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# Effects of Antimycin and 2-Deoxyglucose on Adenine Nucleotides in Human Platelets

## ROLE OF METABOLIC ADENOSINE TRIPHOSPHATE IN PRIMARY AGGREGATION, SECONDARY AGGREGATION AND SHAPE CHANGE OF PLATELETS

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1. Human platelet-rich plasma prelabelled with [$^3$H]adenine was incubated at 37°C with antimycin A and 2-deoxy-D-glucose. Variations in the amounts of ATP, ADP and $P_i$, and in the radioactivity of ATP, ADP, AMP, IMP, hypoxanthine+inosine and adenine were determined during incubation. Adrenaline- and ADP-induced platelet aggregation and the ADP-induced shape change of the platelets were determined concurrently. 2. 2-Deoxyglucose caused conversion of [$^3$H]ATP to [$^3$H]hypoxanthine+inosine. The rate of this conversion increased with increasing 2-deoxyglucose concentration and was markedly stimulated by addition of antimycin, which had no effect alone. At maximal ATP–hypoxanthine conversion rates, the IMP radioactivity remained at values tenfold higher than control, whereas [$^3$H]ADP and [$^3$H]AMP radioactivity gave variations typical for product/substrates in consecutive reactions. The specific radioactivityof ethanol-soluble platelet ATP decreased during incubation to less than one-tenth of its original value. The amounts and radioactivity of ethanol-insoluble ADP did not vary during incubation with the metabolic inhibitors. 3. The rate of ADP- and adrenaline-induced primary aggregation decreased as the amount of radioactive ATP declined, and complete inhibition of aggregation was obtained at a certain ATP concentration (metabolic ATP threshold). This threshold decreased with increasing concentration of inducer ADP. 4. Secondary platelet aggregation (release reaction) had a metabolic ATP threshold markedly higher than that of primary aggregation. 5. Shape change was gradually inhibited as the ATP radioactivity decreased, and had a metabolic ATP threshold distinctly lower than that of primary aggregation, and which decreased with increasing concentration of ADP. 6. A small but distinct fraction of [$^3$H]ATP disappeared rapidly during the combined shape change–aggregation process induced by ADP in platelets incubated with metabolic inhibitors, whereas no ATP disappearance occurred during aggregation in their absence.

In platelets from those species that have been investigated (Mills & Thomas, 1969), metabolic and non-metabolic pools of adenine nucleotides may be distinguished (see Scheme 1 of Holmsen *et al.*, 1972). The metabolic nucleotide pool may be labelled *in vitro* on incubation with radioactive phosphate, adenosine and adenine, and is retained during secretion of various platelet components (e.g. ADP, ATP, 5-hydroxytryptamine, $Ca^{2+}$) in the platelet release reaction (Day & Holmsen, 1971). The non-metabolic nucleotide pool is not labelled by preincubation under these conditions, is probably stored in specific subcellular granules and is secreted during the platelet release reaction. In human platelets approx. 60% of the total adenine nucleotides is present in the non-metabolic (or storage) pool.

Previous studies have demonstrated that platelet functions, such as adhesion, aggregation, release reaction and clot retraction, may be depressed either by pretreatment of the cells with inhibitors of glycolysis and oxidative phosphorylation (Robinson *et al.*, 1963; Skålhegg *et al.*, 1964; Harrison *et al.*, 1966; Mürer *et al.*, 1967; Mürer, 1968, 1969*a*,*b*; Ball *et al.*, 1969) or by depriving the cells of glucose (Lüscher, 1956; Kinlough-Rathbone *et al.*, 1970), indicating the crucial role of cellular ATP synthesis in these responses. Mürer (1969*b*) has demonstrated that incubation of platelets with some of these metabolic inhibitors leads to a decrease in the concentration of metabolic ATP. However, insight into the relationship between inhibition of various platelet functions and a

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decrease in the rate of ATP production requires additional information. First, the effect of the various inhibitors on platelet adenine nucleotide metabolism must be characterized and, secondly, the response of specific platelet functions must be correlated with alterations in platelet adenine nucleotide metabolism which are induced by addition of the inhibitors of glycolysis and oxidative phosphorylation.

In the present paper loss of platelet radioactive ATP, brought about by deoxyglucose+antimycin A, is compared with the ability of platelets to undergo ADP- or adrenaline-induced aggregation, both primary and secondary, and to change shape from discs to spheres with ADP in the presence of EDTA (Born, 1969).

## Experimental

### *Materials*

*Chemicals*, ADP and (−)adrenaline bitartrate were obtained, stored and used as described previously (Holmsen *et al.*, 1972). Solutions of 2-deoxy-D-glucose (Grade II; Sigma Chemical Co., St. Louis, Mo., U.S.A.) (0.18–2M) were prepared in 0.15M-NaCl and stored at −20°C. Appropriate dilutions were prepared by using 0.15M-NaCl. Antimycin A (Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.) was dissolved in 95% (v/v) ethanol (10–25mg/ml) and stored at −20°C. Dilutions were made with 95% ethanol.

*Radiochemicals.* [G-$^3$H]Adenine (NET-063) (3300–6100mCi/mmol) was supplied as an aqueous solution (1mCi/ml). This solution was diluted to 40$\mu$M with 0.15M-NaCl and stored in small portions at −60°C. The solutions were refrozen after use. [G-$^3$H]Inulin (NET-314) (350mCi/g) was dissolved in 0.15M-NaCl to a final concentration of 0.286mg/ml. This solution was stored at −20°C. Both radiochemicals were obtained from New England Nuclear Corp. (Boston, Mass., U.S.A.).

*Biological materials.* Platelet-rich plasma and platelet-poor plasma were obtained as described by Holmsen *et al.* (1972).

*Radioactive platelet-rich plasma.* Platelet-rich plasma (1vol.) was incubated with 0.01vol. of [$^3$H]-adenine (40$\mu$M) for 60min at 23°C. This incubation time allowed for complete conversion of adenine into its metabolites (Holmsen, 1971).

### *Methods*

*Incubation with metabolic inhibitors.* Radioactive platelet-rich plasma was incubated at 37°C with 95% ethanol or antimycin (1.65$\mu$l/ml of platelet-rich plasma) plus either 0.15M-NaCl or deoxyglucose (33.3$\mu$l/ml of platelet-rich plasma). Control samples contained both ethanol and 0.15M-NaCl. Test samples contained in addition either one or both of the inhibitors. Portions were removed from both samples at the times noted for determination of adenine metabolites, phosphate and platelet function.

*Determination of ethanol-soluble adenine metabolite radioactivity and total ATP and ADP.* A 200$\mu$l sample was added to 200$\mu$l of EDTA–ethanol (Holmsen *et al.*, 1971) in an Eppendorf microtube 3810 at 2°C. After mixing the resulting mixture was incubated at 2°C for at least 10min and then centrifuged at approx. 17000*g* for 2min in an Eppendorf microcentrifuge 3200 (Eppendorf Geratebau Metheler and Hinz G.m.b.H., Hamburg, Germany). The supernatant fraction (EDTA–ethanol extract) was analysed directly for metabolite radioactivity as described by Holmsen & Weiss (1970). In some instances the extracts were stored at −60°C for up to 3 days before analysis. A Savant flat-plate electrophoresis system (FP 22A, Savant Instruments, Hicksville, N.Y., U.S.A.) and a Beckman LS 330 liquid-scintillation counter were used in these studies. The scintillation fluid contained 2g of 2,5-diphenyloxazole/litre of toluene. Inosine and hypoxanthine were separated by paper chromatography on Whatman 3MM paper (Randerath & Struck, 1961). ATP and ADP concentrations were determined by using the Du Pont Luminescence Biometer as described by Holmsen *et al.* (1971).

*Determination of amounts and radioactivity of ethanol-soluble and -insoluble adenine nucleotides.* Portions (1.0ml) of test or control samples were mixed with 1.0ml of EDTA–ethanol at 2°C and the resulting mixtures centrifuged at 17500*g* for 10min a 2°C. The supernatant fraction was processed as described above. The sediment was washed once with 2ml of EDTA–ethanol–0.15M-NaCl (1:1, v/v) and was then extracted with 0.5ml of 0.6M-$HClO_4$. The $HClO_4$ extracts were neutralized and analysed for ATP and ADP content as described by Holmsen *et al.* (1971). Determination of metabolite radioactivity in the neutralized $HClO_4$ extracts was performed as for the EDTA–ethanol extracts. The washed sediments were typically stored at −60°C for up to a month before further processing.

Unless otherwise indicated, the radioactivity of a metabolite is expressed as a percentage of the total metabolite radioactivity in the same sample. The adenylate energy charge was calculated from the formula given by Atkinson (1969) by using the total radioactivities of the nucleotides in the ethanol-soluble extracts.

*Platelet inorganic phosphate.* [$^3$H]Inulin (40$\mu$l) was added to 4.0ml of either control or test samples and the platelets were isolated by centrifugation at 17500*g* for 10min at 4°C. The platelet-poor plasma which contained less than $5\times10^6$ cells/ml, was decanted off and extracted with 1vol. of 20% (w/v)

trichloroacetic acid. The centrifuge tube was carefully rinsed with two 5ml portions of 0.15M-NaCl and the packed platelets were then extracted with 3ml of 10% (w/v) trichloroacetic acid. Both extracts were shaken with charcoal (Norit A, N.F.X. Ahmend Drug and Chemical Co., New York, N.Y., U.S.A.) to remove nucleotide phosphate. $P_i$ was measured in the charcoal-treated extracts by the method of Chen *et al.* (1956). Portions of all extracts were spotted on Whatman 3MM filter paper and the [$^3$H]inulin radioactivity was determined as described above. The amount of plasma phosphate trapped in the platelet sediment did not exceed 0.2% as determined from the distribution of [$^3$H]inulin. The $P_i$ values reported may contain some contribution from platelet $PP_i$ since slight hydrolysis (6–7%) of added tetrasodium $PP_i$ occurred during incubation with ascorbic acid+$H_2SO_4$ under the conditions of the phosphate assay. However, 2-deoxyglucose 6-phosphate was not hydrolysed under these conditions.

*Measurement of platelet aggregation and shape change during incubation of platelet-rich plasma with metabolic inhibitors.* Aggregation and shape change were measured at 37°C in a Payton dual-channel aggregation module connected to a Payton vertical two-channel recorder, PHSV (Payton Associates Inc., Buffalo, N.Y., U.S.A.). The platelet-rich plasma-inducer system was stirred at 900rev./min in 1ml cuvettes. When one concentration of inducer was used throughout an experiment, incubation of control and test samples was initiated at the same time. At various times during the incubation, as indicated for each experiment, a portion of the control sample was transferred to one channel of the aggregometer and a portion of the test sample to the other one. Aggregation or shape change was then determined simultaneously in the two channels. Measurements of aggregation and shape change during incubation of the same platelet-rich plasma were made in separate channels calibrated appropriately. In this case, incubation of control and test samples was started 2.5min apart, and aggregation and shape change were determined in portions taken at corresponding stages of incubation of each sample.

When four concentrations of ADP (or ADP+EDTA) were used to induce aggregation (or shape change), the experiment was performed as follows: incubation of the control sample at 37°C was started and one portion was immediately transferred to each channel of the aggregometer and tested for aggregation (or shape change) with the two lowest concentrations of ADP (or ADP+EDTA), one concentration in each channel. After 3min of testing, two new portions from the control sample were introduced in the channels and tested for 3min with the two highest concentrations of ADP (or ADP+EDTA). Then incubation of the test sample was started, and two pairs of samples were tested for aggregation with the four concentrations of ADP (ADP+EDTA) in the same manner as for those from the control mixture. This was repeated with two pairs of portions from the test sample until one concentration of ADP failed to induce aggregation (or shape change) (100% inhibition). Then a portion from the control sample was tested for aggregation (or shape change) with this particular concentration of ADP. Testing of portions from the test sample was then continued with the remaining concentrations of ADP (or ADP+EDTA) until the highest concentration of ADP (or ADP+EDTA) failed to produce aggregation (or shape change). The incubation time for initiation of the aggregation test was recorded, and samples for determination of metabolite radioactivity were taken at recorded times.

For measurement of aggregation, a 0.45ml sample was stirred with 50$\mu$l of the aggregating agent. The channel(s) were calibrated for each platelet-rich plasma as described by Holmsen *et al.* (1972). The aggregation response was measured for 2–5min with a chart speed of 3cm/min.

For measurement of shape change, a 0.2ml sample was mixed with 0.2ml of 0.15M-NaCl and stirred with 50$\mu$l of ADP–EDTA (10–100$\mu$l of 1mM-ADP in water diluted to 2.5ml with 38.5mM-EDTA in 0.075M-NaCl, pH7.4). For each platelet-rich plasma used the channels were calibrated so that the difference in extinction for maximum shape change represented about 20% of maximum recorder deflexion. The shape change response was measured for less than 1min at a chart speed of 12cm/min.

Primary aggregation and shape change were quantified from the initial slope (I.S.) of the respective tracings. In the determination of the initial slope the dilution response resulting from addition of inducer was neglected. Per cent aggregation relative to control at the different stages of incubation with the metabolic inhibitors was calculated as $(I.S._{\cdot test}/I.S._{\cdot control}) \times 100$, where the subscripts represent samples of the respective incubation mixtures tested at the same incubation time for each mixture. The initial slope in control mixtures at a given time of incubation was occasionally computed by interpolation.

Platelet counts were performed in a model $Z_BI$ counter (Coulter Electronics Inc., Hialeah, Fla., U.S.A.).

## Results

### *Effects of antimycin A and deoxyglucose on ethanol-soluble adenine metabolite radioactivity*

On incubation of radioactive platelets with 2-deoxyglucose, the decrease in the radioactivity of ATP was reflected quantitatively as an increase in hypoxanthine without significant change in the $^3$H content of ADP, AMP and IMP (Fig. 1). The rate of

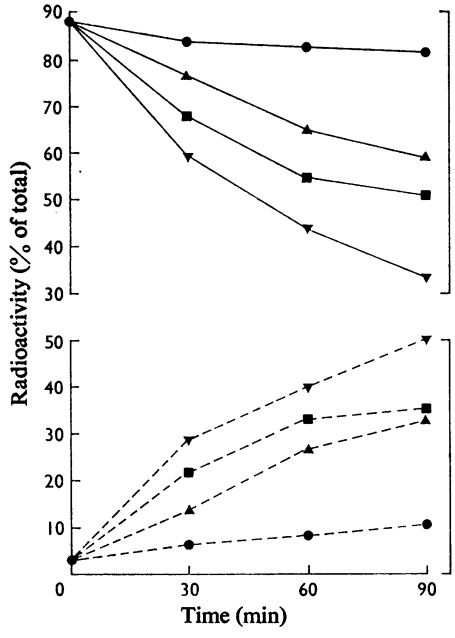


Fig. 1. *Incubation of [$^3H$]adenine-labelled platelet-rich plasma with different concentrations of 2-deoxyglucose*

The effect of incubation time on the concentrations of [$^3$H]ATP (solid lines) and [$^3$H]hypoxanthine+inosine (broken lines) is shown. The incubation mixtures ($4.5 \times 10^8$ cells/ml, 100000c.p.m./ml) were incubated with 0 (●), 8.04 (▲), 24.13 (■) and 48.26 (▼) mM-2-deoxyglucose.

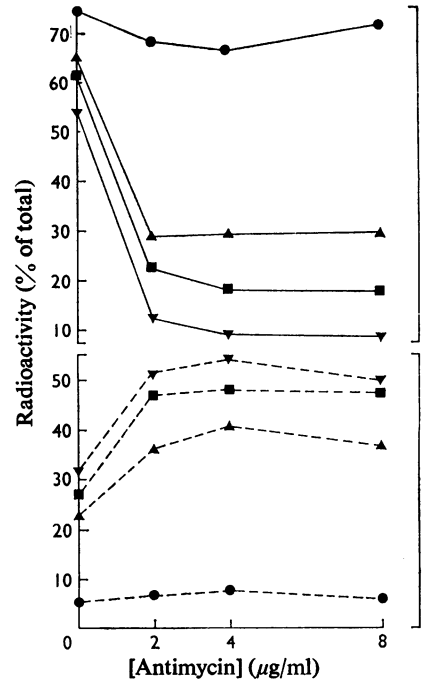


Fig. 2. *Incubation of [$^3H$]adenine-labelled platelet-rich plasma at 37°C with various concentrations of antimycin A and 2-deoxyglucose*

The effect of incubation for 45min on the concentrations of [$^3$H]ATP (solid lines) and [$^3$H]hypoxanthine+inosine (broken lines) is shown. Portions (1ml) of platelet-rich plasma ($2.91 \times 10^8$ cells/ml; 100000c.p.m./ml) were incubated with 0 (●), 6.47 (▲), 12.94 (■) and 33.30 (▼) mM-2-deoxyglucose and various concentrations of antimycin. At the start of incubation, 81.7 and 3.6% of the total radioactivity was present as [$^3$H]ATP and [$^3$H]hypoxanthine+inosine respectively.

conversion of ATP to hypoxanthine increased with increasing deoxyglucose concentration up to at least 50mM in the presence of approx. 5mM-glucose (endogenous plasma concentration). When added alone at concentrations up to 16μg/ml antimycin had no significant effect on the radioactivity in the adenine nucleotides, IMP and hypoxanthine+inosine (Fig. 2). However, in the presence of 2-deoxyglucose, addition of low concentrations of antimycin markedly accelerated the extent of disappearance of [$^3$H]ATP and caused a corresponding, although quantitatively smaller, increase in [$^3$H]hypoxanthine+inosine. At 6.5mM-2-deoxyglucose, the ATP disappearance rate increased linearly with the concentration of antimycin up to 0.31μg/ml of platelet rich plasma; above this concentration no change in the rate occurred. Maximal effects were observed in the presence of 4μg of antimycin/ml of platelet-rich plasma at all deoxyglucose concentrations tested (Fig. 2). The radioactive ATP that was not converted to hypoxanthine+inosine under these conditions could be recovered primarily as AMP and IMP, which increased by an order of magnitude (Fig. 4).

*Effect of low inhibitor concentrations on the decay of radioactive ATP*

To study the effect of the inhibitors on platelet function conditions had to be established such that the decay of [$^3$H]ATP in the test sample was negligible during the period required (2–5min) for measurement of aggregation and shape change. A combination of 5.6mM-deoxyglucose and 4.1μg of antimycin/ml, which was found suitable for such studies, gave the changes in the radioactive adenine metabolites shown in Fig. 3. The $^3$H content of ATP declined progressively and reached a stable value at 12–20% of the initial value after 120min. The increase in $^3$H in hypoxanthine+inosine over this period was equivalent to the decrease in ATP. Little change was observed in the $^3$H content of ADP and IMP during

the incubation period. However, the amount in IMP was increased as compared with control samples. In contrast, the radioactive content of AMP increased slowly to a value after 60–100min of incubation

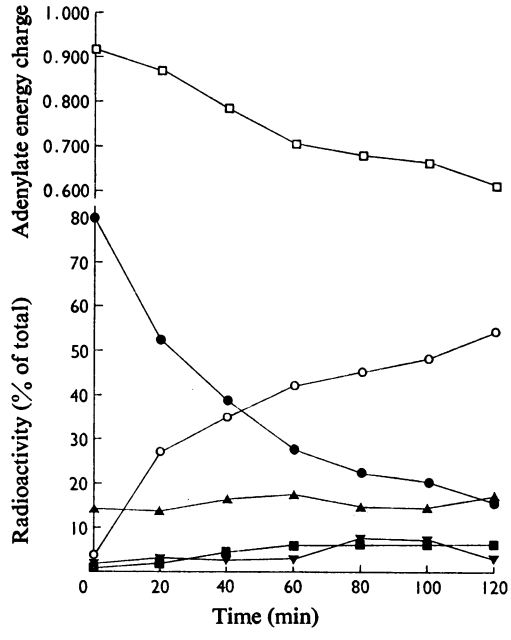


Fig. 3. *Incubation of [$^3$H]adenine-labelled platelet-rich plasma with antimycin A and 2-deoxyglucose at 37°C in concentrations giving ATP disappearance rates suitable for study of aggregation and shape change*

Platelet-rich plasma ($4.2\times10^8$ cells/ml; 136000c.p.m./ml) was incubated with 4.1 $\mu$g of antimycin/ml and 5.6mM-2-deoxyglucose. Samples were analysed at various times for their content of $^3$H in ethanol-soluble ATP (●), ADP (▲), AMP (■), IMP (▼) and hypoxanthine+inosine (○). The upper section gives the corresponding changes in adenylate energy charge as calculated from the radioactivities of ATP, ADP and AMP. Concomitant incubation of this radioactive platelet-rich plasma with the solvents of the inhibitors caused a decrease in [$^3$H]ATP from 77 to 71% and an increase in [$^3$H]hypoxanthine+inosine from 4.5 to 12% in 120min. No changes in the $^3$H content of ADP, AMP and IMP occurred in this control sample.

eightfold greater than that observed at zero time. The adenylate energy charge decreased steadily on incubation with the inhibitors from an initial value of 0.925 to a final constant value of 0.615 (Fig. 3).

When samples from the incubation mixtures were centrifuged and the cells and supernatant plasma extracted separately with EDTA–ethanol, 95% of the [$^3$H]hypoxanthine+inosine was found in the extracellular phase at all stages of the incubation. In contrast, [$^3$H]ATP, [$^3$H]ADP, [$^3$H]AMP and [$^3$H]-IMP were present within the cells. Chromatography of the EDTA–ethanol extracts prepared after 10–20min showed that inosine accounted for up to 20% of the radioactivity in the combined hypoxanthine–inosine spot obtained on separation by electrophoresis. At later times the inosine content decreased, and more than 95% of the radioactivity was present in hypoxanthine. The sum of radioactivity in the extracts was constant throughout the incubation period, indicating that no ethanol-soluble radioactive metabolites other than ADP, ATP, AMP, IMP, hypoxanthine and inosine were formed.

In the absence of the inhibitors (cf. Fig. 1, no dexoyglucose) the slow decrease in the $^3$H content of ATP (0.02–0.1%/min) was reflected as an increase in [$^3$H]hypoxanthine+inosine with little or no change in the $^3$H content of ADP, AMP and IMP. Under these conditions, the adenylate energy charge remained essentially constant for the period of incubation. A similar distribution of the radioactive metabolites between cells and plasma was observed in the presence or absence of the inhibitors. $P_i$ in the platelets increased progressively during incubation with combinations of antimycin and deoxyglucose. Incubation deoxyglucose alone or without inhibitors caused no change in the P′ contents (Table 1); antimycin alone also had no effect.

### *Effect of high inhibitor concentration on the decay of radioactive ATP*

Fig. 4 demonstrates the changes in the $^3$H content of the ethanol-soluble adenine nucleotides on incubation of labelled platelets with a high concentration of deoxyglucose (32.2mM) in the presence of antimycin (4.1 $\mu$g/ml). Under these conditions the $^3$H content of

Table 1. *Changes in platelet inorganic phosphate during incubation of platelet-rich plasma with antimycin and 2-deoxyglucose*

Three samples of platelet-rich plasma ($2.81\times10^8$ cells/ml) were incubated at 37°C separately with 4.1 $\mu$g of antimycin/ml +5.7mM-deoxyglucose, 5.7mM-deoxyglucose alone and with the solvents for the inhibitors. Samples for phosphate determination were taken at the times indicated. Further details are given in the Experimental section.

| Addition | Incubation time (min) ... | $P_i$ ($\mu$mol/$10^{11}$ platelets) 0 | 20 | 40 | 60 | 80 |
|---|---|---|---|---|---|---|
| Solvents | | 8.7 | 8.3 | 8.3 | 8.5 | 8.7 |
| Deoxyglucose | | 8.0 | 7.9 | 7.9 | 8.1 | 8.3 |
| Antimycin+deoxyglucose | | 8.7 | 8.8 | 10.6 | 11.9 | 14.5 |

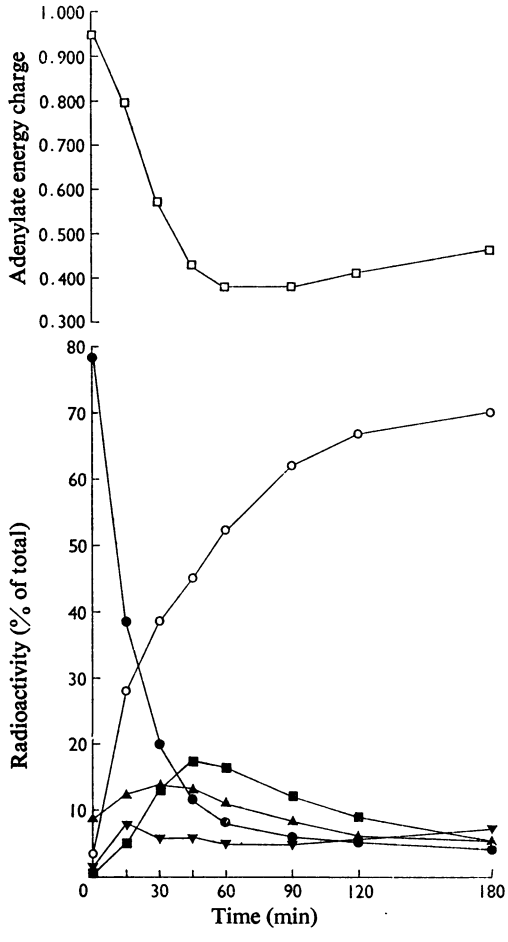


Fig. 4. *Incubation of $^3$H-labelled platelet-rich plasma at 37°C with antimycin A and 2-deoxyglucose in concentrations giving maximal rates of ATP disappearance*

Platelet-rich plasma ($4.2\times10^8$ cells/ml; 101500c.p.m./ml) was incubated with 4.1 $\mu$g of antimycin/ml and 32.2mM-2-deoxyglucose. Samples were analysed at various times for their content of $^3$H in ethanol-soluble ATP (●), ADP (▲), AMP (■), IMP (▼) and hypoxanthine+inosine (○). The upper section gives the corresponding changes in adenylate energy charge as calculated from the radioactivities of ATP, ADP and AMP. Concomitant incubation of the same radioactive platelet-rich plasma with the solvents of the inhibitors caused a decrease in the [$^3$H]-ATP from 78.1 to 69.2 and an increase in the [$^3$H]hypoxanthine+inosine from 3.9 to 13.1% of total radioactivity, during the 180min incubation period. The changes in the $^3$H content of ADP, AMP and IMP were negligible.

ATP decreased by 95% within 2h. Some 85% of the radioactivity was recovered in hypoxanthine+inosine and the remainder was present in AMP and IMP. The $^3$H content of ADP and AMP increased initially and attained maximum values after 30min (ADP) and 50min (AMP) of incubation. After 3h the $^3$H content of ADP was lower, and that of AMP substantially higher, than in the control sample. However, [$^3$H]-IMP increased rapidly to values 8–10 times the control value during the initial phase of incubation with inhibitors but thereafter remained essentially constant. The adenylate energy charge decreased linearly during the first 60min of incubation to a value of approx. 0.38, but thereafter increased somewhat.

After 2h of incubation the concentration of total ATP had decreased to only 35% of its initial value (Table 2). Hence the $^3$H content of ATP which disappeared during incubation of platelets with antimycin+2-deoxyglucose is approximately tenfold greater than that which remains in the platelets under these conditions.

During incubation with the inhibitors the decrease in both the concentration and the $^3$H content of ethanol-insoluble ATP paralleled those of ethanol-soluble ATP. In contrast, both the concentration and the $^3$H content of the ethanol-insoluble ADP (43% of total [$^3$H]ADP) remained constant during incubation with the inhibitors (Table 2).

*Comparison between platelet aggregation and content of radioactive ATP*

At 37°C incubation of platelets with 4$\mu$M-ADP causes a biphasic aggregation response (Fig. 5*a*). The first phase is usually referred to as primary aggregation, whereas the second phase represents occurrence of the platelet release reaction (for references see Holmsen *et al.*, 1972). During incubation in the absence of the inhibitors the primary aggregation response became progressively weaker, and the second-phase response often disappeared entirely at the later stages of incubation. In the presence of the inhibitors the second phase was lost very early in the incubation, whereas the primary aggregation response decreased progressively as incubation proceeded. Fig. 5(*a*) shows that the secondary and primary aggregation responses disappeared completely after incubation for 5 and 45min respectively in the presence of the inhibitors. A 15% decrease in the [$^3$H]ATP was associated with loss of secondary aggregation in the presence of the inhibitors (Fig. 5*b*). In the absence of inhibitors a similar decrease in the $^3$H content of ATP was also usually associated with loss of the secondary aggregation response. In contrast, the primary aggregation response decreased more gradually with the decline in the $^3$H content of ATP and was abolished at a 40% decrease in [$^3$H]-ATP (Fig. 5). Thus, complete inhibition of primary platelet aggregation required a distinctly greater decrease in the metabolic ATP concentration than is necessary for complete inhibition of secondary

Table 2. *Effects of high concentrations of antimycin and 2-deoxyglucose on the ADP and ATP contents of ethanol-soluble and -insoluble fractions of platelet-rich plasma*

Control and test samples were prepared from radioactive platelet-rich plasma ($4.2\times10^8$ cells/ml, 101 500 c.p.m./ml), with the test mixture containing 4.1 $\mu$g of antimycin/ml and 32.23 mM-2-deoxyglucose, and incubated at 37°C. Samples (1 ml) were taken out at the times noted into EDTA–ethanol and analysed for adenine metabolite radioactivity and the ATP and ADP contents of the ethanol-soluble and -insoluble fractions. The results for metabolite radioactivity are given in Fig. 4 as percentages of the total radioactivity in soluble plus insoluble fractions.

| | Incubation time (min) | Amounts ($\mu$mol/$10^{11}$ cells) | | $10^{-5}\times$Radioactivity (c.p.m./$10^{11}$ cells) | | Specific radioactivity (c.p.m./$\mu$mol) | |
|---|---|---|---|---|---|---|---|
| | | Soluble | Insoluble | Soluble | Insoluble | Soluble | Insoluble |
| ATP | | | | | | | |
| Control | 0 | 4.5 | 0.07 | 23.0 | 0.37 | 4632 | 5123 |
| | 120 | 4.8 | 0.08 | 20.9 | 0.40 | 4329 | 5187 |
| Antimycin+deoxyglucose | 15 | 3.1 | 0.07 | 10.2 | 0.34 | 3246 | 4730 |
| | 60 | 2.3 | 0.05 | 2.1 | 0.07 | 911 | 1514 |
| | 120 | 1.9 | 0.03 | 1.3 | 0.02 | 676 | 807 |
| ADP | | | | | | | |
| Control | 0 | 3.8 | 0.27 | 2.2 | 1.61 | 557 | 6051 |
| | 120 | 2.3 | 0.27 | 2.4 | 1.55 | 724 | 5684 |
| Antimycin+deoxyglucose | 15 | 3.7 | 0.29 | 3.5 | 1.64 | 905 | 5641 |
| | 60 | 2.8 | 0.28 | 3.0 | 1.64 | 1006 | 5937 |
| | 120 | 2.3 | 0.26 | 1.6 | 1.40 | 669 | 5438 |

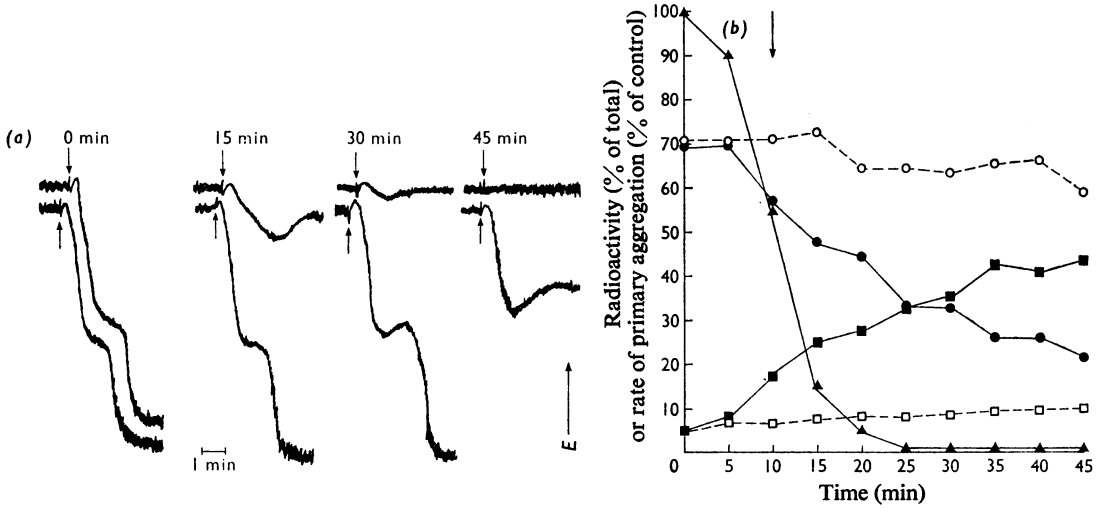


Fig. 5. *Incubation of [$^3H$]adenine-labelled platelet-rich plasma at 37°C with antimycin A and 2-deoxyglucose and concomitant determination of the rate of ADP-induced platelet aggregation*

Platelet-rich plasma ($5.2\times10^8$ cells/ml; 160000 c.p.m./ml) was incubated either with 4.5 mM-2-deoxyglucose and 4.1 $\mu$g of antimycin/ml (upper aggregometer traces, solid lines) or with the solvents of the inhibitors (lower aggregometer traces, broken lines). Samples were analysed at various times for (*a*) aggregation with 5 $\mu$M-ADP and (*b*) content of ethanol-soluble [$^3$H]ATP (●, ○) and [$^3$H]inosine+hypoxanthine (■, □), ▲, rate of primary aggregation relative to the control (see under 'Methods'). The arrow in (*b*) indicates when the secondary aggregation response disappeared in the platelet-rich plasma containing the inhibitors. The arrows in (*a*) indicate addition of ADP.

aggregation. The extent of aggregation in the presence of 4 $\mu$M-ADP was also examined during incubation of platelets with 30 mM-deoxyglucose in the absence of antimycin. Both the primary and secondary aggregation responses varied with the $^3$H content of ATP in a pattern qualitatively similar to that

Table 3. *Effect of the concentration of ADP used to induce platelet aggregation on the radioactive ATP concentration at which aggregation does not occur*

Test and control samples were prepared from radioactive platelet-rich plasma ($4.12 \times 10^8$ cells/ml; 88000 c.p.m./ml) with the test containing 4.1 $\mu$g of antimycin/ml and 5.75 mM-2-deoxyglucose. The samples were incubated at 37°C and portions taken at noted times for determination of metabolite radioactivity and rate of aggregation with different concentrations of ADP. For further details, see the Experimental section. During the 48 min incubation period the ATP radioactivity in the control sample decreased from 82 to 77% of total $^3$H radioactivity. The numbers below show the percentage decrease in radioactive ATP in the test sample as compared with the control, and the numbers in parentheses show the incubation times (in min) required to produce complete inhibition.

| ADP concn. ($\mu$M) | Complete inhibition |
|---|---|
| 1 | 31 (18) |
| 2 | 49 (30) |
| 4 | 56 (39) |
| 8 | 62 (48) |

observed on incubation with 2-deoxyglucose+antimycin as shown in Fig. 5. With 30 mM-2-deoxyglucose the $^3$H content of ATP decreased by 18–30% during a 48 min incubation period (Fig. 1), and complete inhibition of primary aggregation never occurred. Under these conditions, secondary aggregation disappeared completely with a 12–15% decrease in [$^3$H]ATP. The extent of primary aggregation induced by 4 $\mu$M-adrenaline decreased gradually with the $^3$H content of platelet ATP during incubation of platelet-rich plasma with antimycin+2-deoxyglucose. As shown for aggregation induced by ADP (Fig. 5), primary and secondary aggregation responses induced by adrenaline were abolished when the fall in [$^3$H]ATP exceeded 40 and 15% respectively. The secondary aggregation induced by 4 $\mu$M-adrenaline also disappeared completely when the $^3$H content of ATP was decreased by 12–15% during incubation without inhibitors, or with 30 mM-deoxyglucose alone. The relation between the degree of primary adrenaline-induced aggregation and the $^3$H content in ATP was the same whether the amount of [$^3$H]ATP was decreased by incubation with antimycin and deoxyglucose or deoxyglucose alone (30 mM).

However, the degree of inhibition of aggregation platelet ATP is dependent on the concentration of added ADP used to induce aggregation (Table 3). When the ADP concentration used to induce aggregation is decreased, the extent of the decrease in [$^3$H]ATP that is required to produce a given degree of inhibition of aggregation is also decreased. For adrenaline, the extent of primary aggregation at concentrations lower than 4 $\mu$M was too low to permit accurate determination of the rate (initial slope) of aggregation, and therefore the relation between the $^3$H content in ATP and inhibition of primary aggregation could not be established at low concentrations of adrenaline.

*Correlation of the degree of platelet shape change induced by ADP with the amount of radioactivity in platelet ATP*

Fig. 6(*a*) shows recorder tracings of shape change induced by addition of 1 $\mu$M-ADP+7.7 mM-EDTA at different stages of incubation. In the absence of 2-deoxyglucose+antimycin neither the rate nor the extent of the shape change is affected by incubation at 37°C for 120 min. However, the rate of shape change is progressively decreased on incubation in the presence of 2-deoxyglucose (5.6 mM)+antimycin (4.1 $\mu$g/ml). Neither the extinction nor the extent of the oscillations observed before addition of ADP–EDTA changed during incubation with the inhibitors (Fig. 6*a*). Since the extent of the oscillations in particular are indicative of the discoid morphology, it is clear from Fig. 6(*a*) that the presence of the inhibitors did not cause a measurable shape change in the absence of ADP–EDTA.

The rate of shape change decreased gradually as the [$^3$H]ATP content declined. Complete loss of the shape change response is observed when [$^3$H]ATP decreased by 80% below its initial level (Fig. 6*b*). Under the same conditions primary aggregation produced by 4 $\mu$M-ADP was abolished by a 40–50% decrease in [$^3$H]ATP, whereas shape change was only then inhibited by 10% (Fig. 6*b*).

The extent of inhibition of the shape change response which corresponds to a given decrease in the concentration of metabolic ATP was dependent on the ADP concentration used to induce this response. Thus, as shown in Table 4, the decrease in the $^3$H content of platelet ATP required to produce a given percentage inhibition of the shape-change response decreased if a lower concentration of ADP was used to induce the response. This relationship is qualitatively similar to that found for primary aggregation induced by ADP (Table 3).

*Changes in platelet adenine nucleotides on adding ADP to platelets treated with metabolic inhibitors*

Platelets were preincubated in the presence or absence of deoxyglucose (5.6 mM)+antimycin (4.1 $\mu$g/ml) for 15 min at 37°C. Samples were then removed and stirred with 4 $\mu$M-ADP (for preincubation with or without inhibitors) or 0.15 M-NaCl (for preincubation with inhibitors only). At the times indicated, samples were removed from these latter systems for analysis of the $^3$H distribution in ATP, IMP and hypoxanthine+inosine. Negligible changes in the radioactive metabolites occurred during aggregation of platelets incubated without the inhibitors (Fig. 7*b*, curves A).

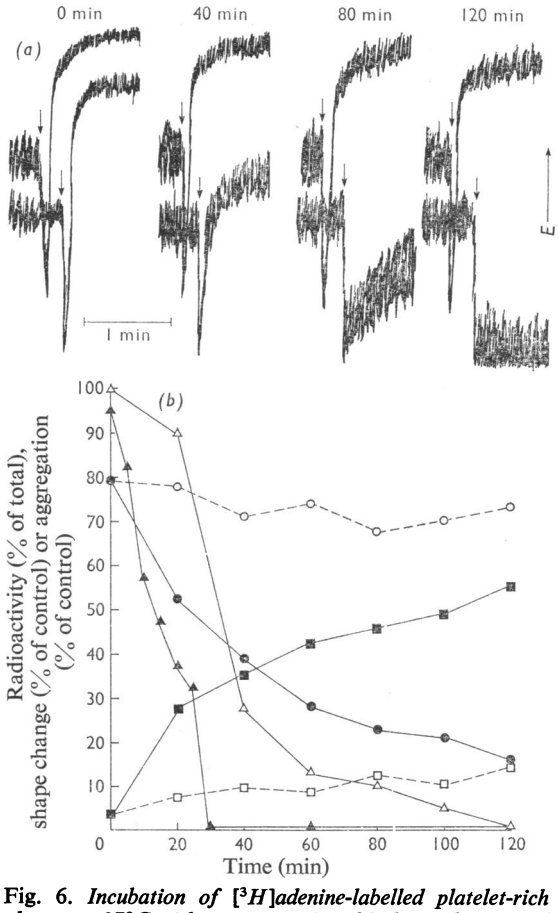


Fig. 6. *Incubation of [$^3$H]adenine-labelled platelet-rich plasma at 37°C with antimycin A and 2-deoxyglucose and concomitant determination of the rate of shape change and aggregation induced by added ADP*

Platelet-rich plasma ($4.2\times10^8$ cells/ml; 136000c.p.m./ml) was incubated either with 4.1$\mu$g of antimycin/ml and 5.6mM-deoxyglucose (upper traces, solid line) or with the solvents of the inhibitors (lower traces, broken lines). Samples were analysed at the times indicated for (*a*) platelet shape change induced with 1$\mu$M-ADP+4.3mM-EDTA, and (*b*) platelet aggregation induced with 4$\mu$M-ADP and content of [$^3$H]ATP (●, ○) and [$^3$H]inosine+hypoxanthine (■, □). ▲, Percentage aggregation; △, shape change relative to control. The arrows in (*a*) indicate addition of ADP–EDTA. The instantaneous fall in extinction on addition of inducer is due to dilution of cuvette content, and the following rapid increase in extinction represents rate of shape change.

In platelets that had been incubated with the inhibitors and stirred with NaCl, ATP decreased steadily with a corresponding increase in hypoxanthine (Fig. 7*b*, curves B). This pattern reflects the continuing action of the inhibitors. However, when platelets preincubated with inhibitors were stirred with ADP, a very rapid fall was observed in [$^3$H]ATP which was reflected in a marked accumulation of $^3$H in IMP over this early period (Fig. 7*b*, curves C). No such rapid fall in [$^3$H]ATP or increase of $^3$H in IMP was observed in the NaCl control (Fig. 7*b*, curves B). The primary platelet aggregation response to ADP is only slightly inhibited by the inhibitors at the concentration used (Fig. 7*a*). A rapid, significant decrease in the ratio of [$^3$H]ATP/[$^3$H]ADP (33%) was observed on addition of ADP to platelets preincubated in the presence of the inhibitors. The decrease is also reflected in the adenylate energy charge which fell from 0.892 before addition of ADP to 0.858 after this addition. This decrease in the ATP/ADP ratio and adenylate energy charge reflects the fall in the $^3$H content of ATP since the $^3$H content of ethanol-soluble ADP did not vary significantly during aggregation.

Table 4. *Effect of the concentration of ADP used to induce shape change of platelets on the radioactive ATP concentration at which shape change does not occur*

Test and control samples were prepared from radioactive platelet-rich plasma ($4.47\times10^8$ cells/ml, 108000c.p.m./ml), and with the test containing 4.1$\mu$g of antimycin/ml and 5.75mM-2-deoxyglucose. The samples were incubated at 37°C and samples taken at noted times for determination of shape change with different concentrations of ADP–EDTA and of adenine metabolite radioactivity. Numbers outside and within parentheses are defined and computed as described in Table 3. During the 60min of incubation the $^3$H content in ATP in the control decreased from 84 to 75% of the total radioactivity.

| ADP concn. ($\mu$M) | Complete inhibition |
|---|---|
| 0.25 | 51 (30) |
| 0.5 | 66 (43) |
| 1.0 | 70 (49) |
| 2.0 | 75 (58) |

## Discussion

### *Action of antimycin and deoxyglucose on platelet adenine nucleotides*

When platelets labelled by incubation with [$^3$H]-adenine are exposed to high concentrations of antimycin and deoxyglucose, an almost complete depletion of [$^3$H]ATP is observed after 120min of incubation. However, a smaller decrease is observed in the total ATP concentration, and after 120min of incubation 35% of the total ATP remains in the cells with a specific radioactivity that is tenfold lower than that of the ATP which disappears under these conditions. During the platelet release reaction, 30–40% of the platelet ATP is released to the extracellular milieu.

When platelets labelled with [$^3$H]adenine or [$^{14}$C]-adenine are used in such release experiments, the specific radioactivity of the released ATP is tenfold lower than that retained in the cells (Holmsen & Day, 1971). Hence incubation with antimycin and deoxyglucose appears to deplete the metabolic ATP pool of platelets in platelet-rich plasma, and has little, if any effect on the non-metabolic (storage) pool.

Deoxyglucose has been shown to produce a concentration-dependent decrease in the total ATP concentration of platelets (Schneider & Niemeyer, 1968; Detwiler, 1971). The data presented here demonstrate that this decrease in platelet ATP is restricted to the metabolic pool. The metabolic ATP is degraded to hypoxanthine via a pathway involving ADP, AMP, IMP and inosine as intermediates, and the final product accumulates extracellularly. Such degradation is irreversible since human platelets are unable to synthesize ATP from IMP, inosine or hypoxanthine (Ireland & Mills, 1966; Rozenberg & Holmsen, 1968), the metabolic sequence which appears most likely to be responsible for the conversion of ATP to hypoxanthine, is ATP → ADP → AMP → IMP → inosine → hypoxanthine, involving adenosine triphosphatases (EC 3.6.1.3), adenylate kinase (EC 2.7.4.3), AMP deaminase (EC 3.5.4.6), 5′-nucleotidase (EC 3.1.3.5) and purine nucleoside phosphorylase (EC 2.4.2.1). Our results (cf. Figs. 3 and 4) are not consistent with this sequence, since [$^3$H]IMP reached a high constant concentration before [$^3$H]-ADP and [$^3$H]AMP had reached their transient maxima during incubation of platelets with antimycin and deoxyglucose (Fig. 4). However, the observed changes may be explained on the basis of the above pathway, since platelet AMP deaminase is regulated by the adenylate energy charge (H. Holmsen, unpublished work) in the same way as the liver enzyme (Chapman & Atkinson, 1973), provided that platelet 5′-nucleotidase, which is responsible for dephosphorylation of IMP, exhibits the high $K_m$ for IMP and the same regulatory properties as in other systems (Fritzon, 1969). The activity of AMP deaminase increases sharply as the adenylate energy charge decreases from 0.95 to 0.60. In platelets the adenylate energy charge falls rapidly and within this range on addition of antimycin and deoxyglucose. Such an effect would explain the rapid, early increase in the concentration of [$^3$H]IMP, which above a certain point may then result in enhancement of the

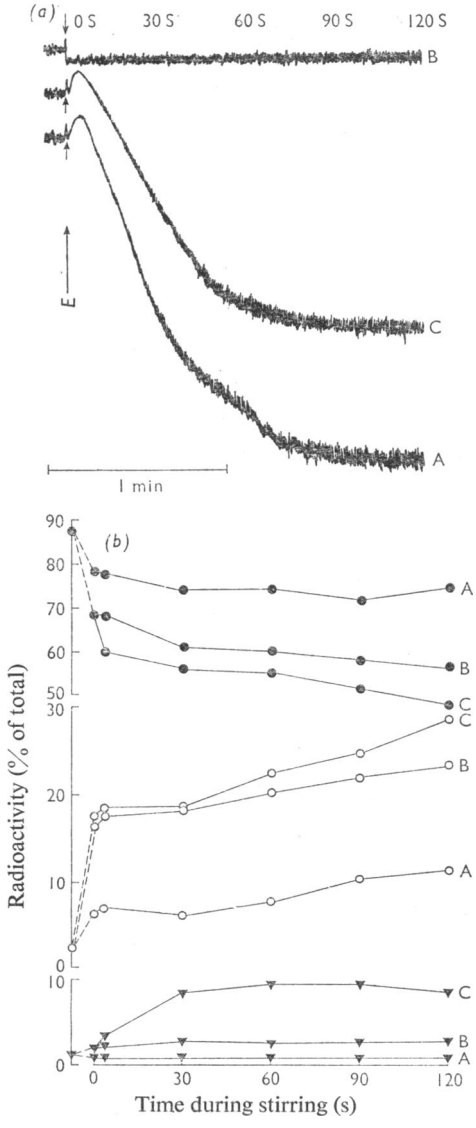


Fig. 7. *ADP-induced platelet aggregation in [$^3$H]adenine-labelled platelet-rich plasma incubated with and without antimycin A+2-deoxyglucose*

Platelet-rich plasma ($4.7\times10^8$ cells/ml; 140000 c.p.m./ml) was incubated at 37°C either with 4.1 $\mu$g of antimycin/ml and 5.6 mM-deoxyglucose, or with the solvents of the inhibitors. A 200 $\mu$l sample of each mixture was taken at the start of incubation for determination of the content of [$^3$H]ATP (●), [$^3$H]IMP (○) and [$^3$H]hypoxanthine+inosine (▼) [points shown on ordinate in (*b*)]. After 15 min two 2.2 ml samples from the platelet-rich plasma–inhibitor mixture were stirred separately with 4 $\mu$M-ADP (C) and 0.15 M-NaCl (B), and a 2.2 ml sample of the platelet-rich plasma without inhibitors was stirred with 4 $\mu$M-ADP (A) in the aggregometer. (*a*) Aggregometer traces. (*b*) Subsamples (200 $\mu$l) were taken from the three mixtures (A, B and C) at the times indicated during stirring and analysed for their content of [$^3$H]ATP (●), [$^3$H]IMP (○) and [$^3$H]hypoxanthine+inosine (▼).

5′-nucleotidase activity, and hence cause the [$^3$H]-IMP concentration to reach a constant steady-state value (cf. Fig. 4).

The key enzyme that regulates the rate of conversion of adenine nucleotides to hypoxanthine is believed to be AMP deaminase, which is activated by ATP and inhibited by GTP and $P_i$ (Lee & Wang, 1968; H. Holmsen, unpublished work). In Ehrlich ascites tumour cells addition of deoxyglucose causes an increase in the rate of degradation of ATP to IMP and other catabolites. The acceleration of this process is believed to result from a decrease in the intracellular $P_i$ observed under these conditions (Overgaard-Hansen, 1965; Yushok, 1971). However, such a mechanism does not appear applicable to the platelet system, since the amounts of intracellular $P_i$ remained constant during incubation with deoxyglucose, and actually increased when deoxyglucose and antimycin were used in combination. This accumulation of $P_i$ may actually cause inhibition of AMP deaminase, since accumulation of AMP was always observed during incubation with deoxyglucose+antimycin. In platelets, addition of antimycin has been shown to inhibit respiration (Mürer, 1969*a*,*b*; Muenzer *et al.*, 1972; Salganicoff & Fukami, 1973) and to enhance the uptake of adenosine and adenine (Rozenberg & Holmsen, 1968; Sixma *et al.*, 1973). Since KCN also stimulates conversion of ATP into hypoxanthine in platelets incubated with either monoiodoacetate (Ball *et al.*, 1969) or with deoxyglucose (Kattlove, 1974), it seems likely that the effect of antimycin is due to inhibition of electron transport (Slater, 1973) and hence of ATP synthesis. Neither KCN nor antimycin has a direct effect on platelet AMP deaminase (H. Holmsen, unpublished work) thus eliminating this mechanism as the cause of stimulation of the conversion of ATP into hypoxanthine. The apparent acceleration of this conversion in the presence of deoxyglucose by antimycin, observed in the present study, may then result from a decrease in the intracellular ATP concentration owing to inhibition of mitochondrial ATP synthesis. When both glycolytic and mitochondrial ATP synthesis are inhibited, a marked decrease in the adenylate energy charge is observed. Since activation of platelet AMP deaminase occurs owing to a decrease in adenylate energy charge (H. Holmsen, unpublished work), this effect might explain the increased rate of conversion of adenine nucleotides into IMP, inosine and hypoxanthine which results from incubation of platelets with antimycin and deoxyglucose.

The metabolic adenine nucleotides in platelets can be subdivided into ethanol-soluble and -insoluble fractions. The radioactive content of ATP and ADP in these two fractions increases in parallel when radioactive adenine is taken up by the intact cell (Holmsen, 1972). However, when the radioactivity in ethanol-soluble ATP is decreased or that in ethanol-soluble ADP is altered, on incubation of platelets with antimycin and deoxyglucose no changes are observed in the radioactivity of the ethanol-insoluble ADP pool. Since this ADP pool is bound to protein (P. C. French & H. Holmsen, unpublished work), the invariance in amount and specific radioactivity of this ADP when ATP synthesis is inhibited suggests that the protein is fully saturated with ADP under the conditions used. ADP may be extracted from this protein fraction if $HClO_4$ rather than ethanol is used. Since protein-bound ADP appears unaffected by inhibition of ATP synthesis, and since this fraction accounts for 20–50% of the total pool of metabolic ADP in human platelets, ethanol extraction rather than $HClO_4$ extraction appears to be the method of choice for studies of the metabolic nucleotide pool.

*Functional consequences of a decrease in the concentration of metabolic ATP induced by inhibition of ATP synthesis in the presence of antimycin and deoxyglucose*

Progressive inhibition of the shape change and (primary) aggregation responses of platelets is observed when the concentration of metabolic ATP is decreased. However, for a given decrease in this ATP pool, the inhibition of the aggregation response is greater than that observed for the shape-change response. This observation indicates that these two functions exhibit different degrees of dependence on the metabolic ATP concentrations. This relationship can be expressed as differences in that concentration of metabolic ATP below which a platelet function cannot occur. This concentration may be defined as the metabolic ATP threshold, characteristic of the particular process. The platelet release reaction, which is expressed as the secondary aggregation response, thus has a metabolic ATP threshold higher than that of primary aggregation, which in turn has a threshold higher than that required for shape change. For ADP the data presented demonstrate that the metabolic ATP threshold for the shape change and aggregation responses decrease as the inducer concentration is increased. Hence the ability to perform these functions is dependent both on the strength of the stimulus and on the concentration of metabolic ATP. This relationship is further substantiated by the observation that the extent of aggregation and shape change decreases gradually with a decrease in metabolic ATP concentration. Hence consumption of ATP appears to be required for performance of the activity *per se* and/or to maintain the platelet in a responsive state for such performance. The rapid fall in adenylate energy charge on addition of ADP to unstirred platelets in the presence of EDTA has been suggested to indicate direct utilization of ATP during the shape-change response (Mills, 1973). However, both Kattlove (1974) and we have failed to demonstrate

similar changes in either metabolic ATP concentrations or the adenylate energy charge during the shape change or aggregation response induced by ADP when stirred platelets are tested in the absence of metabolic inhibitors. This difference between the stirred and unstirred systems suggests that if ATP is consumed during the response, rapid ATP resynthesis completely compensates for such consumption. Hence apparent constancy of the metabolic ATP concentration is observed under such conditions. However, if ATP resynthesis is inhibited, any ATP consumption which occurs during performance of the processes should become apparent. Our data clearly demonstrate such ATP consumption, and suggest that this consumption is related to the performance of the initial phase of the combined shape change–aggregation process, as also concluded by Mills (1973) and Kattlove (1974). It is therefore puzzling that, whereas shape change requires the presence of definitely smaller concentrations of ATP than does primary aggregation, it is during shape change that ATP consumption can be demonstrated. ATP consumption apparently does not occur during aggregation. This could mean that the requirement of the greater concentrations of ATP for aggregation is because ATP is continuously utilized in the resting state to maintain the cells in an aggregation-responsive condition, whereas the ATP requirement for shape change reflects the utilization of ATP during performance of this platelet function.

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