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Secretory Mechanisms

BEHAVIOUR OF ADENINE NUCLEOTIDES DURING THE PLATELET RELEASE REACTION INDUCED BY ADENOSINE DIPHOSPHATE AND ADRENALINE

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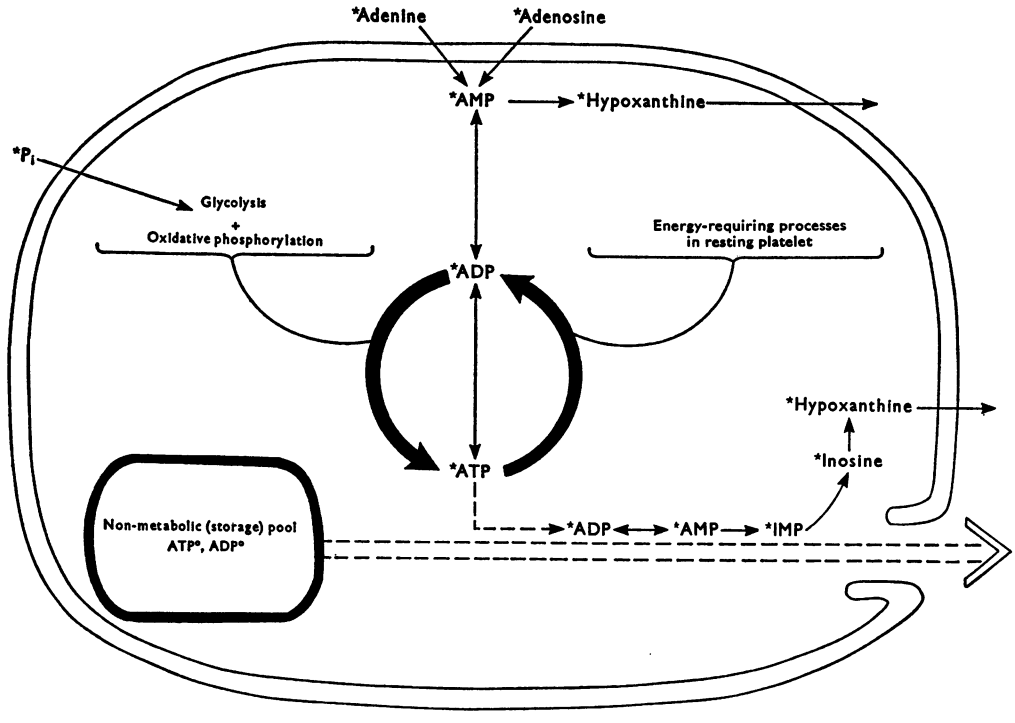
1. Platelets containing adenine nucleotides labelled with ^{3}H and ^{14}C *in vitro* were aggregated biphasically with ADP and adrenaline. Amounts of ATP and ADP as well as the radioactivity of ATP, ADP, AMP, IMP, hypoxanthine and adenine were determined in platelets and plasma at different stages of aggregation. 2. ATP and ADP were released during the second aggregation phase and had a low specific radioactivity compared with the ATP and ADP retained by the cells. The specific radioactivity of intracellular nucleotides increased during release. The parameters observed with ADP and adrenaline as release inducers were the same as for collagen and thrombin. 3. Release induced by all four inducers was accompanied by conversion of cellular [^{3}H]ATP into extracellular [^{3}H]-hypoxanthine. By variation of temperature, inducer concentration, time after blood withdrawal and use of acetylsalicylic acid, the aggregation pattern caused by adrenaline and ADP could be made mono- or bi-phasic. Release or second-phase aggregation was intimately connected with the ATP–hypoxanthine conversion, whereas first phase aggregation was not. 4. The [^{3}H]ATP–hypoxanthine conversion started immediately after ADP addition. With adrenaline it usually started with the appearance of the second aggregation phase. The conversion was present during first phase of ADP-induced aggregation only if a second phase were to follow. 5. When secondary aggregation took place while radioactive adenine was being taken up by the platelets, increased formation of labelled hypoxanthine still occurred, but there was either no change or an increase in the concentration of labelled ATP. 6. Biphasically aggregated platelets converted [^{3}H]adenine more rapidly into [^{3}H]-ATP and -hypoxanthine than non-aggregated platelets. Addition of [^{3}H]adenine at different stages of biphasic aggregation showed that more [^{3}H]hypoxanthine was formed during than after the release step. 7. We conclude that ADP and adrenaline, like thrombin and collagen, cause extrusion of non-metabolic granula-located platelet adenine nucleotides. During release metabolic ATP breaks down to hypoxanthine, and this process might reflect an ATP-requiring part of the release reaction.

Rapid secretion of substances from platelets is induced by numerous compounds (Mustard & Packham, 1970), and may occur by one mechanism, the platelet-release reaction (Holmsen *et al.*, 1969*b*): specific extrusion into the cells' environment of the contents of subcellular platelet granules (adenine nucleotides, 5-hydroxytryptamine, metal ions, acid hydrolases etc.). Platelets thus provide an excellent model for study of two basic steps in secretion: storage and extrusion (Stormorken, 1969).

At 37°C adrenaline (O'Brien, 1963) and ADP (Constantine, 1966; MacMillan, 1966; Hardisty *et al.*, 1970) produce a biphasic aggregation of human platelets; a platelet-aggregating substance, inactivated by ADPase* (Haslam, 1967), accumulates extracellularly during the second phase. Mills *et al.* (1968) showed that both ADP and ATP are released, with the [ATP]/[ADP] ratio lower than in intact platelets, but similar to that of platelet granules (Holmsen *et al.*, 1969*a*). Platelets readily take up and store 5-hydroxy[^{14}C]tryptamine in the dense granules (Minter & Crawford, 1967). Radioactivity from such labelled platelets was released and several non-granular enzymes were retained during the second phase of ADP- and adrenaline-induced aggregation (Mills *et al.*, 1968). These observations support, but do not prove, our view (Holmsen *et al.*, 1969*b*) that ADP and adrenaline induce the platelet release reaction.

Collagen (Holmsen, 1965; Holmsen *et al.*, 1969*a*), thrombin (Ireland, 1967; Holmsen & Day, 1970), latex particles and NaF (Mürer, 1969) release the non-metabolic or storage pool of platelet adenine

* Abbreviation: ADPase, adenosine diphosphatase.



Scheme 1. *Representation of the adenine nucleotide metabolism in resting and release-stimulated platelets*

Radioactive precursors (*P_i, *adenine and *adenosine) can enter the cell and label the metabolic pool of platelet nucleotides; all members of this pool are designated by *. The fairly good balance between ATP resynthesis via glycolysis+oxidative phosphorylation, and the energy-utilizing processes in the resting cell, keeps the concentrations of metabolic ATP, ADP and AMP in the platelet-rich plasma almost constant for hours. However, a small amount of the metabolic nucleotides in the resting cells is catabolized to hypoxanthine, which diffuses into the medium (upper right). In human platelets about two-thirds of the adenine nucleotides form the non-metabolic (storage) pool (°), are stored in granules and do not participate in metabolism. Upon release-stimulation by thrombin and collagen this pool is extruded through channels to the surrounding medium (broken, double-lined arrow). Concomitant with this extrusion a certain amount of metabolic ATP is rapidly degraded to hypoxanthine, which diffuses out of the cell.

nucleotides. This pool (two-thirds of adenine nucleotides in human platelets) is not labelled *in vitro* by radioactive adenosine, adenine or orthophosphate, and is stored in subcellular granules (Holmsen *et al.*, 1969*a*). The metabolic pool present in cytosol, membranes and mitochondria, is labelled *in vitro*, and is retained during the release reaction. During release induced by thrombin and collagen, a certain fraction of metabolic ATP is converted into IMP and hypoxanthine (Ireland, 1967; Holmsen *et al.*, 1969*a*). Scheme 1 summarizes platelet adenine nucleotide compartmentalization and behaviour during the release reaction.

The present study describes the metabolic nature of the adenine nucleotides that are released and retained, as well as their intracellular conversions during ADP- and adrenaline-platelet interaction. Comparison has been made with thrombin- and collagen-platelet interaction. The behaviour of radioactive adenine metabolites during biphasic aggregation at different stages of [^{3}H]adenine uptake has also been studied.

Experimental

Materials

Chemicals. ADP (disodium salt from equine muscle; Sigma Chemical Co., St. Louis, Mo., U.S.A.), was stored in small portions (400μg/ml in 0.15M-NaCl) at −60°C and diluted with 0.15M-NaCl to the desired concentrations immediately before use. The

exact concentration of ADP stock solutions was determined as described by Holmsen & Storm (1969).

L-Adrenaline bitartrate from Winthrop Laboratories (New York, N.Y., U.S.A.) was stored at a concentration of 5mg/ml in 0.15M-NaCl at −60°C and thawed immediately before use. Solutions were kept on ice during the experiment and discarded afterwards.

Acetylsalicylic acid (United States Pharmacopeia grade; Merck, Rahway, N.J., U.S.A.), was dissolved in 0.12M-$NaHCO_3$ and stored at 4°C. Soluble collagen was prepared and stored as described by Holmsen & Weiss (1972). It was used in an undiluted form.

Radiochemicals. [G-^{3}H]Adenine (NET-063, New England Nuclear Corp., Boston, Mass., U.S.A.), 6000mCi/mmol, was supplied as 0.113mg of adenine in 5.0ml of water. It was diluted with 0.15M-NaCl to 16.7 or 40μM, and these stock solutions were stored in portions at −60°C. They were refrozen after use.

[8-^{14}C]Adenosine (NEC-524, New England Nuclear Corp.) 33.5mCi/mmol in aq. 50% (v/v) ethanol, was dried with a N_2 jet (room temperature) and redissolved to a concentration of 308μM in 0.15M-NaCl and kept at −60°C. It was refrozen after use.

[U-^{14}C]Adenine (Schwarz Bio-Research, Orangeburg, N.Y., U.S.A.), 231mCi/mmol, was dissolved to a concentration of 100μM in 0.15M-NaCl and stored at −60°C. A 54μM solution was prepared in 0.15M-NaCl before use.

Enzymes. Bovine thrombin (EC 3.4.4.13) (Topical) was obtained from Parke, Davis and Co. (Detroit, Mich., U.S.A.) and contained 0.1mg of benzethonium chloride per 5000 National Institute of Health units and traces of contaminatory $CaCl_2$, NaCl and glycine. Portions of a stock solution (100 units/ml of 0.15M-NaCl) were stored at −60°C. They were diluted to the desired concentrations, and kept on ice during use, then discarded afterwards.

Biological materials

Platelet-rich plasma. Venous blood (9vol.) from healthy donors, not having taken aspirin within the preceding week, was drawn into 0.11M-disodium citrate (1vol.). The blood was centrifuged at room temperature for 15min at $180g_{max.}$. The supernatant platelet-rich plasma was pipetted off and used immediately.

Platelet-poor plasma. The remainder of the blood after removal of platelet-rich plasma was centrifuged at room temperature in 1ml portions for 2min at 12000rev./min in an Eppendorf 3200 microcentrifuge. The supernatant platelet-poor plasma was pipetted off.

Radioactive platelet-rich plasma. [U-^{14}C]Adenine-labelled platelet-rich plasma. Platelet-rich plasma 50vol.) was incubated with 54μM-[U-^{14}C]adenine at 37°C for various periods of time.

[^{3}H]Adenine-labelled platelet-rich plasma. Two different concentrations of [^{3}H]adenine in platelet-rich plasma were used. Incubation mixture 1 contained 25μl of 16.7μM- or 10μl of 40μM-[^{3}H]-adenine/ml of platelet-rich plasma and incubation mixture 2 contained 75μl of 16.7μM- or 30μl of 40μM-[^{3}H]adenine/ml of platelet-rich plasma. The [^{3}H]adenine in incubation mixture 1 was fully incorporated by the platelets after 45min at 37°C or 60–75min at room temperature. Incubation mixture 2 was used to study adenine uptake during aggregation.

[8-^{14}C]Adenosine-labelled platelet-rich plasma. Platelet-rich plasma (20vol.) was incubated for 60min at 37°C with 308μM-[8-^{14}C]adenosine (1vol.).

Methods

Platelet aggregation. This was measured by reading the changes of light-transmission when light was passed through platelet-rich plasma. The deflection of each platelet-rich plasma was adjusted to 10% of full scale on the recorder with the autologous platelet-poor plasma to give a slightly off-scale (105–120% of the full scale) reading. The experiments were done in an EEL titrator (Evans Electroselenium Ltd., Halstead, Essex, U.K.) provided with a 605 filter and a constant temperature-controlled copper block, and connected via a galvanometer (EEL, Unigalvo, type 200; Evans Electroselenium Ltd.) to one of the following recorders: Heath Servo (model EU 20B), Sargent (model SRL) or Perkin–Elmer (Coleman 165; from Norwalk, Conn., U.S.A.). With the last-named the circuit was fed with a variable backing-off e.m.f. (0–12mV) derived from a mercury cell to obtain full-scale zero suppression. The heating block had several copper adaptors for cuvettes of variable size, to facilitate heat conduction from the heating block to the cuvettes (6–8mm inner diam., round-bottomed). The cuvette contents were stirred by a plastic-covered magnetic stirrer (5mm long × 4.5mm diam.) at the titrator's maximum speed.

Radioactive platelet-rich plasma (1.8–2.0ml) was pipetted into the cuvette, and after temperature equilibration for 1–2min, 0.2ml of ADP or adrenaline was added. At noted times, 200- or 100-μl samples were rapidly transferred into plastic tubes containing 200 or 100μl respectively of EDTA–ethanol (Holmsen *et al.*, 1971). The plasma–EDTA–ethanol mixtures were kept in ice until removal of precipitates by centrifugation (4°C, 10min, $17500g_{max.}$. The supernatants, referred to as EDTA–ethanol extracts, were kept at −20°C or −60°C until analysed, usually within 2–3 days.

The smaller cuvettes could be centrifuged, which made it possible to separate cells and plasma before

Table 1. *Distribution of amounts and radioactivity of ATP and ADP in platelets and plasma from the aggregation experiment with ADP illustrated in Fig. 1*

The values for plasma ADP are corrected for the ADP added externally.

Treatment of platelet-rich plasma	Amount of nucleotide (μmol/10^{11} platelets)				$10^{-3}\times$Total radioactivity (c.p.m./10^{11} platelets)				Specific radioactivity (c.p.m./nmol)			
	Platelets		Plasma		Platelets		Plasma		Platelets		Plasma	
	ATP	ADP	ATP	ADP	ATP	ADP	ATP	ADP	ATP	ADP	ATP	ADP
NaCl (480s)	5.2	2.9	0.10	0.01	21406	4890	78	11	4110	1959	781	106
ADP (120s)	4.9	2.4	0.10	0.11	22990	4410	50	0	4685	1833	502	0
ADP (280s)	4.3	2.0	0.31	0.52	20750	5180	163	0	4829	2595	542	0
ADP (480s)	3.6	1.6	0.60	1.21	20650	5400	188	119	5733	3773	313	99

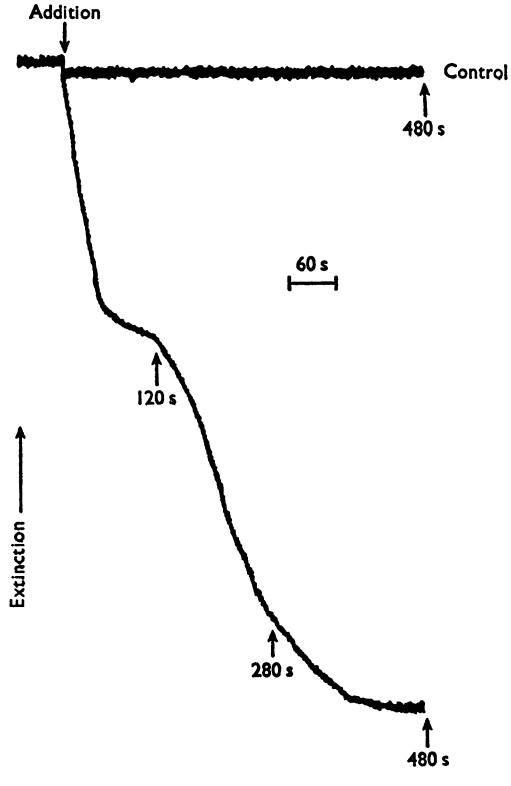


Fig. 1. *Aggregation of platelet-rich plasma caused by ADP*

Changes in extinction during stirring of 1ml of platelet-rich plasma (2.7×10^8 platelets/ml) with 0.1ml of 0.15M-NaCl (control) or 13.2μM-ADP–0.15M-NaCl at 37°C in an EEL platelet-aggregation meter. The plasma was previously incubated at room temperature with 1.7μM-[U-^{14}C]adenine for 100min. At the times indicated the cuvette contents were poured into ice-cooled tubes containing 0.1ml of 0.1M-EDTA. The tubes were centrifuged, and amounts and radioactivities of ATP and ADP were determined; the results are given in Table 1. The tracing shown is that obtained with the sample stirred for 480s, and fitted well with the composite tracing from samples stirred for shorter periods of time.

extraction with EDTA–ethanol. Before centrifugation (4°C, 10min, 17500$g_{max.}$) 0.1ml of 0.1M-EDTA, pH7.4 was added/ml of cuvette contents. After centrifugation, the plasma was decanted off and mixed with aq. 96% ethanol (1:1, v/v). The tubes were drained well and their inside walls were wiped before the cells were suspended in the ice-cold solution described by Haslam (1964) (with the EDTA concentration increased to 4.8mM) in a volume equal to that of the mixture they were isolated from. Finally, one volume of aq. 96% ethanol was added. Both types of ethanol mixtures were kept on ice with occasional stirring for 10min, and the sediments were removed by centrifugation as described above. The supernatants, referred to as 'EDTA–ethanol extracts', were saved and stored as described above.

In some experiments an EEL platelet-aggregation meter (model 169, Evans Electroselenium Ltd.) was used (37°C). The cuvette contents were poured into ice-cold 5ml plastic tubes containing 0.1ml of 0.1M-EDTA/ml of cuvette contents, and centrifuged, then extracted and stored as described above.

Determination of ATP and ADP. The concentrations of the adenine nucleotides were determined in the EDTA–ethanol extracts by a luciferin–luciferase method (Holmsen *et al.*, 1972).

Determination of adenine and adenosine metabolite radioactivity. This was done as described previously (Holmsen & Weiss, 1970).

Platelet counting. Platelets were counted by phase-contrast microscopy or in a Coulter counter, model B.

Table 2. *Distribution of amounts and radioactivity of ATP and ADP in platelets and plasma from the aggregation experiment with adrenaline illustrated in Fig. 2*

For details see Fig. 2 and Fig. 1.

Treatment of platelet-rich plasma	Amount of nucleotide (μmol/10^{11} platelets)				10^{-3} x Total radioactivity (c.p.m./10^{11} platelets)				Specific radioactivity (c.p.m./nmol)			
	Platelets		Plasma		Platelets		Plasma		Platelets		Plasma	
	ATP	ADP	ATP	ADP	ATP	ADP	ATP	ADP	ATP	ADP	ATP	ADP
NaCl (480s)	7.3	5.3	0.4	0.1	43804	7711	510	310	5906	1452	1272	3108
Adrenaline (30s)	7.3	4.7	0.4	0.1	42093	8281	598	0	5759	1763	1498	0
Adrenaline (205s)	4.8	1.9	2.1	3.2	38318	8031	237	200	7990	4222	113	62
Adrenaline (480s)	5.1	2.0	1.8	3.4	39065	9793	361	187	7631	4899	200	55

Results

Release of non-metabolic ATP and ADP during the second phase of ADP- and adrenaline-induced platelet aggregation

Figs. 1 and 2 show recorder tracings of biphasic platelet aggregation produced by ADP and adrenaline respectively in [U-^{14}C]adenine-labelled platelet-rich plasma. Tables 1 and 2 show the amounts and radioactivity of platelet ATP and ADP in platelets and plasma at different stages of aggregation in the corresponding experiments. In both cases, 30–50% of platelet ATP and ADP were released during the second phase of aggregation, and the specific radioactivity of these nucleotides was low compared with that of the nucleotides remaining in the platelets. After release the specific radioactivity of cellular ATP and particularly ADP was higher than in the control cells or during the first phase of aggregation. Use of [8-^{14}C]adenosine- or [^{3}H]-adenine-labelled platelet-rich plasma gave identical results.

Changes in distribution of adenine metabolites after biphasic platelet aggregation caused by ADP and adrenaline in platelet-rich plasma after adenine uptake

Adenine added to platelet-rich plasma is exclusively metabolized by the platelets (Holmsen & Rozenberg, 1968) to cellular ATP, ADP, AMP, IMP and hypoxanthine, the last-named appearing in plasma. This distribution can be seen from Table 3 (NaCl-treated platelet-rich plasma). After biphasic aggregation by adrenaline the intracellular concentration of radioactive ATP had decreased by more than 20%. This was mainly accounted for by an increase in the radioactivity of extracellular hypoxanthine, in particular, and cellular IMP and hypoxanthine (Table 3). Biphasic aggregation induced by ADP gave similar results. The changes in the ADP radioactivity during

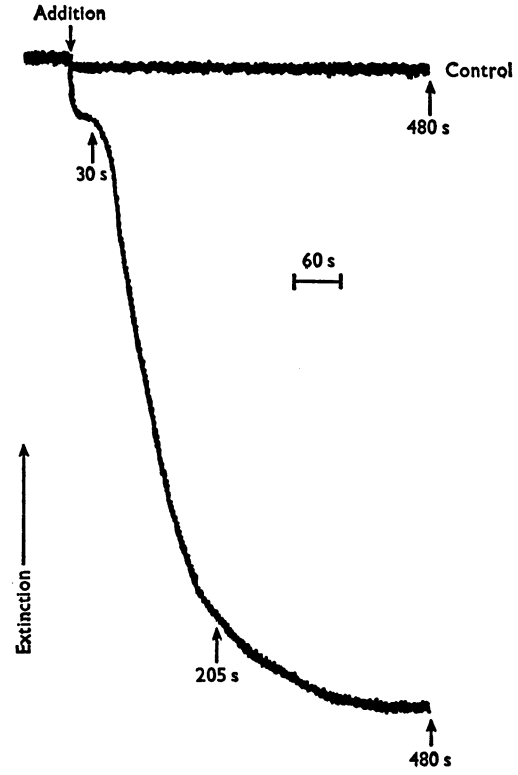


Fig. 2. *Aggregation of platelet-rich plasma caused by adrenaline*

The experiment was the same as in Fig. 1, except that 24.2μM-adrenaline–0.15M-NaCl was added instead of ADP. The platelet-rich plasma contained 3.2×10^{8} platelets/ml and was preincubated with radioactive adenine for 120min. The results of the determinations are given in Table 2.

Table 3. *Effect of adrenaline on [^{14}C]adenine metabolites in platelet-rich plasma*

Platelet-rich plasma (3.83×10^8 platelets/ml) was incubated for 2h at 37°C with [U-^{14}C]adenine (2.7 μM initial concentration). A sample (2ml) of this labelled plasma was stirred in an EEL platelet-aggregation meter with 0.2ml of 22 μM-adrenaline; another 2ml sample was stirred with 0.2ml of 0.15M-NaCl at 37°C for 8min. The reaction was terminated with 0.2ml of 0.077M-EDTA (pH7.4) followed by cooling on ice. The cells and medium were separated by centrifugation and the metabolite radioactivities were determined.

Treatment of platelet-rich plasma		$10^{-3} \times$ Radioactivity (c.p.m./10^{11} platelets)						
		ATP	ADP	AMP	IMP	Hypoxanthine	Adenine	Total
NaCl	Platelets	52675	14281	1088	1559	2073	141	84462
	Plasma	291	195	147	54	11820	138	
Adrenaline	Platelets	40222	12376	1173	2974	3242	80	86070
	Plasma	2360	741	170	85	22510	137	

biphasic aggregation were variable; both decreases (Table 3) and increases (Tables 1 and 2) were noted. Small but varied amounts of ATP and ADP radioactivity were present in extracts of plasma from biphasically aggregated platelet-rich plasma (see Tables 3 and 4). This has been considered to indicate unspecified lysis of few platelets during release (Day & Holmsen, 1971). However, the main changes in the metabolic nucleotides during ADP- and adrenaline-induced release were the cellular breakdown of ATP and the extracellular accumulation of hypoxanthine (Table 3).

The amount of radioactive ATP disappearing intracellularly during release is markedly less in the experiments recorded in Tables 1 and 2 than in that recorded in Table 3. In the two former experiments [U-^{14}C]adenine was not completely taken up by the platelets, whereas in the latter all radioactive adenine added had been consumed before aggregation was started (see below).

Amounts and quality of nucleotides released and degree of ATP–IMP–hypoxanthine conversion: a direct comparison between ADP, adrenaline, thrombin and collagen as release inducers

With samples of a given [^{3}H]adenine-labelled platelet-rich plasma, the same degree of aggregation was achieved by all four inducers within about 5min (Fig. 3). The amounts of ATP and ADP released and the amounts and specific radioactivity of the remaining cellular nucleotides were essentially the same for the four inducers (Table 4). No difference was found between the ability of the inducers to cause conversion of cellular [^{3}H]ATP into extracellular radioactive hypoxanthine (Table 4).

The experiment recorded in Table 4 and Fig. 3 exemplifies a situation with a high degree of platelet lysis during release (presence of radioactive nucleoside phosphates extracellularly after release). Usually, lysis corresponded to less than 2% of the total platelet nucleotide radioactivity appearing extracellularly. Therefore the difference in specific radioactivity between released and retained ATP and ADP was less in the experiment recorded in Table 4 than usual. Nevertheless, the experiment shows that when lysis occurs during the release reaction, it occurs with any of the four release inducers investigated.

Association of the ATP–hypoxanthine conversion with the second phase (release step) of aggregation

Human platelets aggregate biphasically above 30°C and within certain concentrations of ADP and adrenaline. Below the threshold concentrations only the first phase of aggregation occurs, without nucleotide release (Hardisty *et al.*, 1970). Above the upper concentration release occurs, but owing to the maximal rate of primary aggregation, light-transmission recording cannot show the onset of the second phase. Conditions can thus be selected with either pure first-phase aggregation or well-recorded biphasic aggregation by (*a*) variation of aggregation temperature, or (*b*) variation of ADP and adrenaline concentrations.

The ability of platelets to respond with biphasic aggregation to adrenaline and ADP (in particular) varied greatly within the first 3h after blood collection. Thus numerous experiments had to be abandoned because the platelets failed to aggregate biphasically after incubation with radioactive compounds, although they had responded properly before incubation. This failure ('second phase fatigue') was not due to metabolism of the compounds, and provided a third (*c*) condition of studying the association of ATP–hypoxanthine conversion with either phase of aggregation. A fourth way to alter biphasic aggregation is (*d*) use of acetylsalicylate, which blocks the second phase of aggregation induced by adrenaline (O'Brien, 1968*a*) and ADP (Zucker & Peterson, 1968), without affecting the first phase.

(*a*) *Variation of temperature.* [^{3}H]Adenine-labelled platelet-rich plasma was aggregated with ADP or adrenaline at four different temperatures. Variation in ^{3}H-labelled metabolites was determined during

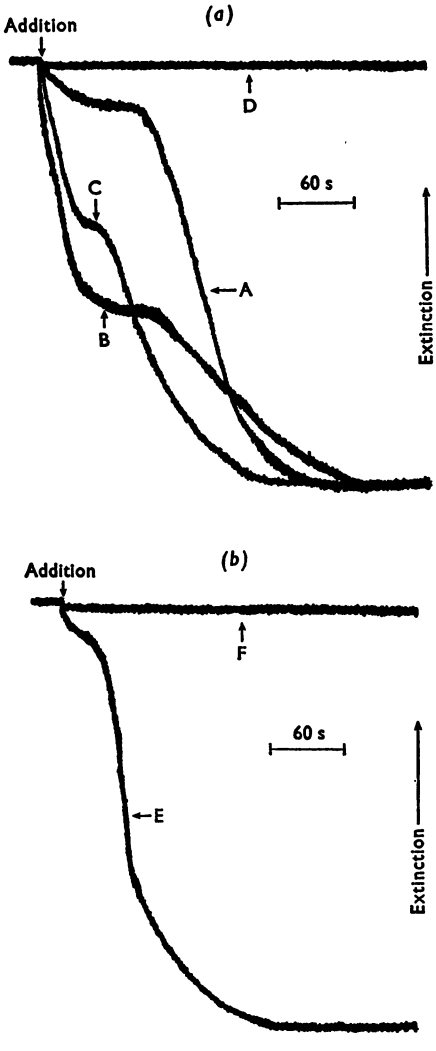


Fig. 3. *Aggregation of platelet-rich plasma caused by adrenaline, ADP, thrombin and collagen*

Extinction changes in platelet-rich plasma from the experiment described in Table 4. (*a*) A, Adrenaline; B, ADP; C, thrombin; D, 0.15M-NaCl. (*b*) E, Collagen; F, 0.016M-acetic acid.

the first 8min, after which platelets and plasma were separated for determination of nucleotide release, measured as the decrease in intracellular ATP and ADP concentrations (it was expected that the enzymic breakdown of ATP and ADP in plasma would vary with temperature). For adrenaline, there was a clear correlation between release and presence of [^{3}H]ATP–hypoxanthine conversion (Table 5,

Table 4. *Comparison of ADP, adrenaline, collagen and thrombin as release inducers in the same platelet-rich plasma*

A sample (10ml) of platelet-rich plasma (4.83×10^8 platelets/ml) was incubated with 0.25ml of [^{3}H]adenine (16.7μM) for 60min at 37°C. Portions (1.0ml) were then transferred to the cuvette of an EEL platelet-aggregation meter and stirred for 7min with 0.1ml of 10.7μM-ADP or 50μM-adrenaline or thrombin (2 units/ml) (all three inducers in 0.15M-NaCl) or collagen (0.5mg/ml in 16.7mM-acetic acid). Portions were also stirred with 0.1ml of the solvents for the release inducers. Recorder tracings of the extinction changes in the plasma during aggregation are shown in Fig. 3. After the aggregation period, 0.1ml of 0.077M-EDTA was added and the cuvette contents were centrifuged. The amounts and radioactivities of ATP and ADP as well as ^{3}H radioactivity in adenine metabolites were determined in EDTA–ethanol extracts of platelets and plasma. The radioactivity in the adenine metabolites includes that of adenine, which was less than 2.1%. The average sum of metabolite radioactivities in the samples was 1455000 c.p.m./10^{11} platelets.

Treatment of platelet-rich plasma	Amount (μmoles/10^{11} platelets)				Specific radioactivity (c.p.m./nmol)				Total radioactivity in adenine metabolites (% of sum of all metabolite radioactivities)											
	ATP and ADP								ATP		ADP		AMP		IMP		Hypoxanthine	
	Platelets		Plasma		Platelets		Plasma											
	ATP	ADP	ATP	ADP	ATP	ADP	ATP	ADP	Platelets	Plasma	Platelets	Plasma	Platelets	Plasma	Platelets	Plasma	Platelets	Plasma
NaCl	4.85	2.51	0.05	0.03	2439	625	2260	2920	67.1	0.1	8.9	0.4	1.4	0.5	1.8	0.2	3.1	13.9
Acetic acid	4.03	2.54	0.05	0.03	2598	636	1420	1760	63.7	0.4	9.8	0.3	1.4	0.5	2.0	0.2	3.3	16.2
ADP	2.43	1.16	1.51	1.57	2636	1637	540	233	35.1	4.5	10.3	2.0	3.0	0.9	2.9	0.6	8.5	30.4
Adrenaline	2.74	1.15	1.26	1.59	2826	1789	486	233	41.9	3.3	12.4	2.0	2.8	1.1	2.6	0.5	7.1	24.2
Collagen	3.15	1.35	1.44	1.32	2943	1523	524	247	42.1	3.7	10.7	1.5	1.8	0.8	5.5	0.5	5.7	26.4
Thrombin	2.46	1.57	1.46	1.40	2693	1408	800	536	36.7	6.4	9.5	4.1	2.0	1.6	3.5	1.6	6.6	26.7

Table 5. *Effect of temperature on [^{3}H]ATP–hypoxanthine conversion and adenine nucleotide release from platelets induced with ADP and adrenaline*

A sample (10ml) of platelet-rich plasma was incubated at room temperature with 0.1ml of 40μM-[^{3}H]adenine for 30min. Portions (2ml) were equilibrated in round-bottomed cuvettes in a thermostatically controlled EEL titrator at the temperatures indicated, and 200μl of 12.8μM-ADP, 50μM-adrenaline or solvent, 0.15M-NaCl, was added. Samples (100μl) of the reaction mixture were transferred into 100μl of EDTA–ethanol at the times indicated. After the 8-min sample had been transferred, 0.2ml of 0.077M-EDTA (pH7.4) was added to the remainder in the cuvettes, and the cuvette was centrifuged. EDTA–ethanol extracts were prepared from cells and plasma as described in the Experimental section. An increase in IMP+hypoxanthine radioactivity was always paralleled by a corresponding decrease in the ATP+ADP radioactivity. For practical reasons the time-sequence of aggregation experiments was from the lowest to the highest temperature, and the NaCl control was always done after aggregation, at the highest temperature. Therefore the zero-time samples contain increasing amounts of [^{3}H]IMP+[^{3}H]hypoxanthine the later in the sequence the aggregation was initiated. The platelet-rich plasma used in the experiment with ADP contained 2.87×10^8 platelets/ml, and that used in the experiment with adrenaline contained 3.40×10^8 platelets/ml. The average sum of metabolite radioactivities was 80500c.p.m./ml of reaction mixture, of which less than 1% was [^{3}H]adenine before aggregation was initiated.

Treatment of platelet-rich plasma	Time after start of aggregation (min) ...	Temperature (°C)	Platelet nucleotides (μmol/10^{11} platelets) ATP	ADP	[^{3}H]IMP+[^{3}H]hypoxanthine (% of total radioactivity) 0	2	5	8
NaCl		38.0	4.91	2.95	7.0	6.6	7.1	7.9
ADP		19.9	4.79	2.94	4.2	3.8	3.9	3.7
		28.7	4.68	2.48	3.8	4.4	8.1	11.7
		35.0	2.76	1.51	5.0	7.8	16.6	18.8
		38.0	3.31	1.25	5.8	7.2	15.3	19.9
NaCl		38.0	3.47	2.88	6.2	6.8	7.5	8.7
Adrenaline		20.1	3.57	2.73	1.4	1.5	1.7	1.9
		29.3	3.56	2.23	2.1	2.1	2.8	2.5
		35.2	2.31	0.94	3.7	4.1	9.1	13.7
		38.0	2.27	0.95	6.3	7.9	14.0	16.5

adrenaline experiment). With ADP considerable amounts of [^{3}H]ATP were converted into IMP and hypoxanthine without significant release (Table 5, ADP experiment, 28.7°C). Distinctly more radioactive IMP+hypoxanthine accumulated when release occurred (Table 5, ADP experiment, 35°C and 38°C). The degree of ADP-induced aggregation in a given plasma preparation (increase of light-transmission in plasma) was the same after 8min of stirring at all temperatures. This further indicated association of ATP–hypoxanthine conversion with the release step (second phase) and not with aggregation.

(*b*) *Variation of the concentration of ADP and adrenaline.* Low concentrations, giving only one phase of aggregation, caused an IMP+hypoxanthine formation slightly above that of the control. With high amounts, giving a distinct secondary aggregation, the IMP+hypoxanthine formation was maximal and constant for a given plasma preparation (it varied in different preparations from 8 to 25% of the total radioactivity) (Fig. 4, Table 6). At intermediate concentrations, giving secondary aggregation with a submaximal rate (Fig. 4*c*), the amount of hypoxanthine+IMP formed varied between 2% and the maximum value.

(*c*) *Occurrence of 'second phase fatigue'.* Platelet-rich plasma, which no longer showed biphasic aggregation with ADP after incubation with isotopes, had insignificant formation of hypoxanthine+IMP (2% of total radioactivity). The biphasic responsiveness to adrenaline did not disappear as often after incubation as did the responsiveness to ADP. When the biphasic response was decreased, but not lost, an IMP+hypoxanthine formation of more than 2% took place. For example, in an experiment with variation of the temperature, 2.91μmol of platelet ATP+ADP per 10^{11} cells was released by ADP at 35°C whereas the release at the 38°C tested 15min later was only

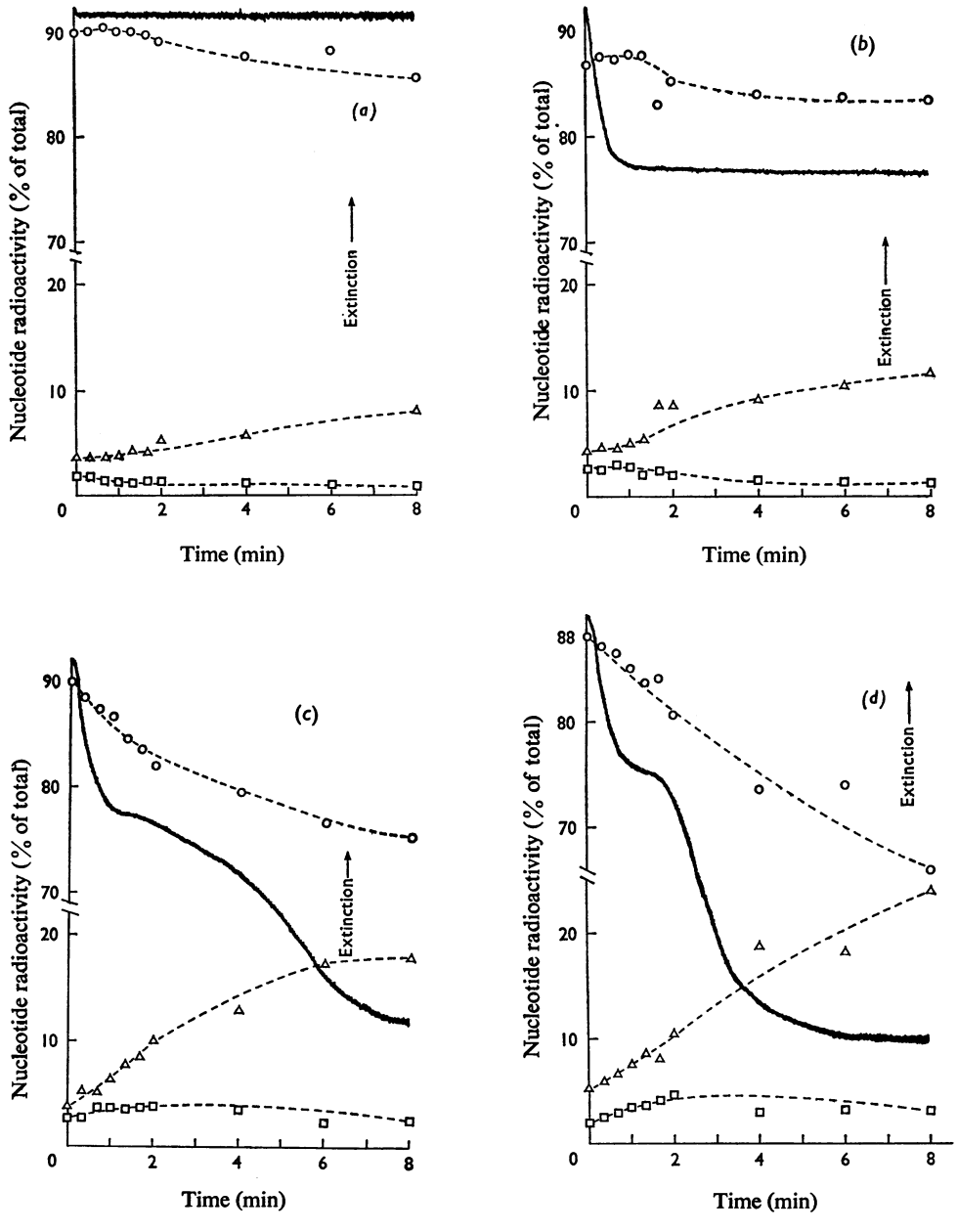


Fig. 4. *Variation in the radioactivity of ATP+ADP* (○), *IMP* (□) *and hypoxanthine* (△) *during stirring of platelet-rich plasma* (1.8 *ml*; 4.02×10^8 *platelets/ml*) *with* 0.2 *ml of ADP*

The final concentrations of ADP in 0.15M-NaCl were: (*a*), 0 μM; (*b*), 0.7 μM; (*c*), 1.4 μM; (*d*), 2.1 μM. In advance, 10.0ml of plasma had been incubated with 0.25ml of 16.7 μM-[^{3}H]adenine at room temperature for 30min, which allowed for complete uptake of [^{3}H]adenine. The reaction was done in an EEL titrator at 37°C and 100-μl samples of the reaction mixture were taken at noted times for determination of metabolite radioactivity (expressed as % of the sum of all metabolite radioactivities; the average sum was 124000c.p.m./ml of reaction mixture). Aggregometer tracings (heavy, solid lines) are superimposed on the Figures. ATP and ADP did not separate well on electrophoresis in this experiment.

Table 6. *Effect of adrenaline concentration on appearance of biphasic platelet aggregation and IMP+hypoxanthine formation*

Platelet-rich plasma (3.75×10^8 platelets/ml) was labelled with [^{3}H]adenine as described in Fig. 4. Samples (1.8ml) were stirred with 0.2ml of adrenaline in (final) concentrations given below for 6min at 37°C in an EEL titrator. Samples (200μl) of the incubation mixture were transferred immediately after adrenaline addition and at 2, 4 and 6min into 200μl of EDTA–ethanol for determination of [^{3}H]adenine metabolite radioactivity (the average sum of metabolite radioactivities was 108000c.p.m./ml of reaction mixture; adenine = 2.3–2.7%). The recorder deflection represents arbitrary units of light-transmission through platelet-rich plasma, 44–47 representing maximal, secondary aggregation. The radioactivity is relative to the zero-time value, in % of total radioactivity.

Adrenaline concentration (μM)	Time ...	Recorder deflection 2min	4min	6min	Increment in IMP+Hypoxanthine radioactivity
0		0	1	1	0.0
1.5		4	4	5	1.5
3.0		6	13	43	7.7
4.5		5	14	44	11.4
6.0		5	18	47	12.8

Table 7. *Effect of aspirin on nucleotide release and ATP–hypoxanthine conversion*

A sample (10ml) of platelet-rich plasma (4.55×10^8 platelets/ml) was incubated at room temperature for 60min with 100μl of 40μM-[^{3}H]adenine. Portions (1.5ml) were incubated with 0.15ml of 0.15M-NaCl or 8mM-aspirin for 10min at 37°C and then stirred with 0.15ml of 0.15M-NaCl, 50μM-adrenaline or 25.6μM-ADP in the EEL titrator. At noted times three 100-μl samples were transferred into 100μl of EDTA–ethanol. After 6min of stirring (which gave maximal secondary aggregation in samples without aspirin) 0.1ml of 77mM-EDTA (pH7.4) was added and cells and plasma were separated. The average total radioactivity was 92000c.p.m./ml and the platelets contained 3.27 and 2.24μmol/10^{11} platelets of ATP and ADP respectively.

Addition in aggregometer	Pretreatment	Time ...	Radioactivity (%) ATP+ADP 0min	ATP+ADP 6min	Hypoxanthine 0min	Hypoxanthine 6min	ATP+ADP released (μmol/10^{11} platelets)
ADP	NaCl		94.3	82.0	3.6	12.0	1.37
	Aspirin		92.4	90.4	4.6	6.8	0.03
Adrenaline	NaCl		90.1	83.3	6.8	14.1	1.57
	Aspirin		92.4	91.7	5.5	5.9	0.04
NaCl	NaCl		91.2	90.5	4.3	5.8	0.04
	Aspirin		92.1	91.6	5.7	6.8	0.03

1.04μmol. The IMP+hypoxanthine formed at the two temperatures was 9.3 and 6.0% respectively of the total radioactivity.

(*d*) *Effect of aspirin.* With adrenaline no [^{3}H]ATP–hypoxanthine conversion took place when release and the second phase of aggregation were blocked by aspirin. The [^{3}H]ATP–hypoxanthine conversion occurring during ADP-induced biphasic aggregation, however, was markedly decreased, but not absent when release and the second phase of aggregation were completely blocked by aspirin (Table 7).

Time-course of ATP–hypoxanthine conversion during optimum biphasic aggregation after all the adenine had been metabolized

On addition of ADP to labelled, stirred plasma, the ATP+ADP radioactivity always decreased immediately and hypoxanthine formation was evident after 20s (Fig. 4*d*). A transient, small accumulation of radioactive IMP sometimes occurred before the rise in hypoxanthine radioactivity, but no changes in the ADP radioactivity were observed. Frequently, adrenaline did not cause ATP–hypoxanthine con-

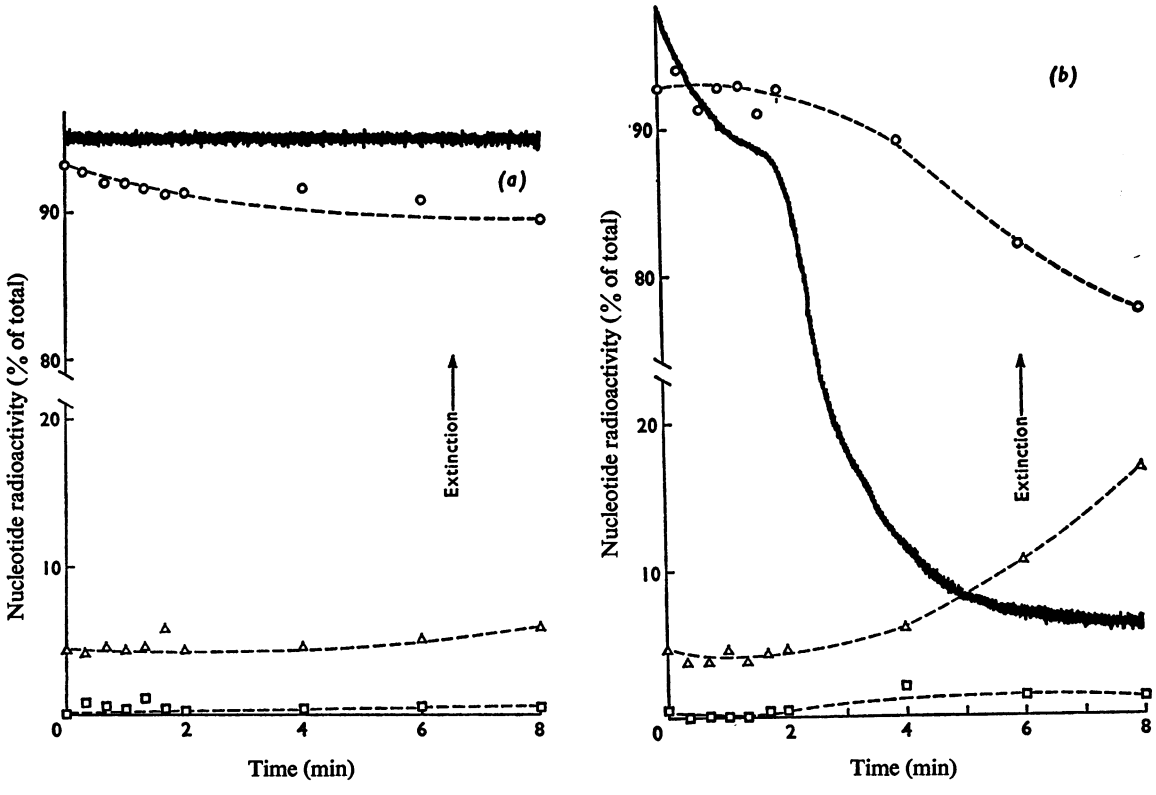


Fig. 5. *Variation in* [^{3}H]*adenine metabolite radioactivities during stirring of platelet-rich plasma* (1.8*ml*; 5.03× 10^8 *platelets/ml*) *with* 0.2*ml of adrenaline*

The procedure and symbols are defined in Fig. 4; (*a*) 0.15M-NaCl; (*b*) 50μM-adrenaline in 0.15M-NaCl.

Table 8. [^{3}*H*]*Hypoxanthine formation during biphasic aggregation in the presence and absence of* [^{3}*H*]*adenine*

A sample (10ml) of platelet-rich plasma (1.5×10^8 platelets/ml) was incubated at 37°C with 0.25ml of 16.1μM-[^{3}H]adenine. Portions of 1.8ml were transferred at recorded times (preincubation times) to the EEL titrator (37°C) and 0.2ml of 50μM-adrenaline or 0.15M-NaCl was added. Samples (200μl) of these stirred incubation mixtures were removed at noted times (aggregometer time) after the last addition into 200μl of EDTA–ethanol, and the radioactivity of the metabolites was determined. The average sum of metabolite radioactivity was 110000c.p.m./ml of reaction mixture.

Preincubation time (min)	Addition	Aggregometer time (min)	Radioactivity (%)	
			Hypoxanthine	Adenine
2.5	ADP	0	2.0	76.1
		9	10.6	37.8
14.5	NaCl	0	2.4	23.2
		9	4.0	8.3
26.5	ADP	0	3.4	3.7
		9	20.6	2.9
38.5	NaCl	0	4.5	1.9
		9	7.6	1.8

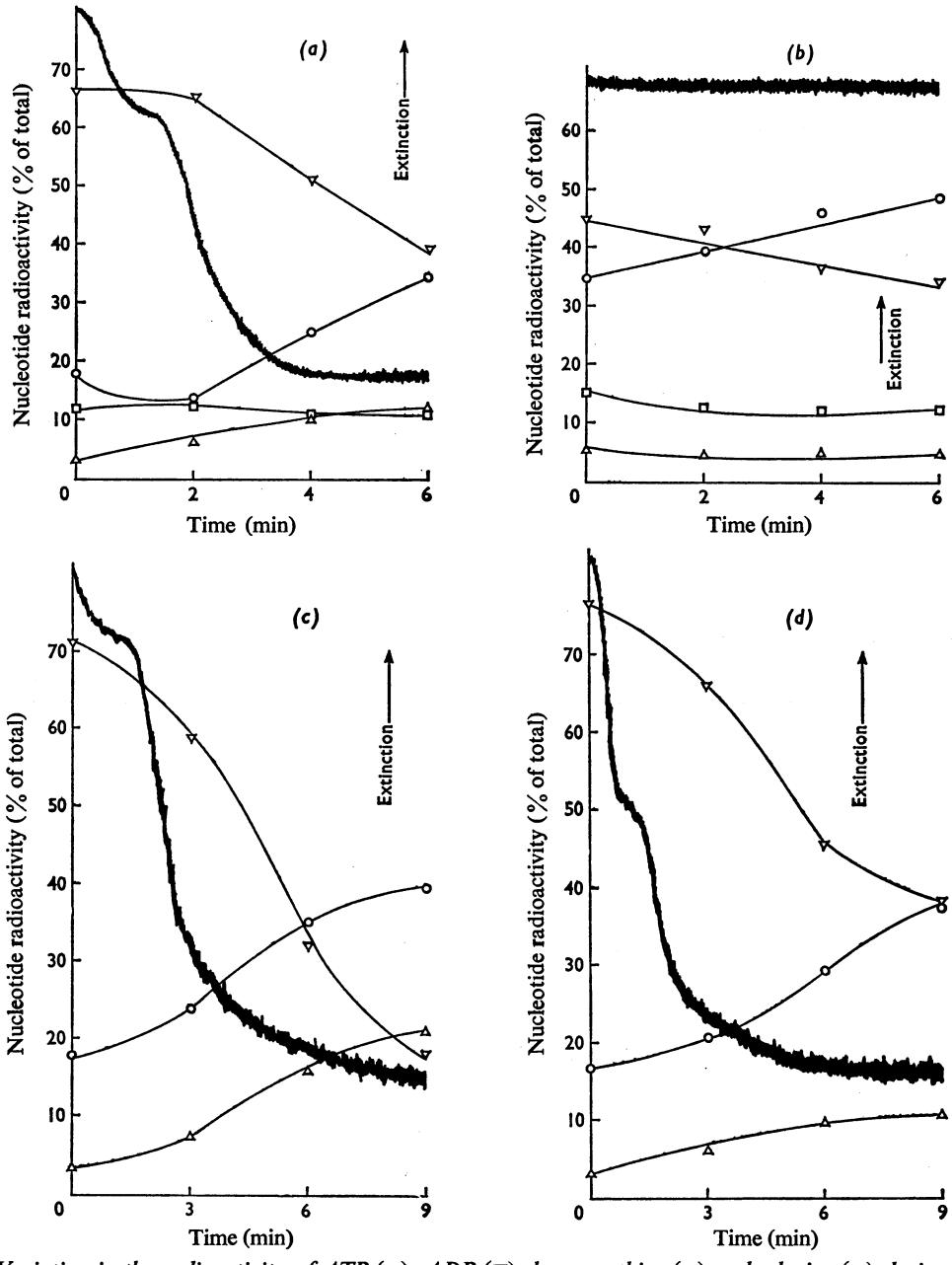


Fig. 6. *Variation in the radioactivity of ATP* (○), *ADP* (□), *hypoxanthine* (△) *and adenine* (▽) *during stirring of 1.8 ml of labelled platelet-rich plasma with 0.2 ml of aggregating agent or its solvent in the EEL titrator at 37°C*

Samples (200 μl) were transferred at noted times into 200 μl of EDTA–ethanol for determination of metabolite radioactivity. For (*a*) and (*b*) platelet-rich plasma (10 ml; 2.94×10^8 platelets/ml) was incubated with 0.75 ml of 16.7 μM-[^{3}H]adenine at 37°C. After 19 min of incubation one sample was stirred with 5 μM-adrenaline (*a*), and after 28.5 min of incubation another sample was stirred with 0.15 M-NaCl (*b*). The zero-time in the above Figures refers to addition of adrenaline or saline. Metabolite radioactivity is expressed in % of the sum of all metabolite radioactivities (average: 380000 c.p.m./ml of reaction mixture). For (*c*) platelet-rich plasma (10 ml; 5.7×10^8 platelets/ml) was incubated with 0.25 ml of 16.7 μM-[^{3}H]adenine at 37°C. After 2.25 min one sample was stirred with 5 μM-adrenaline. Zero-time refers to addition of adrenaline and the average sum of all metabolite radioactivities was 102000 c.p.m./ml of reaction mixture. For (*d*) the experiment was the same as in (*c*) except that the platelet-rich plasma contained 1.5×10^8 platelets/ml and was stirred with 1.1 μM-ADP. The average sum of all metabolite radioactivities was 108000 c.p.m./ml of reaction mixture.

version during the first, but only during the second phase of aggregation (Fig. 5). This difference from ADP-induced aggregation was evident when ADP and adrenaline were used with the same radioactive platelet-rich plasma, and was not dependent on the adrenaline concentration. In some plasma preparations adrenaline did cause a continuous conversion during the first phase of aggregation, as in all experiments with ADP. Although the rate of primary aggregation was the same whether or not the platelets responded mono- or bi-phasically to an aggregating stimulus, the ATP–hypoxanthine conversion only occurred if a second phase were to follow. This is clearly shown in Fig. 4.

ATP–hypoxanthine conversion during biphasic aggregation in the presence of [^{3}H]adenine

An increased formation of [^{3}H]hypoxanthine occurred during biphasic aggregation in the presence of [^{3}H]adenine with both ADP and adrenaline, but there was no or only a transitory decrease in the amount of radioactive ATP (Fig. 6). Aggregation in the presence of [^{3}H]adenine caused a burst in the incorporation of radioactivity into ATP. This usually started during the second phase of aggregation, continued after the light-transmission changes were terminated (Fig. 6) and was preceded by a 'halt' in adenine–ATP conversion. Less radioactive hypoxanthine was formed when biphasic aggregation took place early during adenine uptake, as compared with the hypoxanthine formed after all adenine had been taken up by the platelets (Table 8).

The ^{3}H-labelled metabolite behaviour during aggregation was most outstanding when the adenine uptake rate was greater than 1% of the added radioactivity per min. At lower uptake rates, less impressive changes occurred, as seen from Fig. 7(*a*). When the values were expressed in % of adenine radioactivity taken up by the platelets at any time (i.e. the sum of

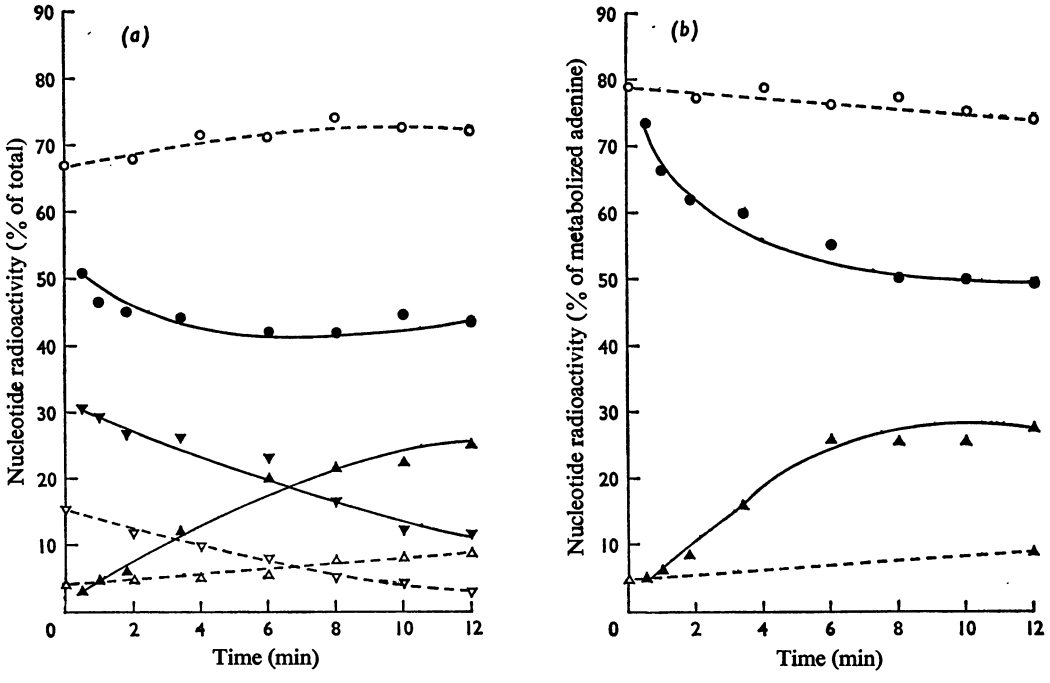


Fig. 7. *Incubation of platelet-rich plasma* (12 *ml*; 3.86×10^8 *platelets/ml*) *at room temperature with* 0.6 *ml* of 16.7 μM-[^{3}*H*]*adenine*

After 60 min of incubation 2 ml of labelled platelet-rich plasma was stirred with 0.2 ml of 12.8 μM-ADP in 0.15 M-NaCl (——) and after 80 min of incubation a 2 ml sample was stirred with 0.2 ml of 0.15 M-NaCl (– – – –). During stirring 100-μl samples were transferred at noted times into 100 μl of EDTA–ethanol and the radioactivities of adenine (▼,▽), hypoxanthine (▲,△) and ATP (●, o) were determined. The average sum of all metabolite radioactivities was 160000 c.p.m./ml of reaction mixture. The metabolite radioactivity has been expressed in (*a*) as % of the sum of all metabolite radioactivities and in (*b*) as % of metabolized adenine (=radioactivity of all metabolites minus radioactivity of adenine).

the radioactivities of ATP + ADP + AMP + IMP + hypoxanthine) the pattern of ATP breakdown and hypoxanthine accumulation (Fig. 7*b*) markedly resembled that obtained without adenine present during aggregation (Figs. 4 and 5). This was not the case at high uptake rates.

Adenine uptake in free and aggregated platelets

The uptake of [^{3}H]adenine by biphasically aggregated platelets was increased as compared with non-aggregated cells (Fig. 8). This increased uptake was accompanied by elevated formation of [^{3}H]-ATP and -hypoxanthine. Although [^{3}H]adenine disappearance and appearance of its metabolites in platelet-rich plasma were linear with non-aggregated cells over the 30-min period studied, these curves were non-linear with aggregated cells: [^{3}H]adenine disappearance became slower, [^{3}H]ATP accumulation ceased, and [^{3}H]hypoxanthine and [^{3}H]AMP formation rates increased as the incubation time increased. The formation of [^{3}H]AMP is noteworthy, since the content

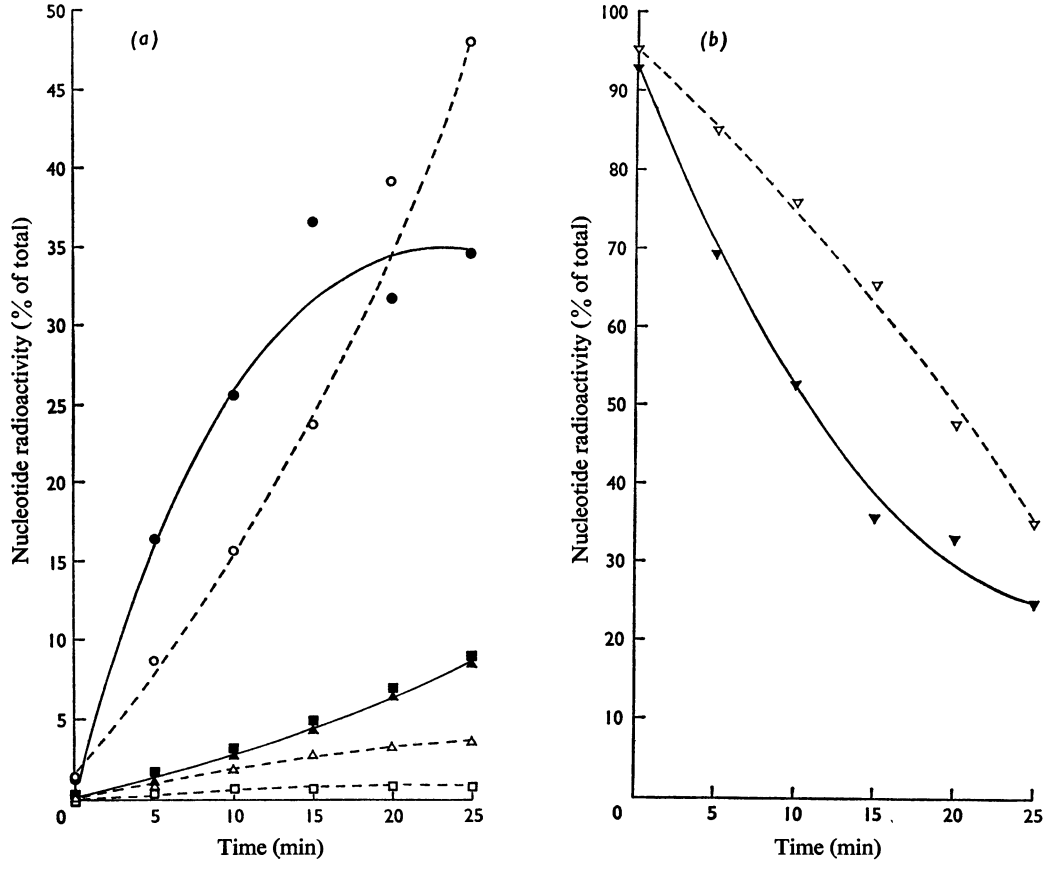


Fig. 8. *Adenine uptake in free and aggregated platelets*

Into the EEL titrator cuvette was pipetted 1.8ml of platelet-rich plasma (3.87×10^8 platelets/ml). After temperature equilibration (37°C) with stirring for 1min, 0.2ml of 50μM-adrenaline (in 0.15M-NaCl) was added. This produced biphasic aggregation, which was terminated 5min after adrenaline addition. Then, 6min after adrenaline addition, 100μl of 16.7μM-[^{3}H]adenine was added and 200μl samples of the reaction mixture were transferred at noted times into 200μl of EDTA–ethanol for measurement of metabolite radioactivity (——; ●, ■, ▲, ▼). Similarly, another 1.8ml sample of platelet-rich plasma was stirred with 0.2ml of 0.15M-NaCl, and [^{3}H]adenine added 6min after saline (– – – –; ○, □, △, △). The metabolite radioactivity is expressed in % of the sum of all metabolite radioactivities (average: 308000c.p.m./ml of reaction mixture). (*a*) Variation in radioactive ATP (●, ○), AMP (■,□) and hypoxanthine (▲,△); (*b*) disappearance of radioactive adenine (▼,▽).

Table 9. *Uptake of [^{3}H]adenine at different stages of the biphasic aggregation*

Samples (1.8ml) of platelet-rich plasma (7.13×10^8 platelets/ml) were equilibrated with stirring in the EEL titrator for 2min at 37°C. Portions (150μl) of 16.7μM-[^{3}H]adenine and 200μl of ADP (28μM in 0.15M-NaCl) or 0.15M-NaCl were added either simultaneously or at different times. Samples (100μl) of the reaction mixture were transferred 10min after [^{3}H]adenine addition into 100μl of EDTA–ethanol for determination of metabolite radioactivity (average sum of metabolite radioactivities = 228680c.p.m./ml of reaction mixture). Biphasic platelet aggregation occurred in all ADP–platelet-rich plasma mixtures with the onset of the second phase and termination of second phase, 1.5 and 5min respectively, after ADP addition. [^{3}H]Adenine added before ADP is designated +; after is designated −; 0 means simultaneously.

Treatment	Relative time of [^{3}H]adenine addition (min)	Radioactivity (%)		
		ATP	Hypoxanthine	Adenine
NaCl	0	18.6	1.3	74.4
ADP	+2	31.5	11.5	52.0
ADP	0	33.8	9.2	44.2
ADP	−3	34.4	5.9	43.1
ADP	−5	38.1	5.2	44.3
ADP	−10	30.3	3.5	51.2

of this nucleotide never varied, being 1–2% of total radioactivity during the release reaction, and was hardly detectable in resting cells.

In view of the above, it was possible that the ATP–hypoxanthine conversion connected with the release reaction was due to a general disturbance of the control of adenine nucleotide concentrations in aggregated cells. However, after radioactive adenine was added together with ADP, only a third of the hypoxanthine formation taking place within 10min did occur after the biphasic aggregation had been fully terminated (Table 9, [^{3}H]adenine addition 10min after ADP). Similar results were obtained with adrenaline.

The accumulation of radioactivity in ATP and disappearance of adenine in platelet-rich plasma treated with ADP or adrenaline was increased (by 80–110% compared with non-aggregated cells) almost independently of the stage of biphasic aggregation (Table 9).

Discussion

The specific radioactivity of ATP and ADP released from labelled platelets by external ADP and adrenaline was negligible compared with that of the ATP and ADP remaining in the cells. The amounts released and the specific radioactivity of released and remaining adenine nucleotides were similar to those released and retained by collagen and thrombin. Thus ADP and adrenaline induce release of the 'storage pool' of adenine nucleotides.

Study of the effects of temperature, the ADP and adrenaline concentrations, 'second phase fatigue' and acetylsalicylic acid shows that the intracellular ATP–hypoxanthine conversion is intimately connected with the release induced by ADP and adrenaline, and not with aggregation. The degree of this conversion was the same as that induced by collagen or thrombin. The ATP consumed has been termed 'release energy ATP' and is thought to energize the release reaction (Holmsen *et al.*, 1969*a*). ATP–hypoxanthine conversion also occurs without release, by simultaneous inhibition of glycolysis and respiration, and is markedly increased when release is conducted in the presence of metabolic inhibitors (Ball *et al.*, 1969). The ATP–hypoxanthine conversion occurring during release could thus represent the ATP that had been utilized, but not immediately compensated for by ATP resynthesis (Holmsen & Day, 1971). Other factors must also be considered such as the increased formation of hypoxanthine during adenine uptake by aggregated platelets after actual release and the increased synthesis of ATP from adenine. The burst in adenine uptake has been shown to be connected with the disc–sphere transformation (Sixma *et al.*, 1972), which takes place during aggregation, and is probably not caused by lowering of the concentration of metabolic ATP. The somewhat catabolic metabolism of adenine during this increased uptake by aggregated platelets might be due to the small fraction of platelets undergoing lysis. Our results indicate that less than one-third of the ATP–hypoxanthine conversion measured during release was due to catabolism in aggregated platelets.

The ATP–hypoxanthine conversion might be used as a marker for intracellular reactions connected with platelet secretion. With ADP it starts immediately, suggesting that some intracellular reactions underlying secretion take place before actual release can be

seen during the first aggregation phase when a second phase is to follow. The conversion was absent during the first phase when the second phase did not follow, further indicating that the ATP–hypoxanthine conversion is not connected with aggregation *per se*. Its presence during the first phase when release was to follow implies that aggregation by ADP is not a prerequisite for release, in contrast with the view (O'Brien, 1968*b*, Massini & Lüscher, 1971) that close cell-contact is the release inducer during biphasic aggregation.

With adrenaline the ATP–hypoxanthine conversion often started after the second aggregation phase became apparent, indicating that the stimulus for release was not received by the platelets until the second phase of aggregation started. Since ADP-induced release always caused immediate ATP–hypoxanthine conversion, it is possible that most of release during the adrenaline–platelet interaction was induced by the ADP appearing extracellularly. It might even be caused by a combination of adrenaline and ADP liberated by lysis of some few platelets, since adrenaline is a powerful potentiator of ADP-induced platelet aggregation (Mills & Roberts, 1967). This also is in agreement with the distinctly smaller degree of first-phase aggregation seen with adrenaline than ADP.

Release reaction was always accompanied by ATP–hypoxanthine conversion, whereas a small conversion without release took place with ADP at 29°C and when release was inhibited by aspirin. This does not necessarily obviate the connexion between release and ATP–hypoxanthine conversion. On release-stimulus the cell answers with a diminished ATP–hypoxanthine conversion, whereas one of the later steps in the secretion process, membrane fusion, might be more severely inhibited, so that emptying of the granule contents does not occur in spite of the intracellular processes.

Less formation of radioactive hypoxanthine took place when aggregation was conducted at an early stage of adenine uptake than at later stages. Thus the ATP just formed from adenine does not equilibrate immediately with the bulk of the metabolically active nucleotide pool, or with that special pool of ATP converted into hypoxanthine, the 'release energy pool'. This was substantiated by the difference in the kinetics of adenine and ATP metabolism at different rates of isotope uptake.

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