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# The immunological generation of a platelet-activating factor and a platelet-lytic factor in the rat

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**Summary.** Antigen challenge of the rat peritoneal cavity which had been prepared with IgGa-rich antiserum generated activities which released [$^{14}$C]-serotonin from pre-labelled human platelets. After adsorption of these activities onto Amberlite XAD-8 and elution in 80% ethanol, two factors of differing polarity were resolved by chromatography on diethylaminoethyl cellulose in organic solvents. The activity eluting in the 7:1 chloroform:methanol solvent contained a platelet-lytic factor (PLF) assessed by the parallel release of lactic acid dehydrogenase and [$^{14}$C]-serotonin; the cytotoxicity of this fraction was confirmed by phase-contrast microscopy examination which demonstrated fragmentation of the exposed platelets. The activity eluting in the 1:1 methanol: aqueous 1·0 M ammonium carbonate solvent was a platelet-activating factor (PAF) as defined by release of [$^{14}$C]-serotonin without lactic acid dehydrogenase. Both the lytic and the activating principles were separable from slow reacting substance of anaphylaxis and polymorphonuclear leucocyte chemotactic activity, and each presented a single activity peak of differing mobility when chromatographed on silica gel H plates. Human eosinophil phospholipase D inactivated the lytic factor by more than 85% in 2 h at 37° without affecting the activity of the activating factor. The release of [$^{14}$C]-serotonin induced by the PAF was not affected by the absence of calcium from the medium or by elevations in the platelet concentrations of cyclic AMP or cyclic GMP that resulted from pre-incubation of platelets with prostaglandin $D_2$ or sodium ascorbate, respectively.

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## INTRODUCTION

A platelet-activating factor (PAF) was recognized as a product of antigen challenge of leucocytes from actively sensitized rabbits (Schoenbechler & Barbaro, 1968; Siraganian & Osler, 1969; Henson, 1969) and was later shown to be generated by the IgE-mediated activation of rabbit basophils (Benveniste, Henson & Cochrane, 1972). Subsequent studies utilizing the same unspecific bioassay, namely the release of platelet amines, have demonstrated platelet-releasing activity to be generated spontaneously by prolonged incubation of preparations of rabbit, human or pig peripheral blood leucocytes at alkaline pH (Benveniste, 1974; Benveniste, Le Couedic, Polonsky & Tence, 1977); by IgE-mediated reactions in rabbit lung (Kravis & Henson, 1975) and in human lung fragments (Bogart & Stechschulte, 1974); and by IgGa-dependent reactions in the peritoneal cavities of rats (Kater, Goetzl & Austen, 1976). Functional heterogeneity of the PAFs was demonstrated by the absence of cross-deactivation between PAFs derived by IgE-dependent reactions involving rabbit lung and peripheral blood leucocytes (Kravis & Henson, 1975). Material generated spontaneously by rabbit peripheral blood leucocytes has an apparent mol. wt of 1100 (Benveniste, 1974) and comparable material from pig peripheral blood leucocytes was subse-

quently described as a 1-lyso-glycerophospholipid (Benveniste *et al.*, 1977). Both this material and that derived from the rat peritoneal cavity (Kater *et al.*, 1976) were inactivated by phospholipase D. The present studies have revealed that the phospholipase-D inactivatable material derived from the rat peritoneal cavity is a platelet-lytic factor which can be chromatographically separated from a phospholipase-D resistant, non-cytotoxic platelet-activating factor.

## MATERIALS AND METHODS

[$^{14}$C]-serotonin (5-[2-$^{14}$C]-hydroxytryptamine binoxalate, 57 mCi/mmol), [$^{3}$H]-arachidonic acid (84 Ci/mmol), [$^{14}$C]-lecithin (dipalmitoyl phosphatidylcholine, L-α-[palmitoyl-1-$^{14}$C], 74 mCi/mmol) and Aquasol Universal LSC Cocktail (New England Nuclear Corp., Boston, Ma), diethylaminoethyl cellulose (DE-52) (Whatman Chemicals, Div. W&R, Balston, Maidstone, Kent), Amberlite XAD-8 (Rohm and Haas, Philadelphia, Pa), silica gel H thin layer plates of 250 μm thickness (Analtech, Inc., Newark, Del.), human thrombin (Fibrindex, Ortho Diagnostics, Inc., Raritan, N.J.), epinephrine (adrenalin 1:1000, Parke, Davis & Co., Detroit, Mich.), human serum albumin and ovalbumin (Miles Laboratories, Inc., Elkhart, Ind.), heparin (sodium heparin injection, USP) and prostaglandin $D_2$ ($PGD_2$) (Upjohn Co., Kalamazoo, Mich.), 5,8,11,14-eicosatetraynoic acid (Hoffman-La Roche, Inc., Nutley, N.J.), silicone for coating glassware (Dricote) (Fisher Scientific Co., Pittsburgh, Pa), sodium-L-ascorbate, adenosine 3′,5′-cyclic monophosphate (cyclic AMP), and guanosine 3′,5′-cyclic monophosphate (cyclic GMP) (Sigma Chemical Co., St Louis, Mo), calcium ionophore A23187 (Eli Lilly and Co., Indianapolis, Ind.), acid alumina (AG 4) and Dowex AG 1-X8 in the formate form (Bio-Rad, Richmond, Ca), [H$^{3}$]-cyclic GMP (21 Ci/mmol) and [$^{3}$H]-cyclic AMP (45 Ci/mmol) (Amersham, Arlington Heights, Ill.), rabbit antiserum to succinyl-cyclic GMP, rabbit antiserum to succinyl-cyclic AMP, $^{125}$I-labelled tyrosine methyl ester of succinyl-cyclic GMP and $^{125}$I-labelled tyrosine methyl ester of succinyl-cyclic AMP (Collaborative Research, Waltham, Mass.) were obtained as noted. Plastic or siliconized glass was used for all platelet processing. All organic solvents used were of nanograde quality (Mallinckrodt, Inc., St Louis, Mo). Batches of Amberlite XAD-8 were prepared by washing 500 ml of resin sequentially with 8 l of propanol and 40 l of distilled water. The XAD-8 was stored in distilled water at 4°. DE-52, which was prepared in 500 ml batches by sequential washing at 37° with 1 l of 1 M HCl, 1 l of 1 M NaOH, 1 l of glacial acetic acid, and 5 l of methanol, was stored at 4° in 7:1 chloroform:methanol (v:v). Portions were washed with fifty volumes of the same solvent mixture immediately before use.

IgGa-rich hyperimmune rat antisera to ovalbumin were prepared by immunizing Sprague–Dawley rats as previously described (Valone & Goetzl, 1978). SRS-A was quantified by bioassay with an atropinized, antihistamine-treated guinea-pig ileum (Orange & Austen, 1976). Polymorphonuclear (PMN) leucocyte chemotactic and chemokinetic factors previously described as being generated in the rat peritoneal cavity (Valone & Goetzl, 1978) were assessed by a modification of the Boyden micropore filter method (Boyden, 1962) utilizing human PMN leucocytes prepared by centrifugation of mixed leucocytes on Ficoll–Hypaque (Boyum, 1968; Valone & Goetzl, 1978). Human eosinophil phospholipase D was extracted from the eosinophils of a patient with rheumatoid arthritis who had a white blood cell count of 8500 containing 90% eosinophils and was sequentially purified by anion exchange chromatography on DE-52 and Sephadex G-100 gel filtration (Kater *et al.*, 1976). Phospholipase D activity was determined by the enneaiodide method (Long, Odavic & Sargent, 1967); 1 unit was defined as the quantity of phospholipase D that generated 1 μmol of choline in 1 h at 37°.

*Generation and purification of platelet-active factors*

One hundred Sprague–Dawley rats weighing 150–200 g were each injected intraperitoneally with 1 ml of 1:1 dilution in Tyrode's buffer of IgGa-rich rat antiserum to ovalbumin. Two hours later the rats were challenged intraperitoneally with 2 mg ovalbumin in 5 ml Tyrode's buffer containing 0·1 g/100 ml of gelatin and 5 units/ml of heparin. After 5 min the animals were killed and the peritoneal fluids were harvested, pooled and centrifuged in 50 ml portions at 400 *g* for 15 min at 4°. The cell-free supernatant was deproteinated by the addition of four volumes of cold absolute ethanol and centrifuged at 10,000 *g* for 15 min at 4°. The deproteinated supernatant was evaporated to dryness in a rotary flask evaporator at 45° under vacuum, resuspended in distilled water, and applied to a 200 ml bed volume column of Amberlite XAD-8. The column was eluted at a flow rate of 2–4 ml per min with two bed volumes of distilled water followed by two bed volumes of 80% ethanol. The 80% ethanol eluate was

evaporated to dryness, resuspended in 7:1 chloroform: methanol and applied to a 5 ml bed volume column of DE-52 equilibrated in 7:1 chloroform:methanol in a modification of previously described methods (Rouser, Bauman, Kritchevsky, Heller & O'Brien, 1961; Takahashi, Webster & Newball, 1976). The column was sequentially developed with 50 ml each of 7:1 (v:v) chloroform:methanol, 7:3 chloroform:methanol, methanol and then a series of 1:1 (v:v) mixtures of methanol and aqueous ammonium carbonate at concentrations of 0·01 M, 0·1 M, 0·3 M, 0·6 M and 1·0 M. The DE-52 eluates were evaporated to dryness, resuspended in 2·5 ml of 0·01 M phosphate buffer, pH 7·0, and stored at −90° until they were assessed for activities.

The DE-52 fractions which released [$^{14}$C]-serotonin from human platelets were lyophilized and applied to silica gel H thin layer plates which had been pre-washed both in hexane:ethyl acetate:acetic acid (1:1:0·005, v:v) and in chloroform:methanol (2:1, v:v), dried, and activated by heating at 110° for 1 h. The plates were developed at room temperature with chloroform:methanol:glacial acetic acid:water (50:30:8:4, v:v) in a covered jar until the solvent front had moved 17·5 cm from the origin. Strips of silica gel of 1·75 cm width were scraped into glass test tubes and eluted sequentially with two 1 ml portions of developing solvent, 1 ml of methanol:acetic acid:water (94:1:5, v:v), and 1 ml distilled water. The eluates from each strip were pooled and evaporated to dryness under nitrogen, reconstituted to their starting volume with distilled water, and assayed for [$^{14}$C]-serotonin releasing activity. Internal standards run on each thin layer plate were [$^{3}$H]-arachidonic acid, [$^{14}$C]-lecithin, and lysolecithin, which yielded Rf values of 1·0, 0·4 and 0·2, respectively.

*Assay of platelet [$^{14}$C]-serotonin release, lactic acid dehydrogenase release, and aggregation*

Platelets in citrated plasma, free of other cellular elements, were prepared as previously described (Valone, Austen & Goetzl, 1974; Kater *et al.*, 1976) from blood of normal volunteers who had not ingested drugs known to affect platelet function for at least 5 days before donation. Platelets were radiolabelled by incubation for 30 min at 37° with 0·4 μmol [$^{14}$C]-serotonin (57 mCi/mmol) per 100 ml platelet-rich plasma. Labelled platelets were sedimented at 1000 *g* for 10 min at room temperature and washed twice in standard platelet buffer (SPB) (pH 7·0, 0·004 M $KH_2PO_4$, 0·006 M $Na_2HPO_4$, 0·1 M NaCl, 0·1% glucose) containing 20% (v:v) citrate anticoagulant (0·225 M, pH 5·2) which had been adjusted to a final pH of 6·5 by the addition of NaOH. Washed labelled platelets were resuspended at a concentration of 0·8–2·5 × $10^8$/ml in buffer composed of SPB containing 0·1 g/100 ml of human serum albumin and 1·8 mM $CaCl_2$ (SPB-HSA). Washed labelled platelets in 250 μl portions were added to 3 ml plastic tubes containing 100 μl of a stimulus or the appropriate control solution. The reaction mixtures were incubated with mild agitation at 37° for 30 min, and sedimented at 1000 *g* for 10 min at 4° to separate the [$^{14}$C]-serotonin released into the supernatant from that remaining in the platelet pellet. One hundred microlitres of each supernatant were transferred to glass scintillation vials and mixed with 10 ml of Aquasol, and radioactivity was quantified on a Searle Mark III Liquid Scintillation Counter. The radioactivity determined in duplicate 250 μl portions of labelled platelets represented 100% radioactivity. The net percentage of stimulated release was calculated by subtracting the percentage of [$^{14}$C]-serotonin which leaked spontaneously from the percentage of [$^{14}$C]-serotonin released from stimulated platelets. All samples were tested as duplicates which varied less than 10% from their mean. The spontaneous leak of [$^{14}$C]-serotonin was 3·6 ± 2·1% (mean ± SD) in ten consecutive experiments.

Lactic acid dehydrogenase (LDH) was quantified in supernatant fluids and lysates of the corresponding platelet pellet by means of a colorimetric assay (LDH-500 reagents, Sigma Chemical Co., St Louis, Mo). The net percentage of stimulated LDH release was calculated by subtracting the percentage of LDH which leaked spontaneously from the percentage released from stimulated platelets.

Platelet aggregation was assessed by a modification of the turbidometric method (Born & Cross, 1963) with a standard aggregometer and recorder (Chronolog, Haverton, Pa). Aggregating stimuli in 100 μl of SPB-HSA were added to 400 μl of washed radiolabelled platelets, 2·2–4·4 × $10^8$/ml, which were being stirred continuously at 37° in 1·0 ml siliconized tubes. Changes in percentage light transmission were recorded continuously for 5 min after addition of the aggregating stimulus. At the end of the 5 min period the platelets were removed and pelleted by centrifugation for 1 min in a Beckman microfuge, and the net percentage release of [$^{14}$C]-serotonin was determined. The extent of aggregation, termed the aggregation index, was measured by the total increase in light transmission during the 5 min interval and was

expressed as the net stimulated increment in percentage transmission after correction for the increment observed for platelets after the addition of buffer alone.

*Measurement of platelet levels of cyclic GMP and cyclic AMP*

Cyclic GMP and cyclic AMP levels were assessed with platelets labelled with [$^{14}$C]-serotonin so that serotonin release could be determined concomitantly for portions of the same platelet suspensions. Two hundred and fifty microlitres of a labelled platelet suspension containing $2{\cdot}5 \times 10^8$ platelets/ml in SPB-HSA were preincubated for 5 min at 37°, followed by the addition of 5 $\mu$l of either 350 mM sodium L-ascorbate in water or 0·7 mM prostaglandin $D_2$ in 95% ethanol, both freshly prepared. Two minutes later a platelet-active factor or 0·14 units of thrombin was added in 100 $\mu$l SPB and replicate reaction mixtures were incubated for either 2 min or 30 min. To assess [$^{14}$C]-serotonin release, reactions were terminated after 30 min by centrifugation for 1 min in a Beckman microfuge; to assess changes in cyclic nucleotides, reactions were terminated after either 2 or 30 min, by the addition of 1 ml of 10% perchloric acid at 4° to the platelet suspension followed by immediate freezing in an ethanol–dry ice bath. After thawing, tracer quantities of radiolabelled cyclic AMP or cyclic GMP were added as internal recovery standards, and the samples were homogenized. Cyclic GMP and cyclic AMP were separated on acid alumina and Dowex-1 columns (Murad, Manganiello & Vaughn, 1971; Schoepflin, Pickett, Austen & Goetzl, 1977), acetylated, and quantified by radioimmunoassay (Harper & Brooker, 1975). Solutions of cyclic nucleotides utilized for the standard radioimmunoassay binding curves were quantified by optical density at 252 nm for cyclic GMP and 262 nm for cyclic AMP. Medium without platelets which had been carried through the same purification steps as the samples was employed as the diluent for establishing standard curves. Both standard curves were linear from 5 or 10 to 160 fmol. Samples which exceeded this range were diluted to enable determination of their cyclic GMP or cyclic AMP concentration within the linear range.

## RESULTS

### Resolution of two platelet-active factors

In the initial studies on the generation of platelet-active material in the rat peritoneal cavity, the activity co-chromatographed with slow reacting substance of anaphylaxis (SRS-A) on neutral Amberlite and silicic acid (Orange, Murphy, Karnovsky & Austen, 1973; Kater *et al.*, 1976). The SRS-A activity was resolved from two platelet-active principles by application of the material eluting from silicic acid to a column of DE-52 cellulose which was developed using organic solvents of increasing polarity. As there was considerable loss of platelet serotonin releasing activity during silicic acid partition chromatography, the material obtained after deproteination and Amberlite XAD-8 chromatography was applied directly to the DE-52 column. Each eluate was divided into four equal portions which were evaporated to dryness and resuspended in 0·5 ml of SPB prior to being assayed for their capacity to release [$^{14}$C]-serotonin, for SRS-A activity

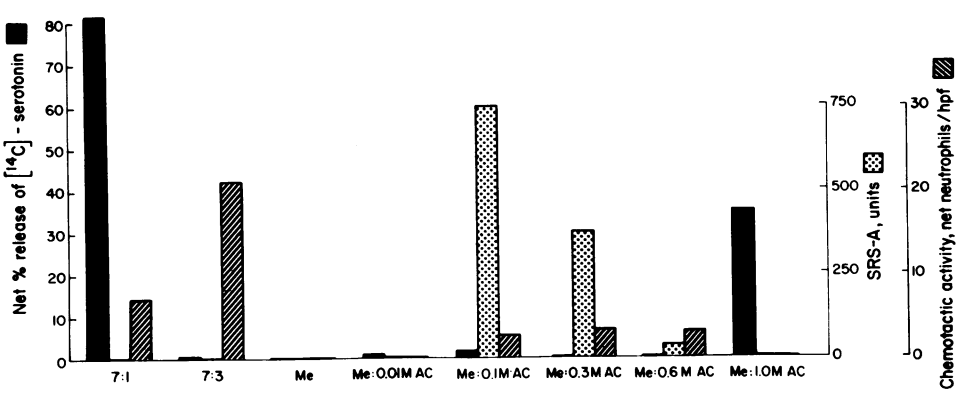


**Figure 1.** Resolution of platelet-active factors, leucocyte chemotactic activity and slow-reacting substance of anaphylaxis by DE-52 cellulose chromatography. The data for platelet serotonin releasing activity are given for 10 $\mu$l samples and for PMN chemotactic activity for 5 $\mu$l samples, and that for SRS-A is expressed as units per 500 $\mu$l.

and for chemotactic activity for human neutrophils (Fig. 1). Each fraction was assessed in the serotonin release assay at 2, 5, 10 and 20 μl. Only the 7:1 chloroform:methanol and the 1:1 methanol:aqueous 0·1 M ammonium carbonate eluates gave net serotonin release, and this release reached a plateau at 10 μl. The SRS-A activity appeared only in the 1:1 methanol: aqueous 0·1 and 0·3 M ammonium carbonate eluates when bioassayed in a dose response fashion. The chemotactic activity was determined with 5 and 10 μl portions and the lower dose yielded the maximum response present in any fraction. The activity for human neutrophils predominated in the 7:3 chloroform:methanol eluate but did contaminate the 7:1 chloroform:methanol eluate containing one of the platelet-active factors.

The platelet-active factors which were widely separated by DE-52 cellulose chromatography, appearing in the first and last fractions, respectively, were examined for functional purity by chromatography on silica gel H thin layer plates using a solvent system which resolves polar lipids (Fig. 2). The platelet-active

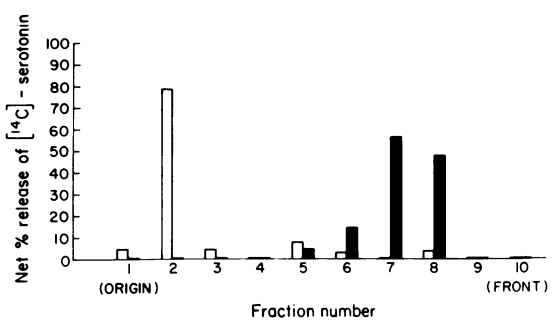


**Figure 2.** Silica gel thin layer chromatography of the two platelet-active factors resolved by DE-52 chromatography. Open columns, material eluting from DE-52 in 7:1 chloroform:methanol; solid columns, material eluting in 1:1 methanol:aqueous 1·0 M ammonium carbonate.

factor eluting from DE-52 in the 7:1 chloroform: methanol fraction exhibited a peak Rf of 0·2 with a recovery of 85%. The platelet-active factor eluting in the 1:1 methanol:aqueous 1 M ammonium carbonate fraction exhibited a peak Rf of 0·7–0·8 with a recovery of 45%.

### Functional characteristics of the platelet-active factors

The release of [$^{14}$C]-serotonin relative to LDH was assessed to determine whether either of the platelet-active factors was cytotoxic in action. Incubation was carried out for 30 min with increasing amounts of the eluates. The platelet-active factor eluting from DE-52 in the 7:1 chloroform:methanol fraction was lytic for platelets as indicated by the dose-related release of both [$^{14}$C]-serotonin and LDH (Fig. 3). While two

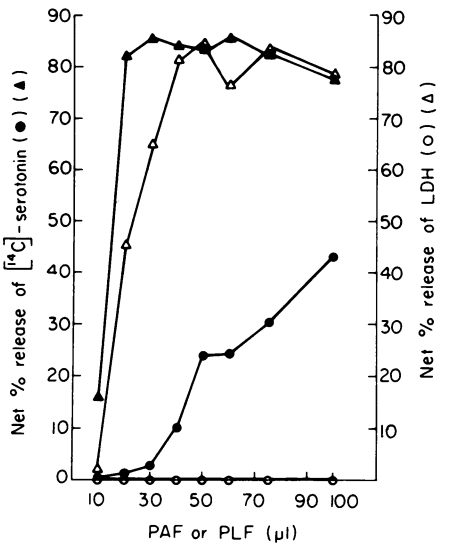


**Figure 3.** Release of [$^{14}$C]-serotonin and lactic acid dehydrogenase from labelled platelets by the two platelet-active factors. Triangles, PLF; circles, PAF.

additional preparations exhibited comparably steep curves for [$^{14}$C]-serotonin release, a 15 μl dose of one released 54% [$^{14}$C]-serotonin and 33% LDH in 30 min. Thus the material in the 7:1 chloroform:methanol eluate was designated a platelet-lytic factor (PLF). Treatment of $9 \times 10^7$ platelets in 400 μl of SPB-HSA with 40 μl of PLF for 15 min at 37° induced platelet fragmentation, as demonstrated by phase microscopy.

The platelet-active factor eluting in the 1:1 methanol: aqueous 1·0 M ammonium carbonate fraction gave a dose-related release of serotonin without detectable release of LDH (Fig. 3), thereby indicating that this eluate contained a secretory principle which was termed platelet-activating factor (PAF). With two additional preparations giving a maximal net serotonin release of 50 and 60%, respectively, with 200 μl of PAF, there was no associated net release of LDH. The time course of serotonin release with 75 μl of PAF was 12% at 2 min, 25% at 5 min and 48% at 15 min; further incubation to 30 and 60 min gave no additional serotonin release. The release of serotonin observed with

50 μl of PAF was not altered by the omission of calcium from the buffer or by the addition of 5 mM Mg-EGTA to calcium-free buffer. Seventy-five and 100 μl of PAF, which gave 38 and 48% release of serotonin, respectively, at 5 min, exhibited an aggregation index of 12 and 19, respectively, at 5 min. Additional studies with other preparations revealed that the aggregation effect of 100 μl of PAF plateaued at 2–5 min without reaching the levels achieved with 0·005 units of thrombin, which, at 5 min, demonstrated both an aggregation index of 60 and net serotonin release of 23%.

Fifty microlitre portions of four different preparations of PAF and PLF, which released between 32 and 55% and 40 and 70% of platelet serotonin, respectively, were each incubated with 5 units of purified human eosinophil phospholipase D in 0·1 M sodium acetate buffer at pH 5·5 for 2 h at 37°. The pH of each reaction mixture was then adjusted to 7·2 by the dropwise addition of 1 M sodium acetate, and residual serotonin releasing activity was assayed with three concentrations of treated and untreated material. Serotonin releasing activity in the control samples incubated with buffer alone was reduced by less than 10%. With four different preparations, phospholipase D inactivated PLF by 85·5 ± 7·3% (mean ± SD) whereas PAF was inactivated by only 6·2 ± 6·5%.

To assess the effect of changes in platelet cyclic nucleotide levels on platelet serotonin release, 50 μl of PAF or 0·14 units of thrombin were added to platelets which had been pre-treated for 2 min at 37° with buffer, with 10 μM $PGD_2$, or with 5 mM sodium ascorbate (Table 1). Platelets pretreated with $PGD_2$, which exhibited a substantial increase in cyclic AMP levels at 2 and 30 min, manifested a net percentage serotonin release of 0% with thrombin and 33% with PAF. In two additional experiments pre-treatment of platelets with 10 μM $PGD_2$ diminished serotonin release by thrombin by 95 and 86%, while diminishing release by PAF by only 10 and 12%. Platelets pre-treated with ascorbate, which showed a ten-fold increase in cyclic GMP levels at 2 and 30 min, exhibited net percentage serotonin release that was comparable to that of platelets pre-treated with buffer alone. The contribution of pathways of arachidonic acid metabolism was assessed by adding 25 μl of PAF or 0·14 units of thrombin to platelets which had been pre-treated for 5 min at 37° with buffer or 30 μM indomethacin. In two experiments, PAF induced a net percentage serotonin release of 40 and 28% from platelets pre-treated with buffer, respectively, and 39 and 27% from platelets pre-treated with indomethacin; thrombin induced 77 and 80% serotonin release from platelets pre-treated with buffer and 79 and 71% release, respectively, from platelets pre-treated with indomethacin. Similarly, in two experiments pretreatment of platelets with 20 μM 5,8,11,14-eicosatetraynoic acid (TYA) in 0·5% dimethyl sulphoxide at 37° for 5 min did not diminish

**Table 1.** Modulation of [$^{14}$C]-serotonin release by cyclic nucleotide levels*

| Platelet pretreatment† | Platelet stimulus | [$^{14}$C]-serotonin‡ (net % release) | Cyclic AMP§ (pmol/$10^8$ platelets) | Cyclic GMP§ (pmol/$10^8$ platelets) |
|---|---|---|---|---|
| Buffer | Buffer | 0 | 3·6/4·8 | 0·4/0·6 |
| | PAF | 39 | 3·2/3·4 | 0·4/0·9 |
| | Thrombin | 28 | 3·4/4·1 | 0·5/0·7 |
| $PGD_2$ | Buffer | 0 | 11·1/8·9 | 0·9/0·5 |
| | PAF | 33 | 15·7/18·9 | 0·4/0·5 |
| | Thrombin | 0 | 2·6/2·8 | 1·5/0·4 |
| Ascorbate | Buffer | 0 | 3·7/3·3 | 4·1/6·4 |
| | PAF | 39 | 5·9/2·8 | 1·1/0·9 |
| | Thrombin | 27 | 6·1/4·4 | 1·1/0·4 |

* All values are the mean of replicate samples.

† Platelets were preincubated with the agonists noted for 2 min before the stimulus was added.

‡ Measured 30 min after stimulus was added.

§ Measured at 2 min (left value) and at 30 min (right value) after stimulus was added.

serotonin release by either 25 μl of PAF or 0·14 units of thrombin.

## DISCUSSION

The lipid platelet-active material that is generated in the peritoneal cavity of the rat upon immunological challenge was separated from amines and peptide mediators by Amberlite XAD hydrophobic chromatography (Kater *et al.*, 1976; Valone & Goetzl, 1978) and was further purified to remove other lipid mediators before its functional characteristics were assessed. Diethylaminoethyl (DE-52) cellulose chromatography in organic solvents of increasing polarity resolved the platelet [$^{14}$C]-serotonin releasing activity into two distinct fractions which were separated from SRS-A and the predominant PMN leucocyte chemotactic activity (Fig. 1). Both principles migrated as single peaks during silica gel H thin layer chromatography in chloroform:methanol:acetic acid:water (50:30:8:4, v:v) and there was no evidence of cross contamination (Fig. 2). The activity eluting from DE-52 cellulose in 7:1 chloroform:methanol was cytotoxic for platelets as revealed by the dose-related release of LDH as well as [$^{14}$C]-serotonin (Fig. 3). Platelets exposed to this fraction for a time that yielded maximal serotonin release were fragmented when examined by phase contrast microscopy and this fraction was thus designated a platelet-lytic factor (PLF). In four experiments this activity was inactivated by 85·5 ± 7·3% (mean ± SD) with phospholipase D isolated from human eosinophils. Earlier studies in which the platelet-active material was not resolved into two fractions revealed almost complete inactivation by treatment with phospholipase D from both human and cabbage sources, presumably because the dilutions of platelet-active material employed to examine the platelet response reflected the lytic rather than the secretory principle. Cabbage phospholipase D has also been reported to inactivate a 1-lysophospholipid derived during alkaline incubation of pig peripheral blood leucocytes which has been designated as a platelet-activating factor because it releases serotonin from rabbit platelets without evidence of cytotoxicity (Benveniste *et al.*, 1977). The solubility characteristics, size and susceptibility to inactivation by phospholipase D of the rat PLF suggest that this activity may represent one or more molecules in the lysophospholipid family. It is possible that phospholipase D would inactivate the platelet effects of either 1-lyso or 2-lysophospholipids and that the nature of the platelet response would differ depending either upon species differences or upon the structural characteristics of the lysophospholipid.

The activity eluting from DE-52 cellulose in 1:1 methanol:aqueous 1·0 M ammonium carbonate gave a dose-related release of serotonin without release of LDH (Fig. 3) and was thus designated a platelet-activating factor (PAF). The time course of the platelet secretory response to PAF was detectable within 2 min and reached a plateau at 15 min. PAF was not susceptible to inactivation by concentrations of phospholipase D which gave 85% inactivation of PLF. The secretory response to PAF was associated with platelet aggregation; the aggregation index, however, was substantially lower than that achieved by a dose of thrombin which released comparable quantities of [$^{14}$C]-serotonin. The simultaneous measurement of human platelet aggregation and secretion has identified two pathways of stimulus–response coupling which can be designated as aggregation-mediated and direct, respectively (Charo, Feinman & Detwiler, 1977). Epinephrine and low concentrations of adenosine diphosphate (ADP), thrombin and ionophore activate platelet secretion by an aggregation-dependent, indomethacin-inhibitable mechanism in which secretion is temporally associated with the second wave of aggregation. Collagen and higher concentrations of ADP, thrombin and ionophore directly initiate secretion with or without concomitant aggregation. The poor aggregating activity of PAF suggested that it would be a direct secretory stimulus; this possibility was supported by the demonstration that its action in releasing serotonin was not inhibited by indomethacin. When thrombin was employed at a concentration which released serotonin in the presence of indomethacin so as to utilize its direct activating effect, the platelet secretory response was inhibited by concentrations of $PGD_2$ which elevated cyclic AMP (Table 1). These concentrations of platelet cyclic AMP, in contrast, had no effect on the direct secretory response to PAF. Rabbit platelet serotonin release induced by crude rabbit PAF occurs without release of LDH but differs from the response of human platelets to rat PAF by its dependence on extracellular calcium and its inhibition by elevation of platelet cyclic AMP (Henson, 1974). While the mechanism of direct stimulus response coupling for rat PAF has not been elucidated, the cyclic AMP and indomethacin resistance of the reaction suggests that rat PAF stimulates non-cytotoxic platelet release through a unique site of action.

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