Br. J. Pharmac. (1977), **59**, 353–366

THE DISTRIBUTION AND METABOLISM OF ARACHIDONIC ACID IN RABBIT PLATELETS DURING AGGREGATION AND ITS MODIFICATION BY DRUGS

G. J. BLACKWELL, W.G. DUNCOMBE, R.J. FLOWER, M.F. PARSONS & J.R. VANE

Wellcome Research Laboratories, Langley Court, Beckenham, Kent, BR3 3BS

1 Gas chromatographic and radio-isotope labelling techniques have been used to establish the origin of the arachidonic acid used by the platelet cyclo-oxygenase for the synthesis of pro-aggregatory prostaglandin endoperoxide derivatives.

2 Measurements of total platelet arachidonate content indicated that more than 95% is esterified in the phosphatide fraction of the cells.

3 During aggregation by collagen or thrombin as much as 80% of the total platelet arachidonate may be liberated and transformed by the platelet enzymes into hydroxyacids and other more polar compounds.

4 The phosphatidylethanolamine, phosphatidylcholine and phosphatidylinositol fractions are major sources of the arachidonate thus used.

5 Indomethacin, which prevents platelet aggregation by inhibiting the cyclo-oxygenase, did not affect this release of arachidonate from the phosphatides but did prevent the transformation of arachidonate to endoperoxide derivatives.

6 Mepacrine, a drug which possesses weak anti-phospholipase activity in platelets, also prevents aggregation by collagen or thrombin, but seems to do so by preventing substrate release from the phosphatide fraction.

7 It is suggested that phospholipase A_2 plays a key role in the initial events during platelet aggregation induced by collagen.

Introduction

Platelets contain at least two enzyme systems which oxygenate arachidonic acid (as well as some other polyunsaturated fatty acids) and a summary of these pathways is depicted in Figure 1. One of the transformations is catalysed by a cytoplasmic lipoxygenase (Hamberg & Samuelsson, 1974; Nugteren, 1975) and results in the formation of a lipid hydroperoxide, and ultimately a hydroxy acid named (by Hamberg & Samuelsson) 'HETE'. The other more complex pathway is catalysed by a membrane bound cyclo-oxygenase complex which in the first instance transforms arachidonate to the prostaglandin endoperoxide intermediates G_2 and H_2 (Hamberg, Svensson, Wakabayashi & Samuelsson, 1974; see also Nugteren & Hazelhof, 1973). These endoperoxides may be further metabolized either to prostaglandins, to a 17-C hydroxy acid ('HHT') or to thromboxane (TX) A_2 or B_2 (Hamberg & Samuelsson, 1974; Hamberg, Svensson & Samuelsson, 1975). This latter pathway is of especial interest; TXA_2 is a potent but highly unstable compound (half-life at 37°C about 32 s) which causes 5-hydroxytryptamine release from platelets and irreversible aggregation, in nanogram quantities. Large amounts of TXA_2 (assayed by its stable metabolite, TXB_2) are formed during aggregation by various agents, and it represents a considerable proportion of total prostaglandin G_2 metabolism (Hamberg & Samuelsson, 1974). As a product of the cyclo-oxygenase pathway, TXA_2 formation is blocked by aspirin-like drugs (Vane, 1971) thus providing an explanation for the observation that aspirin prevents second phase platelet aggregation (O'Brien, 1968). All the available evidence points to a major role for TXA_2 in irreversible platelet aggregation induced by collagen, thrombin and probably other stimuli.

In order to generate TXA_2 the platelet requires arachidonic acid. Platelets aggregate in the presence of

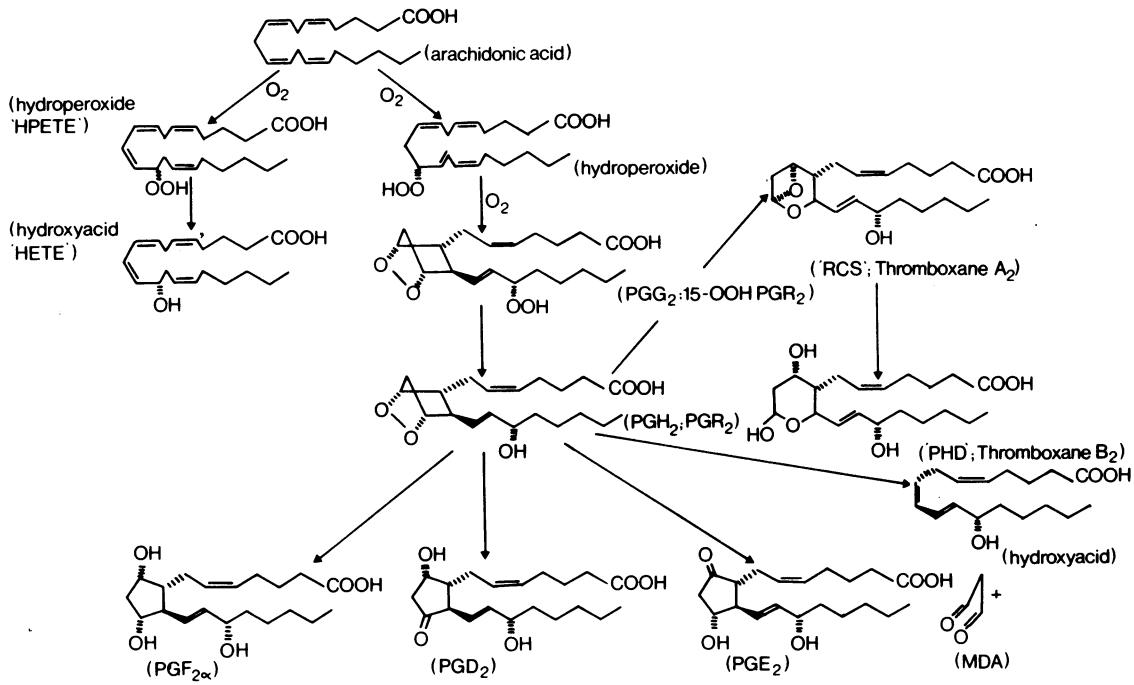

Figure 1 A summary of the known pathways by which arachidonic acid is oxygenated in platelets. See text for references.

this fatty acid (Vargaftig & Zirinis, 1973; Silver, Smith, Ingerman & Kocsis, 1973) but 'spontaneous' aggregation of unstimulated platelets does not occur since the level of free arachidonic acid is apparently very low (Marcus, Ullman & Safier, 1969). Some preliminary reports by ourselves (Flower, Blackwell & Parsons, 1975) and other workers (Schoene & Iacono, 1975; Bills & Silver, 1975) suggested that several aggregating agents trigger cyclo-oxygenase activity by releasing arachidonic acid from the platelet phosphatide fraction. In this paper we describe experiments designed to answer the following questions: how much arachidonic acid is there in platelets, and what is the chief intracellular store? What is the source of the arachidonate used during platelet aggregation? Is phospholipase involved in the mobilization of substrate, and if so, what effect do phospholipase inhibitors have on platelet aggregation?

Whilst this manuscript was in preparation, Bills, Smith & Silver (1976) have published experiments indicating that phosphatidylcholine and phosphatidylinositol are important sources of the arachidonate used during aggregation of human platelets.

Methods

Preparation of platelet rich plasma (PRP)

Male New Zealand white rabbits were anaesthetized with pentobarbitone sodium (25–35 mg/kg). Blood was withdrawn from a cannula placed in the common carotid artery and collected in 10 ml tubes containing 1 ml of 3.8% (w/v) tri-sodium citrate. After mixing the blood was centrifuged at 250 × *g* for 15 min in a bench centrifuge and the PRP removed.

Measurement of platelet aggregation

Platelet aggregation was measured by continuous recording of light transmission through PRP using a Born aggregometer (Born, 1962). Small (10–20 µl) volumes of solutions containing inhibitory drugs or aggregating agents were added to 1 ml aliquots of PRP in siliconized glass cuvettes. Continuous magnetic stirring was used to ensure adequate mixing and to prevent platelet sedimentation.

Preparation of collagen

Acid soluble calf-skin collagen (type IV, Sigma) was homogenized in Tris-saline (pH 7.4) using a motor driven pestle. Suspensions were stored frozen, and thawed in a water bath for 30 min before use.

Preparation of washed platelets

PRP was centrifuged at $300 \times g$ for 20 min to sediment the platelets. The platelet-poor plasma was decanted and the platelets gently resuspended in Tyrode solution or Tris-saline (134 mM NaCl, 15 mM Tris-HCl, 5 mM D-glucose) giving an approximate final concentration of 10^8 platelets/ml.

Extraction of lipids from platelets

Aggregated or control samples (1 ml) of platelets were tipped quickly into 10 ml methanol, cooled with dry ice and the mixture stirred for 10 minutes. Chloroform (20 ml) was added to bring the chloroform/methanol ratio to 2:1 and after shaking, the mixture was placed in the refrigerator (2–4°C) for 1 h to extract the lipids. The platelet extract was then filtered to remove precipitated protein and concentrated under vacuum in a rotary evaporator. The resulting lipid mixture was used for analysis and assay.

Separation into 'neutral' and 'polar' lipid classes

Lipid extracts dissolved in chloroform were separated on 1–3 g (depending on the amount of lipid material) columns of activated silicic acid. Seven column volumes of chloroform eluted the neutral lipids (mono, di, tri-glycerides, fatty acids, hydroxy acids, cholesterol and its esters), and seven column volumes of methanol eluted the polar lipids (all phospholipids and lyso-derivatives, as well as cyclo-oxygenase products).

Hydrolysis of lipid fractions to yield fatty acid methyl esters

In order to estimate the arachidonate content of phosphatides and neutral lipid esters a hydrolytic step must be included in the assay procedure. This was accomplished by the use of the boron tri-fluoride technique of Metcalfe, Schmitz & Pelka (1966). To the dried lipid extract was added 0.4 ml of 0.5 N NaOH in methanol. This was heated in boiling tubes for 10 min at 100°C in a water bath. The boron tri-fluoride solution (14% w/v) in methanol (0.5 ml) was added and the mixture boiled for 2 minutes. After cooling a saturated solution of NaCl was added until an oily layer separated. Hexane (1 ml) was added, the mixture shaken and the top layer containing fatty acid methyl esters removed for assay.

Gas chromatographic assay of arachidonate

Hydrolysed lipid extracts (prepared as above) or the free acid (after methylation with diazomethane) were assayed directly with a Pye Unicam series 104 gas chromatograph. The column used was a 150 cm glass (outside diameter 0.6 cm) packed with 10% DEGS on Gas-chrom Q. A flow rate of 40 ml N_2/min was used. At an oven temperature of 185°C, authentic methyl arachidonate was easily detected by the flame ionization detector (set at 250°C) having a retention time of approximately 18 minutes. All measurements were made isothermally. Estimation of unknown amounts of arachidonate were made by comparing the (peak height × retention time) factor with a standard curve. Labelled arachidonate was used to correct for losses during extraction.

Pulse labelling of platelet suspensions with [1-^{14}C]-arachidonate

Aliquots of washed platelets or in some cases PRP containing about 10^9 platelets/ml were incubated with 1 μCi [1-^{14}C]-arachidonate for 1 h at room temperature. After washing off the excess label, the platelets were resuspended in Tyrode solution giving a final concentration of about 10^8 platelets/ml. After a further 30 min incubation (designed to reduce the concentration of free arachidonate), the labelled platelet suspensions were exposed to aggregating agents or drugs, and extracted as already described.

Separation of phospholipids

For determination of the amount of arachidonate (labelled or unlabelled) in individual phosphatides, the crude platelet lipid extract was first separated into neutral and polar lipids by column chromatography as described. The polar fraction was further separated by thin layer chromatography (t.l.c.) on preparative silica gel plates (250 μm layer). Two solvent systems were used: (I) chloroform/acetone/methanol/acetic acid/water (6:8:2:2:1), and (II) chloroform/methanol/12 N ammonia/water (70:30:4:1). The R_F values (×100) for *authentic* phospholipids were as follows: solvent system I: sphingomyelin (SPH) and lysophosphatidylcholine (LPC), 7.6; phosphatidylcholine (PC), 16.0; phosphatidylinositol (PI), 26.2; phosphatidylserine (PS), 30.7; phosphatidylethanolamine (PE), 47.4. Solvent system II: LPC, 7.2; PS, 14.5; PI, 20.0; SPH, 21.8; PC, 31.5; PE, 48.4. The rank order of R_F values found by us corresponds with those previously published for these solvent systems (Kates, 1972; Cohen & Derksen, 1969).

Phospholipid zones on the developed chromatograms were visualized by brief exposure to iodine vapour. The zones were eluted and hydrolysed with

boron tri-fluoride for estimations of the arachidonate content, or in the case of the radio-isotope experiments the radioactivity in each zone was estimated by liquid scintillation counting.

Enzymatic hydrolysis of phospholipids

To determine the position of the radioactive fatty acid, samples of some phospholipids were hydrolysed with the specific hydrolytic enzyme phospholipase A_2. Extracts containing the phosphatide were dissolved in Tris buffer (100 mM, pH 7.8) containing 5% Triton-X, 20 mM $CaCl_2$ and 100 mM NaCl. Pig pancreas phospholipase A_2 (100 μg enzyme) was added and the mixture incubated at 37°C for 1 hour. The radioactivity was extracted with chloroform/methanol (2:1), excess solvent dried off *in vacuo* and the sample spotted quantitatively onto a silica gel t.l.c. plate (100 μm layer) and developed to a distance of 10 cm in chloroform/methanol/acetic acid (90:5:5). Phosphatides and their lysoderivatives remain at the origin and fatty acids run at the solvent front. The amount of radioactivity in each zone was estimated by liquid scintillation counting.

Measurement of organic phosphorus

Organic phosphorus was measured by the direct colorimetric method of Raheja, Kaur, Singh & Bhatra (1973), using PC as a standard.

Separation of radioactively labelled cyclo-oxygenase and lipoxygenase products by t.l.c.

Two types of procedure were used. During aggregation 1 ml aliquots of labelled platelet suspension were tipped into beakers containing 10 ml ethanol +1% stannous chloride (w/v) to reduce the prostaglandin endoperoxides to prostaglandin $F_{2\alpha}$. Extraction then proceeded in the same way as already described and the constituents were separated by t.l.c. on a silica gel plate (100 μm layer) using ethyl acetate/*iso*-octane/acetic acid/water (11:5:2:10) as a developing solvent. In this system phosphatides remained at the origin, prostaglandin $F_{2\alpha}$ had an R_F value (×100) of about 18, and arachidonic acid an R_F of about 72. Zones containing radioactivity were detected either with a Panax radiochromatogram scanner, or by scraping off the chromatogram in 0.5 cm zones and estimating the radioactivity in each zone by liquid scintillation counting.

No stannous chloride was used for the second method of assaying these products; after extraction in the usual way, the mixture was separated by t.l.c. as above. The phospholipid zone was removed from the origin and the remainder of the radioactive products eluted from the plate with chloroform:methanol (2:1). When the solvent was evaporated the residue was redissolved in ether, the products methylated with ethereal diazomethane and subsequently rechromatographed on t.l.c. using ethyl acetate/*iso*-octane/water (50:100:100) as a developing solvent. Methyl arachidonate has an $R_F \times 100$ value of 81.7 in this system, methylated prostaglandins and other polyoxygenated products remain at the origin and methyl esters of hydroxy acids have an $R_F \times 100$ value of about 43 (HHT) and 51.6 (HETE). Zones containing radioactivity were detected and measured as before.

Preparation of 2-([1-^{14}C]-oleoyl) phosphatidylcholine

PC was prepared from fresh egg yolks and purified by column chromatography (Saunders, 1957), monitored by t.l.c. LPC (1-acyl-glycerylphosphorylcholine) was generated by the action of venom phospholipase A_2 on the PC. To 184 mg PC in 50 ml diethyl ether was added 1 ml borate buffer (0.1 M; pH 7.4) containing 4.4 mg *Crotalus adamanteus* venom (Sigma) and 7 mg calcium acetate (dried). The flask was shaken slowly at 25°C. A white gelatinous precipitate adhering to the flask appeared within 30 min and the shaking was continued for 3 hour. The ethereal supernatant was discarded and the precipitate was washed twice with 10 ml ether, transferred to a small tared flask with chloroform/methanol (1:1), evaporated to dryness, dried in a vacuum desiccator and weighed. The residue (145 mg) was dissolved in 5 ml ethanol + 2 ml dichloroethane and stored at −20°C under nitrogen. It was shown by t.l.c. to consist of LPC with no detectable contamination with lecithin.

The biosynthesis of the specifically labelled PC was carried out as described by Robertson & Lands (1962). The reaction flask contained 250 μCi of [1-^{14}C]-oleic acid (57 mCi/mmol), 2 mg carrier oleic acid and 20 mg LPC. Organic solvents were removed under nitrogen, phosphate buffer (150 ml; 0.1 M; pH 7.5) was added and the mixture sonicated. When the lipids were dispersed the following reagents were added: 200 μmol adenosine triphosphate (ATP) (in a small volume of water, pH adjusted to 6.5), 200 μmol $MgCl_2$, 2 μmol CoA (in water, pH 6.5) and one third (3 ml) of a suspension in 0.25 M sucrose of the microsomes from 11 g of rat liver. Incubation was carried out at 37°C with gentle shaking. At 30 min and 120 min further equal amounts of ATP, $MgCl_2$, CoA and microsomes were added. After 3 h incubation the mixture was extracted with chloroform/methanol (2:1) and the chloroform phase dried over anhydrous $MgSO_4$. The extract was concentrated and applied to a column of 16 g silicic acid + 4 g Hyflo slurried with 10% (v/v) methanol/chloroform. Unreacted fatty acids were eluted with 100 ml of the same solvent. This was followed by 40% methanol/chloroform to elute the labelled phospholipid. The radioactivity of 50 ml fractions was monitored by liquid scintillation

counting and fractions corresponding to the radioactive peak were combined. T.l.c. and radioactive scanning and counting showed that the product was [^{14}C]-PC having a radiochemical purity of about 99% and containing about 70% of the initial activity. Hydrolysis of this product with the snake venom enzyme showed that about 97% of the radioactivity was in the 2-acyl position (assuming that the enzyme was absolutely specific for the 2-position).

Assay of platelet phospholipase A_2 activity

Washed platelet suspensions were lysed by freeze-thawing three times, and the crude preparation thus obtained was used as a source of enzyme. Each reaction set contained the following components: 0.2 ml lysed platelet suspension (about 30 mg/ml protein) homogenized in pH 7.4 Tris buffer (100 mM) containing 0.5% Triton-X; 0.1 ml 200 mM $CaCl_2$; 0.1 ml labelled phosphatide solution (about 50,000 ct/min); 0.1 ml buffer or inhibitory drug. After a thorough mixing the samples were incubated for 1 h at 37°C, the lipids extracted with chloroform-methanol (2:1) and separated by t.l.c. on silica gel plates (100 μm layer) developed in chloroform/methanol/acetic acid (90:5:5). Phosphatides remain on the origin, whereas liberated (radioactive) fatty acids migrate to the solvent front. In one set of experiments labelled phosphatide (0.5 μCi) was incubated with 5 ml aliquots of PRP directly. Two samples one of which contained 50 μM mepacrine were aggregated with thrombin, whilst a third sample served as a non-aggregated control. After 15 min treatment with the aggregating agents, the samples were extracted and the hydrolysis of the labelled phospholipid measured as described.

Measurement of protein

This was done by the biuret method of Gornall, Bardawill & David (1949).

Scintillation counting procedures

The radioactivity in the samples was estimated with a Beckman LS-150 liquid scintillation counter. The scintillation counting fluid used was Beckman 'Cocktail D' (5 g PPO and 100 g naphthalene per litre dioxane). All samples were counted such that $2\sigma \not> 0.5\%\ \bar{x}$. All ct/min values were converted to d/min by the AES ratio method.

The following drugs and chemicals were used: indomethacin (Merck); mepacrine (May and Baker); [1-^{14}C]-oleic acid, 57 mCi/mmol, and [1-^{14}C]-arachidonic acid, 58 mCi/mmol (Radiochemical Centre, Amersham); glass backed t.l.c. plates 2 mm layer of Kieselgel 60 (Merck); Triton-x 100 and *Crotalus adamanteus* venom (Sigma); pig pancreas phospholipase A_2 (Boehringer Mannheim G.m.b.H.); Silicic acid type I 60–200 mesh (Sigma). All other lipid standards were obtained from Sigma or Koch-Light. All other reagents (buffer salts, chromatography solvents) were analar grade or the highest purity available.

Results

Location and content of arachidonic acid in rabbit platelets

In two experiments the total arachidonate content of known numbers of washed platelets was measured in

Table 1 Distribution of arachidonic acid in different platelet lipid fractions

			Distribution (mol %)		
Experiment	*Platelet count*	*Total arachidonate*	*Free acid*	*Neutral esters*	*Phosphatides*
1	2.60×10^8	6.00 μg	~1.0	<1.0	96.8
2	4.07×10^8	6.65 μg	~1.0	<1.0	97.1

Table 2 Distribution of phospholipid phosphorus and arachidonic acid between platelet phosphatide pools (one experiment)

Phosphatide pool	*Phospholipid arachidonate pmol %*	*Phospholipid P g atom %*
Sphingomyelin	Trace	Not assayed
Lysophosphatidylcholine	Trace	Not assayed
Phosphatidylserine	13.12	3.44
Phosphatidylethanolamine	46.65	25.46
Phosphatidylinositol	28.43	21.80
Phosphatidylcholine	11.80	49.30

the three lipid fractions (free acid, neutral esters, phosphatides) after silicic acid column chromatography and hydrolysis with BF_3 in methanol. The results are shown in Table 1, and give an approximate range of 16–23 ng of arachidonate per 10^6 platelets. The distribution studies indicate that 97% of the arachidonate is located in the platelet phosphatide fraction. Rabbit (platelet-poor) plasma itself seems to contain considerable amounts of arachidonate and it is important to ensure that platelets are washed carefully when this type of experiment is undertaken.

Table 2 shows the distribution of phospholipid arachidonate and phospholipid phosphorus between the different phosphatide classes after separation of the polar fraction of the column effluent by t.l.c. In the initial experiments, we experienced great difficulty in obtaining reproducible separation of the different classes of phospholipids, and there appeared to be little correspondence between the platelet extract and the authentic phospholipid markers. The situation was further complicated by the finding that hydrolysis of phosphatides sometimes occurs in solvent system I (which contains acetic acid). The reason for the discrepancy between the 'standards' and the platelet extract was probably due to excess loading of the t.l.c. plates and the presence of other polar materials which tended to retard the migration of the phosphatides. In practice we overcame this by eluting the iodine-positive zones and re-running them separately in the same solvent system. This procedure resulted in a more pure phospholipid preparation and a good correspondence with the authentic standards.

Table 2 shows that SPH and LPC contain virtually no arachidonic acid; approximately half the platelet phospholipid arachidonate resides in the PE fraction, approximately one-third in the PI fraction, and the remainder is distributed equally between the PC and PS fraction. Amongst the major phosphatide stores PC represents about half of the cell phosphatides on a molar basis, PE and PI together represent just less than half, with PS comprising only a few moles per cent.

Effect of collagen and thrombin on platelet phospholipid arachidonate content

Table 3 is a summary of the experiments in which platelet phospholipid arachidonate was assayed by g.l.c. before and after aggregation by collagen or thrombin. Because of the lengthy extraction-purification-assay techniques involved it was not possible to do sufficient experiments in a single group for statistical analysis, however, in all cases (except in experiment 9c) there was a marked decrease in the platelet phospholipid arachidonate content in both washed and PRP platelet suspensions after aggregation by either collagen or thrombin. This decrease of phospholipid arachidonate ranged from a fall of only 6.9% (experiment 9a) to a maximum of 85.5% (experiment 7), with a mean value of 41.6%. Whilst developing this technique we found that from time to time we obtained anomalous results; experiment 9c (Table 3) for example shows an apparent rise in phospholipid arachidonate after aggregation. This may have been due to a mishandling (and consequent hydrolysis) of the phospholipid sample during sample extraction.

Table 3 Effect of aggregating agents on platelet phospholipid arachidonate. Summary of g.l.c. data

		Platelet phospholipid arachidonate (μg)			
Experiment	*Aggregating agent*	*Control*	*Aggregated*	*+Mepacrine (50 μM)*	*+Indomethacin (10 μM)*
1 a (W)*	Collagen 100 μg	6.41	2.24	—	—
b (W)	Collagen 100 μg	7.90	4.90	—	—
2 (W)	Collagen 100 μg	6.01	2.36	6.31	—
3 (W)	Collagen 100 μg	5.92	5.18	5.90	—
4 (W)	Collagen 100 μg	8.41	6.19	7.80	6.52
5 (W)	Thrombin 0.25 u	4.30	2.84	—	—
6 (W)	Thrombin 0.25 u	5.63	4.69	—	4.81
7 (W)	Thrombin 0.25 u	7.60	1.10	—	1.31
8 a (PRP)	Collagen 20 μg	5.25	2.35	—	—
b (PRP)	Collagen 20 μg	5.85	3.35	—	—
9 a (PRP)	Collagen 20 μg	5.80	5.40	7.00	—
b (PRP)	Collagen 20 μg	5.80	2.60	5.61	—
c (PRP)	Collagen 20 μg	8.20	9.40	—	—

* Arachidonate estimations made in washed platelets (W) or in platelet rich plasma (PRP).

Effects of mepacrine and indomethacin on platelet phospholipid arachidonate content

In some experiments (see Table 3) mepacrine (50 μM) and indomethacin (10 μM) were tested. Indomethacin (3 experiments) had virtually no effect on the loss of arachidonate from the phospholipids, but mepacrine (5 experiments) definitely caused a retention of arachidonate within the phospholipid pool, in one instance (experiment 2) there was slightly more (~5%) arachidonate in the mepacrine-treated platelets than in the 'control' samples; again, poor technique may be responsible for this apparently anomalous result. Both indomethacin and mepacrine in the concentrations used here prevented aggregation of the platelets.

Uptake of [1-^{14}C]-arachidonic acid by platelets

Excellent incorporation of [1-^{14}C]-arachidonic acid into rabbit platelets was observed. After analysis of the radioactivity in different lipid fractions it was found that >95% was incorporated into the phosphatide fraction, very small amounts (1–2%) were recovered as neutral lipid esters and the remainder of the label was recovered as free arachidonate. Time course studies indicated that uptake began almost immediately and was linear for as long as 1 to 2 hours. Further analysis of the phosphatide fraction by t.l.c. indicated that the radioactivity was largely associated with the PC, PS and PI fractions with smaller amounts recovered from the PE zone. In order to establish the positional labelling of one radioactive phosphatide (PC), this zone was eluted and incubated with pancreatic phospholipase A_2. After reaction the mixture was extracted and rechromatographed on t.l.c. using chloroform/methanol/acetic acid (90:10:2) as a developing solvent. More than 98% of the radioactivity was liberated indicating that virtually all the label was esterified in the 2′-acyl position. When the liberated fatty acid was recovered, methylated and rechromatographed in hexane/diethyl ether/acetic acid (50:50:2) on t.l.c. plates impregnated with 10% silver nitrate, all the radioactivity co-chromatographed with authentic methyl arachidonate indicating that no change in the chain length or degree of unsaturation of the labelled substrate had occurred, and thus it was still arachidonic acid.

Effect of aggregating agents and drugs on the distribution of [1-^{14}C]-arachidonate

Table 4 shows the results in an initial series of experiments in which the platelet phospholipid [1-^{14}C]-arachidonate was measured before and after aggregation by collagen, and treatment with collagen in the presence of mepacrine. The incorporation of labelled substrate in these experiments was rather less than usual (33% as opposed to about 95%) but after aggregation there was a fall of 36.6% of phospholipid arachidonate compared with the control. In the platelets treated with mepacrine there was a decrease of only 15.3% indicating that mepacrine conserved the phospholipid arachidonate.

The next experiments were designed to find out which phospholipid pool(s) was depleted of [1-^{14}C]-arachidonate during aggregation. Figure 2 is a thin layer chromatogram in which the radioactivity in each phosphatide fraction was measured after separation in chloroform/acetone/methanol/acetic acid/water, (6:8:2:2:1), before and after aggregation by collagen. Only three zones of radioactivity were initially observed; however, upon re-chromatography (see previous section) one zone (first peak on left in Figure 2) was resolved into two separate peaks having mobilities corresponding to PC and PI. It appears that PC and PI in extracts tend to co-chromatograph even though the standards are quite well resolved. This 'rechromatography' technique was routinely employed in our experiments to obtain good resolution of the different classes. Figure 2 does show that there is a marked decrease in the combined PC/PI peak as well as in the PE peak; no incorporation of label into the SPH zone was detected and virtually no change in the PS pool was found. The slight 'shoulder' in the PS peak may have been a very small separate peak but was not further identified.

To obtain more quantitative information about the decrease in arachidonate content of each pool, further experiments were performed using rechromatography to obtain maximum resolution on t.l.c. Table 5 shows an analysis of two such experiments. The quantitative

Table 4 Effect of collagen and mepacrine on platelet phospholipid arachidonate

Sample	*% Radioactivity recovered in phosphatides (n=5)*
Control	33.76 (±1.19)
Aggregated (100 μg collagen)	21.41 (±1.36)
Collagen + mepacrine (50 μM)	28.61 (±1.70)

Results are mean ± s.e. mean.

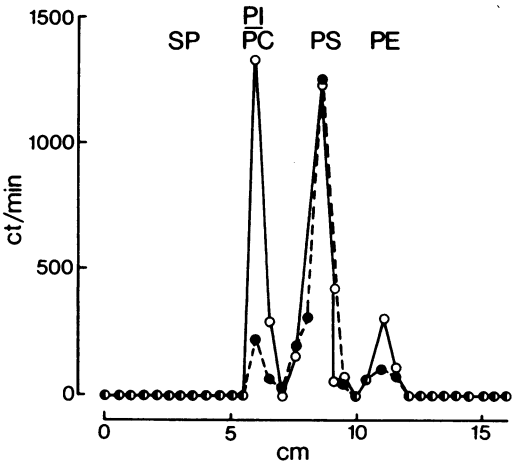

Figure 2 Separation of labelled platelet phosphatides by thin layer chromatography (chloroform/acetone/methanol/acetic acid/water, 6:8:2:2:1). SPH, PC, PS, PE and PI indicate the position of authentic sphingomyelin, phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine and phosphatidylinositol markers. (O) Control samples, (●) samples aggregated with 100 μg collagen. The plate was scraped into 0.5 cm zones and the radioactivity in each zone was estimated by liquid scintillation counting.

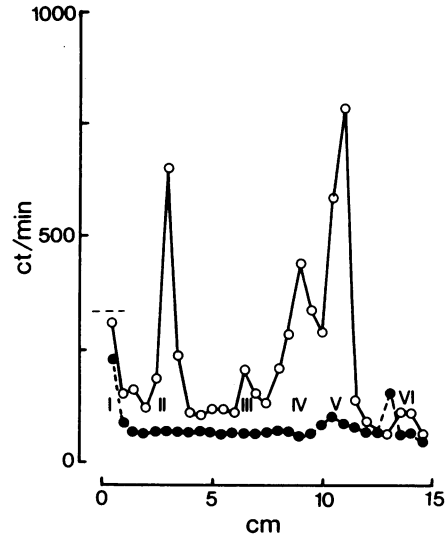

Figure 3 Separation of radioactive arachidonate oxidation products by thin layer chromatography (ethylacetate/*iso*-octane/acetic acid/water, 11:5:2:10) after treatment with 1% $SnCl_2$ in methanol. Peak I, labelled phosphatide fraction (off scale); II, prostaglandin $F_{2\alpha}$; III, unidentified; IV, hydroxyacid—HETE?; V, arachidonic acid; VI, unidentified. (O) Aggregated sample (collagen 100 μg); (●) non-aggregated control. The plate was scraped into 0.5 cm strips and the radioactivity in each estimated by liquid scintillation counting.

data thus obtained were similar to the qualitative data shown in Figure 2. The PC, PE and PI pools all showed large reductions but there was little change in the PS pool. In molar terms almost half the [1-^{14}C]-arachidonate released was derived from PC, PI contributing almost one-third and PE about one-fifth. A small increase in LPC was observed after aggregation.

Metabolic fate of the [1-^{14}C]-arachidonic acid liberated from phospholipids during aggregation

To determine the ultimate metabolic fate of the arachidonic acid liberated during aggregation, thin layer chromatograms of lipid extracts of aggregated or control platelets were prepared. Figure 3 shows the distribution of radioactivity in 0.5 cm zones of a chromatogram developed in ethyl acetate/water/*iso*-octane/acetic acid (11:10:5:2). In this particular experiment stannous chloride (1% w/v) in methanol was added in a tenfold excess to the platelets when they had begun to aggregate. This manoeuvre should result in reduction of the endoperoxide intermediates to prostaglandin $F_{2\alpha}$ (see Hamberg & Samuelsson, 1974). Indeed, one large peak of radioactivity was

Table 5 Distribution of [1-^{14}C]-arachidonic acid between platelet phospholipid fractions before and after aggregation (mean of two experiments)

	pmol [1-^{14}C]-arachidonate			*Decrease in pool size pmol %*	*Fraction of total [1-^{14}C]-arachidonate contributed by*
Phosphatide fraction	*Control*	*Aggregated*	Δ	Δ	*each pool*
Sphingomyelin	Nil	Nil	Nil	Nil	Nil
Lysolecithin?	8.27	13.47	+5.20	—	Nil
Phosphatidylserine	23.80	21.72	−2.80	−8.75	7.21
Phosphatidylethanolamine	15.72	9.22	−6.51	−41.38	22.53
Phosphatidylinositol	28.64	20.37	−8.28	−28.89	28.66
Phosphatidylcholine	29.69	17.68	−12.01	−40.45	41.59

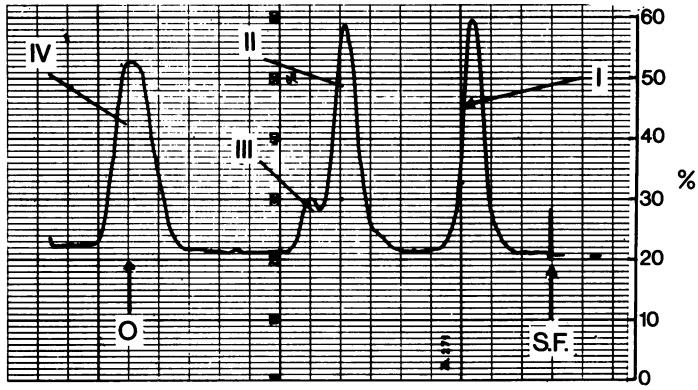

Figure 4 Separation of radioactive methyl esters of poly-oxygenated products of platelet lipoxygenase and cyclo-oxygenase by thin layer chromatography (ethyl acetate/*iso*-octane/water, 50:100:100). The products formed have the same mobility as those formed by human platelets (Hamberg & Samuelsson, 1974). Peak I is unreacted arachidonate; the probable identity of the other products is: II, HETE; III, HHT; IV, cyclo-oxygenase products such as TxB_2 and small amounts of prstaglandins. O=origin, SF=solvent front. Radioactivity was detected by a Panax radiochromatogram scanner.

found to co-chromatograph with prostaglandin $F_{2\alpha}$ (Peak II, Figure 3). Several other zones of radioactivity were also observed; Peak I was the labelled platelet phosphatide fraction (which is not on scale in the figure), peak III was unidentified but could conceivably be the hydroxyacid HHT, peak IV was almost certainly the hydroxyacid HETE—this compound was still formed from arachidonate in the presence of indomethacin, as are products of the lipoxygenase (Hamberg & Samuelsson, 1974). Furthermore, the polarity of the compound is exactly what one would expect for a hydroxyacid. Peak V co-chromatographed with arachidonic acid and Peak VI was unidentified.

Table 6 Effect of aggregating agents and anti-aggregating drugs on the distribution and metabolism of radioactive arachidonate in rabbit platelets—Experiment 1

		pmol %		
Sample	*Phosphatides*	*Free arachidonate*	*HETE*	*TxB_2+PGs*
Control	93.68	2.71	0.86	2.74
	94.29	4.18	0.93	0.60
Aggregated (collagen 100 μg)	77.34	4.17	12.13	6.36
	80.47	2.75	6.25	10.25
Collagen+indomethacin (10 μM)	83.6	0.64	15.47	0.28
Collagen+mepacrine (50 μM)	92.91	2.01	0.46	4.61
	89.64	2.67	0.55	7.14

Table 7 Effect of aggregating agents and anti-aggregating drugs on the distribution and metabolism of radioactive arachidonate in rabbit platelets—Experiment 2

		pmol %		
Sample	*Phosphatides*	*Free arachidonate*	*HETE*	*TxB_2+PGs*
Control	92.76	2.33	1.49	3.41
	92.43	3.21	1.98	2.38
Aggregated (collagen 100 μg)	74.74	5.31	8.07	11.88
	75.18	0.91	21.97	1.93
Collagen+mepacrine (50 μM)	83.60	3.38	2.11	10.9
	91.79	2.60	5.35	0.26

Treatment of platelets with methanolic stannous chloride proved to be a rather unreliable procedure for preparing platelet products for identification; often, sufficient stannous chloride extracted to interfere with the subsequent t.l.c. stages, and anyway chromatography of the free acids did not allow a (simple) complete separation of arachidonic acid and the hydroxyacid HETE. A far better method was to methylate the products after extraction and separate them by development in ethyl acetate/*iso*-octane/water (50:100:100). Figure 4 shows a radiochromatogram scan of the labelled products formed by rabbit platelets from labelled arachidonate derived from phosphatides. Four peaks are seen and these correspond very closely in their distribution to those formed by human platelets as published by Hamberg & Samuelsson (1974). The identity of each zone of radioactivity is based upon a comparison with the chromatogram published by these workers, pharmacological evidence (disappearance with indomethacin denotes it is a cyclo-oxygenase product) and in some cases comparison of R_F values with authentic compounds. The zones of radioactivity have been identified as: I, arachidonic acid; II, HETE; III, HHT; IV, a mixture of polar cyclo-oxygenase products comprising small amounts of prostaglandins E_2 and $F_{2\alpha}$, a very large amount of another compound assumed to be TXB_2 (based on its behaviour in the solvent system, benzene/dioxan, 60:40) and smaller quantities of a very polar unidentified compound. In practice it was convenient to refer to peak IV (which remained on the origin) as 'cyclo-oxygenase products'. It is very difficult to separate the two hydroxy acids HHT and HETE but HHT was such a small component that it was ignored, the only radioactivity quantitated in this zone was HETE. The results which are now presented should be interpreted with these two factors in mind.

Tables 6 and 7 show a quantitation of the total phospholipid arachidonate metabolism. Because of the complexity of the assays only two experiments (in duplicate) were performed.

Experiment 1 (see Table 6) shows that in samples of non-aggregated platelets only very small amounts of HETE and cyclo-oxygenase products were found but these could increase some tenfold during collagen-induced aggregation, the label coming from the phospholipid fraction. The levels of free arachidonate did not increase, presumably because these samples were allowed to aggregate completely and thus use all the arachidonic acid available. Experiment 1 demonstrates that in the presence of indomethacin, there was still a loss of arachidonic acid from the phospholipid fraction but that this was not converted into the pro-aggregatory cyclo-oxygenase products, nor did the substrate accumulate since it was all transformed into HETE. Mepacrine had a completely different effect conserving the substrate within the phospholipid fraction, resulting in a diminished yield of both cyclo-oxygenase and lipoxygenase products, but especially the latter. Experiment 2 (Table 7) shows basically the same results as experiment 1 except that there was a larger degree of variation between the duplicates.

Effect of mepacrine and indomethacin on platelet aggregation

Figure 5 shows an experiment in which collagen and arachidonic acid were used as aggregating agents, and mepacrine and indomethacin as inhibitors. Indomethacin (10 μM) blocked the aggregating activity of both arachidonic acid and collagen. Mepacrine, however, although it easily blocked the aggregation by collagen had no action on the velocity of the aggregation induced by arachidonate (which acts by direct conversion into pro-aggregatory endoperoxides) although it did reduce slightly the degree of aggregation. The concentration of mepacrine is apparently critical for this selective blockade of collagen-induced aggregation since at higher doses this drug also blocked arachidonate-induced aggregation, presumably by inhibition of the cyclo-oxygenase.

In two experiments the anti-aggregating (IC_{50}) concentration of mepacrine and indomethacin were measured against both arachidonate and collagen (Table 8). The IC_{50} of indomethacin was almost the same against both agents suggesting a common mode of action. With mepacrine there was a clear difference the IC_{50} against collagen being about half that required for an equivalent block of arachidonate-induced aggregation. Although this experiment strongly suggests separate modes of action for mepacrine and indomethacin it also illustrates that mepacrine is a poor selective inhibitor, except under rigidly controlled conditions of concentration.

Measurement of platelet phospholipase A_2 activity and the anti-enzyme activity of mepacrine and indomethacin

By the use of the specifically labelled phospholipid enzyme assay described in the methods section, rabbit platelet phospholipase A_2 activity was easily detectable. Time course studies suggested that the reaction was autocatalytic or that some change in the nature of the enzyme was required, since a 'lag' phase was observed of about 10–20 min duration during which hydrolysis was small. This was succeeded by a period of more rapid hydrolysis which was linear for at least 2 hours. In practice, hydrolysis was measured over a period of 1 hour.

Table 9 shows the results of experiments to determine the hydrolysis after 1 h (expressed per mg platelet protein) and the anti-phospholipase activity of the two drugs. Whilst indomethacin had little effect (<3% inhibition) on the enzyme even in concentra-

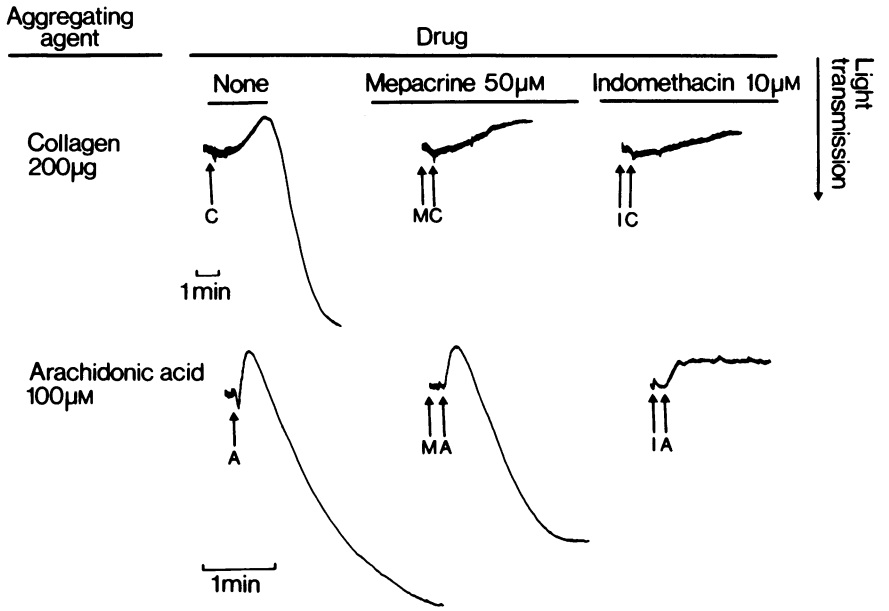

Figure 5 Aggregometer tracings showing the differential blockade by mepacrine of collagen, but not arachidonate, induced aggregation.

Table 8 Inhibition by indomethacin and mepacrine of platelet aggregation induced by collagen and arachidonic acid

	IC_{50} (μM)	
Aggregating agent	*Mepacrine*	*Indomethacin*
Arachidonic acid (100 μM)	71	3.0
Collagen (200 μg)	34	3.5

Table 9 Effect of mepacrine and indomethacin on rabbit platelet homogenate phospholipase A_2 activity

Sample (n=3)	*pmol oleic acid liberated per mg protein (± s.e. mean)*	Δ%
Control	39.20 (±3.12)	—
Mepacrine 50 μM	37.33 (±2.28)	−4.76
500 μM	23.73 (±1.52)	−39.53
Indomethacin 10 μM	39.57 (±1.68)	+0.94
100 μM	38.29 (±2.64)	−2.32

Table 10 Effect of thrombin (0.25 u/ml) on whole platelet phospholipase A_2 activity

Sample (n=3)	*pmol oleic acid liberated per ml PRP/min (± s.e. mean)*	Δ%
Control	25.33 (±5.90)	—
Thrombin aggregated	65.05 (±6.35)	+256.8
Thrombin + 50 μM mepacrine	19.35 (±1.85)	−23.7

tions of 100 μM, mepacrine had a clear blocking action with an IC_{50} value somewhat in excess of 500 μM. When aggregating agents such as collagen or thrombin were added to the platelet homogenate, no change in phospholipase A_2 activity was found.

The experiments in which the labelled phosphatide was added directly to the PRP, however, show a clear stimulation by the aggregating agent thrombin; phospholipase A_2 activity was suppressed (even below basal levels) by 50 μM mepacrine.

Discussion

Two analytical techniques have been used to determine the origin, location and fate of the platelet arachidonic acid. By means of g.l.c. and lipid separation techniques, quantitative determinations of the amounts of arachidonate in different lipid pools of rabbit platelets have been made. These studies indicate that only trace amounts of free arachidonate are found in platelets, and that the amounts present as neutral lipid esters are also very low. Although 16–23 ng of arachidonate were found per 10^6 platelets, about 97% of this was present in the platelet phosphatide fraction. These figures are very similar to estimates of the distribution of arachidonate in human platelets (Marcus *et al.*, 1969).

Following aggregation of platelets with collagen there was a consistent fall in the phospholipid arachidonate content, clearly demonstrating that this was the source of the substrate used for the synthesis of pro-aggregatory endoperoxide derivatives. This 'mobilization' of substrate which occurs during contact of the platelets with the aggregating agent collagen or thrombin is effectively blocked by mepacrine, but not by indomethacin.

Use of the radioactive tracer method for studying the movement of arachidonate during aggregation has disadvantages as well as advantages. Quantitation is much simpler when this technique is used and this (generally) enables larger sample sizes in each group. The use of radioactive arachidonate also enables all the products of the cyclo-oxygenase and lipoxygenase to be detected and measured; however, these assays are rather more complex. There are two theoretical disadvantages to this technique; one is that only lipid pools with relatively quick 'turnover' times are labelled and it is therefore not possible to detect the loss of fatty acids from non-labelled pools. A second objection is the 'isotope dilution' artefact; unlabelled arachidonic acid tends to dilute the labelled acid resulting in a low estimate of the final products formed.

Despite these objections, the results obtained by the two techniques show good corroboration. Labelled arachidonic acid is almost exclusively associated with the phospholipid fraction; in the case of the PC pool this acid is esterified in the 2'-acyl position and is not metabolically altered before incorporation. During contact with collagen the loss of arachidonate from the phosphatide fraction is clearly demonstrated, and analysis of the actual phospholipid pools indicates that this is derived chiefly from the PC, PI and PE fractions. These results are in general agreement with the figures published by Bills *et al.* (1976) for human platelets. Schoene & Iacono (1975) have also published some preliminary results using a tracer incorporation technique with human platelets. However, they concluded that the majority of the arachidonate liberated from platelet phosphatides is derived from the PI fraction.

A fundamental assumption underlying the use of the radioactive tracer experiments is that the incorporated labelled arachidonate behaves in an identical fashion to the endogenous arachidonate. Given that this is correct, a very important concept arises from examination of the data in Tables 2 and 5. Analysis of the data in Table 5 shows that both the PE and PC pools decrease by about 40% during aggregation, however, Table 2 shows that the PE pool contains almost four times as much arachidonate as the PC pool, so when interpreted in terms of the release of endogenous arachidonate PE actually contributes much more substrate. This calculation has been made for all phosphatide pools and is detailed in Table 11. PE contributes the most arachidonate during aggregation followed by PI then PC.

One interesting question is why the PS arachidonate is not liberated during aggregation. This could be because some phospholipase enzymes are specific for individual phospholipids, or because PS is a metabolically distinct pool.

When the oxidation products of the phospholipid arachidonate were examined they appeared to be almost identical to those formed from arachidonic acid by human platelets (Hamberg & Samuelsson, 1974). During aggregation the labelled arachidonate was transformed into two main classes of compound, a hydroxy acid fraction and another very polar fraction. The former is almost certainly HETE, a hydroxy acid formed by a soluble lipoxygenase enzyme in platelet cytoplasm (Hamberg & Samuelsson, 1974; Nugteren, 1975), and the latter is most probably TXB_2 together with smaller amounts of prostaglandins (Hamberg & Samuelsson, 1974). The amount of radioactivity found in each fraction was approximately equivalent (see Tables 6 and 7) except in the presence of indomethacin. This drug prevented the conversion of radioactive arachidonate into the cyclo-oxygenase products (TXB_2 and prostaglandins) and there was a concomitant increase in the amount of HETE formed. In the presence of mepacrine, there was a retention of arachidonate within the phosphatide fraction. This again confirms the results obtained with the g.l.c. and provides an explanation

Table 11 Estimation of the total contribution of each phospholipid pool to the total amount of endogenous arachidonate released by platelets during aggregation

Phosphatide pool	*% Decrease in* [1-^{14}C]-arachidonate during aggregation*	*Phosphatide arachidonate pool size, mol %***	*Mol endogenous arachidonate released during aggregation*	*% Contribution from each endogenous pool*
Sphingomyelin	Nil	Nil	Nil	Nil
Phosphatidylcholine	40.45	11.80	4.77	14.27
Phosphatidylinositol	28.89	28.43	8.21	24.56
Phosphatidylserine	8.75	13.12	1.14	3.41
Phosphatidylethanolamine	41.38	46.65	19.30	57.74

* Data from Table 5; ** Data from Table 2.

for the effect of mepacrine on platelet aggregation shown in Figure 5. The results suggest that collagen acts by liberating arachidonic acid from the platelet phosphatide fraction and that this step is mepacrine-sensitive. How is this effect of mepacrine brought about? One possibility is that the liberation of arachidonate from platelets is under the control of a phospholipase enzyme and that this is mepacrine-sensitive. In view of the positional labelling of the PC, this enzyme is presumably phospholipase A_2. The blocking action of mepacrine on this enzyme has already been reported by Vargaftig & Dao Hai (1972) and supported by other indirect evidence (Flower & Blackwell, 1976). Platelet phospholipase A_2 activity was easily detectable in lysed platelets but the reaction rate was slow, incubation periods of about 1 h being required to obtain good hydrolysis. This could be because the membrane bound enzyme obviously does not mix properly with a water soluble substrate. Mepacrine was found to block the enzyme activity but the concentrations required were some tenfold higher than those required to block collagen-induced aggregation; when the labelled phosphatide was incubated directly with PRP (i.e. whole platelets) hydrolysis was more rapid and was stimulated by the addition of thrombin, and blocked by lower concentrations of mepacrine. This discrepancy in activity might be explained on the basis of differences between lysed and whole platelets, or it could be that the inhibition of phospholipase is only an epiphenomenon and that mepacrine exerts its action by another distinct mechanism. A non-specific 'membrane stabilization' might be one such alternative mechanism although, as Figure 5 shows, platelets continue to respond to arachidonic acid when the effect of collagen is blocked. Even if mepacrine acts by blocking phospholipase A_2, it is unlikely that it will find a place as a selective inhibitor of this enzyme, for the IC_{50} against the cyclo-oxygenase is only two fold greater than the IC_{50} against phospholipase. Indomethacin, even at high concentrations, does not inhibit phospholipase A_2.

We believe this is the first study in which the metabolism of arachidonic acid has been followed from individual phosphatide pools to poly-oxygenated end products, and in which the two rate-limiting steps, phospholipase A_2 and the cyclo-oxygenase, have been shown to be targets for different types of drugs. As interest in poly-oxygenated fatty acid derivatives gathers momentum, studies such as the ones we report here will become valuable in determining the cellular origin of the substrate. In many respects, platelets are well suited for these studies as we have shown that there is very little arachidonate outside the phosphatide fraction; in other cells additional complications exist due to the fact that other lipid pools contain arachidonic acid (see Flower & Blackwell, 1976).

Reprint requests and correspondence to R.J.F.

References

BILLS, T.K. & SILVER, M.J. (1975). Phosphatidylcholine is the primary source of arachidonic acid utilized by platelet prostaglandin synthetase. *Fedn Proc.*, **34**, 790.

BILLS, T.K., SMITH, J.B. & SILVER, M.J. (1976). Metabolism of [1-^{14}C]-arachidonic acid by human platelets. *Biochim. biophys. Acta*, **241**, 303–314.

BORN, G.V.R. (1962). Quantitative investigations into the aggregation of blood platelets. *J. Physiol. Lond.*, **162**, 67P.

COHEN, P. & DERKSEN, A. (1969). Comparison of phospholipid and fatty acid composition of human erythrocytes and platelets. *Br. J. Haematol.*, **17**, 359–371.

FLOWER, R.J. & BLACKWELL, G.J. (1976). The importance of phospholipase A_2 in prostaglandin biosynthesis. *Biochem. Pharmac.*, **25**, 285–291.

FLOWER, R.J., BLACKWELL, G.J. & PARSONS, M.F. (1975). Mechanism of collagen induced platelet aggregation. *Proc. VI Int. Cong. Pharmac., Helsinki, Finland* p. 292.

GORNALL, A.G., BARDAWILL, C.J. & DAVID, M.M. (1949). Determination of serum proteins by means of the biuret reaction. *J. biol. Chem.*, **177**, 751–766.

HAMBERG, M. & SAMUELSSON, B. (1974). Prostaglandin endoperoxides. Novel transformations of arachidonic acid in human platelets. *Proc. natn. Acad. Sci. U.S.A.*, **71**, 3400–3404.

HAMBERG, M., SVENSSON, J. & SAMUELSSON, B. (1975). Thromboxanes: A new group of biologically active compounds derived from prostaglandin endoperoxides. *Proc. natn. Acad. Sci. U.S.A.*, **72**, 2994–2998.

HAMBERG, M., SVENSSON, J., WAKABAYASHI, T. & SAMUELSSON, B. (1974). Isolation and structure of two prostaglandin endoperoxides that cause platelet aggregation. *Proc. natn. Acad. Sci. U.S.A.*, **71**, 345–349.

KATES, M. (1972). In *Techniques in Lipidology*. p. 552. Amsterdam, London and New York: North-Holland and American Elsevier.

MARCUS, A.J., ULLMAN, H.L. & SAFIER, L.B. (1969). Lipid composition of subcellular particles of human blood platelets. *J. Lip. Res.*, **10**, 108–114.

METCALFE, L.D., SCHMITZ, A.A. & PELKA, J.R. (1966). Rapid preparation of fatty acid esters from lipids for gas chromatographic analysis. *Analyt. Chem.*, **38**, 514–515.

NUGTEREN, D.H. (1975). Arachidonate lipoxygenase in blood platelets. *Biochim. biophys. Acta*, **380**, 299–307.

NUGTEREN, D.H. & HAZELHOF, E. (1973). Isolation and properties of intermediates in prostaglandin biosynthesis. *Biochim. biophys. Acta*, **326**, 448–461.

O'BRIEN, J.R. (1968). Effect of anti-inflammatory agents on platelets. *Lancet*, **i**, 894–895.

RAHEJA, R.K., KAUR, C., SINGH, A. & BHATIA, I.S. (1973). New colorimetric technique for the quantitative estimation of phospholipids without acid digestion. *J. Lip. Res.*, **14**, 695–697.

ROBERTSON, A.F. & LANDS, W.E. (1962). Positional specificities in phospholipid hydrolysis. *Biochemistry*, **4**, 804–810.

SAUNDERS, L. (1957). Some properties of mixed sols of lecithin and lysolecithin. *J. Pharm. Pharmac.*, **IX**, 834–840.

SCHOENE, N.W. & IACONO, J.M. (1975). Stimulation of platelet phospholipase activity by aggregating agents. *Fedn Proc.*, **34**, 257.

SILVER, M.J., SMITH, J.B., INGERMAN, C. & KOCSIS, J.J. (1973). Arachidonic acid induced human platelet aggregation and prostaglandin formation. *Prostaglandins*, **4**, 863–875.

VANE, J.R. (1971). Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs. *Nature, New Biol.*, **231**, 232–235.

VARGAFTIG, B.B. & DAO HAI, N. (1972). Selective inhibition by mepacrine of the release of 'rabbit aorta contracting substance' evoked by the administration of bradykinin. *J. Pharm. Pharmac.*, **24**, 159–161.

VARGAFTIG, B.B. & ZIRINIS, P. (1973). Platelet aggregation induced by arachidonic acid is accompanied by the release of potential inflammatory mediators distinct from PGE_2 and $PGF_{2\alpha}$. *Nature, New Biol.*, **244**, 114–116.

(Received August 13, 1976.)