Effect of Thrombin on the Radioactive Nucleotides of Human Washed Platelets

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Radioactive ATP and ADP were found in platelets after incubation of human platelet-rich plasma with either [8-¹⁴C]adenosine or [8-¹⁴C]ADP. Treatment of the labelled and washed platelets with thrombin indicated that, though considerable amounts of ATP and ADP were released to the supernatant, radioactive ATP and ADP remained predominantly in the cellular fraction. Breakdown of radioactive ATP took place to form mainly IMP and hypoxanthine, the latter compound appearing in the supernatant. The results indicate the presence of at least two pools of nucleotide in platelets. Evidence is given that the two pools contain approximately the same amounts of ATP plus ADP, and that the ratio of ATP to ADP in the pool released to the supernatant by the action of thrombin is about 0.7-0.8.

Grøtte (1962) showed that thrombin induces the release from washed pig platelets of serotonin, adenine nucleotides, P_i, protein and amino acids. Thrombin also caused the aggregation of platelets in the presence of Ca²⁺, and Haslam (1964, 1966) has shown that, with human platelets, the aggregating action of thrombin is probably mediated through ADP released from the platelets. Holmsen (1965*a,b*) described the uptake of ³²P by platelets, in human and rabbit platelet-rich plasma, to form radioactive organophosphates, including ATP and ADP. On collagen treatment, Holmsen found that the radioactive ATP content of the platelets decreased, whereas the radioactive ADP content remained almost unchanged. With rabbit platelets (Holmsen, 1965*b*), little or no radioactive ATP or ADP was detected in the extracellular fluid after collagen treatment, whereas a considerable amount of non-radioactive ADP was released. These results indicated the presence of at least two adenine nucleotide pools in platelets.

The incorporation of radioactive adenosine into platelets to form radioactive adenine nucleotide, mainly ATP and ADP (Ireland & Mills, 1966), offers a means for further investigation of some of these reactions. A preliminary report of part of this work has been given (Ireland, 1966).

MATERIALS

Chemicals. GTP, GDP, GMP, ATP, ADP, AMP and adenosine were from Sigma Chemical Co., St Louis, Mo., U.S.A.; ITP, IDP, IMP, inosine, hypoxanthine and adenine were from Koch-Light Laboratories Ltd., Coln-

brook, Bucks.; [8-¹⁴C]ATP (28.2 mc/m-mole and 32 mc/m-mole), [8-¹⁴C]ADP (34 mc/m-mole), [8-¹⁴C]IMP (22 mc/m-mole) and [8-¹⁴C]inosine (21.8 mc/m-mole) were from Schwarz BioResearch Inc., Orangeburg, N.Y., U.S.A.; [8-¹⁴C]adenosine (28.4 mc/m-mole) was from The Radiochemical Centre, Amersham, Bucks; tris, glycine and glycyglycine were from British Drug Houses Ltd., Poole, Dorset; HClO₄ (sp.gr. 1.70) was from Hopkin and Williams Ltd., Chadwell Heath, Essex. Laboratory chemicals were of A.R. grade, and other chemicals used were as described by Ireland & Mills (1966).

METHODS

Enzymes and test kits. The kit ADP/AMP-K15980, used for determining ADP and AMP, and the kit ATP-J15979, used for determining ATP, were from Boehringer Corp. (London) Ltd., London, W. 5. Xanthine oxidase and urate oxidase were from Koch-Light Laboratories Ltd.; 0.1 ml. of the (NH₄)₂SO₄ suspension of xanthine oxidase was diluted to 0.4 ml. with 0.6 M-glycylglycine buffer, pH 8.2. The solution was stored frozen at -5° to -10°, and 0.1 ml. was diluted to 1 ml. with 60 mM-glycylglycine buffer, pH 8.2, before use. The contents of one ampoule of freeze-dried solid containing 100 Praetorius units (Praetorius, 1963) of urate oxidase were dissolved in 0.25 ml. of 60 mM-glycine buffer, pH 9.3, stored at 2-4° and used as required. Bovine thrombin was from Parke, Davis and Co., Detroit, Mich., U.S.A.

Apparatus. Incubation and centrifuge tubes were either polycarbonate tubes (10.5 cm. × 1.5 cm.) from Fisons Scientific Apparatus Ltd., Loughborough, Leics., or transparent nylon tubes (11.6 cm. × 2.8 cm.) supplied by A. Gallenkamp and Co. Ltd., London, E.C. 2. A Warburg water bath with a horizontal shaking movement (H. Mickle, Gomshall Surrey) was used for incubations. Other apparatus has been described by Ireland & Mills (1966).

Isolation of washed platelets. Blood was taken from the antecubital vein of male volunteers either by drip through a serum needle (17 gauge) attached to polythene tubing, or by 50ml. disposable plastic syringes (hypodermic needle, 20 gauge). Samples of blood (45 ml.) were added to 5 ml. of 3.13% (w/v) citrate contained in silicone-treated 100ml. glass vials. The blood was immediately centrifuged at room temperature for 15 min. at 180g in a swing-out head, and about the top two-thirds of the resultant platelet-rich plasma was removed by a plastic Pasteur pipette. The platelet-rich plasma was cooled to 0°, and EDTA at 0° was added to give a final concentration of 8.6mm-EDTA (0.125 ml. of 77mm-disodium EDTA, pH 7.4/ml. of plasma). Subsequent operations were performed at 0°. The platelet-rich plasma plus EDTA was centrifuged in polycarbonate tubes for 20 min. at 750g in an angle head. The supernatant platelet-poor plasma was discarded and the platelet pellet was resuspended by gently sucking the cells up and down with a plastic Pasteur pipette in one-quarter the plasma volume of washing solution (0.139M-NaCl-12.3mm-tris-HCl-1.54mm-EDTA, pH 7.4). The suspension was centrifuged for 3 min. at 750g in a swing-out head, a small pellet of platelets and erythrocytes was discarded and the supernatant was centrifuged again for 3 min. at 750g. The resultant supernatant was centrifuged for 25 min. at 750g to sediment the rest of the platelets. Normally, two 3 min. centrifugations were sufficient to remove the erythrocytes. The final platelet pellet was resuspended in the appropriate incubation medium, and normally contained 2×10^8 – 5×10^8 platelets/ml. of suspension. Erythrocytes comprised less than 0.1% of the cell population. Platelets were counted by microscopy in a haemocytometer with 1:20 dilutions of platelet suspensions in formalized saline (Zucker & Borrelli, 1954).

The medium referred to as full saline was used for all incubations unless otherwise indicated and contained NaCl (0.12M), tris-HCl, pH 7.4 (13.3mm), KCl (15.4mm), $MgSO_4$ (1.54mm), $CaCl_2$ (0.55mm) and D-glucose (6mm). An incubation medium without Ca^{2+} and Mg^{2+} and containing EDTA was also used [NaCl (0.119M), tris-HCl, pH 7.4 (13.2mm), KCl (15.4mm), EDTA (1.54mm) and D-glucose (6mm)].

Preparation of labelled platelets. Platelet-rich plasma, prepared as described, was incubated at 37° for the time noted with either [^{14}C]adenosine or [^{14}C]ADP. These compounds were added in aqueous solution to the plasma to give an initial concentration of 2–6 μ M of the radioactive compound. The volumes added were 1% or less of the plasma volume. Subsequent isolation of washed platelets was as described above. ADP is degraded in plasma to adenosine, which is taken up by the platelets (Ireland & Mills, 1966).

Incubation of washed platelets with thrombin. Suspensions of washed platelets were incubated in the Warburg water bath at 37° for 2–3 min. at 80–100 strokes/min., and then amounts of thrombin in 0.9% NaCl were added as noted. At various times later the reaction was stopped either by adding 10% (w/v) trichloroacetic acid containing carrier compounds (see below) or by cooling the mixture in ice.

Preparation of supernatant and cellular fractions of cell suspensions. All operations were performed at 0°. The cell suspension was centrifuged for 10 min. at 3000g and the supernatant was removed. To portions of the supernatant was added 10N-HClO₄ (0.15 ml./2.85 ml. of supernatant),

and after 10 min. the solution was centrifuged for 10 min. at 3000g to remove traces of protein. The whole of the supernatant was neutralized with 5N-KOH (0.3 ml.). After centrifugation for 10 min. at 3000g to remove insoluble KClO₄, the solution was stored at –20°. To the cellular residue from 3 ml. of original cell suspension was added 2.85 ml. of full saline. To the resuspended platelets was added 10N-HClO₄ (0.15 ml./2.85 ml. of suspension), the cells were broken up with a glass rod, and after 10 min. the suspension was centrifuged for 10 min. at 3000g. The whole of the acid extract was removed, neutralized and stored at –20°. In some instances the cellular residue was washed, before acidification, with 1 ml. of tris-buffered NaCl containing EDTA.

Assays. Neutralized extracts of the supernatant and cellular fractions of the platelet suspensions were assayed for the following substances.

ADP and AMP were assayed by the use of pyruvate kinase, lactate dehydrogenase and adenylate kinase as described in the relevant test-kit, except that 3 ml. samples were tested and the solution containing 0.43M-triethanolamine buffer and 0.55M-K₂CO₃ was omitted. Known amounts of ADP added to extracts obtained after thrombin treatment were recovered to within 10% of the added amount.

ATP was assayed by the use of phosphoglycerate kinase and glyceraldehyde phosphate dehydrogenase as described in the relevant test-kit, except that 3 ml. samples were tested and 0.25 ml. of a solution containing triethanolamine buffer, pH 7.6 (1M), $MgSO_4$ (40mm) and 3-phosphoglycerate (60mm) was added to the cuvette. Known amounts of ATP added to extracts obtained after thrombin treatment were recovered to within 10% of the added amount. Radioactive ATP was present in some of the samples that were assayed for total ATP. After the determination of total ATP, some of these samples were treated with trichloroacetic acid containing carrier compounds and subjected to charcoal treatment and chromatography in solvent 1 with EDTA (see below). Results showed that 98% of the initial radioactive ATP was converted into radioactive ADP during the enzymic assay procedure.

Hypoxanthine plus xanthine was assayed by oxidation with xanthine oxidase to uric acid and the measurement of the uric acid formed with urate oxidase at 292.5m μ (Jørgensen, 1963). Since the uric acid content of washed platelet suspensions after thrombin treatment was very small, preliminary incubation of samples with urate oxidase was omitted. To 3 ml. portions of the sample were added 0.01 ml. of 1.6N-NaOH, 0.37 ml. of 0.6M-glycylglycine buffer, pH 8.2, and 0.02 ml. of xanthine oxidase solution. $E_{292.5}$ was measured before, and 1 hr. and 2 hr. after, the addition of xanthine oxidase. To the solution in the cuvette were added 0.05 ml. of 1.6N-NaOH, 0.34 ml. of 0.6M-glycine buffer, pH 9.3, and 0.01 ml. of urate oxidase solution. Readings were taken before and 30 min. after the addition of urate oxidase. The results of determinations with urate oxidase were taken as representing the hypoxanthine plus xanthine content of the sample.

Hypoxanthine was assayed by oxidation with xanthine oxidase to uric acid and measurement of the uric acid formed at 280m μ , at which wavelength xanthine is not measured (Petersen, Jørgen & Jørgensen, 1965). The conditions used were as described for the assay of hypoxanthine plus xanthine. The amount of xanthine in eight

samples of supernatant from platelets incubated with thrombin, determined as the difference between hypoxanthine plus xanthine and hypoxanthine alone, was $0.37 \pm 0.35 \mu\text{mole/ml.}$ of solution (mean $\pm$ S.E.M.). This is not significantly different from zero. Uric acid, if present, would have been included in this value.

Inosine plus inosine nucleotide was determined after acid hydrolysis by conversion of the hypoxanthine formed into uric acid with xanthine oxidase and measurement of the uric acid formed by urate oxidase. To a 3ml. portion of sample was added 0.13ml. of 11.4N-HCl; after 1.5 hr. in a boiling-water bath, the solution was cooled and 0.15ml. of 10N-NaOH added. Portions (3ml.) of the neutralized solution were taken for the determination of the hypoxanthine content, as described for the assay of hypoxanthine plus xanthine. The value obtained for the hypoxanthine content of the sample after hydrolysis minus that obtained before hydrolysis was taken as the inosine plus inosine nucleotide content of the sample. The samples assayed also contained adenine nucleotide, and it was found by radioactivity determinations that on acid hydrolysis about 0.7% of the adenine nucleotide was converted into hypoxanthine. Since in some samples 0.7% of the adenine nucleotide content amounted to 20–30% of the value attributed to inosine plus inosine nucleotide, a correction was applied by subtracting 0.7% of the total ATP, ADP and AMP content of the sample from the value obtained for inosine plus inosine nucleotide content. It was found that the adenine formed by acid hydrolysis of the adenine nucleotide in the samples did not interfere in the assay. Klenow (1952) showed that adenine, in the presence of large amounts of xanthine oxidase, is converted into 6-amino-2,8-dihydroxypurine, but that urate oxidase has no action on the latter substance. The method described probably measures xanthosine and xanthine nucleotide as well as ITP and IDP. No information is available on the content of these substances in the platelet. Since radioactive ITP and IDP were not detected in platelet extracts but radioactive IMP and, to a lesser extent, inosine were formed after thrombin treatment (see below), it is likely that the major portion of the values obtained may be attributed to IMP plus inosine.

All results obtained by the assay methods given were corrected for blank values, obtained by treating relevant amounts of incubation medium as described for test samples.

Radioactivity of the nucleotides, nucleosides and purines from either platelet suspensions or supernatant and cellular fractions of platelet suspensions was measured after adsorption on and elution from charcoal (Ireland & Mills, 1966). Portions (0.5 or 1.0 ml.) of cell suspensions or 2.5 ml. portions of neutralized extracts were added to 4.5 ml. of 10% (w/v) trichloroacetic acid containing carrier compounds. The carrier solution contained about 25 μmole s of each of ATP, ADP, AMP, IMP, inosine, adenosine and hypoxanthine in 4.5 ml. of 10% (w/v) trichloroacetic acid. After charcoal treatment and separation of substances by chromatography or electrophoresis, radioactivity was measured by scintillation counting. The counting efficiency was about 50%. Blank values were obtained by counting areas of paper containing no applied radioactivity. The recovery of nucleotides, nucleosides and purines from charcoal varied with the substance. For certain mixtures of these compounds recovery factors

have been determined (Ireland & Mills, 1966). In the experiments reported here as much as 60 μmole s of ATP, 30 μmole s of ADP and 20 μmole s of hypoxanthine additional to the standard carrier amounts of these substances could be present in the extracts applied to charcoal. The recoveries from charcoal of ATP, ADP and hypoxanthine, as determined by measurement of the recoveries of small amounts of radioactive ATP, ADP and hypoxanthine added to the extracts, were not, however, significantly altered from those given for the carrier compounds alone by Ireland & Mills (1966). The recovery of IMP from charcoal, given as 55% by Ireland & Mills (1966), was found to be too low. In the present paper, corrections for loss of IMP on charcoal are based on a recovery value of 85%. When small amounts of each of radioactive ATP, ADP and hypoxanthine were added to the extracts and applied to duplicate charcoal columns, then the amount of radioactivity recovered for each substance was within 5% of the mean recovery for that substance. The factors applied for loss of radioactivity on charcoal were: ATP, $\times 1.25$; ADP, AMP, IMP and inosine, $\times 1.15$; adenosine and hypoxanthine, $\times 1.4$.

Chromatography and electrophoresis. These were as described by Ireland & Mills (1966), except that in some experiments EDTA was included in the solvent system of Randerath & Struck (1961), giving the composition: butan-1-ol–acetone–acetic acid–5% (v/v) aq. ammonia (sp.gr. 0.88)–water–0.1M-EDTA (45:15:10:10:19:1, by vol.). Solvent 1 refers to this system of Randerath & Struck (1961); if EDTA was included this has been noted in parentheses. Solvent 2 refers to the isobutyric acid system described previously (Ireland & Mills, 1966). The time of solvent development of chromatograms was 16–18 hr. at 20–26°. The two solvent systems were used for two-dimensional chromatography where noted; in this event, the chromatograms were dried at room temperature for 4 hr. between the runs. With solvent 1, small amounts of radioactive GTP and GDP were counted along with ATP, and the value obtained has been corrected by application of the factor used for ATP. With solvent 1 (EDTA), very small amounts of GDP were counted along with ATP, and this has been similarly corrected.

Purification of [8-¹⁴C]ATP. [8-¹⁴C]ATP (32 mc/m-mole) as supplied was contaminated with about 34% of radioactive ADP, as shown by electrophoresis. It was partially purified as follows. Two samples each containing 1.5 μC were evaporated to dryness in silicone-treated evaporation flasks, and each residue was dissolved in 20 $\mu\text{l.}$ of water and applied to paper. After chromatography in solvent 1 (EDTA), the areas of the paper containing radioactive ATP were located by radioautography for 4 hr., cut out and eluted with 3 ml. of water for 1 hr. at room temperature. Paper fibres were removed by centrifugation. The resulting solution contained about 6.4% of the radioactivity as ADP.

Preparation of [8-¹⁴C]hypoxanthine. [8-¹⁴C]Hypoxanthine was prepared from [8-¹⁴C]inosine by acid hydrolysis (Ireland & Mills, 1966).

EXPERIMENTAL AND RESULTS

Identity of radioactive substances. Ireland & Mills (1966) reported that, when platelet-rich plasma was incubated with radioactive adenosine, the

subsequently washed platelets contained radioactive ATP, ADP and AMP. Radioactive adenosine, inosine and hypoxanthine, present in the plasma, were not found intracellularly.

The presence of three other radioactive substances was detected in extracts of labelled suspensions of platelets during the present work. One of these substances was identified, by two-dimensional chromatography (Fig. 1) and by mobility on electrophoresis, as IMP. The other two substances were tentatively identified by two-dimensional chromatography as GTP and GDP. GTP was not separated from ITP, and GDP was not separated from IDP, in the systems used, but the radioactivity of the unknown substances occupied only about half of the relevant carrier areas (Fig. 1), and the one-dimensional chromatography of GTP, ITP, GDP and IDP in both solvent systems showed that

the R_F values of the guanine compounds were slightly less than those of the inosine compounds. The relevant radioactive areas shown in Fig. 1 therefore probably indicate guanine rather than inosine nucleotides.

It was confirmed that little radioactive adenosine is found intracellularly after platelets incubated with radioactive adenosine or ADP had been washed (see below).

Effect of thrombin on labelled platelet suspensions. Platelet-rich plasma was incubated at 37° for 2 hr. with an initial concentration of about $2\mu\text{M}$ -[8- ^{14}C]ADP. The platelets were then washed and resuspended in full saline and portions were incubated at 37° with and without thrombin for various times. The reactions were stopped with trichloroacetic acid and the radioactivity of the separated substances was determined. The results of an experiment in which platelet suspensions were

Fig. 1. Diagram of two-dimensional paper chromatogram of an acid extract of labelled platelets. Platelet-rich plasma was incubated at 37° for 2 hr. with an initial concentration of $2\mu\text{M}$ -[8- ^{14}C]ADP, and washed platelets were prepared as described in the text. The washed platelets (about 7×10^8) were extracted with trichloroacetic acid containing about $25\mu\text{M}$ moles of each of carrier ATP, ADP, AMP and IMP and, after charcoal treatment, the neutralized and concentrated extract was subject to chromatography. About $25\mu\text{M}$ moles of each of GTP, GDP, ITP and IDP were applied to the origin just before the test sample. The first dimension was run in solvent 2, and the second dimension in solvent 1 (EDTA) (see the Methods section). The compounds indicated, enclosed by continuous lines, were detected under u.v. light. The hatched areas indicate areas of radioactivity, detected by means of X-ray film applied to the chromatogram for 3 days. O, origin.

Fig. 2. Incubation of labelled washed platelets with and without thrombin at 37°. Portions (1 ml.) of platelet suspension (4.32×10^8 platelets/ml.), prepared as described in the text, were incubated at 37° with shaking for 2 min., and then either $10\mu\text{l.}$ of 0.9% NaCl or $10\mu\text{l.}$ of 0.9% NaCl containing 0.2 unit of thrombin was added to the relevant suspensions. Incubation was continued for the times indicated, the reactions were stopped by addition of trichloroacetic acid containing carrier compounds, and radioactivity was determined after chromatography in solvent 1 (EDTA). The results shown for zero time were obtained from a sample treated with trichloroacetic acid immediately after the platelets had been suspended in the incubation medium, and the results shown to the right of the time scale were obtained from a sample kept at 0° for about 1 hr. The incubation medium contained tris-HCl, pH 7.4, Na^+ , K^+ , Mg^{2+} , Ca^{2+} and glucose. Corrections were made for loss on charcoal, as described in the Methods section. The radioactivity attributed to hypoxanthine+adenosine was probably mainly hypoxanthine (see the text). ■, ATP; ●, ADP; ○, AMP; △, IMP; □, hypoxanthine+adenosine. —, With thrombin; - - - -, without thrombin.

Table 1. *Effect of various concentrations of thrombin on the radioactive ATP content of washed platelets incubated at 37°*

Portions (1 ml.) of labelled platelet suspensions were incubated at 37° with shaking for 2 min., and then 10 μ l. of 0.9% NaCl containing the amounts of thrombin indicated were added. Incubation was continued for the times noted. Experimental conditions were as described for Fig. 2.

Thrombin concentration (units/ml. of suspension)	Incubation time (min.) ...	Radioactivity present as ATP (% of total counts)					
		0	1	4	8	16	30
	Concn. of platelets ($10^{-8} \times$ platelets/ml. of suspension)						
0.04	4.32	75	64	52	48	45	—
0.2	4.32	75	53	44	44	38	—
1.0	2.39	80	61	48	—	39	—
5.0	2.39	80	48	42	—	40	—
5.0	2.78	74	—	—	—	37	32

incubated with 0.2 unit of thrombin/ml., and without thrombin, are shown in Fig. 2.

After the platelets had been washed and re-suspended at 0°, most of the radioactivity was present in the platelets as ATP and ADP. Small amounts of AMP and IMP were also present. The amount of radioactivity detected in hypoxanthine was less than 0.5% of the total counts. There was no significant change in the distribution of radioactivity if platelets were kept at 0° for 1 hr. Incubation at 37°, however, caused the breakdown of appreciable amounts of radioactive ATP. In the experiment shown in Fig. 2, 16% of the radioactive ATP had disappeared after 16 min. incubation, and in another experiment the equivalent value was 28%. Radioactive IMP was formed and then decreased in amount, but the radioactive hypoxanthine content increased with increasing incubation time. Radioactive ADP and AMP did not change significantly. With thrombin treatment (0.2 unit/ml. of suspension), the breakdown of radioactive ATP was accentuated, 50% of the radioactive ATP disappearing after 16 min. incubation. About 17% of the total radioactivity was present as IMP after 1 min. incubation with thrombin, but this value had decreased to about 10% after 16 min. incubation. Radioactivity in hypoxanthine increased during the incubation, attaining a value of about 23% of the total counts by 16 min. ADP and AMP radioactivity increased slightly. Small quantities of radioactive inosine were also detected, but accounted for less than 0.8% of the total radioactivity at any incubation time. In the experiment illustrated by Fig. 2, separation of radioactivity was achieved by chromatography with solvent 1 (EDTA). This system does not separate hypoxanthine from adenosine, but, in similar experiments in which separation of radioactive substances was effected

by electrophoresis, little or no adenosine was detected.

The effect of various concentrations of thrombin on the radioactive ATP content is shown in Table 1. Decreasing the concentration of thrombin from 0.2 unit/ml. to 0.04 unit/ml. resulted in a reduction of the rate and extent of breakdown of radioactive ATP. Increasing the concentration of thrombin to 5 units/ml. may have caused an increase of the rate of breakdown of radioactive ATP, but the amount of radioactive ATP after 16 min. incubation was similar to that found with 0.2 unit/ml. Incubation with 5 units of thrombin/ml. for 30 min. showed a further small decrease in the radioactive ATP content.

The results of an incubation of labelled platelets at 15° with 0.2 unit of thrombin/ml., and without thrombin, are shown in Fig. 3. There was no breakdown of radioactive ATP in the control samples; indeed at 7.5 min. and 15 min. incubation times there appeared to be a slight increase of radioactive ATP content. There was no significant change in radioactive ADP, AMP, IMP or hypoxanthine content. The addition of 0.2 unit of thrombin/ml. of suspension caused a decrease of radioactivity in ATP of about 10% of the total counts over the 30 min. incubation period. Small increases of radioactive ADP, AMP, IMP and hypoxanthine contents occurred as compared with the corresponding control values.

Distribution of radioactivity between cellular and supernatant fractions. Table 2 shows the distribution of radioactivity between the cellular and supernatant fractions of labelled platelet suspensions with and without thrombin treatment. Portions of the suspensions were incubated for 10 min. at 37° with 0.04 unit of thrombin/ml., and without thrombin (controls).

Of the total radioactivity, 61% and 76% were

found in the cellular fractions of the thrombin-treated and the control incubation respectively. The major portion of the radioactivity found in the

Fig. 3. Incubation of labelled washed platelets with and without thrombin at 15°. Conditions were as described in Fig. 2, except that the temperature of incubation was 15° and the suspension contained 2.28×10^8 platelets/ml. ■, ATP; ●, ADP; ○, AMP; △, IMP; □, hypoxanthine + adenosine. —, With thrombin; - - - - -, without thrombin.

supernatant fractions was present as hypoxanthine. Of the total radioactivity, 28% and 17% was present as hypoxanthine in the supernatant after the thrombin-treated and control incubations respectively. Nucleotides accounted for 11.7% and 6.7% of the total radioactivity in the thrombin-treated and the control supernatant respectively. The radioactive ATP:radioactive ADP ratio in both the thrombin-treated and the control supernatant fraction was less than 1, whereas the corresponding ratio in the cellular fractions was greater than 3.

For the five experiments the mean results of which are shown in Table 2, five platelet-rich plasmas were incubated with [8- 14 C]adenosine at 37°. Concentrations of radioactive adenosine of 2–6 μ M were added and incubated with platelets for 45–80 min. The results of the individual experiments showed that these variations of incubation conditions did not significantly alter the percentage radioactivity of the substances found in the relevant fractions.

Effect of thrombin on the total and the radioactive ATP and ADP of platelets. Labelled washed platelets were incubated for 10 min. at 37° with 0.04 unit of thrombin/ml., and without thrombin (controls), and the total and the radioactive ATP and ADP contents of the supernatant and cellular fractions were determined. Results are shown in Table 3.

Table 2. Radioactivity of cellular and supernatant fractions of labelled platelet suspensions with and without thrombin treatment

Five samples of platelet-rich plasma were incubated at 37° with 2–6 μ M-[8- 14 C]adenosine for 45–80 min. Washed platelets, suspended in full saline, were prepared and portions were incubated for 10 min. at 37° with and without thrombin (0.04 unit/ml.). The platelet suspensions were separated by centrifugation at 0° into cellular and supernatant fractions, trichloroacetic acid containing carrier compounds was added, and after charcoal treatment and chromatography in solvent 1 the radioactivities of the separated substances were determined. Platelet suspensions contained 1.14×10^8 – 4.68×10^8 platelets/ml. (mean, 3.07×10^8 platelets/ml.). The results are mean values obtained from the five experiments and have been corrected for loss on charcoal. Included in the radioactivity shown as ATP are small amounts of radioactivity attributed to GTP and GDP.

Fraction	Substance	Radioactivity (counts/min./ 10^8 platelets)		
		Control	Thrombin	Change
Cells	ATP	6051	4738	– 1313
	ADP	1539	1556	+ 17
	IMP	155	281	+ 126
	AMP	83	175	+ 92
	Total	7828	6750	– 1078
	ATP:ADP ratio	3.93	3.04	
Supernatant	ATP	218	466	+ 248
	ADP	247	490	+ 243
	IMP	117	193	+ 76
	AMP	111	143	+ 32
	Hypoxanthine	1767	3061	+ 1294
	Total	2460	4353	+ 1893
	ATP:ADP ratio	0.88	0.95	
Cells + supernatant	Total	10288	11103	+ 815

Table 3. *Effect of thrombin on the total and the radioactive ATP and ADP contents of washed platelets*

Three samples of platelet-rich plasma were incubated at 37° with about 2 μ M-[8-¹⁴C]adenosine for 50–60 min. Washed platelets suspended in full saline were prepared and portions were incubated for 2 min. at 37°. Then either thrombin in 0.9% NaCl to give an initial concentration of 0.04 unit/ml. of suspension, or the same volume of 0.9% NaCl (control), was added. Incubation was continued for 10 min. The suspensions were separated by centrifugation at 0° into supernatant and cellular fractions, and after acid extraction and neutralization portions of each extract were assayed for total ATP and ADP and radioactive ATP and ADP. Radioactivity was measured after addition of trichloroacetic acid containing carrier compounds, charcoal treatment and separation of radioactive substances by chromatography in solvent 1; the results are corrected for loss on charcoal. Results are mean values obtained from the three experiments. The mean platelet count was 2.53×10^8 platelets/ml. of suspension. Included in the radioactivity shown as ATP are small amounts of radioactivity attributed to GTP and GDP.

Substance	Fraction	Concn. of substance (μ moles/ 10^8 platelets)		Radioactivity of substance ($10^{-3} \times$ counts/min./ 10^8 platelets)		Specific radioactivity ($10^{-3} \times$ counts/min./ μ mole)	
		Control	Thrombin	Control	Thrombin	Control	Thrombin
ATP	Cells	4.783	3.083	3.82	3.33	0.80	1.08
	Supernatant	0.395	2.253	0.25	0.32	0.63	0.14
	Total	5.178	5.336	4.07	3.65	0.79	0.68
ADP	Cells	3.281	1.264	1.01	1.02	0.31	0.81
	Supernatant	0.435	2.806	0.34	0.38	0.78	0.13
	Total	3.716	4.070	1.35	1.40	0.36	0.34
ATP:ADP ratio	Cells	1.46	2.44	3.78	3.26		
	Supernatant	0.91	0.80	0.74	0.84		
	Total	1.39	1.31	3.01	2.61		

The supernatants derived from the thrombin-treated platelets contained 6.5 and 5.7 times as much of the total ATP and ADP, respectively, as the corresponding control supernatants. After thrombin treatment, 42% and 69% of the total ATP and ADP, respectively, were found in the supernatant fractions. The supernatants derived from the thrombin-treated platelets contained little more radioactive ATP and ADP than the corresponding control supernatants. After thrombin treatment, 9% and 27% of the total radioactive ATP and ADP, respectively, were found in the supernatant fraction.

After thrombin treatment therefore the main portion of the radioactive ATP and ADP remained in the cells, although considerable proportions of the total ATP and ADP were found in the corresponding supernatants.

Total and released ATP and ADP of platelets after thrombin treatment at 15° and 37°. Thrombin at various concentrations was added to suspensions of washed platelets and incubated for 15 min. or 30 min. at 15° or 37°. Cellular and supernatant fractions of these suspensions were prepared, as well as corresponding fractions of portions of the platelet suspensions that were kept at 0° without thrombin treatment. The fractions were assayed for either ATP or ADP content. The sum of the ATP or ADP values of the supernatant and cellular fractions kept at 0° was taken as the initial ATP or

ADP content of the system. Results are shown in Table 4. With 0.2 unit of thrombin/ml., release of ATP and ADP occurred whether the incubation was at 15° or 37°, but, whereas at 37° a loss of total ATP was observed, no loss of total ATP occurred at 15°. At 37°, maximum release of ATP was attained with an initial concentration of 0.07 unit of thrombin/ml. Approximately twice as high a percentage of ADP was released as of ATP.

Effect of thrombin on total and radioactive ATP, ADP and hypoxanthine in the presence and absence of bivalent cations. Labelled washed platelets, suspended either in full saline (containing Ca^{2+} and Mg^{2+}) or in salt solution lacking Ca^{2+} and Mg^{2+} and containing 1.54 mM-EDTA, were treated with thrombin, and the total and the radioactive ATP, ADP and hypoxanthine contents of the cellular and supernatant fractions were determined. Thrombin (0.2 unit/ml.) was added to the suspensions, which were incubated for 15 min. at 37°. Results are shown in Table 5.

Platelets suspended in the incubation medium containing EDTA did not visibly aggregate on addition of thrombin, and ATP and ADP were released to the supernatant in amounts not greatly different from the amounts released when Ca^{2+} and Mg^{2+} were present. After thrombin treatment in the presence of Ca^{2+} and Mg^{2+} , about 51% of the total ATP and 78% of the total ADP present in the cellular plus supernatant fractions were found in

Table 4. *Effect of various concentrations of thrombin on the released and total ATP and ADP contents of washed platelets at 37° and 15°*

Portions (3 ml.) of washed platelets were incubated with shaking at either 37° or 15° for 2 min., the amounts of thrombin indicated were added in 10 μ l. amounts of 0.9% NaCl and incubation was continued for the times noted. The samples were rapidly cooled in ice and the supernatant and cellular fractions were separated by centrifugation at 0°. These fractions were assayed for ATP or ADP content as described in the text. Portions of platelet suspension not incubated with thrombin and kept at 0° throughout were also separated into the two fractions. The sum of the values obtained for the latter fractions were taken as the initial ATP or ADP content of the system.

Substance assayed	Thrombin concentration (units/ml. of suspension)	Temp.	Time of incubation (min.)	Concn. of platelets ($10^{-8} \times$ platelets/ml. of suspension)	Nucleotide content (% of initial total)			
					Supernatant	Cells	Total	Change
ATP	0	0°	—	2.76	0	100	100	0
	0.07	37	15	2.76	37	31	68	-32
	0.33	37	15	2.76	37	31	68	-32
	1.67	37	15	2.76	34	24	58	-42
	0.20	37	15	2.49	35	41	76	-24
	0.20	37	30	2.49	29	38	67	-33
	0.20	15	15	2.28	28	75	103	+ 3
	0.20	15	30	4.95	40	63	103	+ 3
ADP	0	0	—	7.35	2	98	100	0
	0.20	37	15	7.35	73	22	95	- 5
	0	0	—	2.45	7	93	100	0
	0.20	15	15	2.45	60	41	101	+ 1
	0.20	15	30	2.45	73	30	103	+ 3

Table 5. *Effect of thrombin on the total and the radioactive ATP, ADP and hypoxanthine contents of washed platelets, incubated with and without bivalent cations*

Two samples of platelet-rich plasma were incubated at 37° with about $2 \mu\text{M}$ -[8- ^{14}C]ADP for 2 hr. Two lots of washed platelets were prepared, one containing 5.73×10^8 platelets/ml. of full saline (see the Methods section), the other containing 4.62×10^8 platelets/ml. of solution containing Na^+ , K^+ , glucose and EDTA (see the Methods section). Portions of both suspensions were incubated at 37° for 2 min.; then thrombin was added to give an initial concentration of 0.2 unit/ml. of suspension. Incubation was continued for 15 min. The suspensions were separated by centrifugation at 0° into supernatant and cellular fractions and, after acid extraction and neutralization, portions of each extract were assayed for total and radioactive ATP, ADP and hypoxanthine. Radioactivity was measured after addition of trichloroacetic acid containing carrier compounds, charcoal treatment and separation of radioactive substances by chromatography in solvent 1 (EDTA); the results are corrected for loss on charcoal. Included in the radioactivity shown as ATP were small amounts of radioactivity attributed to GTP and GDP. Results shown for total hypoxanthine content are the mean values obtained from assays that measured hypoxanthine and hypoxanthine plus xanthine (see the Methods section).

Incubation medium	Substance	Concn. of substance ($\mu\text{moles}/10^8$ platelets)			Radioactivity of substance ($10^{-3} \times$ counts/min./ 10^8 platelets)			Specific radioactivity ($10^{-3} \times$ counts/min./ μmole)		
		Supernatant	Cells	Total	Supernatant	Cells	Total	Supernatant	Cells	Total
+ Mg^{2+} and Ca^{2+}	ATP	2.77	2.69	5.46	0.44	4.61	5.05	0.16	1.71	0.92
	ADP	2.43	0.68	3.11	0.54	1.22	1.76	0.22	1.79	0.57
	Hypoxanthine	1.22	—	1.22	2.44	—	2.44	2.00	—	2.00
	Total	6.42	3.37	9.79	3.42	5.83	9.25			
	ATP:ADP ratio	1.14	3.96	1.76	0.81	3.78	2.87			
+ EDTA	ATP	2.29	3.74	6.03	0.82	5.37	6.19	0.36	1.44	1.03
	ADP	3.42	0.82	4.24	0.22	1.36	1.58	0.06	1.66	0.37
	Hypoxanthine	0.95	—	0.95	2.47	—	2.47	2.60	—	2.60
	Total	6.66	4.56	11.22	3.51	6.73	10.24			
	ATP:ADP ratio	0.67	4.56	1.42	3.73	3.95	3.92			

the supernatant. The corresponding values for the medium containing EDTA were 38% and 81% respectively. The ratio of total ATP to total ADP in the supernatant fractions was about 1 in the presence of Ca^{2+} and Mg^{2+} , and about 0.7 in the absence of bivalent cations.

About 13% of the total radioactivity present as ATP plus ADP was found in the supernatant fractions of both incubation mixtures. However, the ratio of radioactive ATP to radioactive ADP was 3.7 for the supernatant fractions containing EDTA and 0.8 when Ca^{2+} and Mg^{2+} were present.

The values obtained for the specific radioactivities of the ATP and the ADP of the cellular fractions, after incubation of each medium with thrombin, were similar (Table 5). The hypoxanthine that appeared in the supernatant fractions after thrombin treatment had a slightly greater specific radioactivity than the ATP and ADP that remained in the cells.

Comparison of total and radioactive nucleotide contents before and after thrombin treatment. Labelled washed platelets suspended in full saline were divided at 0° into two portions. One portion was kept at 0° and the other was incubated at 37° for 15 min. with 0.2 unit of thrombin/ml. of suspension. The latter portion was cooled to 0° and both

portions were separated into supernatant and cellular fractions. The total and the radioactive ATP, ADP, AMP, IMP + inosine and hypoxanthine contents of the fractions were determined. The mean results of two experiments are shown in Table 6.

After thrombin treatment, the sum of the values obtained by assays of total amounts of the substances was about 10% greater than the sum of the equivalent values obtained before thrombin treatment. This might indicate that thrombin treatment caused the formation of one or more of these substances from precursors not assayed by the methods used, but the sum of the values obtained by assays of radioactivity of the substances was also about 10% greater after thrombin treatment than before it. Since radioactivity cannot be gained, the differences noted are most likely to be due to an error in dividing the two portions of the platelet suspension.

After thrombin treatment the ATP content of the system (supernatant + cellular fractions) had decreased by about 16%. As described above, considerable amounts of ATP and ADP were found in the supernatant fraction after thrombin treatment, but most of the radioactive ATP and ADP remained in the cellular fraction. About 82% of the

Table 6. *Comparison between total and radioactive nucleotide contents of washed platelets before and after thrombin treatment*

Platelet-rich plasma was incubated at 37° for 1 hr. with about $2\mu\text{M}$ -[8- ^{14}C]adenosine. After cooling to 0° and addition of EDTA, the platelets were washed, resuspended in full saline and divided into two portions. One portion was kept at 0°. The other was incubated at 37° for 2 min., and then thrombin was added to give an initial concentration of 0.2 unit/ml. of suspension. Incubation was continued for 15 min., the aggregated platelets were rapidly cooled to 0° and, along with the other non-aggregated portion of platelets, separated into supernatant and cellular fractions. The cellular fractions were washed with tris-NaCl-EDTA solution, which was discarded. After acid extraction and neutralization, portions of each extract were assayed for total and radioactive ATP, ADP, AMP, IMP + inosine and hypoxanthine. Other conditions were as given in Table 5. Results shown are the mean values of two experiments. The suspensions contained 5.41×10^8 and 4.77×10^8 platelets/ml. (mean 5.09×10^8 platelets/ml.).

		Concn. of substance ($\mu\text{moles}/10^8$ platelets)			Radioactivity of substance ($10^{-3} \times \text{counts/min.}/10^8$ platelets)			Specific radioactivity ($10^{-3} \text{ counts/min.}/\mu\text{mole}$)		
	Substance	Supernatant	Cells	Total	Supernatant	Cells	Total	Supernatant	Cells	Total
Before thrombin	ATP	0.24	7.67	7.91	0.27	8.63	8.90	1.13	1.13	1.13
	ADP	0.23	4.82	5.05	0.16	1.91	2.07	0.70	0.40	0.41
	AMP	0.19	0.92	1.11	0.06	0.20	0.26	0.32	0.22	0.23
	IMP + inosine	0.06	0.30	0.36	0.04	0.34	0.38	0.67	1.13	1.06
	Hypoxanthine	—	—	—	0.04	0.03	0.07			
	Total	0.72	13.71	14.43	0.57	11.11	11.68			
	ATP:ADP ratio	1.04	1.59	1.57	1.69	4.52	4.30			
After thrombin	ATP	3.38	3.28	6.66	0.37	4.92	5.29	0.11	1.50	0.79
	ADP	4.04	1.11	5.15	0.55	1.53	2.08	0.14	1.38	0.40
	AMP	1.34	0.30	1.64	0.25	0.32	0.57	0.19	1.07	0.35
	IMP + inosine	0.42	0.50	0.92	0.55	0.92	1.47	1.31	1.84	1.60
	Hypoxanthine	1.58	—	1.58	3.34	0.13	3.47	2.11	—	2.20
	Total	10.76	5.19	15.95	5.06	7.82	12.88			
	ATP:ADP ratio	0.84	2.95	1.29	0.67	3.22	2.54			

AMP present after thrombin treatment was found in the supernatant fraction; the corresponding value for radioactive AMP was 44%. About 47% of the IMP + inosine present after thrombin treatment was found in the supernatant fraction; the corresponding value for radioactive IMP + inosine was 36%. Hypoxanthine was not assayable in the cellular fraction after thrombin treatment. A small amount of radioactive hypoxanthine was, however, detected in this fraction, even though the cells were washed during the extraction procedure.

The values obtained for the specific radioactivities of the ATP, ADP and AMP found in the supernatant fraction after thrombin treatment were similar to one another but were about 7–19 times less than the specific radioactivities found for IMP + inosine and hypoxanthine in the supernatant fraction. The latter values were similar to those found for all the substances in the cellular fraction after thrombin treatment.

Expressed as a percentage of the relevant total value of the substances found in the cellular fraction after thrombin treatment, 63.2% was found as ATP and 63.0% as radioactive ATP. The corresponding values for the other substances were: ADP, 21.4% and 19.6%; AMP, 5.8% and 4.1%; IMP + inosine, 9.6% and 11.8%, respectively.

DISCUSSION

Haslam (1966) showed that once-washed human platelets treated with bovine thrombin (0.017 unit/ml.) had aggregated completely after 15 min. incubation at 37°, and had by this time released to the supernatant about 24% of the ATP and 42% of the ADP present after this period of incubation. The ATP:ADP ratio appearing in the supernatant was about 0.73. The ATP content of the system decreased by some 8% of the initial value and the ADP content increased by about 8% of the initial value during incubation. With higher concentrations of thrombin, there was a greater decrease of the initial ATP content but no equivalent increase in ADP content. The results reported in the present paper showed that about 42% of the ATP and 69% of the ADP remaining after 10 min. incubation at 37° with 0.04 unit of thrombin/ml. were found in the supernatant (Table 3). The corresponding values for ATP and ADP after 15 min. incubation at 37° with 0.2 unit of thrombin/ml. of suspension were 51% and 78% respectively (Table 6). The ATP:ADP ratios in the supernatants were 0.8–1.1, and with 0.2 unit of thrombin/ml. there was a decrease of 16–24% of the initial ATP content (Tables 4 and 6).

In contrast, of the radioactive ATP and ADP present after incubation at 37° with 0.04 and 0.2 unit of thrombin/ml., only some 7–9% of the radio-

activity in ATP and 26–31% of the radioactivity in ADP were found in the supernatant (Tables 3, 5 and 6). Part of the radioactive ATP was degraded during thrombin treatment, predominantly to IMP and hypoxanthine (Fig. 2 and Table 2). This degradation of radioactive ATP also occurred to a lesser extent in platelets incubated at 37° without thrombin, and indicates an inherent instability of ATP in washed platelets (Fig. 2 and Table 2).

The failure of thrombin to release the radioactive ATP and ADP from platelets in the same proportion as the total ATP and ADP were released implies the presence of at least two nucleotide pools, one containing most of the radioactive ATP and ADP, which is not released, and another containing less radioactive ATP and ADP, which is released by the action of thrombin. This finding agrees with that reported by Holmsen (1965*a,b,c*), in which ^{32}P incorporated into ATP and ADP in human and rabbit platelets suspended in plasma was measured; treatment of these platelets with collagen caused a decay of radioactive ATP but not of radioactive ADP in the cellular fractions, whereas the supernatant fractions contained considerable amounts of ADP and serotonin but little radioactive ATP and ADP.

The question arises whether the radioactive ATP and ADP found in the supernatant, which was about 8–9% of the total radioactivity, was part of the nucleotide pool specifically released by thrombin or was due to leakage from the other pool, perhaps caused by damage to the platelets. The results shown in Table 5 suggest the latter explanation. Grette (1962) and Zieve, Gamble & Jackson (1964) showed that washed platelets, when treated with thrombin at 37° in a medium lacking bivalent cations, released adenine nucleotide. With labelled human platelets under similar conditions but with much lower thrombin concentrations, both ATP and ADP were found to be released in amounts not greatly different from those obtained with an incubation medium containing Ca^{2+} and Mg^{2+} (Table 5). The extracellular ATP:ADP ratio was 0.7–1.1 irrespective of the incubation medium. The radioactive ATP:radioactive ADP ratio of the extracellular fluid, however, was about 0.8 if Ca^{2+} and Mg^{2+} were present in the medium but was 3.7 if EDTA was present and the bivalent cations were omitted. The latter value was similar to that obtained for the radioactive and the total ADP:ADP ratios for the fractions remaining in the cells. It appears that an enzyme(s) is available to the supernatant that in the presence of Ca^{2+} and Mg^{2+} causes the conversion of part of the radioactive ATP appearing in the supernatant into ADP, to give the observed radioactive ATP:radioactive ADP ratio of about 0.8. The total ATP and ADP released appear in the supernatant at about this

ratio whether the enzyme is inhibited by EDTA or not. Hence it seems likely that the ATP and ADP released were present in the platelets in these proportions before thrombin treatment, whereas the radioactive ATP and ADP found in the supernatant were originally in the platelets at the ratio of about 3.8:1, which was subsequently converted into a ratio of 0.8:1 during thrombin treatment. This indicates that the radioactive ATP and ADP in the supernatant may arise through leakage from the cells rather than from the ATP and ADP pool released by the action of thrombin. If this is so, the latter ATP and ADP is devoid of radioactivity and thus this pool is not in rapid equilibrium with externally applied [8-¹⁴C]adenosine.

If all of the ATP and ADP of the non-radioactive pool were released by thrombin, the specific radioactivities of the ATP and ADP remaining in the cells would be the same if there is one pool remaining or two pools remaining, each having the same ATP:ADP ratio. The results given in Tables 5 and 6 show that, with 0.2 unit of thrombin/ml., the specific radioactivities of the ATP and ADP remaining were similar.

Radioactive hypoxanthine appeared in the supernatant after thrombin treatment. The specific radioactivity of the hypoxanthine was greater than the specific radioactivities of the ATP, ADP, AMP and IMP + inosine found for the cellular fraction after treatment with thrombin (Tables 5 and 6). This indicates that the hypoxanthine is formed from the pool or pools of readily labelled nucleotide, and is not to any great extent formed from the pool of nucleotide released by the action of thrombin. The value of the specific radioactivity of the IMP + inosine appearing in the supernatant after thrombin treatment also indicates that these substances are derived from the readily labelled nucleotide pool, but the value of the specific radioactivity of the AMP in the supernatant indicates that AMP is derived largely from the non-labelled pool released by the action of thrombin (Table 6).

Since the value of the specific radioactivity of hypoxanthine appeared to be higher than the values of the specific radioactivities of ATP and ADP remaining in the cells, it is probable that two radioactive pools exist.

The results given in Table 6 show that during thrombin treatment about 16% of the initial ATP content was lost. The ADP content probably remained unchanged and the AMP, IMP + inosine and hypoxanthine contents increased. The size of the labelled pool in terms of ATP + ADP should be

approximately given by the sum of the ATP and ADP contents remaining in the cells after thrombin treatment and the amounts of hypoxanthine and IMP + inosine formed. A small amount of ATP and ADP from the labelled pool was assumed to have leaked from the platelets during thrombin treatment and this amount was calculated from the mean specific radioactivity of ATP and ADP found in the cellular fraction and added to the other values. The size of the labelled pool, thus calculated from the values given in Table 6, was about 55% of the initial ATP + ADP content.

The breakdown of ATP in the labelled and non-released pool of nucleotide is not necessarily connected with the release action of thrombin, since after incubation at 15° there appears to be little or no breakdown of ATP, and yet large amounts of ATP and ADP were released to the supernatant (Fig. 3 and Table 4). Similar results were obtained by Grette (1962) with washed pig platelets. Platelets depleted of ATP by thrombin treatment are, however, capable of supporting clot retraction (Zieve *et al.* 1964), a process that depends on the availability of ATP or an ATP-generating systems.

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