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Secretion, subcellular localization and metabolic status of inorganic pyrophosphate in human platelets

A major constituent of the amine-storing granules

Miriam H. FUKAMI, Carol A. DANGELMAIER, Jean S. BAUER and Holm HOLMSEN
Specialized Center for Thrombosis Research and Department of Pharmacology, Temple University Medical School, Philadelphia, PA19140, U.S.A.

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The platelet content of PP_i is $1.90 \pm 0.22 \mu mol/10^{11}$ platelets (S.E.M., $n = 19$) or about 10.5 nmol/mg of protein, several hundred times that found for rat liver. Some 80% of this PP_i is secreted by platelets treated with thrombin with a time course and dose–response relationship similar to secretion of ATP, ADP and 5-hydroxytryptamine (serotonin) from the platelet dense granules. During platelet aggregation induced by ADP and adrenaline, substantial amounts of PP_i were secreted, but no release of acid hydrolases was observed. Subcellular-fractionation studies showed that the PP_i is highly enriched in the same fraction that contains the storage organelles which store ATP, ADP, Ca^{2+} and 5-hydroxytryptamine. Inorganic pyrophosphatase was present mainly in the soluble fraction and in the mitochondria. Secretion studies done with platelets prelabelled with [^{32}P]P_i showed that the sequestered PP_i was relatively metabolically inactive, as is the ATP and ADP in the storage organelles. The possible participation of PP_i in the formation of a bivalent-cation–nucleotide complex associated with amine storage is discussed.

The main role of PP_i metabolism, especially the rapid removal of PP_i by inorganic pyrophosphatase (EC 3.6.1.1), was long thought to be that of ensuring the irreversibility of certain biosynthetic reactions (Stetten, 1960). More recently it has been shown that PP_i can serve as an energy source in micro-organisms (for review, see Wood, 1977). A similar role for PP_i in mammalian tissues has not been clearly demonstrated, although a pyrophosphatase coupling factor associated with PP_i synthesis has been described in ox heart mitochondria (Mansurova *et al.*, 1977). In rat liver the PP_i content was found to be inconsistent with the pyrophosphatase equilibrium, and a regulatory function for PP_i in other reactions has been proposed (Guynn *et al.*, 1974). In another cell type, the blood platelet (human), the PP_i concentration has been reported to be as high as 1.4–3 nmol/10^8 cells (about 8–17 nmol/mg of platelet protein) (Silcox *et al.*, 1973) or several hundred times those in rat liver (Guynn *et al.*, 1974). Certainly the role of PP_i in platelets must be different from those functions considered above.

Silcox *et al.* (1973) found that PP_i appeared to be one of several substances that are secreted by platelets exposed to thrombin (for review on platelet secretion, see Holmsen & Weiss, 1979). Secretion experiments and observations on platelets deficient in the storage pool of adenine nucleotides led Silcox *et al.* (1973) to conclude that PP_i and the storage nucleotides had a common storage site different from that of Ca^{2+} and 5-hydroxytryptamine (serotonin). This conclusion is inconsistent with the now well-established localization of secretable Ca^{2+}, 5-hydroxytryptamine and storage nucleotides in the same type of organelle, the dense granule (Holmsen & Weiss, 1979).

Our interest in the exact storage site of platelet PP_i is related to our current studies on the storage mechanism for amines (e.g. 5-hydroxytryptamine), bivalent cations and purine nucleoside tri- and di-phosphates in the platelet dense granules. It has been suggested by Berneis *et al.* (1970) that stored nucleotides and bivalent cations in platelet storage organelles form a complex that is able to bind 5-hydroxytryptamine. Studies with high-resolution ^{31}P n.m.r. have shown that the nucleotides in human platelet storage granules are not in free solution, but appear to be complexed in a manner such that the tumbling of their phosphate groups is markedly restricted (Ugurbil *et al.*, 1979). PP_i, if

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present in these same granules, may also participate in the formation of such complexes, since it also does not show detectable resonances in high-resolution ^{31}P-n.m.r. spectra of intact platelets.

Therefore we have investigated the localization of human platelet PP_i by secretion and subcellular-fractionation studies. Inorganic pyrophosphatase distribution was also determined in these subcellular fractions. In addition, the metabolic reactivity of the stored PP_i was evaluated in platelets pre-labelled with [^{32}P]P_i. A simple, rapid method for PP_i determination based on the Malachite Green–phosphomolybdate method for P_i (Hess & Derr, 1975) was developed for these studies.

Materials and methods

Platelet-rich plasma was prepared from blood taken by venipuncture from healthy donors into sodium citrate as described previously (Holmsen *et al.*, 1972*a*).

Gel-filtered platelets were isolated from platelet-rich plasma as described by Lages *et al.* (1975), by using a modified Tyrode's solution without Ca^{2+} and with 0.2% bovine serum albumin; in addition, P_i was omitted in order to facilitate the PP_i measurements.

For the preparation of 5-hydroxy[^{14}C]tryptamine-labelled platelets, platelet-rich plasma was incubated with 1 μM-5-hydroxy[2-^{14}C]tryptamine for 15 min at 37°C before gel filtration.

^{32}P-labelled platelets were prepared by incubating platelet-rich plasma with 0.5 mCi of [^{32}P]P_i/ml for 120 min before gel filtration. Excess [^{32}P]P_i that was not taken up by the platelets was eluted from the column after the labelled platelets were eluted from the Sepharose 2B columns.

In the thrombin-secretion experiments, 1.0 ml of gel-filtered platelets was incubated at 37°C for 3–5 min; then 0.1 vol. of thrombin in saline (0.9% NaCl) or saline alone was added to the platelets and the mixtures were incubated for a given time. The suspension was then added to an ice-cooled Eppendorf tube containing 0.1 vol. of saline and centrifuged in an Eppendorf centrifuge 3200 at 12000 *g* for 1 min. The supernatant fraction was divided as follows for assay of released constituents: 0.015 ml of 10% Triton X-100 was added to 0.15 ml of supernatant and frozen for β-*N*-acetylglucosaminidase and β-galactosidase determinations (Holmsen *et al.*, 1975); 0.1 ml of a 10 mM-EDTA/87% (v/v) ethanol mixture was added to 0.1 ml of supernatant for ATP and ADP determinations by the firefly luciferase assay (Holmsen *et al.*, 1972*b*); 0.07 ml of 6.6 M-$HClO_4$ was added to 0.7 ml of the supernatant and centrifuged at 12000 *g* for 1 min, after which the supernatants were neutralized with 2 M-K_2CO_3 for P_i and PP_i determinations. When 5-hydroxytryptamine was used as a marker for dense-granule secretion, hydroxy[^{14}C]tryptamine-labelled platelets were used and the amount of 5-hydroxy[^{14}C]-tryptamine released was measured by conventional liquid-scintillation methods (Holmsen *et al.*, 1973). In secretion experiments with ADP, adrenaline or collagen as inducers, 0.1 ml of platelet-poor plasma (dialysed overnight against 22 mM-trisodium citrate in 0.9% NaCl, pH 7.4, at 4°C) was added to 0.9 ml of gel-filtered platelets before addition of inducer.

PP_i was assayed in neutralized $HClO_4$ extracts as follows: the samples were divided into two portions of 300 μl each, and 150 μl of inorganic pyrophosphatase (1.7 units/ml in 0.2 M-Tris/HCl, pH 8.0, with 0.5 mM-$MgSO_4$) was added to one portion and 150 μl of buffer was added to the other. After the mixtures were incubated at 37°C for 15 min, 45 μl of 6.6 M-$HClO_4$ was added to each sample. The total P_i was then determined in the samples with and without pyrophosphatase by adding 495 μl of 'phosphate reagent'; after 5 min at room temperature the samples were read at 660 nm against a processed blank and compared with standards. The difference between the samples with and without pyrophosphatase was taken as the PP_i value. The 'phosphate reagent', a modification of that in the method of Hess & Derr (1975), consisted of a mixture of 3 vol. of 0.1% Malachite Green and 1 vol. of 4.2% (w/v) ammonium molybdate in 4 M-HCl stirred for 30 min and filtered; the mixture was prepared freshly on each day of use.

Pyrophosphatase was assayed by incubating 50 μl of sample and 400 μl of a medium consisting of 45 mM-Tris/maleate, pH 7.9, 25 mM-$MgCl_2$ and 3 mM-sodium pyrophosphate for 4 min at 37°C. Then 0.1 ml of the reaction mixture was added to 0.5 ml of 0.72 M-$HClO_4$. The amount of PP_i that was liberated was determined after the addition of an equal volume of 'phosphate reagent', as described above. Samples incubated without substrate were used for spectrophotometric blanks.

For determination of the specific radioactivity of PP_i, a two-dimensional paper-chromatography system was used for separations; the solvent system for the first direction was 0.1 M-EDTA (8 ml), isobutyric acid (500 ml), 28% (v/v) NH_3 (21 ml) and water (279 ml) (Krebs & Hems, 1953); the solvent system for the second direction was butan-1-ol (40 ml), propan-1-ol (20 ml), acetone (25 ml), 80% (v/v) formic acid (25 ml), 35% (w/v) trichloroacetic acid (15 ml) and 0.1 M-EDTA (5 ml) (Wood, 1968). Extracts of ^{32}P-labelled platelets and supernatants from the secretion experiments were co-chromatographed with unlabelled PP_i and P_i. The spots were detected by spraying with a molybdate spray, which consisted of 5 ml of conc. $HClO_4$, 10 ml of 1 M-HCl, 25 ml of 4% ammonium molybdate and 60 ml of water (Hanes & Isherwood, 1949). The

spots were cut out and counted for radioactivity in a liquid-scintillation fluid consisting of 2g of 2,5-diphenyloxazole/litre of toluene at room temperature in a Beckman LS230 liquid-scintillation counter. When PP_i standards (5–30μM) were processed together with 30μM each of ATP, ADP and fructose 1,6-bisphosphate, no P_i contribution from these substances was found.

Platelet subcellular fractions were prepared as described previously (Fukami *et al.*, 1978).

Specific chemicals and reagents were obtained from the following sources and stored as described: [^{32}P]P_i (code NEX, carrier-free) from New England Nuclear Corp., Boston, MA, U.S.A.; 5-hydroxy-[*side-chain*-2-^{14}C]tryptamine binoxalate (48.5Ci/mol) from New England Nuclear Corp.; Sepharose 2B-XL from Pharmacia, Piscataway, NJ, U.S.A.; L-adrenaline bitartrate from Winthrop Laboratories, New York, NY, U.S.A., prepared and stored as described by Holmsen *et al.* (1972*a*); collagen type I from bovine achilles tendon, Sigma Chemical Co., St. Louis, MO, U.S.A., prepared and stored as described by Holmsen *et al.* (1973); disodium ADP from horse muscle, Sigma, stored as described by Holmsen *et al.* (1972*a*); luciferin–luciferase obtained as part of the 'reagent kit' from E. I. Dupont Co., Wilmington, DE, U.S.A., and dissolved in 10ml of 0.01M-sodium 4-morpholinepropanesulphonate/10mM-$MgSO_4$, pH7.4; inorganic pyrophosphatase from baker's yeast, type III, Sigma Chemical Co., made up as 333units/ml in 0.15M-NaCl and stored at 4°C.

Results

Secretion studies

The total platelet content of PP_i was 1.90 ± 0.22 (S.E.M., $n = 19$) μmol/10^{11} platelets, or about 10.5nmol/mg of protein (by using the conversion factor of 180mg of protein/10^{11} platelets; Marcus & Zucker, 1965). Under conditions of maximal secretion with 5 NIH units of thrombin/ml for 5min at 37°C, some 80% of the total platelet PP_i (1.53 ± 0.21 μmol/10^{11} platelets; S.E.M., $n = 15$) was released extracellularly with other secreted substances. The time course for secretion of 5-hydroxytryptamine, ATP and ADP, PP_i and the two acid hydrolases with 0.1 unit of thrombin/ml is shown in Fig. 1. The 5-hydroxytryptamine, ATP plus ADP and PP_i were fully released within 60s under these conditions, whereas acid hydrolases were released to only about 70% of the maximally secreted amounts at 60s; maximal release of acid hydrolases was not reached until 5min after thrombin addition. P_i, which was determined as part of the PP_i method, also increased in the intracellular medium, with a biphasic time course; about 50% of the total P_i released appeared extracellularly within 10s of incubation, and the remainder appeared slowly and linearly with time thereafter.

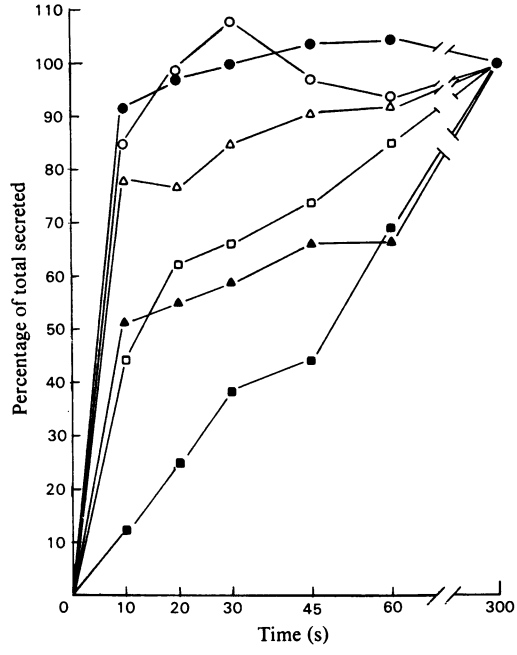

Fig. 1. *Time course of secretion of PP_i compared with other released substances*

Thrombin (0.7 unit in 0.1ml of saline) was added to 7ml of a stirred suspension of gel-filtered platelets at 37°C. Samples were taken at the times indicated and processed for determination of released constituents as described in the Materials and methods section. The secreted components shown are ATP and ADP (○), 5-hydroxytryptamine (●), PP_i (△), P_i (▲), *β-N*-acetylglucosaminidase (□) and *β*-galactosidase (■). The secreted amounts are given as percentages of the amount released when a separate sample of the same platelet preparation was exposed to a high dose of thrombin (5units/ml) for 5min.

This difference in release kinetics between the three groups of secreted compounds was seen even more clearly in a thrombin dose–response experiment (Fig. 2). With 0.05 unit of thrombin, nearly all of the 5-hydroxytryptamine, ATP, ADP and PP_i was secreted in the observation period of 20s. In this same period, less than 30% of the releasable *β-N*-acetylglucosaminidase and *β*-galactosidase was secreted with the same amount of thrombin. Again, release of P_i was somewhat intermediate between the two groups of secreted substances for a given amount of thrombin.

Secretion of PP_i also occurred during aggregation of platelets induced by ADP, adrenaline and collagen (see Table 1). Both ATP + ADP and 5-hydroxytryptamine were released to the same

extent. No release of acid hydrolases took place with ADP and adrenaline as inducers, but substantial amounts were released with collagen (results not shown).

Metabolic status of secreted PP_i

When platelets prelabelled with ^{32}P (as described in the Materials and methods section) were treated with thrombin, the PP_i that was released into the extracellular medium had a specific radioactivity which was less than 3% of that of the platelet-bound PP_i (55 c.p.m./nmol of PP_i, compared with 1853 c.p.m./nmol of PP_i for platelet-bound PP_i; Table 2). P_i was also released into the supernatant and had a specific radioactivity which was 5% of that of the platelet P_i (794 compared with 15570 c.p.m./nmol of P_i in whole platelets).

Distribution of PP_i in subcellular fractions (Fig. 3)

In the primary fractions the PP_i was found both in the granule fractions and in the soluble fraction. 5-Hydroxytryptamine in these preparations was found mainly in the granule fraction. When these granules were further fractionated on a sucrose density gradient (Fukami *et al.*, 1978), the PP_i and the 5-hydroxytryptamine were most enriched in the

Table 1. *PP_i secretion induced by ADP, adrenaline and collagen*

The secretion experiments were carried out as described in the Materials and methods section; 0.1 vol. of platelet-poor plasma was added to the gel-filtered platelet suspensions. The reactions were monitored at 37°C in a Payton dual-channel aggregometer module (Payton Associates, Buffalo, NY, U.S.A.) with constant stirring at 900 rev./min and allowed to proceed until full aggregation had occurred. The amount of PP_i released was compared with total platelet PP_i. Platelets from different donors were used for Expts. I and II.

	Percentage release of PP_i	
Inducer	Expt. I	Expt. II
ADP (5 μM)	40	68
Adrenaline (5 μM)	36	47
Collagen (1 μg/ml)	34	48
Collagen (5 μg/ml)	56	81

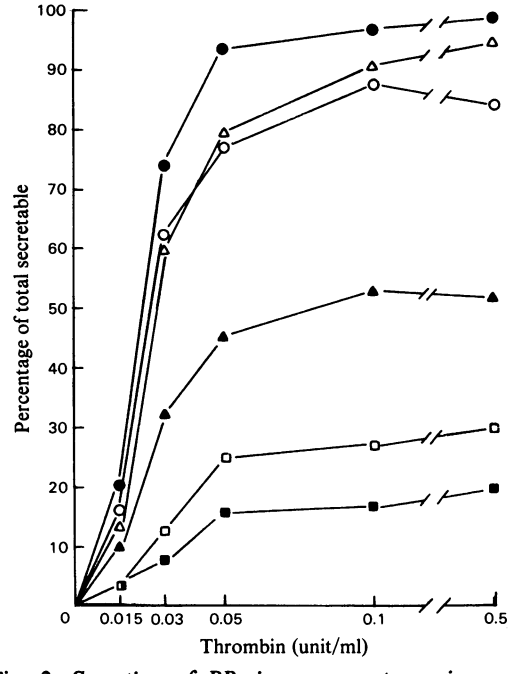

Fig. 2. *Secretion of PP_i in response to various concentrations of thrombin compared with other released substances*

Various amounts of thrombin in 0.1 ml of saline were added to 0.9 ml samples of gel-filtered platelets; each reaction was carefully mixed and then rapidly cooled and centrifuged at 12000 *g* for 2 min after 20 s at 37°C. The released constituents were assayed as described in the Materials and methods section (the symbols are the same as in Fig. 1). The percentage of total secreted is that fraction of the amounts released by a separate sample of the same platelet preparation exposed to 5 units of thrombin/ml for 5 min.

Table 2. *Specific radioactivity of PP_i and P_i released by platelets pre-labelled with [$^{32}P_i$]P_i*

^{32}P-labelled platelets were treated with thrombin (final concn. 5 units/ml) as described for thrombin-secretion experiments in the Materials and methods section. PP_i and P_i in the supernatant portions were separated by paper chromatography for specific-radioactivity determinations. For the details of the separation, see the Materials and methods section. This experiment is representative of three similar experiments.

	Concn. (μM)	Radioactivity (c.p.m./ml)	Sp. radioactivity (c.p.m./nmol of P_i)
Platelet suspension			
P_i	9.51	148078	15570
PP_i	2.35	8737	1853
Supernatant of thrombin-treated platelet suspension			
P_i	3.78	3003	793
PP_i	2.05	228	55

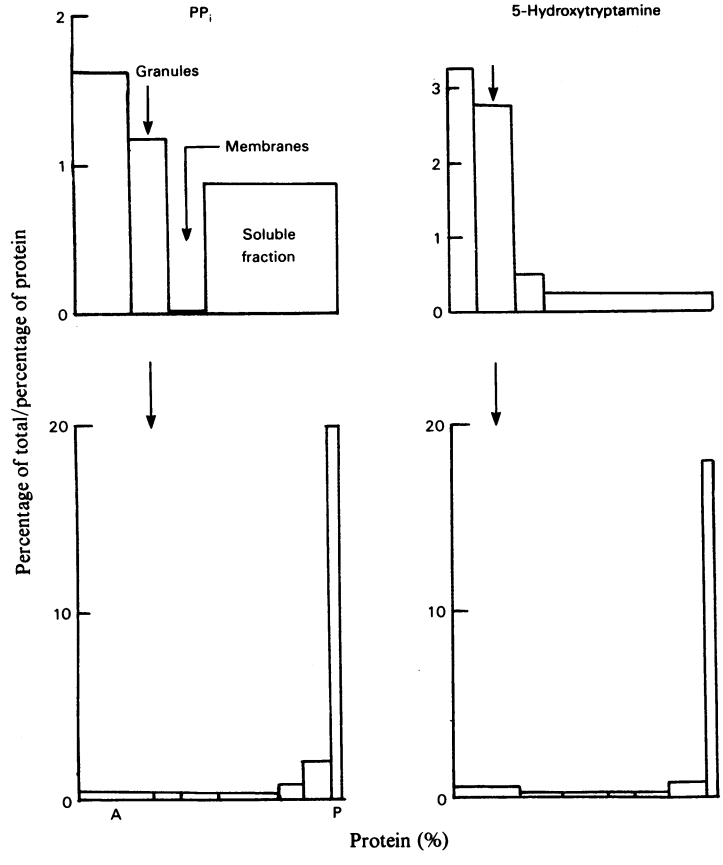

Fig. 3. *Distribution of PP_i in subcellular fractions compared with 5-hydroxytryptamine*

The upper two histograms show the distributions of PP_i and 5-hydroxytryptamine in the primary fractions obtained after differential centrifugation of platelet homogenates prepared as previously described (Fukami *et al.*, 1978). The lower two histograms show the distribution of PP_i and 5-hydroxytryptamine on a sucrose density gradient after subfractionation of the granule fraction. The sucrose gradient ranged from 0.8 to 2.0 M-sucrose in 0.2 M steps; subfractions A, B, C, D, E and P were collected; subfraction P is the pellet under the 2.0 M-sucrose step and is the fraction most enriched in dense storage organelles (Fukami *et al.*, 1978). The abscissae, which span 0–100%, represent percentage of total protein in each fraction and the ordinates the percentage of total PP_i or 5-hydroxytryptamine in each fraction divided by the percentage of total protein in that fraction, after the convention of de Duve *et al.* (1955). The values shown on the histograms represent the mean of four preparations, with S.E.M. values of about ±25% (see Table 2 for dense-granule PP_i values).

Table 3. *PP_i and P_i contents in whole platelets, in the extracellular medium after induction of secretion by thrombin (5 units/ml) and in isolated dense storage organelles*

The data are expressed in nmol/mg of protein, ±S.E.M.

	PP_i	P_i
Whole platelets	10.48 ± 1.22 ($n = 19$)	22.38 ± 4.14 ($n = 11$)
Secreted amounts	8.50 ± 1.17 (81%) ($n = 15$)	8.75 ± 2.01 (39%) ($n = 4$)
Dense granules	236 ± 53 ($n = 4$)	248 ± 65 ($n = 4$)

pellet heavier than the 2 M-sucrose step. The actual amounts of PP_i and P_i in intact platelets and in the isolated dense granules are shown in Table 3, as well as the amounts secreted. The value for the P_i in the dense granules is not completely reliable, since the recoveries of P_i on the sucrose gradients were about 200%. However, it was quite clear that P_i was most concentrated in the dense granules.

Pyrophosphatase distribution

The activity of inorganic pyrophosphatase in human platelet homogenates was 80.3 ± 6.6 nmol/

min per mg of platelet protein (S.E.M., $n = 3$). When the homogenate was fractionated by differential centrifugation, 11–19% of the pyrophosphatase was found in the 10000*g*-10min pellet, 6–9% in the 100000*g*-60min pellet and 72–83% in the 100000*g*-60min supernatant. The 10000*g* pellet, which contains platelet organelles, was further subfractionated on a sucrose density gradient as described above. As much as 40% of the organelle-bound pyrophosphatase was found in the subfraction most enriched in mitochondria, with specific activities of 923–1095nmol/min per mg of protein or 11–13 times the homogenate activity. Less than 5% of the pyrophosphatase in the 10000*g* pellet was present in the dense granules.

Discussion

The PP_i assay described in the present paper requires no separation of nucleotides and other phosphate esters, because it is run under mild conditions over a short incubation period so that no hydrolysis occurs. Furthermore, the sensitivity of the final P_i determination with Malachite Green (Hess & Derr, 1975) is 8 times greater than with the Fiske & Subbarow (1925) method. The only limitation is that the cell-incubation medium must be kept phosphate-free in order to keep the background P_i low. We attempted to use the isotope-dilution method described by Silcox *et al.* (1973), but were unable to obtain reproducible results with it; however, the platelet PP_i concentrations that they obtained are comparable with our determinations. Since the present study was initiated, other sensitive methods for PP_i analysis without pre-separation and which are independent of the presence of P_i have been published (Drake *et al.*, 1979).

The secretion studies showed that the time course and the dose–response relationship to thrombin was the same for PP_i, 5-hydroxytryptamine, ATP and ADP release. Although we did not measure Ca^{2+} in these experiments, it has been established in earlier studies using the same conditions as described here that the pattern of Ca^{2+} secretion is identical with that of 5-hydroxytryptamine, ATP and ADP (Lages *et al.*, 1977). Thus our secretion studies indicate that PP_i, storage nucleotides, Ca^{2+} and 5-hydroxytryptamine originate from the same subcellular localization. These findings are in contrast with the conclusions of Silcox *et al.* (1973), that PP_i and adenine nucleotides have a common storage site different from that of Ca^{2+} and 5-hydroxytryptamine. The discrepancy may have resulted from the fact that they did not analyse all the kinetic parameters in actual samples, but compared their data for some components with that for other components taken from the literature.

The time course of P_i release was different from that of the dense-granule contents and similar to *β-N*-acetylglucosaminidase release, in that it showed an early, rapid phase and a later, slow phase. The subcellular-fractionation data (see below) indicate that significant amounts of P_i are present in the dense granules. Since the initial burst in the appearance of P_i coincided with maximal secretion of adenine nucleotides, PP_i and 5-hydroxytryptamine, it is most likely that this burst represents exocytosis of P_i from the dense granules. The late, slow appearance of extracellular P_i might be due to hydrolysis of secreted nucleotides (Rossi, 1971), cytoplasmic ATP breakdown during platelet secretion (Holmsen, 1965*b*), or possibly phospholipid breakdown.

In studies with other secretagogues (Table 1), PP_i was released by ADP, adrenaline and collagen. Adrenaline and ADP are known to induce secretion of only dense-granule and α-granule contents and not of acid hydrolases (Holmsen & Weiss, 1979).

The metabolic reactivity of the secreted platelet PP_i was evaluated by determining how much label was incorporated into PP_i secreted by thrombin treatment of platelets preincubated with ^{32}P. Under these conditions, the released PP_i had a low specific radioactivity compared with total platelet PP_i; the storage pool of adenine nucleotides is similarly inactive with respect to labelling *in vitro* (Holmsen, 1965*a*; Ireland, 1967). The specific radioactivity of PP_i in whole platelets, 1853c.p.m./nmol of P_i, was also very much less than that of P_i in whole platelets, indicating that most of the PP_i turns over more slowly than the P_i.

The subcellular fraction most enriched in 5-hydroxytryptamine and PP_i was that containing the dense storage granules. It has been shown previously that the storage pools of ATP and ADP are also present in the same fraction as 5-hydroxytryptamine (Fukami *et al.*, 1978). The concentration of P_i in the dense granules appeared to be 10 times enriched over that in intact platelets, per mg of protein.

The activity of inorganic pyrophosphatase in platelet homogenates was about one-third of that in rat liver (Guynn *et al.*, 1974). Although most of the platelet inorganic pyrophosphatase was present in the soluble part of a platelet-fractionation preparation, about 5–7% was associated with mitochondria. The specific activity of the enzyme associated with mitochondria was 10–13 times enriched over the activity observed in the homogenate and about 20 times that reported for ox heart mitochondria (Mansurova *et al.*, 1977). The mitochondria constituted the only fraction in which the relative specific activity of inorganic pyrophosphatase compared with homogenate was significantly more than 3.

It can be concluded from these results that most

of the PP_i is stored in the dense granules of plateletes, well shielded from inorganic pyrophosphatase. Like the storage pool of ATP and ADP, PP_i is probably packaged into the organelles at some early stage in platelet formation, probably in the megakaryocyte, and is relatively metabolically inert, in contrast with 5-hydroxytryptamine, which is rapidly taken up into the storage organelles by the circulating platelet. We therefore consider that PP_i and P_i should be included with bivalent metal ions and nucleotides in investigations of the mechanisms for storage of biogenic amines in secretory granules.

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