI of the stimulated platelets 20 min after the addition of ADP were 3 ± 3 and -2 ± 2 % respectively, statistically indistinguishable from the control values.

Aggregation studies in which ADP was added to platelet suspensions 30 min after the first ADP addition

A second addition of ADP to the platelets 3 min after the first addition of ADP when they had partially deaggregated caused slight aggregation of the platelets but no further decrease in the ^{32}P -labelling of TPI (Table IV). The labelling of DPI and PA did not increase significantly after the second addition of ADP. However, when the second addition of ADP was made 30 min after the first addition of ADP (when platelets had deaggregated and had regained their disc shape), a decrease

TABLE IV. EFFECT OF SECOND ADDITION OF ADP ON THE 32 P-CONTENT OF PHOSPHOINOSITIDES AND PHOSPHATIDIC ACID

Time of second addition of ADP	Phospho-lipid	First addition	Second addition	% change in 32 P content 1.5 minutes after the addition of the second stimulus*	Significance of difference between means
Study I (3 min)	TPI	(1) Tyrode	Tyrode	100 (12)	(1) vs (2) NS
		(2) ADP	Tyrode	97.8 (11)	(1) vs (3) NS
		(3) ADP	ADP	94.8 (12)	(2) vs (3) NS
	DPI	(1) Tyrode	Tyrode	100 (11)	(1) vs (2) P < .001
		(2) ADP	Tyrode	123.8 (11)	(1) vs (3) P < .001
		(3) ADP	ADP	135.3 (12)	(2) vs (3) NS
	MPI	(1) Tyrode	Tyrode	100 (11)	(1) vs (2) P < .01
		(2) ADP	Tyrode	111.6 (10)	(1) vs (3) P < .05
		(3) ADP	ADP	110.6 (11)	(2) vs (3) NS
	PA	(1) Tyrode	Tyrode	100 (11)	(1) vs (2) P < .001
		(2) ADP	Tyrode	187.6 (10)	(1) vs (3) P < .001
		(3) ADP	ADP	231.8 (11)	(2) vs (3) NS

continued

TABLE IV continued.

Study II (30 min)	TPI	(1) Tyrode	Tyrode	100 (19)	(1) vs (2)	NS
		(2) ADP	Tyrode	103.3 (19)	(1) vs (3)	P < .05
		(3) ADP	ADP	94.9 (19)	(2) vs (3)	P < .01
DPI	(1) Tyrode	Tyrode	100 (19)	(1) vs (2)	P < .01	
	(2) ADP	Tyrode	111.3 (19)	(1) vs (3)	P < .001	
	(3) ADP	ADP	133.1 (19)	(2) vs (3)	P < .01	
MPI	(1) Tyrode	Tyrode	100 (19)	(1) vs (2)	P < .001	
	(2) ADP	Tyrode	121.5 (18)	(1) vs (3)	P < .001	
	(3) ADP	ADP	122.2 (19)	(2) vs (3)	NS	
PA	(1) Tyrode	Tyrode	100 (19)	(1) vs (2)	NS	
	(2) ADP	Tyrode	110.6 (18)	(1) vs (3)	P < .001	
	(3) ADP	ADP	220.6 (19)	(2) vs (3)	P < .001	

*Numbers in parentheses in column 5 indicate the number of samples. At least three experiments were done for each time interval. ADP was added initially to give a final concentration of 4 μ M. Three min (Study I) or 30 min (Study II) after the first addition an equal amount of ADP was added. The platelet suspensions were subjected to lipid extraction 1.5 min after the second addition.

in ^{32}P -content in TPI occurred (Table IV). Addition of ADP 30 min after the first addition caused an increase of ^{32}P -labelling in DPI and PA. The ^{32}P -content in MPI continued to rise during the 30 min following the first addition of ADP and remained elevated after a second exposure to ADP.

Inhibition of platelet aggregation by AMP

The results are shown in Table V. AMP (0.7 mM) inhibited ADP-induced platelet aggregation, but itself had no effect on the ^{32}P -labelling in PA or the phosphoinositides. In these studies, ADP (0.9 μM) caused changes in ^{32}P -labelling in the phospholipids similar to that described above. The decrease of ^{32}P -labelling in TPI and the increase of ^{32}P -content in PA and DPI was abolished by AMP.

3. . Discussion

Our results show a relationship between the decline in ^{32}P -labelling of TPI and the aggregation of platelets after the addition of ADP; the maximum decrease of radioactivity in TPI was observed when platelet aggregation reached its maximum. Labelling in TPI increased as soon as platelet deaggregation commenced and returned to prestimulation levels between 10 and 30 min after exposure to ADP.

The decrease of ^{32}P -labelling in TPI is unlikely to be caused by a depletion of the precursor pool (γ - ^{32}P) ATP for the phosphates on the inositol ring of TPI; platelets have an abundant source of metabolic ATP, the specific activity of which is several hundred-fold greater

TABLE V. INHIBITORY EFFECT OF AMP ON ADP-INDUCED CHANGES IN ³²P-LABELLING OF PHOSPHOINOSITIDES AND PHOSPHATIDIC ACID IN RABBIT PLATELETS.

Phospho-lipid	First Addition	Second Addition	% ³² P-Radioactivity (mean ± S.E.M.)	P Values *
TPI	(1) Tyrode	Tyrode	100.0 (20)	
	(2) Tyrode	ADP	96.3 ± 0.89 (14)	(1) vs. (2) P < 0.005
	(3) AMP	Tyrode	101.23 ± 1.33 (21)	(1) vs. (3) N.S.
	(4) AMP	ADP	104.31 ± 1.46 (19)	(1) vs. (4) P < 0.010
DPI	(1) Tyrode	Tyrode	100.0 (20)	
	(2) Tyrode	ADP	119.33 ± 4.03 (14)	(1) vs. (2) P < 0.001
	(3) AMP	Tyrode	99.74 ± 2.53 (20)	(1) vs. (3) N.S.
	(4) AMP	ADP	96.64 ± 2.23 (19)	(1) vs. (4) N.S.
MPI	(1) Tyrode	Tyrode	100.0 (20)	
	(2) Tyrode	ADP	103.88 ± 3.08 (10)	(1) vs. (2) N.S.
	(3) AMP	Tyrode	98.10 ± 1.76 (20)	(1) vs. (3) N.S.
	(4) AMP	ADP	97.55 ± 2.20 (19)	(1) vs. (4) N.S.
PA	(1) Tyrode	Tyrode	100.0 (20)	
	(2) Tyrode	ADP	233.60 ± 16.96 (10)	(1) vs. (2) P < 0.001
	(3) AMP	Tyrode	99.35 ± 2.96 (20)	(1) vs. (3) N.S.
	(4) AMP	ADP	99.80 ± 3.13 (19)	(1) vs. (4) N.S.

The second stimulus was added one minute after the addition of the first stimulus. The platelet suspensions were subjected to lipid extraction 1½ min after addition of the second stimulus. Final concentration of ADP: 0.9 µM. Concentration of AMP: 0.7 mM. Numbers in parenthesis in the fourth column represent the number of samples.

*Calculated using a paired difference analysis, paired with the Tyrode, Tyrode samples.

than the specific activity of TPI for the duration of the experiment. Furthermore, it has been demonstrated by Lloyd et al. (1972) that there are no significant changes in the specific activity of ATP in platelets aggregated by ADP. The observation over a prolonged period showed that the decrease in ^{32}P -labelling in TPI is reverted during deaggregation indicates that there is an available source of (γ - ^{32}P)ATP.

In other cells, the metabolism of the monoesterified phosphates of TPI has been shown to involve the enzymes TPI phosphomonoesterase (Keough and Thompson, 1970) and DPI kinase (Kai et al., 1968). Since most of the ^{32}P -incorporated into TPI resides in the monoesterified phosphates of this lipid (Hokin and Hokin, 1964), small changes in ^{32}P -labelling in TPI could therefore represent the net effect of degradation and synthesis of TPI by these enzymes. (Platelets do contain DPI kinase (Kaulen and Gross, 1976); the presence of TPI phosphomonoesterase has not yet been demonstrated directly). The decline of ^{32}P -labelling in TPI during ADP-induced platelet aggregation could thus be due either to inhibition of phosphorylation of DPI or to enhancement of hydrolysis of TPI. Inhibition of phosphorylation of DPI caused by ADP with unaltered TPI hydrolysis would result in a decrease in the labelling of TPI in the ADP-treated sample. Alternatively, enhanced hydrolysis of TPI caused by ADP without a change in phosphorylation of DPI would also result in a decrease in ^{32}P -labelling of TPI. Hydrolysis of TPI could also be caused by increased activity of polyphosphoinositide phosphodiesterase

(Thompson and Dawson, 1964), which leads to a release of diglyceride and a decrease in ^{32}P -labelling in TPI. The results from the present experiments do not distinguish these possible mechanisms.

It is also possible that ADP causes a simultaneous acceleration of phosphorylation of DPI and hydrolysis of TPI. If this were the case, it seems likely that the ^{32}P -content of TPI in the ADP-treated platelets would not be significantly different from the control during the time course of these experiments. The hydrolysis of TPI in the first 60 sec after ADP addition to platelets was confirmed by a similar decrease in the ^{14}C -arachidonic acid and ^3H -inositol labelling in this phospholipid. The loss in the radioactivity of various isotopes from TPI during platelet aggregation most likely represents hydrolysis of TPI to DPI and that return of ^{32}P -labelling to prestimulation level in TPI during platelet deaggregation reflects conversion of DPI back to TPI. The increase in the labelling of $^{32}\text{PO}_4$, ^{14}C -arachidonic acid and ^3H -inositol in DPI during ADP-induced platelet aggregation and the return of ^{32}P -labelling of DPI toward prestimulation level during platelet deaggregation are compatible with this hypothesis. Although the enzyme TPI phosphomonoesterase has not been directly demonstrated in platelets, it was shown to be a very active enzyme in many mammalian tissues (Michell, 1975). Kaulen and Gross (1976) demonstrated the presence of DPI kinase in platelets. Although synthesis of DPI from MPI is possible during ADP-induced platelet aggregation, the decrease in labellings of ^{14}C -glycerol,

^{14}C -arachidonic acid and ^3H -inositol in MPI argue against this possibility.

It may seem strange that the ^{32}P -labelling in MPI did not fall as did the labelling with other isotopes during ADP-induced aggregation. This paradox can be explained by the cyclical conversion of MPI. It has been shown in many mammalian tissues that MPI undergoes phosphodiesteratic cleavage to form 1,2-diacylglycerol upon stimulation (Michell, 1975). This compound is then phosphorylated to PA with ATP. PA is further converted to MPI with the formation of CDP-diacylglycerol as an intermediate. During this cyclical conversion, the diacylglycerol backbone of MPI is conserved, but its phosphate moiety can be renewed, possibly by $^{32}\text{PO}_4$ from (γ - ^{32}P)ATP. In this way, the ^{32}P -labelling in MPI is sustained. However, the decrease in ^{14}C -AA and ^3H -inositol isotopic labelling in MPI was limited, indicating that phosphodiesteratic cleavage is not a major reaction during ADP-induced platelet aggregation. In contrast, TPI and DPI interconversion appears to be a major reaction during ADP-induced platelet aggregation. Experimental evidence of DPI to TPI interconversion in stimulated mammalian tissue was first provided by Torda (1972). Recently, hydrolysis of TPI was also demonstrated in electrically or acetylcholine-stimulated crab nerves (Tret'jak et al., 1977) and in rabbit iris smooth muscle (Abdel-Latif et al., 1977).

AMP is a specific inhibitor of ADP-induced platelet aggregation. It inhibits platelet aggregation by a competitive mechanism (Mustard and Packham, 1970). AMP not only inhibited the decrease in ^{32}P -labelling of TPI in response to ADP stimulation, it slightly increased the content of this isotope in TPI. AMP also abolished the rise in

^{32}P -content in DPI. These findings indicate a relationship between TPI degradation and the functional changes of platelets. The changes in ^{32}P labelling of PA was also inhibited by AMP, further substantiating a close relationship of PA and phosphoinositide metabolism and platelet aggregation.

When platelets are refractory to ADP, the relationship between DPI and TPI may be altered. For some time after the initial addition of ADP the deaggregated platelets either did not aggregate or aggregated poorly to further ADP stimulation (refractory period). During this period the ^{32}P -labelling in DPI and TPI returned toward the control values. Stimulation with ADP during this period did not cause any further changes in MPI, DPI, TPI or PA. When the platelets had regained their disc shape and their responsiveness to ADP, a decrease in ^{32}P -labelling of TPI and an increase in ^{32}P -labelling of DPI and PA could again be elicited. Thus, the loss of refractoriness of platelets is associated with restoration of ^{32}P -labelling in TPI, DPI and PA towards their prestimulation values. This indicated that returning of inositol phospholipid and PA metabolism to control levels is part of the restoration of platelet sensitivity to ADP.

The changes in the phosphoinositides labelled with isotopes in the present experiments are comparatively less than the changes observed in other mammalian tissues (Tret'jak et al. 1977; Abdel-Latif et al., 1977; Jones and Michell, 1974; Hokin-Neaverson, 1974). In these latter experiments, however, tissues were incubated with the

appropriate stimulus for at least 10 min to 1 hour. In the present experiments the data were collected within 1 min following addition of ADP. The differences in the experimental planning and in the magnitude of changes in phosphoinositides between these experiments and those of other investigators reflect the fundamental differences between platelets and other tissues in their response to stimuli and the types of stimuli used. Since in experiments with thrombin (Chapter 6) we observed much larger and rapid changes in the radioactivity of isotopes in PA and the phosphoinositides, the smaller changes in these lipids in the ADP-stimulated platelets could also be due to the nature of ADP as a less potent stimulus.

Platelets possess a highly fluid membrane. They demonstrate visible shape change 7-9 sec after the addition of ADP and attain maximum aggregation in approximately 1 min. It is possible that the extremely acidic phosphoinositides and phosphatidic acid are located on the inner surface of the platelet membrane (Shick et al., 1976) and distributed mainly around the Na^+/K^+ or Ca^{2+} channels. Hydrolysis of TPI with concurrent release of bound Ca^{2+} could therefore change the charge properties of these channels. Thus the amount of TPI hydrolysed need not be very large before membrane permeability is altered considerably, according to Hendrickson and Reinertsen (1971). In addition, Diamond and Wright (1969) indicated that pore charge was of greater importance than pore size in the control of selective cation permeability in a biological system. Thus, it is possible that the changes in PA and inositol phospholipids in the present studies,

although small, may be closely related to the alteration of membrane permeability during platelet aggregation induced by agents such as ADP.

CHAPTER FIVE

Experiment C

Comparison of Changes in Isotopically Labelled Phosphatidic Acid
and Phosphoinositides of Platelets in Response to Ionophore A23,187 and

ADP

1. Introduction

In a medium containing Ca^{2+} the divalent cation ionophore A23,187 causes contraction of rabbit aorta (Pressman, 1973), elevation of the resting potential and twitching tension of rat diaphragm (Levy et al., 1973), and uncoupling of oxidative phosphorylation in mitochondria (Reed and Lardy, 1972). The compound also elicits the release reaction from a variety of secretory cells, including the adrenal medulla (Ricci et al., 1975), rat neurohypophysis (Nordmann and Currell, 1975), parotid slices (Selinger et al., 1974) and pancreatic α and β cells (Wollheim et al., 1975; Williams and Lee, 1974). In human platelets it has been shown that A23,187-induced aggregation requires the presence of Ca^{2+} but the release reaction can take place in the presence or absence of Ca^{2+} (Feinman and Detwiler, 1975; White et al., 1974). Previous studies have been shown that the ionophore can induce platelet aggregation independent of the release of ADP as well as independent of the formation of endoperoxides or thromboxane A_2 (Packham et al., 1977).

A23,187 also causes changes in membrane lipids. Allan and associates (1976) using human erythrocytes found that the incorporation of $^{32}\text{PO}_4$ into phosphatidic acid was enhanced and that the amount of membrane diacylglycerol was also increased by five to seven fold following incubation of human erythrocytes with the compound for 1 hour. On the other hand, ^{32}P -labelling decreased in TPI and DPI in the A23,187-treated erythrocytes (Lang et al., 1977). In platelets labelled

with radioactive arachidonic acid, A23,187 caused a rapid formation of free arachidonic acid and a corresponding loss in this fatty acid from phospholipids, indicating activation of phospholipase A₂ in platelets (Pickett et al., 1977). Significant loss of arachidonic acid occurred in phosphatidylcholine, phosphatidylethanolamine and monophosphoinositide. Plasmalogen phosphatidylethanolamine was the only phospholipid shown to have gained a significant amount of radioactivity (Rittenhouse-Simmons and Deykin, 1977). Thrombin-treated platelets demonstrated similar changes in these phospholipids (Rittenhouse-Simmons et al., 1976, 1977; Bills et al., 1976).

The mechanism(s) involved in platelet aggregation is not yet clearly defined. Despite the diversified nature of the stimuli, the manifestations of functional changes in platelets remain the same, i.e. shape change and aggregation or the release reaction, depending upon the conditions of the experiments. However, the pathways for A23,187-induced platelet aggregation would seem to be different from that for aggregation induced by thrombin or ADP. The latter two stimuli are considered to interact with specific receptors on the platelet surface, causing changes in membrane structures and/or activation of a 'second messenger' system (Detwiler et al., 1975). Ionophore A23,187 on the other hand can cause platelet aggregation by carrying divalent cations directly across the membrane and/or by mobilization of Ca²⁺ from intracellular binding sites (Reed and Lardy, 1972; Pressman, 1973; Friedman and Detwiler, 1975). It was previously observed that AMP,

a specific inhibitor of ADP-induced platelet aggregation had no effect on the action of A23,187 and that dibutyryl cyclic AMP which abolishes the effect of thrombin had no effect on the reaction to A23,187.

With these differences in mind it is reasonable to expect that variations of phospholipid metabolism may be present early in the response of platelets to stimuli and that experimental observations obtained subsequent to these initial changes might, therefore, fail to detect such differences. This is probably also true of the experiments using other mammalian tissues which do not show functional changes as readily as those detected in platelets. Therefore, despite the similarity in the functional changes of platelets and the reported changes in platelet phospholipids in response to various stimuli, we have decided to examine the metabolism of phosphatidic acid and the phosphoinositides early in the interaction between platelets and A23,187. In addition, we compared these changes to those elicited by ADP described in the previous experiments. The present experiments were carried out with washed rabbit platelet suspension labelled with $^{32}\text{P}\text{O}_4$ or ^{14}C -arachidonic acid. Thrombin-degradulated platelets were used in some of the experiments.

2. Results

Table VIa shows that MPI, DPI and TPI of unstimulated normal platelets had similar ^{14}C -arachidonic acid specific radioactivity and that their specific radioactivity values were many fold higher than that of PA. When platelets labeled with ^{14}C -arachidonic acid were

TABLE VIa. SPECIFIC RADIOACTIVITY OF ^{14}C -ARACHIDONIC ACID
IN PA AND THE PHOSPHOINOSITIDES OF UNSTIMULATED WASHED
RABBIT PLATELETS.

Phospholipids	Specific Radioactivity dpm/nmole (mean \pm S.E.M.)
PA	136 \pm 22
MPI	994 \pm 22
DPI	866 \pm 71
TPI	1182 \pm 54

The data represent the average values of five samples.
Platelets were incubated with 1- ^{14}C -arachidonic acid for
an hour, washed once in calcium free Tyrode solution and
resuspended in Tyrode albumin solution with Ca^{2+} .

stimulated with A23,187, the labelling was significantly increased in PA, DPI and TPI at 30 sec and 60 sec. In contrast, MPI demonstrated a loss of ^{14}C radioactivity (Figure 20).

When MPI was assayed for phosphorus, there was a significant loss of the phospholipid 3 min after the addition of the ionophore at concentrations of 0.6 μM and 1.2 μM A23,187 (Table VIb).

There was no initial rapid rise in ^{32}P -labelling in PA and no decrease of radioactivity in TPI in both the thrombin-degranulated and the non-degranulated platelets (Figure 21). Degranulated platelets were used to avoid the effect of the released substances such as ADP on phospho metabolism. At 60 sec, there was a significant increase in ^{32}P -labelling in PA, DPI and TPI in both normal and thrombin-degranulated platelets, but for DPI the percentage change was greater in the non-degranulated platelets.

With rabbit platelets, A23,187 caused aggregation and the release (80% of ^3H -serotonin) in the presence of calcium. The ionophore did not cause aggregation in the absence of Ca^{2+} 60 sec after the addition of the ionophore (Figure 22). The amount of ^{14}C -arachidonic acid in PA, DPI and TPI was higher in platelets stimulated with A23,187 in the presence of calcium than in its absence (Table VII). The radioactivity in MPI, was lower in platelets in the presence of external calcium (Table VII).

3. Discussion

Comparison of the effect of A23,187 and ADP on platelet phosphatidic acid and phosphoinositide metabolism within the first 60 sec have brought forth the following observations.

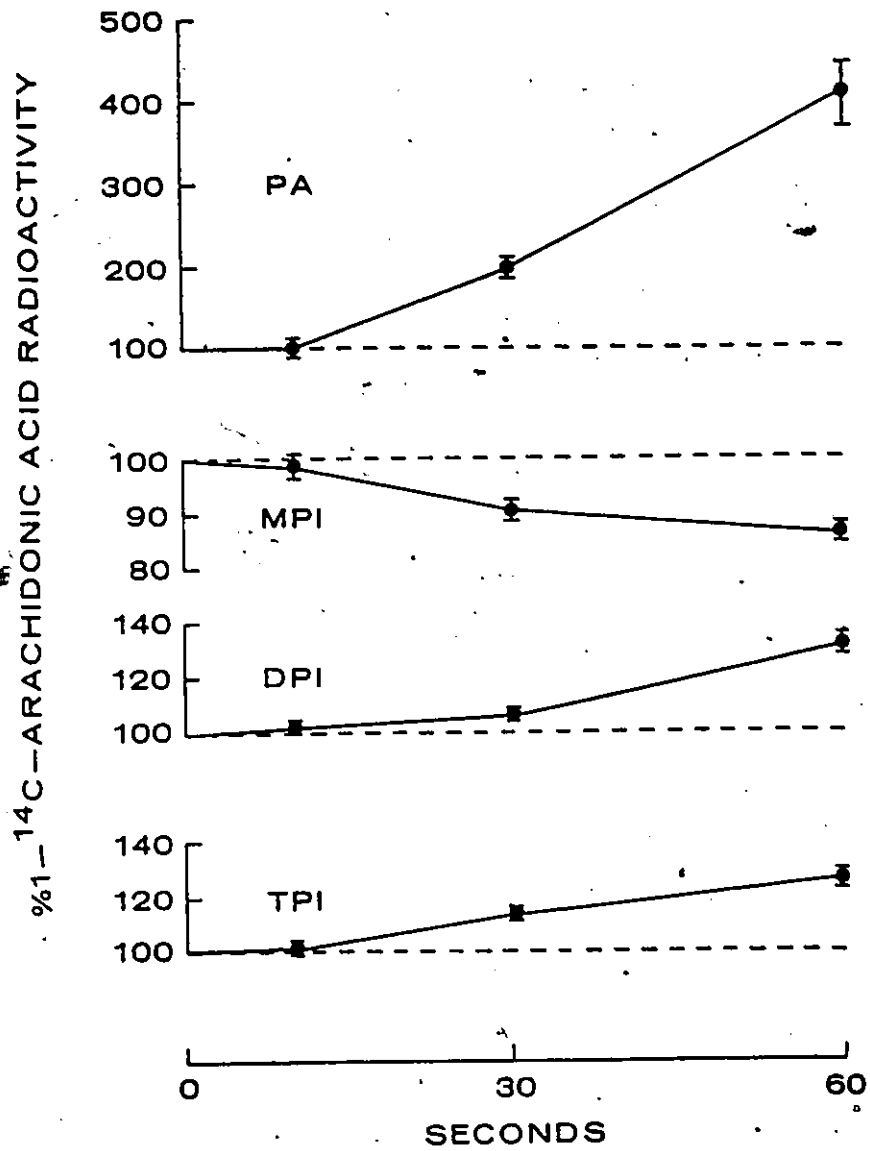


Figure 20.

Figure 20. Changes in $1-^{14}\text{C}$ -arachidonic acid labelling in rabbit platelets in response to A23,187 (6.7×10^{-7} M). The percentage changes (mean \pm S.E.M.) were obtained from two experiments, each containing 5 pairs of samples. The P values derived from paired-t-test are as follows:

	<u>10 sec</u>	<u>30 sec</u>	<u>60 sec</u>
PA	NS	<0.001	<0.001
MPI	NS	<0.001	<0.001
DPI	NS	<0.025	<0.001
TPI	NS	<0.005	<0.025

NS = not significant

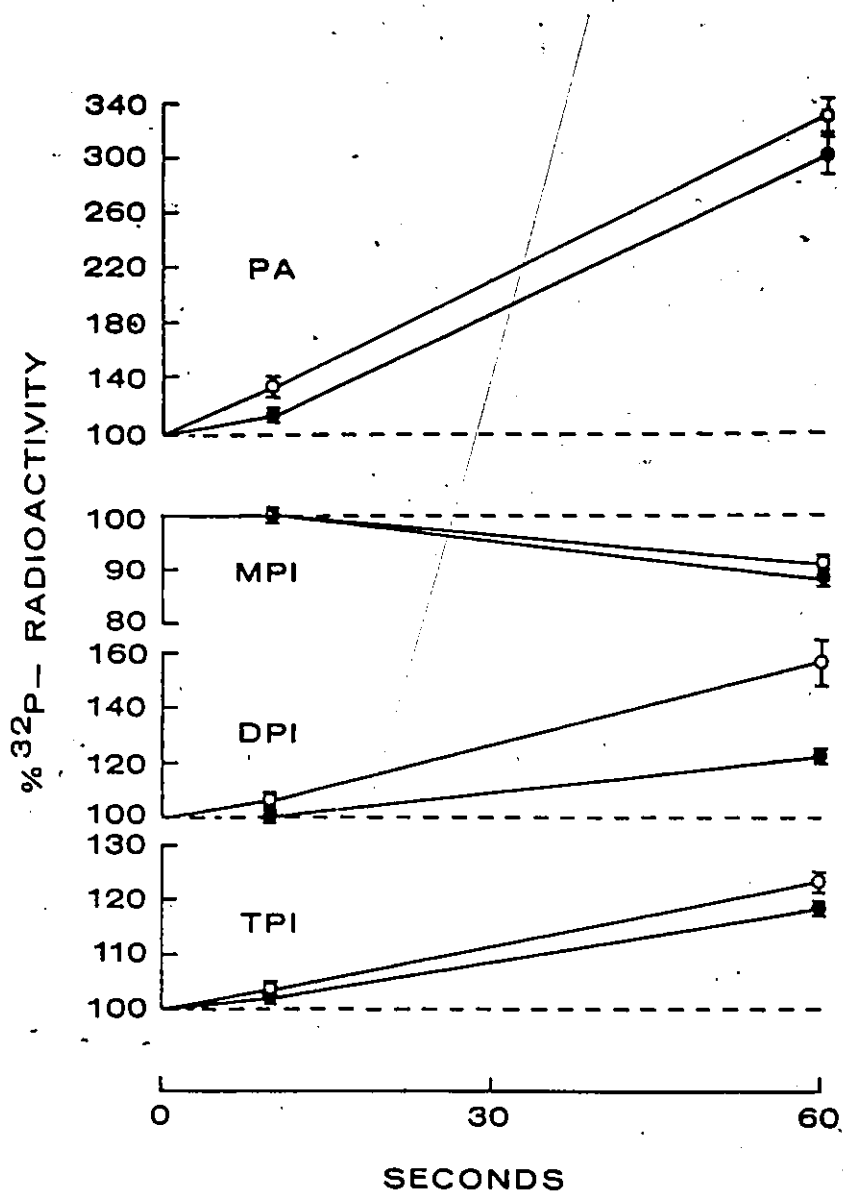


Figure 21.

Figure 21. Changes in ^{32}P -labeling in PA and phosphoinositides in response to A23,187 (6.7×10^{-7} M) in thrombin-degranulated platelets (●) and non-degranulated platelets (○). The percentage (mean \pm S.E.M.) for the thrombin-degranulated platelets were obtained from 28-33 pairs of samples of six experiments, using the degranulated platelets as controls. For the non-degranulated platelets, 14-18 pairs of samples from four experiments were used for the calculations, using non-degranulated platelets as controls. The P values derived from paired-t-test are as follows:

	<u>Thrombin-degranulated platelets</u>		<u>Non-degranulated platelets</u>	
	<u>10 sec</u>	<u>60 sec</u>	<u>10 sec</u>	<u>60 sec</u>
PA	<0.005	<0.001	<0.005	<0.001
MPI	NS	<0.001	NS	<0.005
DPI	NS	<0.001	<0.02	<0.005
TPI	NS	<0.001	NS	<0.001

NS = not significant

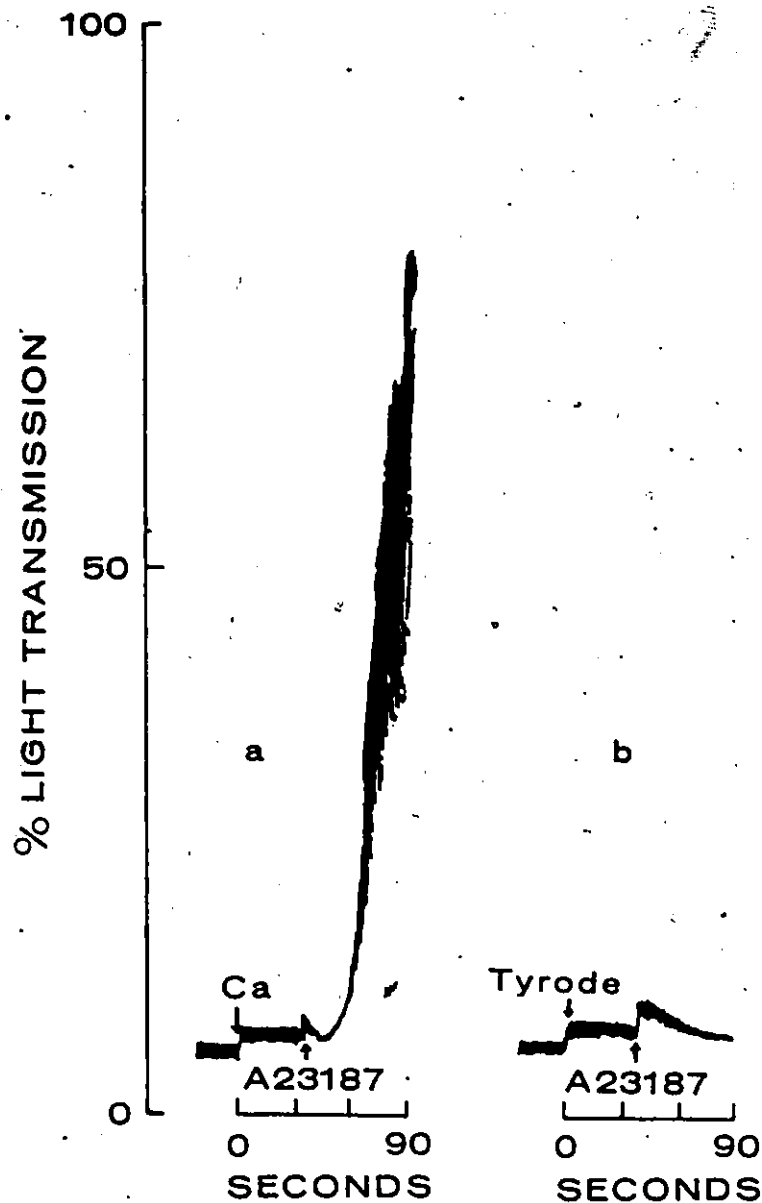


Figure 22. Aggregometer tracing of A23,187-induced platelet changes in the presence and absence of external Ca^{2+} . (a) A23,187 (6.7×10^{-7} M) induced shape change, aggregation in the presence of CaCl_2 (6.6×10^{-3} M). (b) Without Ca^{2+} , A23,187 caused shape change without aggregation of platelets.

TABLE VII. REDUCTION OF MPI IN PLATELETS IN RESPONSE TO A23,187.

	MPI nmoles/10 ⁹ platelets (mean ± S.E.M.)		Length of stimulation (min)	P values **
	A23,187*	Control		
Experiment A	8.80 ± 0.32	9.89 ± 0.32	3	<0.025
Experiment B	7.76 ± 0.32	9.89 ± 0.32	3	<0.001

The data were obtained from phosphorus assay of MPI of 7 pairs of samples in 3 experiments. The amount of phosphorus was corrected for recovery and converted to nmoles/10⁹ platelets.

* Concentration of A23,187 in Experiment A was 0.6 μM and that in Experiment B was 1.2 μM. The platelet suspensions treated with A23,187 of these 2 concentrations were run simultaneously with one control suspension to which Tyrode was added.

** P values were calculated by the 2-tailed-paired-t-test on the A23,187-stimulated samples and the controls.

TABLE VII. EFFECT OF IONOPHORE A23,187 ON THE 1-¹⁴C-ARACHIDONIC ACID LABELLING IN PA AND PHOSPHOINOSITIDES IN THE PRESENCE AND ABSENCE OF EXTERNAL Ca⁺⁺.

Phospholipids	Total Radioactivity dpm/10 ⁹ platelets		P Value
	A23,187 + Ca ⁺⁺	A23,187 - Ca ⁺⁺	
PA	492 ± 44	155 ± 12	<0.001
MPI	6074 ± 352	7270 ± 193	<0.001
DPI	1873 ± 89	1106 ± 52	<0.001
TPI	1492 ± 14	1153 ± 45	<0.001

The data represent mean ± S.E.M. of five samples from one of the two similar experiments. The platelets were subjected to lipid extraction 60 sec after the addition of A23,187 (6.7×10^{-7} M). The concentration of Ca²⁺, when present, was 6.6×10^{-3} M. P values were calculated by paired-difference test. See also

Figure 22.

In contrast to ADP, treatment of platelets with the ionophore caused an increase in ^{32}P -labelling as well as ^{14}C -arachidonic acid in TPI during the first 60 sec. Both aggregating agents caused increased isotope labellings in DPI.

In contrast to ADP, A23,187 caused a small increase in ^{32}P -labelling of PA in both normal and thrombin-degranulated platelets without significant change in ^{14}C -arachidonic acid labelling at 10 sec.

Also, ADP caused a more rapid increase in ^{14}C -arachidonic acid labelling of PA than did A23,187 even though both agents induce aggregation at about the same time. Increase in ^{32}P -labelling of PA was detected as early as 2 sec after the addition of ADP by previous investigators (Lloyd et al., 1973). Sixty seconds following addition of the ionophore, the ^{32}P - and ^{14}C -arachidonic acid labelling in PA of the stimulated platelets increased to three times that of unstimulated platelets. At this time, however, radioactivity of PA labelled with $^{32}\text{PO}_4$ and ^{14}C -arachidonic acid of the ADP-aggregated platelets had declined. (Fig. 15, 16)

Ionophore A23,187 caused a significant decrease in ^{32}P - and ^{14}C -arachidonic acid labelling in MPI at 60 sec. Assay for the amount of phosphorus in MPI 3 min after addition of the ionophore showed a considerable reduction in this phospholipid. In contrast, 60 sec following addition of ADP to a platelet suspension, there was no significant change in the ^{32}P -labelling in MPI and only a small decrease in ^{14}C -arachidonic acid and ^3H -inositol in this lipid.

In mammalian tissues stimulated with appropriate agents, MPI can undergo three pathways of metabolism: a phospholipase C type cleavage to form diacylglycerol, conversion to DPI and TRI or hydrolysis by phospholipase A₂ to yield free fatty acid and lyso MPI. It is possible that all three of these pathways were stimulated in A23,187-treated platelets, with each pathway leading to a loss of MPI.

Phosphodiesteratic cleavage of MPI is suggested by a moderate increase in ¹⁴C-arachidonic acid in PA, a phospholipid with ¹⁴C-arachidonic acid specific radioactivity many times lower than that of the phosphoinositides. This pathway appeared to occur more slowly than when ADP was used as the aggregating agent. Furthermore, unlike ADP, A23,187 apparently did not cause as much conversion of PA to MPI mediated by CDP-diacylglycerol formation since in contrast to ADP-stimulation during the first minute the ³²P-labelling in MPI significantly declined even at 30 sec. The moderate increase in ³²P-labelling in PA may, therefore, reflect some stimulation of the phosphatidate phosphohydrolase and diacylglycerol kinase activities. It could also be interpreted that the amount of 1,2-diacylglycerol formed from the phosphodiesteratic-cleavage of MPI in the ionophore-treated platelets was of limited extent in the first 60 sec. Evidence for phosphodiesteratic cleavage of MPI in stimulated mammalian tissues was first provided by Durwell and Garland (1969) in acetylcholine-stimulated brain homogenate and was further substantiated by the studies of HoKin-Neaverson (1974) and her associates (Geison et al., 1976) with pancreas as well as by

Jones and Michell (1974) working with parotid gland fragments.

The increased labelling of DPI and TPI in platelets prelabelled with $^{32}\text{PO}_4$ and ^{14}C -arachidonic acid may indicate synthesis from MPI. The failure to demonstrate a fall in radioactivity in TPI may indicate that the ionophore does not cause increased hydrolysis of TPI or that the increased hydrolysis is offset by the increased synthesis from DPI. It is possible that the divalent cation ionophore by either mobilizing internal calcium or by transporting calcium ions directly into the cell, does not require hydrolysis of TPI to free membrane calcium in these reactions.

Although in the present experiments measurements of the formation of free arachidonic acid and lyso MPI were not carried out, there are reasons to believe that activation of phospholipase A_2 of platelet membrane is an important cause for the ionophore-induced loss of isotopic labelling and the loss of phosphorus from the MPI fraction. Pickett and associates (1977) reported that A23,187 caused the release of five times more arachidonic acid from platelets than did thrombin at concentrations which produce a maximum stimulation of O_2 consumption. Significant loss of arachidonic acid occurred in MPI of A23,187 treated platelets (Rittenhouse-Simmons and Deykin, 1977).

Although ADP does not cause the release reaction of washed rabbit platelets (Packham et al., 1971) and ionophore does, the observed differences in the incorporation of isotopes into PA, MPI

and TPI by these two agents are likely not due to the effect of released ADP. The rationale being that the changes in ^{32}P -labeling in these phospholipids induced by the ionophore were similar in the thrombin-degranulated and non-degranulated platelets. The latter platelets have no releasable ADP (Kinlough-Rathbone et al., 1975).

The differences could be related to the mechanisms of platelet aggregation induced by these agents. ADP is considered to cause platelet aggregation by a primary mechanism by interaction with specific receptors on the platelet surface (Mustard et al., 1975). The nature of the receptors has not been clearly defined. It has been shown that interaction of ADP and platelets caused influx of Na^+ into the platelets (Feinberg et al., 1977). This is compatible with the recent observation of hydrolysis of TPI in association with increased K^+ permeability in acetylcholine-stimulated crab nerve (Tretjak et al., 1977). A number of investigators have suggested interconversion of TPI and DPI may regulate of membrane permeability toward monovalent cations (Hawthorne, 1970; Hendrickson and Reinertsen, 1971).

Ionophore A23,187, on the other hand, is considered to cause platelet aggregation by at least 3 mechanisms - the release reaction, formation of prostaglandin endoperoxides and thromboxane A_2 , and an unknown mechanism(s). The first two reactions may occur secondary to or concurrent with the third mechanism (Packham et al., 1977). This third mechanism is possibly due to disruption of membrane organization by the ionophore molecules

as they move the Ca^{2+} from intracellular structures. The loss of MPI from stimulated platelets in the present experiments is compatible with a secondary role of A23,187-induced endoperoxide formation in the mechanism of platelet aggregation caused by this agent, 'secondary' in the sense that the precursor of endoperoxides which are potent platelet aggregation agents, is formed from MPI after activation of phospholipase A_2 by Ca^{2+} either released from the intracellular binding sites or transported into platelets by the ionophore. Hydrolysis of MPI, however, may occur very early in the aggregation process.

The importance of Ca^{2+} in the activation of phospholipase A_2 may be illustrated by the present observation that loss of ^{14}C -radioactivity ^{14}C -arachidonic acid labelled MPI of rabbit platelets was greater in the presence than in the absence of external calcium. Although Pickett and associates (1977) reported that greater amounts of ^{14}C -arachidonic acid were lost from human platelet phospholipids with A23,187 in the absence of external Ca^{2+} than in its presence, it does not exclude the importance of Ca^{2+} in the activation of platelet phospholipase A_2 . Kinlough-Rathbone and associates (1976) also demonstrated that in the absence of external Ca^{2+} , human platelets underwent second phase aggregation in response to ADP and second phase aggregation did not occur in the presence of Ca^{2+} . Furthermore, indomethacin which abolished the

second phase aggregation induced by ADP also inhibited loss of ^{14}C -arachidonic acid from MPI (unpublished observations). What these experiments have illustrated is that human platelets require external Ca^{2+} for membrane stabilization. Removal of Ca^{2+} from one side of the membrane may generate a membrane potential across the membrane and render it less stable, as has been documented with phospholipid bilayers by Papahadjopoulos (1973). These changes may favor the exposure of substrates to phospholipase A_2 . Therefore, when the ionophore molecules make their way across the platelet membrane, causing further disruption of membrane architecture, and the release of Ca^{2+} from the intracellular binding sites (Feinman and Detwiler, 1974; White et al., 1974), the membrane phospholipase A_2 would be readily activated by the free Ca^{2+} . The role of Ca^{2+} in the activation of platelet phospholipase A_2 , depolymerization of microtubules and activation of actomyosin ATP-ase have recently been summarized by Haslam and Associates (1978).

CHAPTER SIX

Experiment D

Early Degradation of Monophosphoinositide and Triphosphoinositide of
Platelets in Response to Thrombin Stimulation

1. Introduction

Thrombin is a strong activator of platelets in haemostasis. It induces aggregation and the release reaction of platelets. At low concentrations, thrombin causes only shape change. At high concentrations, thrombin induces the release reaction of human platelets in the absence of calcium without the onset of platelet aggregation (Kinlough-Rathbone et al., 1975).

In addition, thrombin influences the metabolism of platelet phospholipids during platelet aggregation and release. It enhances the incorporation of $^{32}\text{PO}_4$ into PA, DPI and TPI 10 sec after addition of thrombin to washed human platelets prelabelled with $^{32}\text{PO}_4$, and into MPI in 1-2 min (Kaulen and Gross, 1976). Thrombin also prevents incorporation of ^3H -glycerol into phosphatidylcholine and phosphatidylethanolamine, indicating inhibition of de novo synthesis or a net loss of these phospholipids (Lewis and Majerus, 1969). This is consistent with the recent findings that thrombin causes a decrease in ^{14}C -arachidonic acid content from several major phospholipids of platelets, including PC, PE and MPI (Bill et al., 1976; Rittenhouse-Simmons et al., 1976). Furthermore, ^{14}C -arachidonic acid is transferred to plasmalogen phosphatidylethanolamine during thrombin-induced platelet aggregation (Rittenhouse-Simmons et al., 1976). In these studies, however, the metabolic relationship among the various phospholipids was not studied. Furthermore, the apparent differences in the response of platelet MPI metabolism to thrombin in terms of $^{32}\text{PO}_4$ and ^{14}C -arachidonic acid

incorporation requires some clarification.

It has been observed in other mammalian tissues that there are three pathways for MPI metabolism. MPI can be degraded to form 1,2-diacyl-glycerol (Durell and Garland, 1969; Hokin-Neaverson, 1974; Jones and Michell, 1974) and inositol-1,2-cyclic phosphate (Michell and Lapitina, 1972) as a result of the action of MPI phosphodiesterase. 1,2-Diacylglycerol is rapidly phosphorylated to form PA which in turn, can be converted to MPI through an intermediate CDP-diacylglycerol. It is also possible for MPI to be phosphorylated with ATP by specific kinases to form DPI and TPI. There are also specific hydrolytic enzymes that can convert TPI to DPI and DPI to MPI (Dawson and Thompson, 1964). Furthermore, as a result of phospholipase A₂ activity, MPI may release a fatty acid and form lyso-MPI. Thus far, the possibility that all three pathways could occur simultaneously in stimulated tissues has not been reported.

We have in our previous experiments observed that ADP did not cause the release reaction of washed rabbit platelets, while thrombin did. Moreover, ionophore A23,187 and thrombin appear to cause platelet aggregation and the release reaction by different pathways (Luscher and Massini, 1975). This has raised the question if thrombin elicits a pattern of phosphoinositide metabolism different from the other two platelet aggregating agents. Moreover, do all three pathways mentioned for MPI metabolism operate during thrombin-induced platelet aggregation and release reaction? And if this occurs, which pathway may predominate?

TABLE VIII. DISTRIBUTION OF RADIOACTIVITY OF $^{32}\text{P}\text{O}_4$, 1- ^{14}C -ARACHIDONIC ACID, 2- ^3H -GLYCEROL AND 2- ^3H -INOSITOL IN PLATELET PHOSPHOLIPIDS.

ISOTOPES	PA	MPI	DPI	TPI	PC	PE	PS	FPA	DC	TG
$^{32}\text{P}\text{O}_4$	3.1**	17.2**	22.8***	53.0**	1.5***	1.7***	0.6***	-	-	-
1- ^{14}C -arachidonic acid	0.27**	14.2**	2.1**	2.6**	47.9**	27.7**	2.1***	1.7**	0.05*	1.0***
2- ^3H -glycerol	3.0**	11.5**	4.0**	3.1**	49.7***	18.9**	0.76***	0.17**	0.6*	7.8**
2- ^3H -inositol	-	79.5*	11.4**	9***	-	-	-	-	-	-

* S.E.M. < 4%, ** S.E.M. between 4-7%, *** S.E.M. between 10-20%

The data represent % distribution of radioactive isotopes in lipids approximately 3 hours after the addition of the isotopes to the platelet suspensions. Preparation of ^{32}P -labeled platelets was carried out by washing the platelets obtained from platelet-rich plasma in a calcium-free, phosphate-free Tyrode-albumin solution. The platelets were then incubated with ^{32}P -ortho-phosphate in a phosphate-free medium containing calcium for an hour and was again washed in a calcium-free and phosphate-free medium before resuspending in the final medium. Preparation of platelets labeled with 1- ^{14}C -arachidonic acid, 2- ^3H -glycerol or 2- ^3H -inositol were carried out similarly, except that the media contained unlabelled-phosphate. The radioactivity of individual lipids was expressed as percentage of the total lipid-bound radioactivity. The data were obtained from six samples.

FPA= free fatty acid, DC = diacylglycerol, TG= triacylglycerol.

The present experiments are designed to answer these questions by measuring the changes in platelet PA and phosphoinositides labelled with different isotopic precursors as well as by phosphorus analysis of these phospholipids after thrombin stimulation. In addition, emphasis was placed on the biochemical reactions of PA and phosphoinositides during the first 9 sec of thrombin stimulation for the reason that the events occurring in the first few seconds are the most pertinent to the mechanism of platelet aggregation in response to thrombin.

2. Results

Table VIII shows the distributions of various isotopes in lipids of stimulated platelets approximately 3 h after the addition of the isotopes to platelet suspension, a time when the aggregation studies were conducted. With $^{32}\text{PO}_4$ as precursor, 95% of radioactivity in lipids was located in PA and the phosphoinositides; a negligible amount of $^{32}\text{PO}_4$ was found in the major phospholipids. However, PC and PE together comprised 70% or more of total ^{14}C -arachidonic acid or ^3H -glycerol radioactivity. Although the amount of MPI in platelets is only 1/8 that of PC, the amount of isotopes incorporated into MPI (14% of the ^{14}C -arachidonic acid and 11% of the ^3H -glycerol) was between 1/2 - 1/4 that incorporated into PC. In addition, MPI constitutes about 60% of the inositol phospholipids yet it incorporated about 80% of the ^3H -inositol. Thus, all components in the MPI molecule turn over quickly. Indeed, MPI has a specific radioactivity in ^{14}C -arachidonic acid twice as high as

TABLE IX. 1-¹⁴C-ARACHIDONIC ACID LABELING IN THE MAJOR PHOSPHOLIPIDS AND DI- AND TRI-ACYLGLYCEROL OF RABBIT PLATELETS BEFORE AND AFTER EXPOSURE TO THROMBIN.

LIPIDS	TOTAL RADIOACTIVITY		P VALUE
	CONTROL	THROMBIN	
PC	33923 ± 837	31209 ± 440	<0.001
PE	19601 ± 516	18620 ± 431	<0.05
PS	1450 ± 45	1493 ± 38	N.S.
DG	38 ± 4	198 ± 14	<0.001
TG	711 ± 16	675 ± 17	N.S.

The values in this table represent the mean ± S.E.M. of five samples from a typical experiment. Three experiments were performed with comparable results.

The concentration of thrombin was 0.33 u/ml and the duration of stimulation was 60 sec.

Abbreviations: PC = phosphatidylcholine, PE = phosphatidylethanolamine, PS = phosphatidylserine, DG = 1,2-diacylglycerol, TG = triacylglycerol.

that in PC (approximately 1000 vs 500 dpm/nmole). The specific radioactivity of MPI is shown in Table XII and that of PC was calculated from the radioactivity shown in Table IX and the phosphorus assay of PC (about 81 nmole/ 10^9 platelets).

The results also demonstrated a low content of ^{14}C -arachidonic acid in PA, di- and triacylglycerol and a more extensive incorporation of ^3H -glycerol into these lipids (Table VIII). PS had the least active metabolism since it incorporated negligible amounts of various isotopes. The synthesis of PS by the base exchange reactions of PC or PE could therefore be a very slow process in platelets.

Figure 23 shows the aggregometer tracing of thrombin-induced platelet shape change, aggregation and the release reaction. Maximum shape change took place within 9 sec of thrombin addition. At this time, there was a significant increase in the ^{14}C -arachidonic acid in PA and a significant decrease in ^{14}C -arachidonic acid in MPI (Figure 24). No significant changes in DPI and TPI and other phospholipids neutral lipids were observed at this time. During the first 1 min, large quantities of ^{14}C -arachidonic acid became incorporated into PA and the radioactivity in diacylglycerol increased 500%. There was a significant increase in radioactivity in DPI and TPI (Figure 24). There was also a small but significant loss in ^{14}C -arachidonic acid from PC and PE 60 sec following thrombin stimulation but there was no significant change in triacylglycerol (Table IX). With ^3H -glycerol-labelled platelets a significant increase in radioactivity

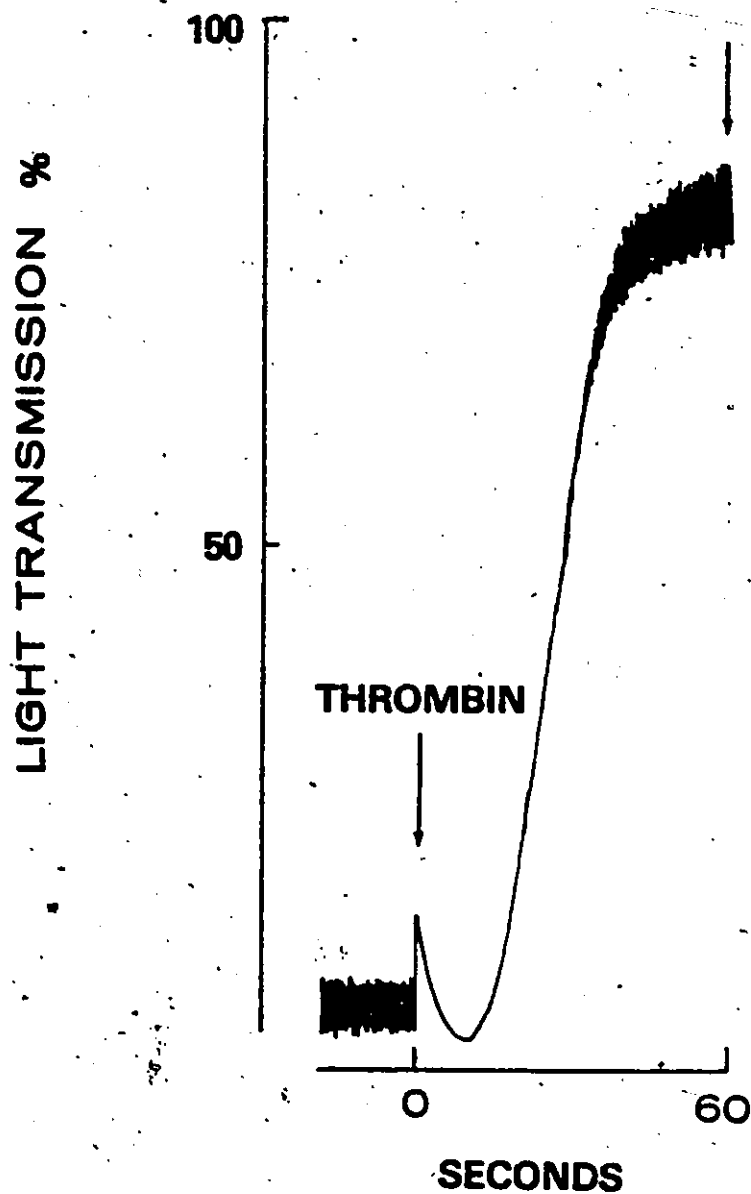


Figure 23. Aggregometer tracing of platelet aggregation induced by thrombin (0.33 u/ml). Maximum swelling and platelet shape change occurred at 9 s. The platelet release reaction was 70% at 60 s in platelets labelled with ^{14}C -serotonin.

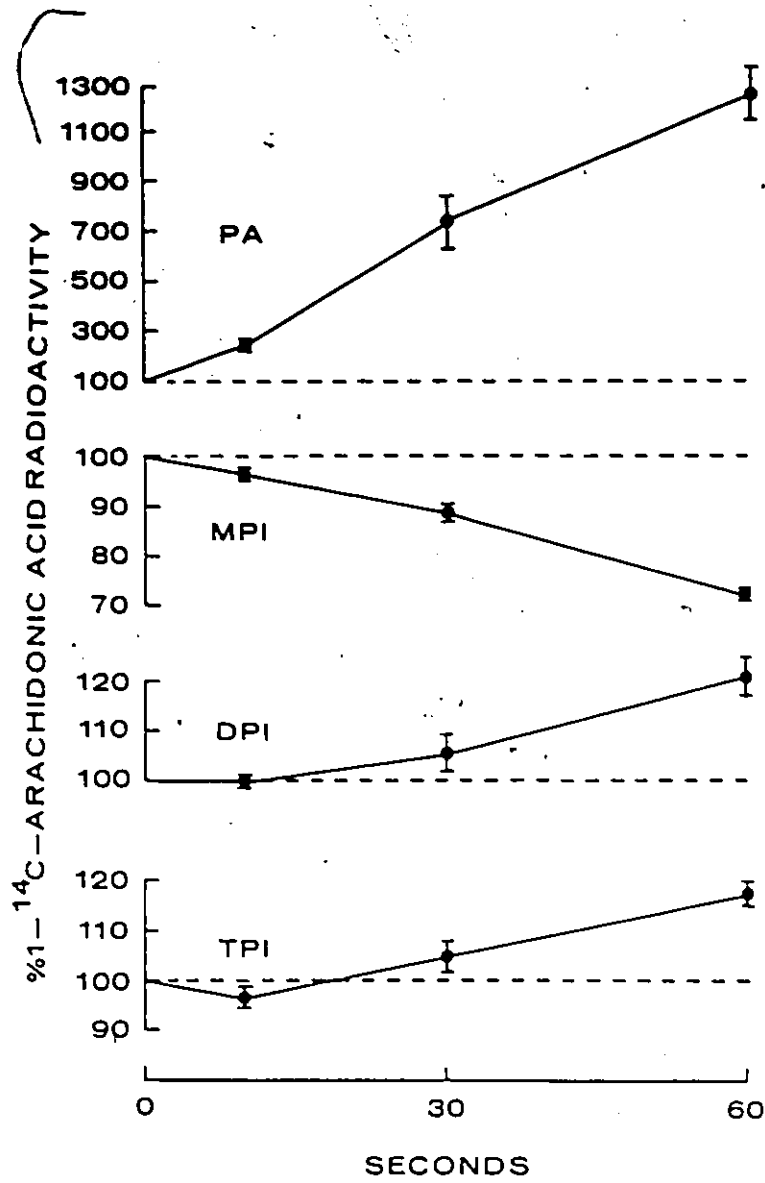


Figure 24.

Fig. 24 Changes in 1-¹⁴C-arachidonic acid labeling in PA and phosphoinositides during thrombin(0.33 u/ml)-induced platelet aggregation and the release reaction. The amount of radioactivity in the control samples was taken as 100%. The amount of radioactivity in the phospholipid of the stimulated samples was expressed as a percentage of the radioactivity in the corresponding control sample. The data (mean \pm S.E.M.) for each time interval were calculated from 10-15 pairs of samples of three experiments. The statistical significance(P value) between the stimulated samples and the control samples derived from the paired difference test is as follows :

	<u>9 sec</u>	<u>30 sec</u>	<u>60 sec</u>
PA	< 0.001	< 0.001	< 0.001
MPI	< 0.005	< 0.001	< 0.001
DPI	N.S.	N.S.	< 0.005
TPI	N.S.	N.S.	< 0.001

N.S. = not significant.

The distribution of radioactivity in PA and the phosphoinositides of the control samples in a typical experiment is shown in Table 12. The average net % increase in ¹⁴C-arachidonic acid labelling in 1,2-diacylglycerol at 60 sec was $422 \pm 37\%$ (P < 0.001).

associated with PA and a decrease in MPI radioactivity were observed at 30 sec (Figure 25). These changes, together with a 75% increase in radioactivity with diacylglycerol are statistically significant at 30 sec. At 60 sec, there was a significant increase in ^3H -glycerol radioactivity in PA, DPI and TPI. The ^3H -glycerol radioactivity of MPI was still significantly decreased at 60 sec. Significant changes were not observed in PC, PE or PS during the 60 sec period.

In ^3H -inositol-labelled platelets, a significant fall in MPI labelling was found at 30 sec (Figure 26). At 60 sec there was a further fall in ^3H -inositol with MPI. The amount of increase in radioactivity with DPI and TPI was significant at 1 min.

Nine seconds after the addition of thrombin to platelets, an increase in ^{32}P -radioactivity in PA and a significant decrease in TPI were observed (Figure 27). At 60 sec, PA had accumulated an amount of ^{32}P phosphate twelve-fold that of the control. The labelling in TPI and DPI were also significantly above the control value. The ^{32}P -labelling in MPI was increased but was not significantly different from the values for the control samples.

Phosphorus analysis performed on platelet TPI and DPI 9 sec after thrombin addition demonstrated a significant fall in TPI concentration (-8%) and a corresponding rise in DPI (+6%) (Table X). At 60 sec there were significant increases in the concentrations of PA (130%), DPI (33%) and TPI (13%). In contrast, there was a 16% decrease in the concentration of MPI (Table XI).

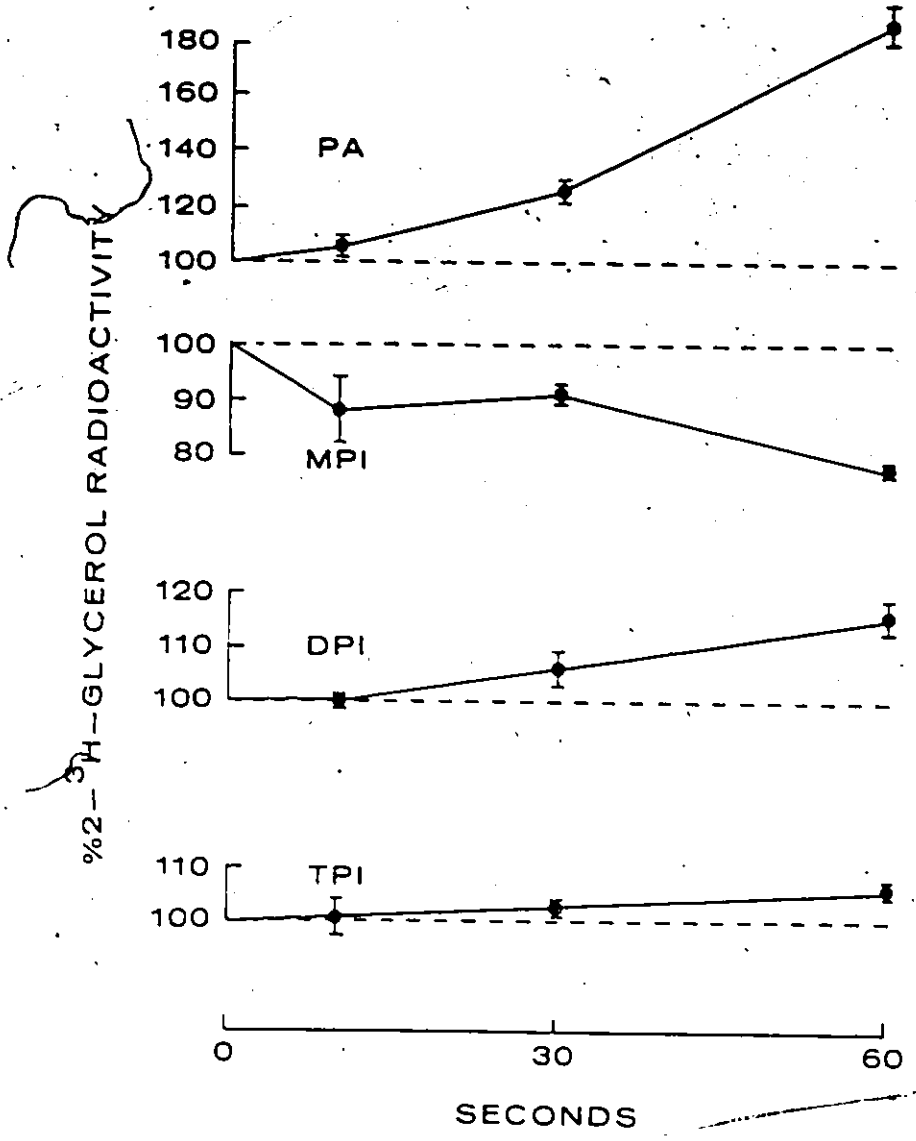


Figure 25.

Figure 25. Changes in 2-³H-glycerol labeling in PA and phosphoinositides during thrombin (0.33 u/ml)-induced platelet aggregation and the release reaction. Data are presented in a way explained in Figure 24. Data (mean ± S.E.M.) for each time interval were calculated from 10-15 pairs of samples of three experiments. P values calculated from the paired-difference-test are as follows:

	<u>9 sec</u>	<u>30 sec</u>	<u>60 sec</u>
PA	NS	<0.001	<0.001
MPI	NS	<0.001	<0.001
DPI	NS	NS	<0.005
TPI	NS	NS	<0.05

NS = not significant

The distribution of radioactivity in PA and the phosphoinositides of the control samples in a typical experiment is shown in Table 13. The average net increase in ³H-glycerol radioactivity in 1,2-diacylglycerol at 30 sec was 75 ± 10% (p < 0.01).



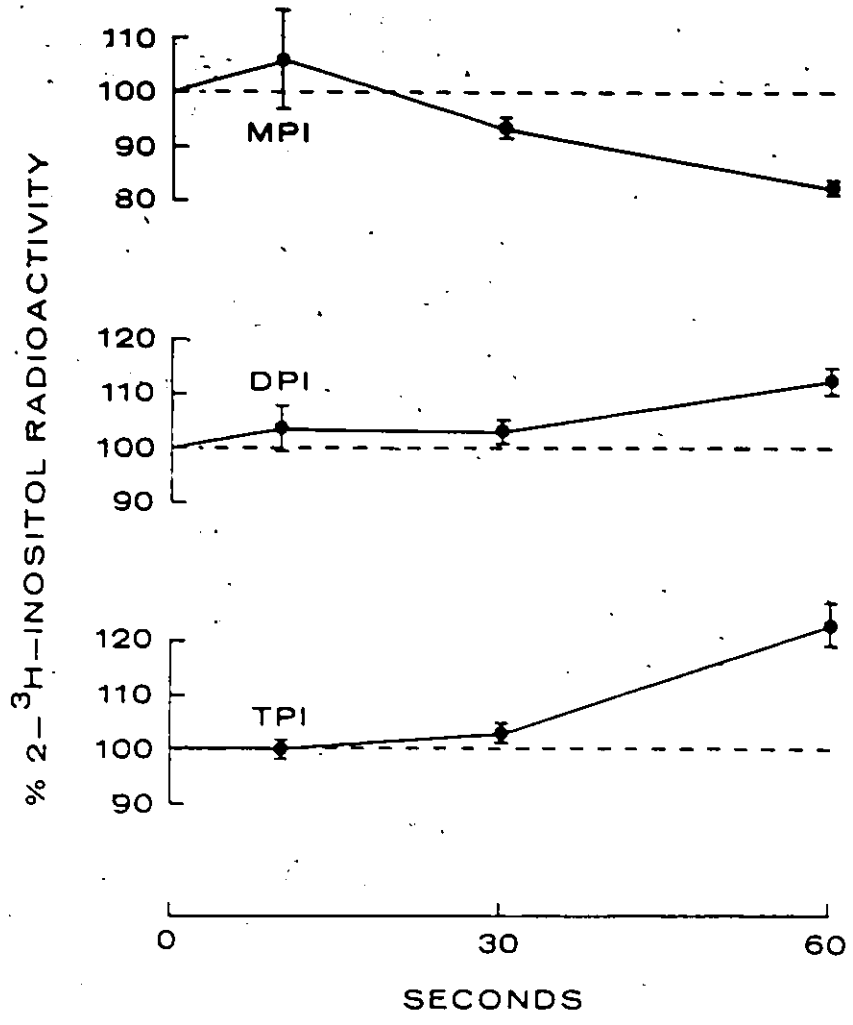


Figure 26.

Figure 26. Changes in myo-2-³H-inositol labelling in the phosphoinositides during thrombin (0.33 u/ml)-induced platelet aggregation and the release reaction. Data represent mean \pm S.E.M. of 10 samples from two experiments, each with 5 pairs of samples. Data are presented in the same way as described in Figure 24. P values are calculated by the paired-difference-test:-

	<u>9 sec</u>	<u>30 sec</u>	<u>60 sec</u>
MPI	NS	<0.02	<0.001
DPI	NS	NS	<0.005
TPI	NS	NS	<0.001

NS = not significant

The distribution of radioactivity in the individual phosphoinositides of the control samples in a typical experiment is shown in Table 14.

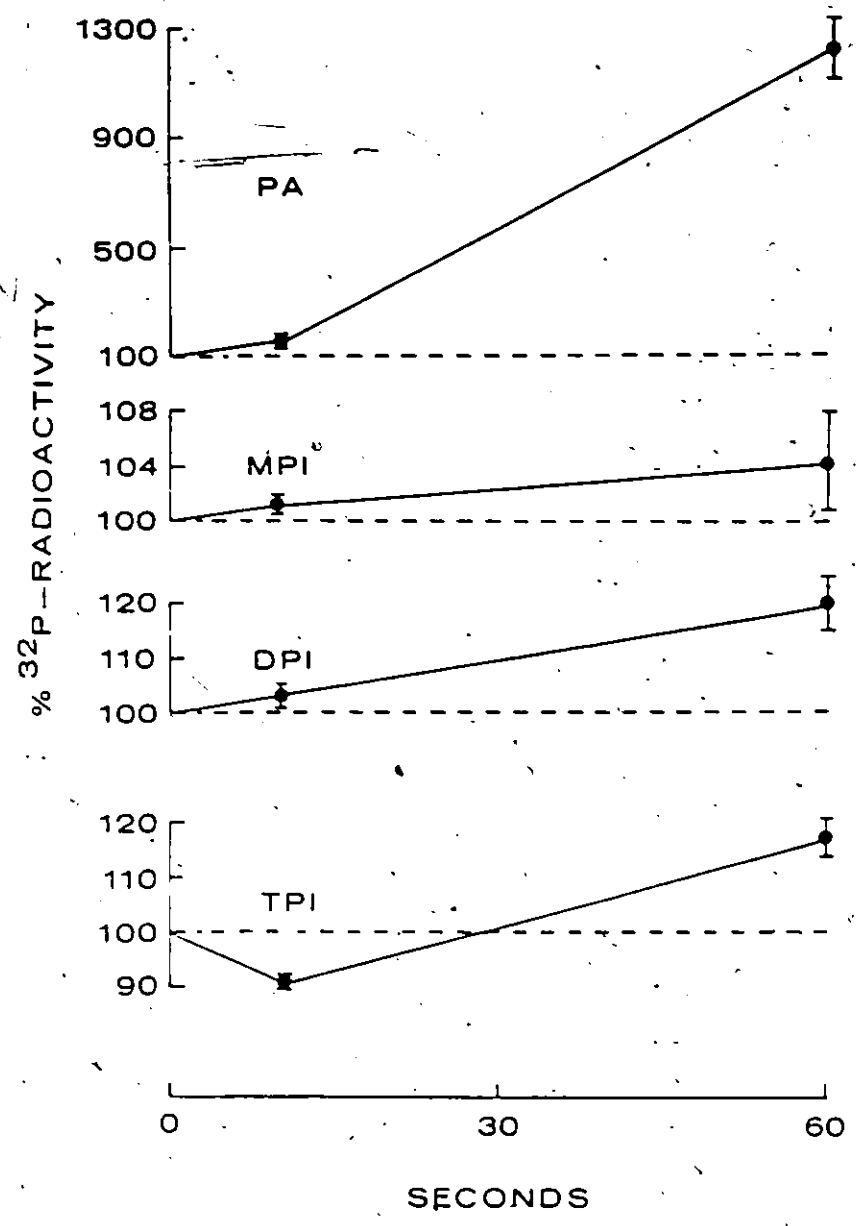


Figure 27.

Fig. 27. Changes in ^{32}P -labeling in PA and phosphoinositides during thrombin(0.33u/ml)-induced platelet aggregation and the release reaction. Data are presented in the same way as in Fig. 24. Data for MPI, DPI and TPI at 9 sec were calculated from 30 pairs of samples of four experiments. Other data were calculated from 10-15 pairs of samples of three experiments. P values calculated by the paired-difference-test are shown as following :

	<u>9 sec</u>	<u>60 sec</u>
PA	< 0.01	< 0.001
MPI	N.S.	N.S.
DPI	N.S.	< 0.025
TPI	< 0.025	< 0.001

N.S. = not significant.

The distribution of radioactivity in PA and the phosphoinositides of the control samples in a typical experiment is shown in Table 15.

TABLE X. CHANGES IN THE AMOUNT OF PHOSPHORUS OF DPI AND TPI NINE SECONDS FOLLOWING ADDITION OF THROMBIN TO THE PLATELET SUSPENSION.

Phospholipid	µg Phosphorus (Mean ± S.D.) *		P Value **
	control	thrombin	
DPI	1.60 ± 0.20	1.72 ± 0.20	<0.05
TPI	2.21 ± 0.10	2.02 ± 0.06	<0.001

* The data represent the average values of five samples from a typical experiment of phosphorus assay. Three experiments were performed with comparable results. The amount of phosphorus in DPI of the control samples ranged from 1.42 to 1.92 µg phosphorus and that of the thrombin stimulated samples from 1.51 to 2.12 µg phosphorus. There was an increase in each of the thrombin stimulated samples with respect to its own control. Similarly, the value of the TPI control samples ranged from 2.08 to 2.33 µg phosphorus and for the thrombin-treated samples, 1.96 to 2.10 µg phosphorus. There was a decrease in each of the thrombin-stimulated samples with respect to its own control. The amount of phosphorus of each sample was pooled from three spots on the thin-layer chromatography plates and was derived from a total of approximately 1.6×10^{10} platelets. The mean percentage increase in DPI calculated from 14 samples of three experiments was $5.35 \pm 1.77\%$, $P < 0.01$. The mean percentage decrease in TPI calculated from 15 samples of three experiments was $5.64 \pm 1.55\%$, $P < 0.005$.

** The P values were calculated from the paired-difference test between the control and the thrombin-stimulated platelets. The concentration of thrombin was 0.33 u/ml.

TABLE XI. PLATELET PA AND PHOSPHOLIPIDS IN RESPONSE TO THROMBIN

PHOSPHOLIPID	n MOLES/10 ⁹ PLATELETS (MEAN ± S.E.M)		NUMBER OF SAMPLES	P VALUE
	CONTROL	THROMBIN		
PA	0.72 ± 0.18	1.66 ± 0.42	3	<0.05
MPI	10.87 ± 0.20	9.14 ± 0.47	3	<0.005
DPI	1.51 ± 0.11	2.01 ± 0.06	4	<0.01
TPI	1.48 ± 0.10	1.67 ± 0.04	4	<0.025

The platelets were stimulated with thrombin (0.33 u/ml) for 60 seconds. The data represent the average of three samples from one of the two experiments that yielded similar results. The concentrations of the phospholipids have been corrected for percentage of recovery, and the number of phosphate groups in DPI and TPI have been accounted for. The P values were calculated by a paired-difference-test.

To examine the metabolic relationship of PA and the phosphoinositides upon thrombin stimulation of platelets, the actual amounts of radioactivity and the specific radioactivities of these lipids in the controls and stimulated samples were compared 60 sec after thrombin stimulation. With ^{14}C -arachidonic acid labelled platelets the specific activity of PA before thrombin stimulation was one sixth that for the inositol phospholipids (Table XII). Following thrombin stimulation, the specific activity in PA approached that in MPI, which was significantly diminished from its pre-stimulation value. The decrease in specific activity of DPI was not significant but the increase in specific activity of TPI was significant.

With ^3H -glycerol, the radioactivity of PA was 90% greater than before thrombin stimulation, but its specific radioactivity became much smaller (Table XIII). Although there was a significant decrease in the radioactivity of ^3H -glycerol with MPI following thrombin treatment, there was no decrease in its specific radioactivity. The specific radioactivity of TPI and DPI was not significantly changed.

Although thrombin stimulation of ^3H -inositol labelled platelets caused a decrease in labelling of MPI it did not cause a significant decrease in the specific radioactivity of MPI. The specific activity of DPI was significantly decreased and the specific activity of TPI was not significantly increased (Table XIV).

Thrombin caused a 12-fold increase in ^{32}P -labelling in PA, a small increase in DPI and TPI and no significant change in MPI.

TABLE XII. CHANGES IN 1-¹⁴C-ARACHIDONIC ACID LABELLING IN PLATELET PA AND PHOSPHOINOSITIDES IN RESPONSE TO THROMBIN.

Phospho-lipids	Total Radioactivity dpm/10 ⁹ platelets		P Value	Specific Radioactivity dpm/nmole		P Value
	control	thrombin		control	thrombin	
	PA	92 ± 13	1329 ± 98	<0.001	136 ± 22	860 ± 64
MPI	10082 ± 227	7632 ± 243	<0.001	994 ± 22	896 ± 26	<0.01
DPI	1218 ± 100	1457 ± 66	<0.02	866 ± 71	777 ± 42	<0.1
TPI	1521 ± 74	1846 ± 60	<0.001	1182 ± 54	1271 ± 38	<0.05

The data represent mean ± S.E.M. of five samples from a typical experiment of three which yielded comparable results. Platelets were subjected to lipid metabolism 60 sec after the addition of thrombin (0.33 u/ml). P values were calculated by the paired-difference-test.

TABLE XIII. CHANGES IN 2-³H-GLYCEROL LABELLING IN PLATELET PA AND PHOSPHOINOSITIDES IN RESPONSE

TO THROMBIN.

Phospho-lipids	Total Radioactivity dpm x 10 ⁻² /10 ⁹ platelets		P Value	Specific Radioactivity dpm x 10 ⁻² /nmole		P Value
	control	thrombin		control	thrombin	
PA	39 ± 3	64 ± 6	<0.001	54 ± 5	39 ± 3	<0.005
MPI	184 ± 16	141 ± 16	<0.005	16 ± 1	15 ± 1	<0.1
DPI	26 ± 3	31 ± 0	<0.01	17 ± 2	15 ± 0	<0.1
TPI	29 ± 1	32 ± 1	<0.02	20 ± 1	19 ± 0	<0.3

Data represent the mean ± S.E.M. of 5 samples from a typical experiment of three that yielded similar results. Platelet suspensions were subjected to lipid extraction 60 sec after the addition of thrombin (0.33 u/ml). P values were calculated by the paired-difference-test.

TABLE XIV. CHANGES IN 2-³H-INOSITOL LABELLING IN PLATELET PA AND PHOSPHOINOSITIDES IN RESPONSE TO THROMBIN.

Phospho-lipids	Total Radioactivity		P Value	Specific Radioactivity		P Value
	dpm x 10 ⁻² /10 ⁹ platelets	thrombin		dpm x 10 ⁻² /nmole	thrombin	
MPI	697 ± 59	554 ± 30	<0.005	64 ± 5	60 ± 3	<0.03
DPI	68 ± 4	78 ± 3	<0.001	45 ± 2	39 ± 1	<0.001
TPI	61 ± 3	79 ± 8	<0.02	41 ± 2	47 ± 5	<0.2

Data represent the mean ± S.E.M. of five samples from a typical experiment of two that yielded similar results. Platelet suspensions were extracted for lipids 60 sec after addition of thrombin (0.33 u/ml). P values were calculated by the paired-difference-test.

TABLE XV. CHANGES IN ³²P-LABELLING IN PLATELET PA AND PHOSPHOINOSITIDES IN RESPONSE TO THROMBIN.

Phospho-lipids	Total Radioactivity		P Value	Specific Radioactivity		P Value
	dpm x 10 ⁻³ /10 ⁹ platelets	thrombin		dpm x 10 ⁻³ /nmole	thrombin	
PA	13 ± 4	182 ± 29	<0.001	19 ± 6	109 ± 17	<0.001
MPI	73 ± 5	76 ± 7	<0.5	6 ± 0	8 ± 0	<0.01
DPI	66 ± 1	95 ± 3	<0.001	44 ± 0	47 ± 1	<0.02
TPI	167 ± 4	209 ± 13	<0.005	113 ± 2	125 ± 7	<0.025

Data represent the mean ± S.E.M. of five samples from a typical experiment of three that yielded comparable results. Platelet suspensions were subjected to lipid extraction 60 sec after the addition of thrombin (0.33 u/ml). P values were calculated by the paired-difference-test.

TABLE XVI. % CHANGES OF $^{32}\text{PO}_4$ -RADIOACTIVITY OF PA AND PHOSPHOINOSITIDES OF PLATELETS IN RESPONSE TO LOW CONCENTRATION OF THROMBIN

PHOSPHOLIPID	NET % CHANGE IN $^{32}\text{PO}_4$ -RADIOACTIVITY (MEAN \pm S.E.M.)		
	9 SECONDS	P VALUE	5 MINUTES
PA	14 \pm 11	N.S.	174 \pm 57
MPI	- 2 \pm 2	N.S.	16 \pm 6
DPI	16 \pm 8	N.S.	28 \pm 5
TPI	- 1 \pm 1	N.S.	- 8 \pm 1
			P VALUE
			<0.01
			<0.05
			<0.01
			<0.001

The net % change of individual phospholipids was calculated by subtracting 100% of the control radioactivity from a % value of the thrombin-stimulated sample. The latter was derived from the ratio of ^{32}P -radioactivity in the stimulated and the control samples multiplied by 100%. The data were calculated from 12 samples of three experiments. The concentration of thrombin was 0.01 u/ml. This concentration of thrombin caused only shape change of platelets.

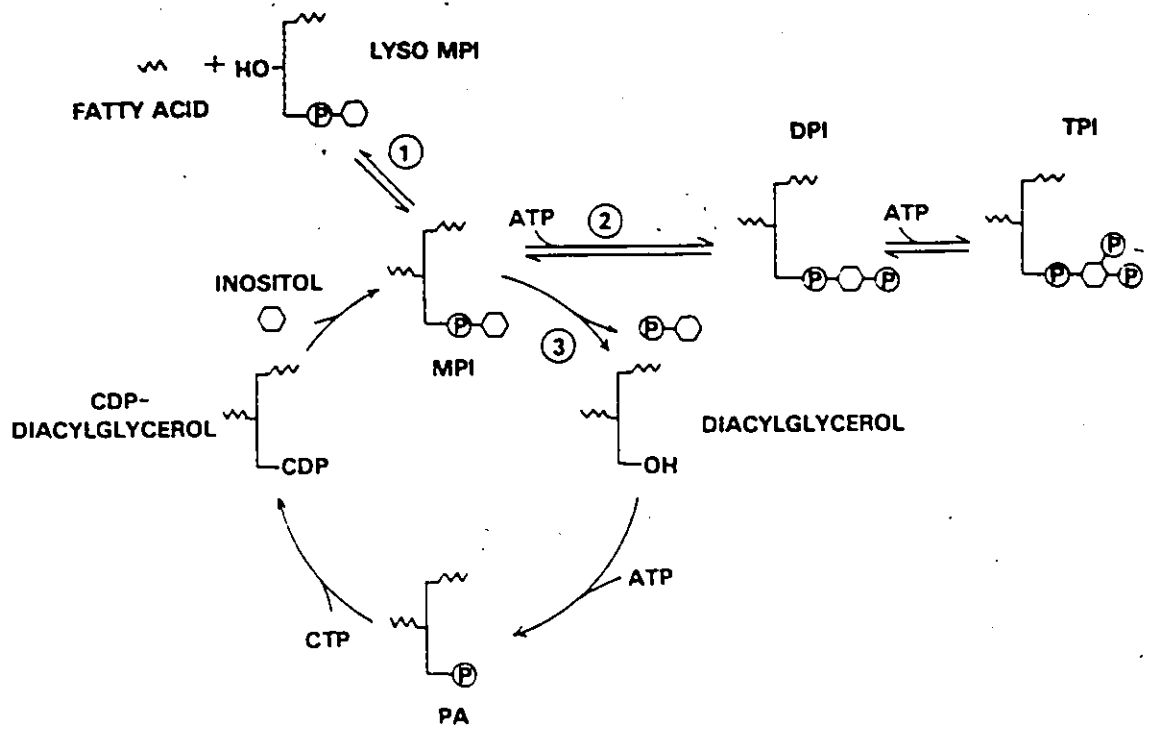


Fig.28. Metabolic pathways of phosphatidic acid and phosphoinositides.

The specific radioactivity of PA, MPI, DPI and TPI were all increased (Table XV).

Low concentration of thrombin which caused only platelet shape change induced no significant change in the ^{32}P -labelling in any of the phospholipids at 9 sec (Table XVI). Yet at 5 min, it caused significant increase in the radioactivity in PA, MPI, DPI and a significant loss of $^{32}\text{PO}_4$ from TPI. This low concentration of thrombin caused only 3% release reaction in 5 min in ^{14}C -serotonin labelled platelets.

3. Discussion

The following discussion is based on the metabolic pathways of PA and phosphoinositides summarized in Figure 28.

The present results have shown a significant decrease in the ^{32}P -labelling of TPI and an increase in DPI 9 sec after thrombin stimulation of platelets. These changes were accompanied by a reduction in the amount of TPI and an increase in DPI as demonstrated by the phosphorus assay experiments. These changes in TPI were only transient. As platelet aggregation and the release reaction proceeded, the amount of TPI increased at 60 sec, which was accompanied by a rise in the radioactivity of $^{32}\text{PO}_4$, ^3H -glycerol, ^3H -inositol and ^{14}C -arachidonic acid. The observations at 9 sec of TPI changes are consistent with the hypothesis that hydrolysis of TPI to DPI is closely related to the response of platelets to thrombin. This is further supported by the observation that TPI hydrolysis persisted in platelets which

only underwent shape change during the treatment with low concentrations of thrombin. The possibility of TPI hydrolysis with concurrent release of Ca^{2+} and alteration of pore size and pore charge has been proposed as a regulatory mechanism for membrane permeability changes toward Na^+/K^+ (Hendrickson and Reinertsen, 1971). The recent findings of early Na^+ influx into platelets during ADP-induced platelet aggregation by Feinberg and associates (1977) is consistent with this possible role of TPI-DPI interconversion in platelet shape change.

Hendrickson and Reinertsen (1971) also estimated that the hydrolysis of TPI would not need to be extensive to affect membrane permeability especially if the enzymes or the polyphosphoinositide were located near such channels. The relationship of TPI hydrolysis and membrane depolarization was recently demonstrated in crab nerve fibers in response to acetylcholine (Tretjak et al., 1977).

Another way by which TPI hydrolysis could mediate platelet shape change is by releasing Ca^{2+} from the membrane. This Ca^{2+} might initiate the activity of the contractile proteins associated with the membrane in platelets and activate phospholipase A_2 to bring about the shape change. The importance of Ca^{2+} in the initiation of activity in platelet contractile proteins has been well established (Gerrard et al., 1977). It has also been shown that TPI has very high affinity for Ca^{2+} and Mg^{2+} and that hydrolysis of TPI to DPI reduces this affinity by 60% (Hendrickson and Reinertsen, 1971).

The present experiments have also demonstrated a reduction in the amount of MPI during thrombin-induced platelet aggregation and the release reaction, which was accompanied by a loss of radioactivity of ^{14}C -arachidonic acid, ^3H -glycerol and ^3H -inositol. The decrease for the ^{14}C -arachidonic acid was detectable at 9 sec, as well as in ^3H -glycerol and ^3H -inositol at 30 sec. It is possible for MPI to be metabolised by any one of the three pathways outlined in Figure 28 during thrombin-induced functional changes of platelets. Conversion of MPI to DPI and TPI is indicated by an increase in the amount of DPI and TPI, as well as by an increase of radioactivity in $^{32}\text{P}\text{O}_4$, ^3H -glycerol, ^3H -inositol and ^{14}C -arachidonic acid in these two lipids. Thus in contrast to the previous findings by Kaulen and Gross (1976), there is net synthesis of DPI and TPI during thrombin stimulation of platelets. This accumulation of polyphosphoinositides could be the result of compensatory activities of platelets in response to the earlier hydrolysis of TPI during platelet shape change. The significant increase in the specific radioactivity of ^{32}P -labelled DPI and TPI possibly reflects the enhanced turnover of the monoesterified phosphates in these molecules. The enzymes responsible for the phosphorylation of MPI to DPI and further to TPI, namely the MPI kinase and DPI kinase, have been shown to be present in platelets (Kaulen and Gross, 1976). The causes for the differences observed in the present experiments and that by Kaulen and Gross (1976) was not clear. It could be a consequence of different methods for platelet preparation.

Phosphatidic acid of platelets has little amount of arachidonic acid but it has a highly efficient system for the turnover of its glycerol and phosphate moieties, as demonstrated by the distribution of isotopes in this phospholipid (Table VIII). Phosphoinositides on the other hand, are rich in arachidonic acid (Baker and Thompson, 1972; Marcus et al., 1969) and have the highest specific radioactivity in ^{14}C -arachidonic acid. However, the glycerol moiety of these lipids turns over slowly with a specific radioactivity of ^3H -glycerol much lower than that in PA. During thrombin-induced platelet aggregation and the release reaction, the ^{14}C -arachidonic acid labelling in PA and in 1,2-diacylglycerol increased many times and the specific radioactivity of ^{14}C -arachidonic acid in PA increased to that of MPI (Table IX and XII). At the same time, the specific radioactivity of ^3H -glycerol in PA decreased considerably despite a moderate gain in the labelling of this isotope. This evidence is consistent with the proposal that phosphodiesterase cleavage of MPI occurred in thrombin stimulated platelets, and that the diacylglycerol backbone of this phosphoinositide was conserved during its conversion to PA. There was further conversion of PA to MPI via the formation of an intermediate, cytidine-diacylglycerol phosphate. This cyclic conversion is indicated by an increase in the specific radioactivity of $^{32}\text{PO}_4$ in MPI. This paradoxical finding of an increase in the specific radioactivity and total radioactivity of $^{32}\text{PO}_4$ in MPI on the one hand, and a loss in the amount as well as the labelling of

^3H -inositol, ^3H -glycerol and ^{14}C -arachidonic acid of this phospholipid on the other can be explained as follows. In the experiments with $^{32}\text{P}\text{O}_4$, the precursor (γ - ^{32}P)ATP had a specific radioactivity several thousand-fold higher than that of PA. This abundance of ^{32}P -precursor with a higher specific radioactivity than in PA led to a five-fold increase in ^{32}P -specific radioactivity of PA by phosphorylation of 1,2-diacylglycerol in the thrombin-stimulated platelets. Thus, one would expect that MPI newly synthesized from PA would have a much higher specific radioactivity than MPI which is broken down to 1,2-diacylglycerol. The balance between the resynthesis of small amounts of high specific radioactivity MPI and the breakdown of larger amounts of low specific radioactivity MPI, could lead to a net accumulation of counts in the MPI fraction and an increase in specific radioactivity.

It is not likely that phospholipids other than MPI would be a major source for the observed changes in PA during the first 60 sec of thrombin stimulation of platelets. Although there was some decrease in the ^{14}C -arachidonic acid radioactivity in PC and PE, it is important to note that the specific radioactivity for PC and PE was less than 50% of that of MPI. The lack of changes in ^3H -glycerol or ^{14}C -arachidonic acid labelling of triacylglycerol during the 60 sec period of observation would argue against this lipid being a source for PA. Finally, a fall in the specific radioactivity of ^3H -glycerol in PA served as evidence against de novo synthesis of this phospholipid. Cyclic conversions between MPI and PA has been postulated by Hokin-Neaverson and associates (1976; Geison et al., 1976) working with mouse pancreas. They found a selective increase

in stearic acid and arachidonic acid in PA with a stoichiometric loss of these fatty acids from MPI during acetylcholine stimulation of the pancreas. The changes were reversed following addition of acetylcholine antagonist. Phosphodiesterase cleavage of MPI of mammalian tissues in response to stimulation was however first proposed by Durell and Garland (1969), and the formation of 1,2-diacylglycerol as the intermediate of this conversion was observed by a number of investigators (Jones and Mitchell, 1974; Banschbach et al., 1974).

Although it is possible that MPI could be transformed to lyso-MPI and free arachidonic acid, we were unable to quantitate the amount of lyso-MPI formed in these experiments. If one postulates that the ^{14}C -arachidonic acid and ^3H -glycerol in MPI can be incorporated into PA through 1,2-diacylglycerol as a result of thrombin stimulation, it would be estimated that about 60% of the MPI that was lost, appeared in PA. From the increase in DPI and TPI at 60 sec, it is possible to calculate that approximately 20-30% of the MPI lost was used in the synthesis of DPI and TPI. It could be concluded that about 20% of the ^{14}C -arachidonic acid lost from MPI could be the result of its being converted to lyso-MPI and free arachidonic acid by the activation of phospholipase A_2 and its effects on MPI. These observations suggest that individuals studying ^{14}C -arachidonic acid loss from phospholipids must not assume that a decrease in the ^{14}C -arachidonic acid in a phospholipid necessarily indicates cleavage of

arachidonic acid from the phospholipid since the phosphodiesterase could cleave the phosphate group leading to the formation of 1,2-diacylglycerol.

The role of the changes in MPI and PA during thrombin-induced aggregation and release are not clear but these data do demonstrate that these changes are associated with the very early responses of platelets to thrombin. It can be inferred from the experiment with other mammalian tissues that myo-inositol-1,2-cyclic phosphate formed from phosphodiesteratic cleavage of MPI could be a cyclic-AMP-like mediator of cellular responses to stimuli (Michell and Lapetina, 1972). 1,2-Diacylglycerol might accumulate in cell membrane and potentiate shape change. The free arachidonic acid cleaved from MPI consequent to activation of phospholipase A₂ is a precursor for prostaglandin synthesis (Bills et al., 1976). Some intermediates of these oxygenated products, namely the endoperoxides and thromboxane A₂, are extremely powerful aggregating agents (Hamberg et al., 1973).

We have previously demonstrated that ADP-treatment of platelets has little effect on MPI breakdown during ADP-induced platelet aggregation and that A23,187 does not cause net hydrolysis of TPI. Thrombin, however, brings forth both of these changes. These findings thus substantiate other studies that there are fundamental differences in the interaction of platelets with various aggregating agents.

CHAPTER SEVEN

General Discussion

I. Turnover of phospholipids in unstimulated platelets.

All molecular components are periodically replenished, both in growing and non-growing cells. Platelets do not have a nucleus. They nevertheless undergo active metabolic reactions.

Analysis of phospholipid composition in this project and by other investigators (Marcus et al., 1969; Lewis and Majerus, 1969; Lloyd et al., 1972) has demonstrated PC to be the most abundant phospholipid in platelets, followed by PE, sphingomyelin and PS. Phosphatidic acid and the phosphoinositides make up less than 10% of total lipid phosphorus. The quantity of DPI and TPI is however, as high as that in the brain.

The incorporation studies with various isotopic precursors have revealed that some phospholipid species turn over components slower than the others. In addition, some parts of a given molecule are renewed more frequently than other parts. In general, PS has the lowest replacement rates of all its components among glycerol phospholipids, including its glycerol backbone, fatty acids and the phosphate moiety. In contrast, PA and phosphoinositides have very high replacement rates of all components, particularly their phosphates. Some components in PC and PE turn over very fast as indicated by rapid incorporation of ^{14}C -arachidonic acid and ^3H -glycerol into these lipids. On the other hand, the phosphate group in PC and PE turn over rather slowly. The variations in the turnover rates of phospholipids can be explained on the basis of metabolic pathways, chemical nature and their distribution in platelet membrane.

1. Incorporation of ^{32}P -orthophosphate

Among all phospholipids investigated, PA and the phosphoinositides have the highest incorporation rates of $^{32}\text{PO}_4$. This is because $^{32}\text{PO}_4$ can be incorporated into these phospholipids by 3 different pathways - de novo synthesis, cyclical interconversion of phosphoinositides mediated by PA formation, and interconversion of phosphoinositides (Figure 3b). In these pathways as well as those of major phospholipids, any $^{32}\text{PO}_4$ appearing in the phospholipids is donated by (γ - ^{32}P)ATP which has a much higher specific radioactivity than that of PA and the inositol phospholipids.

The de novo pathway in which glycerophosphate is acylated to form PA accounts for some of the diesterified $^{32}\text{PO}_4$ in the phosphoinositides and some of the ^{32}P -radioactivity in PA. Most of the ^{32}P -radioactivity located in the diesterified phosphate of the inositol lipids however, appear to be the consequence of cyclical interconversion. In this pathway, a phospholipase C type enzyme(s) specific for the inositol phospholipids attacks the glycerol-phosphate bond of the molecules to yield 1,2-diacylglycerol. The latter may then be phosphorylated with (γ - ^{32}P)ATP to form PA which is further converted to MPI, mediated by the formation of CDP-diacylglycerol. MPI may be phosphorylated to DPI and TPI. In the above two pathways, the only common reactions are the conversion of PA to CDP-diacylglycerol and the reaction of CDP-diacylglycerol with inositol to form MPI.

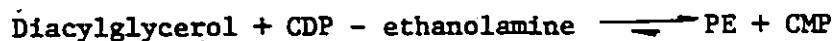
Interconversions between the phosphoinositides account for most of the ^{32}P -radioactivity in DPI and TPI. These two phospholipids

possessed over 90% of the total lipid $^{32}\text{PO}_4$ in platelets one hour following addition of ^{32}P -orthophosphate to the platelet suspensions. It was documented by Hokin and Hokin (1964) that over 80% of ^{32}P -radioactivity in DPI and TPI of erythrocytes labeled with (γ - ^{32}P)ATP resided in the monoesterified phosphates of these lipids. Similarly, most of the ^{32}P -radioactivity in PA is incorporated by interconversion between PA and 1,2-diacylglycerol. Except for TPI phosphomonoesterase and the phosphodiesterase, all other enzymes involved in the above reactions have been demonstrated in platelets (p. 49), and all enzymes involved have been shown in other animal tissues. Among them, TPI phosphomonoesterase, DPI kinase, PA phosphohydrolase and diacylglycerol kinase have been demonstrated to have extremely high activities.

The scarcity of ^{32}P -labelling in the major phospholipids is mainly attributed to their biosynthetic pathways (Figure 2). To start with, the phosphate in these phospholipids does not come from PA despite PA being the parent compound for all phospholipids. The $^{32}\text{PO}_4$ in PC (PE) comes from phosphorylation of choline (ethanolamine) with (γ - ^{32}P)ATP which is a much slower process than incorporation of $^{32}\text{PO}_4$ into MPI mediated by rapid interconversion between PA and diacylglycerol. PA only provides the 1,2-diacylglycerol portion of the PC (PE) molecules. Furthermore, topographical studies on membrane phospholipid distribution in platelets (Shick et al., 1976) showed that most of the platelet PC is located on the outer leaflet of the lipid bilayer, almost all PS is hidden in the inner leaflet and PE is mainly

found in the inner surface. This distribution pattern is similar to the one in erythrocytes (Zwaal et al., 1973). It is therefore possible that the polar head groups of PC molecule facing the aqueous environment are less accessible to enzyme action. This hypothesis is consistent with a recent finding by van Deenen's group (Renvoij et al., 1976) who reported that incorporation of radioactive fatty acids into phospholipids of erythrocytes occurred predominantly at the inside of the membrane. By studying the exchange reactions of ^{32}P -labelled PC molecules between the outer and inner leaflet of rat erythrocyte membrane, they estimated the half time for flip-flop of PC molecules from the outer leaflet to the inner leaflet to be 4-4.5 hours, a value much shorter than that of previous investigators who studied flip-flop rates of phospholipids with liposomes (McNamee and McConnell, 1973; Smith and Green, 1974). We therefore propose that accumulation of $^{32}\text{PO}_4$ in PC of washed platelets was achieved by incorporation of $^{32}\text{PO}_4$ into the PC molecules present on the inner leaflet. These molecules then flip-flop to the outer leaflet. This process is slow and our observation that incorporation of $^{32}\text{PO}_4$ into PC of washed platelets became significant approximately 4-6 hours after the pulse labelling is compatible to the half time for flip-flop of PC molecules in erythrocyte membrane calculated by Renvoij et al., (1976).

Since some PE is located in the outer leaflet of platelet membrane (Shick et al., 1976) the slow incorporation of $^{32}\text{PO}_4$ into PE may be attributed to causes similar to that of PC. In addition, the last reaction in the biosynthesis of PE



is far less reversible than that of the PC reaction (Thompson, Jr., 1973). This reduces the chance of $^{32}\text{PO}_4$ turnover in PE molecules. Furthermore, since platelet PE is rich in plasmalogens (Broekman et al., 1976) which are relatively resistant to enzymic degradation and therefore have a very slow turnover of the phosphate group, and since we did not separate plasmalogen PE and diacyl PE, the observed $^{32}\text{PO}_4$ incorporation into platelet PE in the present experiments was therefore slower than that of PC which is rich in the diacyl species.


Incorporation of $^{32}\text{PO}_4$ into PS is the slowest among glycerol phospholipids. This is because in mammalian tissues PS is synthesized by the base exchange reaction of serine with PE and there is little free serine available in unstimulated platelets. Furthermore, PS molecules are readily decarboxylated to form PE. It is thus not surprising that PS was the only glycerophospholipid species which exhibited the slowest rates of incorporation of all the isotopes employed in the present experiments.

It thus becomes clear that the major reason for previous investigators (Cohen et al., 1971; Lloyd et al., 1972) to observe $^{32}\text{PO}_4$ incorporation into the major phospholipids of platelets many hours following intravenous infusion of ^{32}P orthophosphate into human or animals but not following one or two hours incubation of washed platelets with ^{32}O -orthophosphate, was the rather slow turnover rates of the phosphates in PC, PE and PS. In the present experiments longer incubation hours were allowed for the in vitro experiments before harvesting the platelets. Thus the phosphate groups of all phospholipids turn over in platelets in vitro,

some more rapidly than others. This is because in the *in vivo* experiments the major phospholipids of platelets derive their ^{32}P -radioactivity mostly from megakaryocytes in the bone marrow, as well as by exchanging the phospholipid molecules in the outer leaflet of platelet membrane with the ^{32}P -labelled phospholipid molecules in the plasma (Joist et al., 1976). Such plasma phospholipids were not available for exchange with washed platelets. From the above discussion on phosphate turnover in platelet phospholipids, it has become obvious that the major factor contributing to the observed discrepancy of ^{32}P -incorporation into the major platelet phospholipids under *in vivo* and *in vitro* conditions was the longer incubation hours allowed for the *in vivo* experiments before harvesting the platelets in comparison to the *in vitro* experiments. Thus the phosphate groups of all phospholipids turn over, some more rapidly than the others.

2. Incorporation of ^3H -glycerol.

Incorporation of ^3H -glycerol into platelet phospholipids represents *de novo* synthesis (Lewis and Majerus, 1969). Comparison between the percentage distribution of lipid phosphorus (Table I) and the percentage distribution of ^3H -glycerol (Table VIII) in various platelet phospholipids has revealed that phospholipids were synthesized at different rates. For example, the amount of PC, PE, PS and MPI were approximately in the proportions of 8:6:2:1. Yet the ratios of their ^3H -glycerol content were close to 4:1.6:0.1:1. These data indicate that biosynthesis of MPI was approximately twice as rapid as PC, four times as rapid as PE and many times more rapidly than PS. This variation in the rates of phospholipid biosynthesis is compatible with



the knowledge that enzymes of phospholipid metabolism exhibit preferential substrate specificity (Dawson, 1973). The data also support the 'compartmentation' hypothesis of phospholipid biosynthesis (Soto et al., 1977).

3. Incorporation of ^{14}C -arachidonic acid.

The results of ^{14}C -arachidonic acid incorporation experiments (Table VIII) show that PC, PE and the phosphoinositides possess an abundance of this fatty acid. However, the phosphoinositides have much higher turnover rates of arachidonate than the others and over 80% of their C-2 position fatty acid is arachidonate (Baker and Thompson, 1972; Marcus et al., 1969). In contrast, PA contained negligible amounts of ^{14}C -radioactivity. This is much lower than one would expect from PA being the precursor of all phospholipids, less to mention its being the precursor of the arachidonate-rich phosphoinositides. There are two possible explanations to account for this discrepancy. One is based on the deacylation-reacylation cycle independent of the biosynthesis pathway. Another is based on selective conversion into phosphoinositides of certain PA molecules that have arachidonate.

Acylation of l-acyl lyso MPI with pigeon pancreatic microsomes was first reported by Keenan and Hokin (1964, 1962). They also showed that the acyl transferase had a preference of oleoyl-CoA over the saturated palmitoyl-CoA. Laker, Baker and Thompson (1972) reported a highly selective acylation enzyme system in rat brain microsomes that

preferentially transfer arachidonoyl-CoA to 1-acyl lyso MPI 2 to 5 times more effectively than it transfers linoleoyl-, linolenoyl- or eicosatrienoyl-CoA. They also observed that incorporation of arachidonoyl group into MPI proceeded at 2 to 3 times the rate for PC. This value is consistent with our finding that the ^{14}C -arachidonic acid specific radioactivity of MPI was approximately twice that of PC within a few hours of pulse labelling of platelets. Since it was previously shown with rat liver in vivo or with liver slices that radioactivity from ^{14}C -glycerol, ^{32}P -orthophosphate or ^3H -inositol appeared more rapidly in the monoenoic and dienoic species of MPI than in the tetraenoic species (Holub and Kuksis, 1972, Holub and Kuis, 1971), it could mean that during the biosynthesis of MPI, the newly formed molecules would have the same fatty acid compositions as that of PA. The high concentration of arachidonic acid in MPI could then be achieved by desaturation reactions of the monoenoic and dienoic species and by the deacylation-reacylation process. Judging from the rapid incorporation of ^{14}C -arachidonic acid into platelet MPI, DPI and TPI, the deacylation-reacylation reaction appears to be a highly important reaction in the regulation of the major molecular species of platelet phosphoinositides.

Another mechanism by which phosphoinositides enrich their arachidonate content is mediated by selective conversion of arachidonate-containing phospholipid molecules into phosphoinositides. Selection can take place at the level of CTP: diacylglycerophosphate cytidyl

transferase(s) or at the level of CDP-diacylglycerol:inositol phosphatidyl transferase(s). The former enzyme catalyses the conversion of PA to CDP-diacylglycerol while the latter converts CDP-diacylglycerol to MPI. Thompson and MacDonald (1975) found the fatty acid compositions of beef liver CDP-diacylglycerol similar to that of MPI, characterized by a high level of stearate and of arachidonate. It is thus possible that some selective process takes place at the CTP-diacylglycerophosphate cytidil transferase level.

The biological significance of diversity in phospholipid compositions has been discussed previously (p. 53). Although the question why certain lipids are replaced more rapidly than others remains to be discovered, it can be safely said that the turnover process provides the platelets with means of introducing structural changes, thereby to modify its membrane properties to meet changing environmental conditions. An example of interest is that PC molecules which are predominantly located on the outer leaflet of platelet membrane and constantly subject to the wear-and-tear motion of blood flow, are capable of renewing their components through a slow yet steady flip-flop process.

II. Phosphatidic acid and phosphoinositide metabolism in platelets
in response to stimuli.

The rapid metabolism of phosphatidic acid and phosphoinositide in mammalian tissues and their possible roles in the cellular responses to stimuli have been a topic of intensive investigation in the last twenty years. The relationship between these phospholipid changes and the biological responses such as secretory function or impulse conduction remains unclear. Platelets are one of the few mammalian tissues which are suitable candidates for these studies because of availability as pure preparations, richness in these minor phospholipids and readily measurable morphological and physiological changes. Therefore, the importance of phosphoinositide metabolism in relation to platelet aggregation in response to stimuli depends on whether the changes are primarily involved in aggregation or secondary to this process. It means whether the detectable changes in the phosphoinositides occur rapidly enough after the addition of aggregating agents to be possibly primarily involved in the mechanism of aggregation, as well as whether the changes can be meaningfully associated with some biological phenomena connected to platelet functions. Although there are only minute amounts of PA and phosphoinositides in platelets, study of the possible changes in these lipids of stimulated platelets as compared to their controls was facilitated by the improved methods on sample collection, thin-layer chromatography separations developed earlier in the present project.

Previous reports on the metabolism of PA and phosphoinositides in platelets treated with aggregation stimuli mentioned rapid increase of ^{32}P -labelling in PA with ADP, thrombin or collagen (Lloyd et al., 1973, 1974; Kaulen and Gross, 1976). Some of the changes were detected within 10 seconds. The labelling of DPI was also rapidly elevated by ADP and thrombin. Kaulen and Gross (1976) reported an increase in ^{32}P -labelling in TPI by thrombin but Lloyd and associates (1973) could not find consistent changes in TPI of ADP-stimulated platelets. Both investigators observed an increase in ^{32}P -labelling in MPI with ADP or thrombin and agreed that the changes were not significant until 1 to 2 min after addition of stimulus. In addition, Kaulen and Gross (1976) reported that with ^{14}C -glycerol no phosphoinositides other than MPI were labelled and no changes of this isotope were observed in any of these phospholipids during the 4 min of thrombin stimulation. These authors therefore concluded that phosphoinositides with rapid incorporation of phosphate groups were not as rapidly synthesized de novo and only the phosphorylating reactions were stimulated by thrombin. Lloyd and associates (1973) also considered that the increase in labelling of $^{32}\text{PO}_4$ in PA and the inositides did not involve quantitative changes in these phospholipids. In contrast, the present experiments have not only demonstrated much earlier changes in the concentrations as well as isotopic labelling of TPI and MPI, but have also distinguished the different patterns of phospholipid metabolism induced by ADP, thrombin as well as ionophore A23,187. Furthermore, we compared the

relative predominance of the metabolic pathways of PA and of all phosphoinositides in thrombin-induced platelet aggregation and the release reaction.

The most distinctive features of the present data are the identification of TPI-DPI interconversion as well as hydrolysis of MPI in platelets in response to stimuli. Interconversion between TPI and DPI occurred with ADP and thrombin, but was not detected during the ^{initial} ~~initial~~ ^{initial} stages of aggregation with A23,187. On the other hand, hydrolysis of MPI took place extensively with A23,187 and thrombin, but only to a small extent with ADP. These observations suggest differences in the nature of response of platelets to these stimuli and are compatible with the current knowledge that ADP and A23,187 appear to cause platelet aggregation by different mechanisms while thrombin shares some common pathways with both reagents.

1. ADP-induced TPI-DPI interconversion and platelet ADP-receptors.

Degradation of TPI to DPI occurred during ADP-induced platelet aggregation as indicated by significant decreases in ³²P-labelling, ¹⁴C-arachidonic acid and ³H-inositol labelling in TPI at 60 sec, and a gain of these isotopes in DPI (Figure 14-17). Conversion of DPI back to TPI happened during platelet deaggregation and restoration of their disc shape. During this period, ³²P-labelling of DPI and TPI returned to the prestimulation levels (Figures 18,19).

The only proposed mechanism by which ADP causes aggregation of washed platelets is a primary mechanism which involves interaction between stimulus and the receptor molecule on platelet surface. The nature of ADP receptors has not been fully defined (p. 14). However, regardless of the true identity of the ADP receptors, if TPI-DPI interconversion is to play a role in this primary mechanism, either the lipids or the enzymes metabolising these lipids must be spatially associated with the ADP-receptors so that any conformational or functional changes imposed on the receptors by ADP would initiate TPI-DPI interconversion. Torda (1972) once proposed TPI phosphomonoesterase as the acetylcholine receptor in the post synaptic membrane of nerve cells and further attempted to characterise the receptor nature of this enzyme. In platelets, however, TPI phosphomonoesterase is unlikely to be a receptor for aggregating agents. The reason being that many agents that cause TPI hydrolysis and also platelet aggregation by stimulus-receptor interaction may have very different molecular structures. On the other hand, evidence from experiments with platelets and other mammalian tissues support a close spatial relationship between polyphosphoinositides and membrane receptors. For example, the polyphosphoinositides are

extremely acidic and polar lipids, and interact strongly with proteins and calcium (Hendrickson, 1969). The interaction with proteins is so strong that strong acidic lipid solvent is required for extracting them from cell membranes. It was recently shown that all TPI and DPI in human erythrocyte membrane could be recovered from a proteolipid fraction that was enriched in PS, MPI and PE, but did not contain sphingomyelin and PC. This trace amount of DPI and TPI contained all of the phospholipid ³²P-radioactivity (Redman, 1972). LaTorre and associates (1970) isolated from the electric tissue of fishes a proteolipid that appeared to possess receptor abilities in that it bound (+)-tubocurarine, 5-HT and atropine. In rabbit iris smooth muscle, the acetylcholine-stimulated breakdown of TPI is considered to be mediated through muscarinic receptors since the breakdown is inhibited by atropine but not by tubocurarine. Furthermore, only muscarinic agonists, but not nicotinic agonists, were found to induce an acetylcholine-stimulated breakdown of TPI (Abdel-Latif et al., 1977). In addition, Abdel-Latif and coworkers (1978) reported TPI degradation mediated through α -adrenergic receptors but not β -receptors in the norepinephrine-stimulated rabbit iris smooth muscle. In platelets, we observed that TPI-DPI interconversion was inhibited by AMP, a

specific inhibitor that abolishes ADP-induced platelet shape change and aggregation. Association of polyphosphoinositides with receptor could be such that the phospholipid molecules are located on the inner surface of the platelets. The supportive evidence being that enzymes involved in the metabolism of TPI and DPI are mainly located on the cytoplasmic surface of erythrocytes (Garrett and Redman, 1975) and that polyphosphoinositides of the impermeable inside-out erythrocyte vesicles are labelled with (γ - ^{32}P)ATP. Whereas DPI and TPI of the impermeable right-side out vesicles are not labelled.

2. Thrombin-induced TPI-DPI interconversion and platelet thrombin-receptors.

Degradation of TPI occurred much faster with thrombin. Decreases of concentration as well as ^{32}P -labelling in TPI were detected 9 sec following addition of thrombin, a time when platelet shape change was maximum. The ^{32}P -labelling and concentration of DPI increased accordingly (Table X and Figure 27). However, degradation of TPI was reversed once aggregation and the release reaction commenced. Synthesis of TPI mediated by DPI formation became substantial at 60 sec. The radioactivity of $^{32}\text{PO}_4$, ^3H -glycerol, ^{14}C -arachidonic acid and

³H-inositol as well as the concentrations of these lipids significantly increased. These observations therefore illustrate a close time relationship between TPI-DPI interconversion and the response of platelets to thrombin.

Thrombin causes aggregation associated with release of ADP as well as prostaglandin endoperoxide formation (Willis et al., 1974). The ADP which is released from the granules augments the aggregation response to thrombin, and the endoperoxides themselves also cause aggregation and further release of ADP. Neither the aggregation and ADP-release induced by high concentration of thrombin can be inhibited by CP/CPK and indomethacin, singly or in combination. In addition, the combined use of PGE₁ with caffeine, CP/CPK, indomethacin and EGTA abolishes aggregation and the release reaction of washed rabbit platelets but not the shape change induced by thrombin. These observations led Packham and colleagues (1977) to conclude that a third mechanism is likely in operation through which thrombin can cause platelet aggregation and the release reaction. Since thrombin is a large peptide which cannot go across membrane as ionophore A23,187 molecules do and there have been strong evidence for thrombin-receptor interaction on platelet surface (Detwiler et al., 1975; Majerus et al., 1976), it is reasonable to expect that the primary pathway by which thrombin initiates biochemical changes in platelets is mediated by interaction with the thrombin receptors. Therefore, if the TPI

degradation observed during the thrombin-induced platelet shape change is to play a role in this mechanism, the polyphosphoinositide molecules must be located within the structural domain of the thrombin receptors, a situation similar to the one proposed for the receptors of ADP.

3. Metabolism of MPI and response of platelets to A23,187 and thrombin.

The present data on metabolism of MPI have revealed that both thrombin and ionophore A23,187 caused substantial loss of platelet MPI. But the major biochemical pathways contributing to the MPI loss appear to be different for these two stimuli. It has been mentioned previously (p. 46) that MPI can be metabolised by 3 different pathways - phosphodiesteratic cleavage of the compound to yield 1,2-diacylglycerol, cleavage by phospholipase A₂ to form lyso-MPI and free fatty acid, and phosphorylation of MPI to DPI and TPI.

With thrombin the decreases in MPI concentration and labelling of ¹⁴C-arachidonic acid, ³H-glycerol and ³H-inositol (Table XI, Figures 24-26) were mainly attributed to the phosphodiesteratic cleavage pathway, which accounted for 60% of the amount of MPI that disappeared from MPI fraction 60 sec following addition of thrombin to platelet suspensions. The computation of these data is explained in page 197. This resulted in a many-fold increase in the labellings of ¹⁴C-arachidonic acid, ³²PO₄ and ³H-glycerol labelling in PA as well as several-fold increase in ¹⁴C-arachidonic acid in 1,2-diacylglycerol (Table IX). The concentration

of PA was also significantly increased at 60 sec. Hydrolysis by phospholipase A₂ also contributed to some loss of MPI, but to a much smaller extent. Phosphorylation of MPI to DPI and TPI occurred to approximately the same extent as cleavage of MPI by phospholipase A₂. Each pathway accounted for approximately 20% of the isotopic labellings (¹⁴C-arachidonic acid, ³H-glycerol and ³H-inositol) as well as 20% lipid phosphorus lost from MPI 60 sec following addition of thrombin to platelet suspensions. We have therefore concluded that phosphodiesteratic cleavage is the predominant pathway to account for the loss in MPI during the first 60 sec in thrombin-stimulated platelets.

Ionophore A23,187 caused significant loss of ¹⁴C-arachidonic acid and ³²P-labelling as well as concentration of MPI. But the increase in ³²P-labelling and ¹⁴C-arachidonic acid in PA was much smaller and slower than that of thrombin-treated platelets. It therefore appears that cleavage of MPI by phospholipase A₂ to form free fatty acid and lyso-MPI is the predominant pathway of MPI metabolism in the first 60 sec of A23,187 reaction. Although we did not measure these products, ionophore A23,187-induced formation of free arachidonic acid and prostaglandin endoperoxide derivatives has been well documented by others (Pickett et al., 1977; Rittenhouse-Simmons et al., 1977). Pickett and associates (1977) reported that five times more arachidonic acid were lost from phospholipids in A23,187-treated platelets than in the thrombin-treated platelets at stimulus concentrations which

caused maximum oxygen consumption with ionophore-treated platelets. Synthesis of DPI and TPI was significant but resynthesis of MPI from PA appeared to be inhibited, resulting in a rapid loss of ^{32}P -labelling from MPI (Figure 21). Loss of ^{32}P -labelling from MPI did not occur with ADP or thrombin-treated platelets.

It is generally accepted that A23,187 and thrombin cause platelet aggregation and the release reaction by some common mechanisms (ADP release and prostaglandin endoperoxide formation) as well as by a third mechanism(s) which may be common to each of the reagents (Packham et al., 1977). A23,187 does not act by interaction with surface receptors on platelets; rather, it acts by transporting Ca^{2+} into platelets and/or by causing redistribution of internal platelet calcium (White et al., 1974; Feinman and Detwiler, 1974). Therefore, the third mechanism(s) by which A23,187 causes platelet aggregation may be related to its ability to mobilize calcium. Since phospholipase A_2 is dependent on Ca^{2+} for activation (De Haas et al., 1968), degradation of MPI by phospholipase A_2 may therefore be subsequent to the calcium-mobilizing activity of the ionophore. This reaction is possibly made

by the more favorable exposure of substrate molecules to phospholipase A₂ as a result of disruption of phospholipid matrix packing when the A23,187 molecules pass to and fro across the membrane. Disruption of membrane phospholipid packing by A23,187 presumably stems from the rather large size molecules of the ionophore oligopeptide and the hydrophobicity of the external surface of the cage-like ionophore molecules. This hydrophobicity may allow the A23,187 molecules to interact strongly with the membrane cholesterol molecules, causing aggregate formation in the membrane. Supportive evidence comes from the demonstration of ultrastructural alterations (formation of aggregates of membrane-associated particles and craters on the inner fracture face induced by amphotericin B in the plasma membrane of a fungus by freeze-etch electron microscopy (Nozawa et al., 1974). Amphotericin B is also an antibiotic ionophore which increase membrane permeability toward cations (Paphadjopoulos, 1973). Similarly, phosphodiesteratic cleavage by the Ca²⁺-dependent MPI phosphodiesterase is enhanced (Lapetina and Michell, 1973; Quinn, 1975).

The amount of MPI in platelet membranes, although small, is many times more than TPI and DPI. It is conceivable that distribution of MPI is not as restricted as the polyphosphoinositides and some MPI is associated with certain surface receptors of platelets. In this way, interaction of thrombin and receptor would influence phosphoinositide metabolism in a way similar to that described for TPI-DPI interconversion. However, the exact nature of the interaction between thrombin and

platelet surface components is not known. It can be certain that the type of stimulus-receptor interaction with thrombin is not the same as that with ADP because after all thrombin is a well characterized proteolytic enzyme. The much more extensive phosphodiesteratic hydrolysis of platelet MPI with thrombin as stimulus compared to that with ADP or A23,187 may possibly reflect this difference. Phosphodiesteratic cleavage of MPI in association with membrane receptors was described in a number of tissues (Michell, 1975). The acetylcholine-stimulated cyclical conversion of MPI in peripheral tissues is considered to be mediated by muscarinic receptors as this response is inhibited by atropine and not by tubocurarine. Phosphodiesteratic cleavage of MPI associated with other receptors in response to various stimuli has been summarized by Michell (1975).

4. Biological significance of MPI hydrolysis and TPI-DPI interconversion in platelet shape change and aggregation.

The roles of MPI hydrolysis and TPI-DPI interconversion can be discussed in terms of the properties of products derived from cleavage of the phosphoinositides, the effect of the reaction proper on platelet membrane, as well as the possibility of TPI as a source of Ca^{2+} which serves as second messenger in many cellular functions.

(a) Products of MPI hydrolysis.

The most extensively documented product of MPI hydrolysis is the free arachidonic acid. MPI has been known as a rich source of arachidonate (Marcus et al., 1969). The arachidonic acid cleaved from MPI may be immediately converted to prostaglandin endoperoxides and

thromboxane A_2 by platelet cyclo-oxygenase. These products are some of the most potent platelet-aggregating agents (Hamberg et al., 1974; Smith et al., 1975).

Another product of phospholipase A_2 activity is lyso-MPI, a very powerful lytic agent. Involvement of lysophospholipids in the fusion of erythrocytes or fusion of fibroblasts with erythrocytes to form polykaryocytes was demonstrated by Poole and colleagues (1970). Presumably the lyso-compounds create phase separation in the cell membrane, converting the bilayer molecular arrangement of phospholipid molecules into micellar aggregates at certain locations (Lucy, 1970). Similar mechanisms may be responsible for granule-plasma membrane fusion in secretory cells (Lucy, 1970). It is thus possible that formation of lyso-MPI assists in the release reaction of platelets.

Diacylglycerol and phosphorylinositol formation was observed in A23,187-stimulated human erythrocytes and acetylcholine-treated mouse pancreas (Allan and Michell, 1975; Banschbach et al., 1974). Accumulation of diacylglycerol in the A23,187-treated erythrocytes has been cited as the cause for the transformation of disc-shaped erythrocytes into echinocyte (Allan and Michell, 1975). This is probably due to the generation of an imbalance of the amphipathic forces as a result of the removal of the polar phosphorylinositol moiety from MPI. In addition, greater than 50% of the phosphorylinositol group cleaved from MPI is present as myoinositol-1,2-cyclic phosphate, the rest as inositol-1-phosphate. The cyclic compound has been

suggested by Michell and Lapetina (1972) to act as an intracellular second messenger for those stimuli which provoke enhanced MPI turnover. However, evidence for this hypothesis has been inconclusive. In platelets, the extensiveness of diacylglycerol production from phosphodiesteratic cleavage of MPI during thrombin-induced platelet aggregation and the release reaction almost surely reflects a special response of platelets to this stimulus. At the present, however, the role of these products in platelets is totally unknown.

(b) TPI-DPI interconversion and platelet membrane fluidity and permeability.

Phosphoinositides, especially TPI, complex readily with divalent metal ions such as Ca^{2+} and Mg^{2+} (Hendrickson and Fullington, 1965). In the absence of divalent metal ions these lipids are largely water soluble but become water insoluble upon the addition of metal ions. Phosphoinositides complex readily with a variety of proteins forming in most cases water soluble complexes in the absence of divalent metal ions and water-insoluble complexes in the presence of these ions (Hendrickson, 1969). The calcium bound to the acidic phospholipids has considerable ability to restrict the motion of their fatty acids in the membrane as indicated by the spin-labelling technique (Schnepel et al., 1974). In addition, PS and PA molecules in a PS-PA

or PA-PC lipid mixture that contains minor quantity of anionic phospholipids laterally condense into independent arrays if Ca^{2+} is present (Ohnishi and Ito, 1974; Ito and Ohnishi, 1974).

Furthermore, if divalent cations are presented to only one side of an anionic model membrane, a membrane potential is generated which is dependent on the calcium concentration (Papahadjopoulos, 1972). From this information, it can be inferred that removal of Ca^{2+} from the inner platelet membrane subsequent to degradation of phosphoinositides would increase the solubility of membrane lipids and the motion of their acyl chains, thereby increasing membrane fluidity. It would also create instability in the membrane.

Permeability of cell membrane is influenced by environmental factors such as pH, temperature and the presence of Ca^{2+} . It is therefore not surprising to find that the monoesterified phosphates of TPI bind Ca^{2+} most strongly at physiological pH (Joo and Can, 1967) and that the permeability of cell membrane is at its minimum at the same pH values (Papahadjopoulos, 1973). During cellular excitation, TPI may be hydrolysed to DPI with release of the bound Ca^{2+} , thus altering the permeability of cell membrane (page 193). Definite experimental evidence was provided by Tret'jak and

associates (1977). They reported a decrease of TPI content 5 min after acetylcholine treatment of crab nerve fibers. The loss of TPI was accompanied by a simultaneous efflux of $^{32}\text{PO}_4$ as well as efflux of Ca^{2+} from the nerve fibers and a delayed efflux of K^+ . On the other hand, we observed significant hydrolysis of TPI at 60 sec in ADP-stimulated platelets and at 9 sec in thrombin-stimulated platelets. A close time relationship between TPI-DPI interconversion and membrane permeability control toward Na^+ is indicated by the recent finding by Feinberg and associates (1977). They observed that addition of ADP to platelet-rich plasma resulted in platelet aggregation and a rapid rise (within seconds) in $^{22}\text{Na}^+$ -radioactivity within the platelets, which was not accompanied by increased influx of ^{36}Cl into platelets. Furthermore, they reported that 300 sec after addition of ADP, the increase of $^{22}\text{Na}^+$ in the platelets diminished toward control levels. This was also the time when we observed deaggregation of washed rabbit platelets previously aggregated by ADP, as well as the time when the $^{32}\text{PO}_4$ labellings in TPI and DPI returned toward their prestimulation values.

(c) Role of TPI as source of Ca^{2+} ions.

The role of Ca^{2+} in cellular response is widely recognized.

The secretory activities of many tissues, including mast cells, parotid gland, chromaffin cells do not occur unless Ca^{2+} is present in the medium. Similarly, platelets do not aggregate in a medium lacking Ca^{2+} . However, unlike other tissues, human platelets only undergo release reaction even in the absence of external Ca^{2+} if they are stimulated with the appropriate aggregation agents such as thrombin and ionophore A23,187. This observation with the latter agent have led a number of investigators to suggest that A23,187 acts by mobilization of internal calcium from intracellular binding sites (White et al., 1974; Feinman and Detwiler, 1974, 1975; Kinlough-Rathbone et al., 1975). These Ca^{2+} -binding sites in platelets however, have never been clearly defined. In view of the previous discussion on the properties of polyphosphoinositides, it is possible that TPI of platelet membrane takes part in the regulation of intracellular Ca^{2+} concentrations. Further evidence are that phospholipids bind many times more Ca^{2+} than membrane proteins do (Anghileri, 1972). Definite evidence that polyphosphoinositides control the binding of Ca^{2+} to the erythrocyte membrane has been obtained by Buckley and Hawthorne (1972). The tightly bound Ca^{2+} increased in 1:1 molar ratio with polyphosphoinositide monophosphate. The activity of the Ca^{2+} -activated adenosine triphosphate was also higher than in normal membranes. In addition, the bound Ca^{2+} is readily released through hydrolysis of TPI to DPI. The reaction is catalysed by TPI-

phosphomonoesterase which in fresh brain has an activity almost as high as that of acetylcholinesterase (Kai and Hawthorne, 1969). Furthermore, the activity of this enzyme is independent of Ca^{2+} (Dawson and Thompson, 1964).

Therefore, taking into consideration the scheme on the roles of Ca^{2+} in platelet functions as summarized by Haslam and associates (1978), the roles of TPI-DPI interconversion and MPI hydrolysis in platelet aggregation can be envisaged as follows: interaction of stimulus with membrane receptor molecules triggers hydrolysis of TPI to DPI with concurrent release of Ca^{2+} and opening of Na^+ channels. Influx of Na^+ causes depolarization of membrane. Meanwhile, the free Ca^{2+} would interact with several cellular systems, including the dense tubular system to release more Ca^{2+} , activation of the membrane phosphorylase kinases, depolymerization of microtubules and activation of acto-myosin ATPase. The latter two changes possibly mediate immediate shape change and aggregation of platelets. The free Ca^{2+} also activates phospholipase A_2 and the MPI phosphodiesterase to yield diacylglycerol, inositol-1,2-cyclic phosphate, lyso MPI, and free arachidonic acid which may be converted to prostaglandin endoperoxides and thromboxane A_2 . These products further enhance aggregation and the release reaction. Although in the scheme described by Haslam and coworkers (1978) dense tubular system (White, 1972b) was suggested as a storage place for Ca^{2+} in platelets (Feinstein et al., 1976) because of its anatomical resemblance to the sarcoplasmic reticulum system in muscle cells, it should be noted that TPI may

provide the Ca^{2+} -binding sites for these membranes as well.

The present project has therefore shown that phosphoinositide metabolic reactions are among the earliest detectable biochemical changes in platelets in response to stimuli. The very early hydrolysis of TPI during thrombin-induced platelet shape change, and the proposed roles of TPI in the regulation of membrane permeability and as a source of free Ca^{2+} possibly qualify TPI hydrolysis as part of the primary mechanism of platelet aggregation. On the other hand, although some of the changes of hydrolysis of MPI could be detected very early in the thrombin-stimulated platelets, hydrolysis of MPI is likely to occur subsequent to the release of Ca^{2+} by TPI degradation. Synthesis of TPI during platelet deaggregation following ADP-stimulation, and during thrombin or A23,187-induced platelet aggregation and the release reaction, as well as resynthesis of MPI mediated by the cyclical conversion may represent the repair mechanisms of platelets to compensate for the loss of these phospholipids. The roles of PA accumulation, except for its being the precursor for all phospholipids, remains unknown. The only thing can be said about the increase in concentration as well as isotopic labellings in this phosphoglyceride of platelets in response to stimuli is that these increases must be secondary to the phosphodiesteratic cleavage of MPI.

SUMMARY

SUMMARY

The aims of these studies were to determine whether phosphate turnover of phospholipids in platelets occurs with different patterns under in vitro and in vivo conditions. It was also intended to determine whether changes in synthesis or breakdown of PA and the phosphoinositides had a role in platelet aggregation, changes in platelet shape and the release reaction. The following contributions to knowledge were made:

- (1) A two dimensional thin-layer chromatography method was developed to give satisfactory separation among PC, PS, MPI, PE, PA, lyso PE and sphingomyelin.
- (2) It was found that the major phospholipids (PC, PE, PS) of platelets labelled for one hour with ^{32}P -orthophosphate, followed by in vitro incubation incorporated significant amounts of ^{32}P -labelling into all phospholipids after 4-6 hours. The incorporation pattern was comparable to the one in which ^{32}P -labelled platelets were infused back into the circulation of rabbits. Therefore, there is no difference in the ability of washed platelets to incorporate $^{32}\text{PO}_4$ into the major phospholipids compared to platelets in vivo. Thus, the phosphate moiety of all cell phospholipids turn over, but that of the major phospholipids turn over more slowly.

- (3) The glycerol, acyl chains and phosphate components in any given phospholipid species of platelets turn over at different rates, which could be explained in terms of metabolic pathways and the asymmetric distribution of these lipids in platelet membranes.
- (4) The patterns of metabolism of PA and the phosphoinositides differ in platelets in response to ADP, thrombin and ionophore A23,187. This is compatible with the generally accepted differences in the mechanisms of platelet aggregation caused by these stimuli. The major metabolic pathway in ADP-induced aggregation of washed platelets was TPI-DPI interconversion. Hydrolysis of TPI to DPI was observed during platelet aggregation as indicated by a loss of ^{32}P , ^3H -inositol and ^3H -glycerol in TPI and a gain of these isotopes in DPI. Resynthesis of TPI from DPI occurred during platelet deaggregation as the ^{32}P -labelling in these inositol lipids returned to their prestimulation levels. Hydrolysis of TPI was abolished with AMP, a specific agent that inhibited ADP-induced shape change and aggregation of platelets. Some time after platelet deaggregation, platelets were refractory to further addition of ADP. ADP added at this time caused little or very weak platelet aggregation and the $^{32}\text{PO}_4$ in TPI did not change further. However, ADP

added to platelets which had completely restored their disc shape and sensitivity to ADP, would induce TPI hydrolysis again. These studies demonstrated a close relationship between TPI-DPI interconversion and platelet aggregation as well as the importance of TPI in platelet membrane sensitivity to ADP.

- (5) Thrombin also caused hydrolysis of TPI to DPI, as early as 9 sec when platelet shape change was at a maximum. Both the concentrations and isotopic labellings of these lipids changed. There was no hydrolysis of TPI demonstrable in A23,187-treated platelets. It is thus possible that TPI-DPI interconversion is associated with stimulus-receptor interaction, which occurs with ADP and thrombin, but not with A23,187.
- (6) Both thrombin and A23,187 caused substantial loss of MPI from platelets. Very little loss of MPI occurred in ADP-induced platelet aggregation. The major pathways contributing to the loss of MPI however, was different with thrombin and A23,187. With thrombin, phosphodiesteratic cleavage was the main cause while activation of phospholipase A₂ appeared to be the most important cause for A23,187-induced loss of the MPI fraction. Both stimuli caused conversion of MPI to DPI and TPI, measurable by concentrations as well as changes in the distribution of radioactivity in platelets labelled with ¹⁴C-arachidonic acid, ³H-glycerol, ³H-inositol or ³²P₄.

Phosphodiesteratic cleavage in thrombin-aggregated platelets is possibly related to the stimulus-receptor interaction mechanism, while the effects of A23,187 is likely associated with its directly transporting calcium into the platelets and/or mobilizing intracellular calcium. From the close time relationships between the changes in PA and phosphoinositide metabolism and the functional changes in platelets (shape change, aggregation and the release reaction), as well as based on the current knowledge and hypothesis on the properties and roles of phosphoinositides in cell membranes, it is conceivable that TPI may be an important source of Ca^{2+} for platelet functions and that TPI-DPI interconversion may be part of the primary mechanism in platelet aggregation. On the other hand, the Ca^{2+} -dependent nature of the enzymes involved in MPI hydrolysis suggests that both phosphodiesteratic cleavage of MPI and phospholipase A_2 activation for hydrolysis of MPI to lyso MPI and free arachidonic acid must occur subsequent to the Ca^{2+} release and membrane permeability changes following TPI hydrolysis, although the products of MPI degradation may play an important role in the enhancement of platelet aggregation and the release reaction. Furthermore, it was concluded that resynthesis of TPI during

deaggregation of platelets previously aggregated by ADP, or during thrombin and A23,187-induced platelet changes as well as resynthesis of MPI mediated by thrombin of PA was secondary to platelet aggregation. Similarly, the ³²P-labelling in PA observed by previous investigators in thrombin and ADP induced platelet aggregation must be interpreted as secondary to the phosphodiesteratic cleavage of MPI. We have therefore, demonstrated that net synthesis and breakdown of PA and the phosphoinositides occur in platelets in response to aggregation agents.

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