*Biochem. J.* (1969) **114**, 669
*Printed in Great Britain*



# Effect of some Inhibitors of Platelet Aggregation on Platelet Nucleotides

By G. Ball, Mary Fulwood, D. M. Ireland and Patricia Yates
*Glaxo Research Ltd., Greenford, Middx.*

(*Received* 17 *July* 1969)

ADP-induced adhesion and aggregation of platelets (Mürer, Hellem & Rozenberg, 1967), thrombin-induced release of platelet nucleotide (Mürer, 1968) and clot retraction (Mürer, 1969) are dependent on platelet ATP.

Human platelets contain at least two pools of ATP and ADP, one that readily incorporates radioactivity from [$^{32}$P]phosphate or [$^{14}$C]adenosine and one that does not. Thrombin or collagen treatment of labelled platelets causes both a breakdown of radioactive ATP, the nucleoside and purine products of which are found extracellularly, and a release of non-radioactive ATP and ADP (Holmsen, 1965; Ireland, 1967). The released ADP causes platelet aggregation (Haslam, 1967).

This communication describes the effect of some inhibitors of collagen-induced aggregation on radioactive platelet ATP and on release of non-radioactive ATP and ADP. Radioactivity was incorporated in the platelets by incubation with [$^{14}$C]adenine (Holmsen & Rozenberg, 1968).

*Methods and materials.* Citrated platelet-rich plasma was prepared at 37° from freshly withdrawn human blood (Ireland, 1967) and incubated at 37° with [U-$^{14}$C]adenine (25nc/ml. of plasma) for 30min., by which time only trace amounts of radioactivity were present as adenine. The plasma was kept at 37° and aggregation was followed in a modified absorptiometer (Mills & Roberts, 1967*a*,*b*), after initiation with bovine collagen [Sigma (London) Chemical Co. Ltd., London S.W.6]. The collagen was prepared in 0·9% NaCl as described by Evans, Packham, Nishizawa, Mustard & Murphy (1968).

For radioactivity assays of platelet-rich plasma, incubations were terminated by adding 4·5ml. of 10% (w/v) trichloroacetic acid containing carrier compounds to the plasma and radioactivity was measured by scintillation counting (Packard Tri-Carb 3320) after charcoal treatment and chromatography with solvent system 1 (Ireland & Mills, 1966; Ireland, 1967). The counting efficiency was about 65%. For assays of ATP and ADP released to the supernatant, incubations were terminated by adding 0·1ml. of 77mM-disodium EDTA, pH 7·4, to the plasma and cooling the mixture to 0° in ice–methanol (−5°). Cells were removed by two centrifugations at 2–4°, each for 15min. at 2410*g*. Portions of the supernatants were mixed with equal volumes of ethanol and after centrifugation ATP and ADP were measured in the supernatant by a firefly bioluminescence method (Holmsen, Holmsen & Bernhardsen, 1966). For assays of radioactivity released to the supernatant, incubations were terminated with EDTA and cooling. After centrifugation, 1ml. of platelet-free plasma was added to 4·5ml. of trichloroacetic acid containing carrier compounds and treated as before.

[U-$^{14}$C]Adenine (231mc/m-mole) was supplied by The Radiochemical Centre, Amersham, Bucks.; PCMBS* was supplied by Sigma (London) Chemical Co. Ltd.; NEM, monoiodoacetic acid and ouabain were supplied by British Drug Houses Ltd., Poole, Dorset; amitriptyline hydrochloride was supplied by Allen and Hanburys Ltd., Ware, Herts.; acetylsalicylic acid (aspirin) was supplied by Albright and Wilson Ltd., Birmingham.

*Results and discussion.* The results are summarized in Table 1. Collagen caused a non-reversible aggregation (see Constantine, 1967) accompanied by release of ATP and ADP and by breakdown of radioactive ATP. Curves of ATP breakdown plotted against time were asymptotic, and 4min. after the addition of collagen 21–23% of the radioactive ATP present in untreated samples had been degraded. The products were found as ADP, AMP, IMP, inosine and hypoxanthine. Inosine and hypoxanthine were present in the supernatant, but most of the radioactivity present as nucleotide remained in the cells, only about 4% of the initial radioactive nucleotide occurring as nucleotide in the supernatant. When aggregation was inhibited by addition of test compounds this value decreased to about 0·5%, which was the amount found without collagen treatment. The pattern of breakdown of ATP in these experiments was thus similar to that found with platelets labelled with [$^{14}$C]-adenosine, suspended in saline and treated with thrombin (Ireland, 1967).

Zucker & Jerushalmy (1967) reported that iodoacetate and KCN together inhibited collagen-induced aggregation, although neither was effective alone. We found that iodoacetate caused the breakdown of radioactive ATP to hypoxanthine and some ADP, AMP, IMP and inosine, and also inhibited collagen-induced aggregation and release of ATP

* Abbreviations: PCMBS, *p*-chloromercuribenzenesulphonate; NEM, *N*-ethylmaleimide.

Table 1. *Effect of inhibitors of collagen-induced aggregation on platelet radioactive ATP and release of ATP and ADP*

Portions (1 ml.) of labelled plasma containing $2{\cdot}2 \times 10^8$–$3{\cdot}7 \times 10^8$ platelets/ml. were stirred in the absorptiometer at 37° either with or without added test compound. Test compounds were added in aqueous solution (15–75 $\mu$l.) except for aspirin, which was added in 0·1% (w/v) $NaHCO_3$ solution (30–100 $\mu$l.). Collagen suspension (20 $\mu$l.) was added as noted 3 min. after addition of test compound. Incubations were terminated 4 min. later and assays for radioactivity and released nucleotide were as described in the text. The degree of aggregation was measured as the change in light transmittance ($\Delta\%T$) that had occurred and the results given are the mean values of two determinations. Samples to which no addition was made were run at the beginning and end of each experiment and the results given are the mean values obtained.

| Treatment | Concn. of compound in plasma ($\mu$M) | $10^{-3}\times$ Radioactivity of substance (c.p.m./$10^8$ platelets) | | | | | | | | Degree of aggregation ($\Delta\%T$) | Nucleotide released (nmoles/$10^8$ platelets) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | ATP | ADP | AMP | IMP | Inosine | Hypoxanthine | Total | ATP/ADP ratio | | ATP | ADP | ATP/ADP ratio |
| No addition | | 8·26 | 1·05 | 0·06 | 0·03 | 0·09 | 0·91 | 10·40 | 7·9 | 0 | 0·04 | 0·05 | 0·8 |
| KCN | 189 | 8·18 | 1·11 | 0·07 | 0·05 | 0·08 | 0·90 | 10·39 | 7·4 | 0 | 0·06 | 0·09 | 0·7 |
| Iodoacetate | 943 | 5·68 | 1·25 | 0·15 | 0·28 | 0·37 | 2·79 | 10·52 | 4·5 | 0 | 0·02 | 0·05 | 0·4 |
| Iodoacetate + KCN | 943 + 189 | 1·14 | 1·77 | 1·53 | 2·32 | 0·82 | 3·14 | 10·72 | 0·6 | 0 | 0·02 | 0·02 | 1·0 |
| Collagen | | 6·52 | 1·64 | 0·17 | 0·20 | 0·08 | 1·48 | 10·09 | 4·0 | 53·9 | 1·88 | 1·82 | 1·0 |
| KCN + collagen | 189 | 5·10 | 1·54 | 0·20 | 0·56 | 0·10 | 1·98 | 9·48 | 3·3 | 47·3 | 1·53 | 2·58 | 0·6 |
| Iodoacetate + collagen | 943 | 1·52 | 1·77 | 0·71 | 2·38 | 0·96 | 3·52 | 10·86 | 0·9 | 10·7 | 0·36 | 0·68 | 0·5 |
| Iodoacetate + KCN + collagen | 943 + 189 | 0·99 | 1·73 | 1·65 | 1·99 | 0·86 | 3·46 | 10·68 | 0·6 | 0 | 0·02 | 0·10 | 0·2 |
| No addition | | 7·83 | 1·03 | 0·06 | 0·03 | 0·04 | 0·41 | 9·40 | 7·6 | 0 | 0·03 | 0·05 | 0·6 |
| NEM | 330 | 7·85 | 0·94 | 0·08 | 0·02 | 0·06 | 0·58 | 9·53 | 8·4 | 0 | 0·01 | 0·01 | 1·0 |
| Collagen | | 5·96 | 1·47 | 0·15 | 0·54 | 0·07 | 1·28 | 9·47 | 4·1 | 54·6 | 1·55 | 1·85 | 0·8 |
| NEM + collagen | 142 | 6·72 | 1·42 | 0·11 | 0·20 | 0·06 | 1·06 | 9·57 | 4·7 | 33·9 | 0·84 | 0·99 | 0·9 |
| NEM + collagen | 189 | 6·98 | 1·37 | 0·08 | 0·14 | 0·04 | 0·72 | 9·33 | 5·1 | 10·2 | 0·39 | 0·65 | 0·6 |
| NEM + collagen | 330 | 7·39 | 1·29 | 0·09 | 0·05 | 0·06 | 0·68 | 9·56 | 5·7 | 1·4 | 0·15 | 0·28 | 0·5 |
| No addition | | 9·22 | 1·21 | 0·06 | 0·04 | 0·06 | 0·54 | 11·13 | 7·6 | 0 | 0 | 0 | 0 |
| PCMBS | 685 | 8·95 | 1·14 | 0·06 | 0·04 | 0·08 | 0·49 | 10·76 | 7·9 | 0 | 0·01 | 0·01 | 1·0 |
| Collagen | | 7·08 | 1·69 | 0·16 | 0·22 | 0·13 | 1·76 | 11·04 | 4·2 | 55·5 | 1·34 | 1·53 | 0·9 |
| PCMBS + collagen | 288 | 7·59 | 1·65 | 0·13 | 0·18 | 0·11 | 1·38 | 11·04 | 4·6 | 45·3 | 0·91 | 1·15 | 0·8 |
| PCMBS + collagen | 320 | 8·03 | 1·60 | 0·10 | 0·18 | 0·08 | 1·24 | 11·23 | 5·0 | 21·0 | 0·51 | 0·74 | 0·7 |
| PCMBS + collagen | 685 | 8·61 | 1·27 | 0·09 | 0·10 | 0·10 | 0·66 | 10·83 | 6·8 | 2·5 | 0·12 | 0·26 | 0·5 |
| No addition | | 8·55 | 1·23 | 0·09 | 0·04 | 0·08 | 0·91 | 10·90 | 7·0 | 0 | 0·01 | 0·05 | 0·2 |
| Amitriptyline | 327 | 8·69 | 1·21 | 0·08 | 0·04 | 0·09 | 1·11 | 11·22 | 7·2 | 0 | 0·03 | 0·06 | 0·5 |
| Collagen | | 5·70 | 1·85 | 0·22 | 0·71 | 0·11 | 2·16 | 10·75 | 3·1 | 52·8 | 1·16 | 1·99 | 0·6 |
| Amitriptyline + collagen | 164 | 5·88 | 1·72 | 0·20 | 0·67 | 0·10 | 2·18 | 10·75 | 3·4 | 42·8 | 1·19 | 1·67 | 0·7 |
| Amitriptyline + collagen | 234 | 6·44 | 1·64 | 0·12 | 0·53 | 0·06 | 1·53 | 10·32 | 3·9 | 18·3 | 0·58 | 0·82 | 0·7 |
| Amitriptyline + collagen | 327 | 8·10 | 1·36 | 0·12 | 0·17 | 0·09 | 1·24 | 11·08 | 6·0 | 1·9 | 0·14 | 0·27 | 0·5 |
| No addition | | 13·18 | 2·29 | 0·13 | 0·07 | 0·08 | 0·76 | 16·51 | 5·8 | 0 | 0·02 | 0·04 | 0·5 |
| Aspirin | 820 | 13·50 | 2·15 | 0·14 | 0·06 | 0·11 | 0·88 | 16·84 | 6·3 | 0 | 0·01 | 0·01 | 1·0 |
| Collagen | | 10·29 | 3·23 | 0·29 | 1·04 | 0·16 | 2·55 | 17·56 | 3·2 | 49·9 | 1·00 | 1·16 | 0·9 |
| Aspirin + collagen | 246 | 11·19 | 3·17 | 0·27 | 0·78 | 0·11 | 1·79 | 17·31 | 3·5 | 30·9 | 0·69 | 0·78 | 0·9 |
| Aspirin + collagen | 820 | 11·72 | 2·34 | 0·66 | 0·18 | 0·12 | 1·81 | 16·83 | 5·0 | 21·0 | 0·39 | 0·65 | 0·6 |

and ADP. KCN alone had no effect, although, like iodoacetate, it enhanced the breakdown of radioactive ATP induced by collagen. KCN also enhanced the effects of iodoacetate, leading to an almost complete depletion of radioactive ATP and to complete inhibition of aggregation and nucleotide release by collagen. Assay of ATP in platelets depleted of radioactive ATP by treatment with iodoacetate and KCN for 7 min. indicated that non-radioactive ATP was not degraded. Mürer (1969) showed with [$^{32}$P]phosphate-labelled platelets that iodoacetate caused the disappearance of radioactive ATP and the formation of radioactive fructose 6-phosphate and fructose 1,6-diphosphate, which he interpreted as inhibition of glycolysis. It appears that the rapid loss of radioactive ATP and accumulation of IMP, inosine and hypoxanthine is due to inhibition of ATP synthesis by iodoacetate.

Other compounds shown to inhibit platelet aggregation or the release of nucleotides are NEM (Zucker & Jerushalmy, 1967), PCMBS (Aledort, Troup & Weed, 1968), amitriptyline (Mills & Roberts, 1967*a*,*b*) and aspirin (Weiss & Aledort, 1967; Evans *et al.* 1968). We found that these compounds inhibited collagen-induced aggregation, release of adenine nucleotide and breakdown of radioactive ATP. PCMBS inhibits platelet $Mg^{2+}$-dependent adenosine triphosphatase and $Na^+ + K^+$-dependent ouabain-sensitive adenosine triphosphatase, both of which enzymes probably occur in platelet membranes since PCMBS does not penetrate the platelet and does not inhibit clot retraction (Aledort *et al.* 1968), a process dependent on ATP-regenerating systems (Mürer, 1969). Ouabain at concentrations up to 1·0 mM in the plasma did not inhibit collagen-induced aggregation, release of adenine nucleotide or breakdown of radioactive ATP.

On the basis of these results, the inhibitors of collagen-induced platelet aggregation can be divided into two groups. The metabolic inhibitors, iodoacetate and iodoacetate plus cyanide, inhibit aggregation and nucleotide release to an extent proportional to the degree of depletion of radioactive ATP they produce. This suggests that the release process is dependent on the availability of this fraction of platelet ATP. By contrast, amitriptyline, aspirin and the thiol-blocking reagents do not affect the distribution of radioactivity in platelets but produce a degree of inhibition of aggregation and nucleotide release proportional to the extent to which they inhibit the collagen-induced breakdown of radioactive ATP. Hence the release of nucleotides from platelets appears to be an energy-dependent process and inhibitors of release may work by interfering either with the supply of energy or with the reactions by which it is made available.

We are most grateful to Mr D. C. B. Mills for his interest and helpful suggestions.

Aledort, L. M., Troup, S. B. & Weed, R. I. (1968). *Blood*, **31**, 471.

Constantine, J. W. (1967). *Nature, Lond.*, **214**, 1084.

Evans, G., Packham, M. A., Nishizawa, E. E., Mustard, J. F. & Murphy, E. A. (1968). *J. exp. Med.* **128**, 877.

Haslam, R. J. (1967). In *American Lecture Series: Physiology of Hemostasis and Thrombosis, Detroit*, p. 88. Ed. by Johnson, S. A. & Seegers, W. Springfield, Ill.: Charles C. Thomas.

Holmsen, H. (1965). *Scand. J. clin. Lab. Invest.* **17**, 537.

Holmsen, H., Holmsen, I. & Bernhardsen, A. (1966). *Analyt. Biochem.* **17**, 456.

Holmsen, H. & Rozenberg, M. C. (1968). *Biochim. biophys. Acta*, **157**, 266.

Ireland, D. M. (1967). *Biochem. J.* **105**, 857.

Ireland, D. M. & Mills, D. C. B. (1966). *Biochem. J.* **99**, 283.

Mills, D. C. B. & Roberts, G. C. K. (1967*a*). *Nature, Lond.*, **213**, 35.

Mills, D. C. B. & Roberts, G. C. K. (1967*b*). *J. Physiol.* **193**, 443.

Mürer, E. H. (1968). *Biochim. biophys. Acta*, **162**, 320.

Mürer, E. H. (1969). *Biochim. biophys. Acta*, **172**, 266.

Mürer, E. H., Hellem, A. J. & Rozenberg, M. C. (1967). *Scand. J. clin. Lab. Invest.* **19**, 280.

Weiss, H. J. & Aledort, L. M. (1967). *Lancet*, ii, 495.

Zucker, M. B. & Jerushalmy, Z. (1967). In *American Lecture Series: Physiology of Hemostasis and Thrombosis, Detroit*, p. 249. Ed. by Johnson, S. A. & Seegers, W. Springfield, Ill.: Charles C. Thomas.