Biologically active concentrations of potently vasoactive and platelet-active adenine nucleotides are generated in plasma by a variety of pathophysiological mechanisms. Although there is evidence that ATP and ADP are inactivated by endothelial ectonucleotidases, there has been little attempt to study the metabolic routes of their catabolism in blood or to assess the contribution of this process to their clearance in vivo. Therefore, we have studied the rates and patterns of catabolism of ATP, ADP, and AMP in whole blood, plasma, and isolated blood cells. Rates of degradation of each nucleotide in cell-free plasma ranged from 0.07-0.12 nmol/min/ml with 1 μM substrates to 1.1-3.6 nmol/min/ml with 100 μM substrates. The pattern of catabolism indicated that sequential dephosphorylation from ATP→ADP→AMP→adenosine occurs. In whole blood, the pattern was similar although ATP and ADP (but not AMP) breakdown was more rapid. This was due to leukocyte ectonucleotidase activity. The use of selective inhibitors demonstrated that catabolism was not due to nonspecific phosphatase activity and that plasma 5'-nucleotidase is distinct from ATPase or ADPase. In leukocytes, ATPase and ADPase activities were distinguishable, and each contributed substantially to the rates of catabolism in whole blood. Leukocyte 5'-nucleotidase did not measurably contribute to AMP dephosphorylation in blood. By comparison, ecto-ATPase and ecto-ADPase activities on cultured human umbilical vein endothelial cells were similar to those on leukocytes while endothelial 5'-nucleotidase per 10^9 cells was equivalent to the soluble activity in 1 ml of blood or plasma. We conclude that in microvascular beds, endothelial catabolism of circulating nucleotides predominates (as expected from the short half-lives of added nucleotides in vivo), but in larger blood vessels or at sites of impaired blood flow such as within a forming thrombus, leukocyte ecto-ATPase and ecto-ADPase and soluble 5'-nucleotidase may be important regulators of the concentrations of vasoactive nucleotides. (Circulation Research 1989;65:531-537)

Extracellular adenosine and adenine nucleotides modulate vascular tone and platelet function. The vasoactive properties of adenosine and ATP were first recognized over 50 years ago,1,2 and receptor-mediated vasodilation induced by adenosine acting on vascular smooth muscle has now been well characterized.3 More recent work has demonstrated that dilatation is induced in a separate manner by ATP or ADP, which acts at specific P2 purinoceptors on vascular endothelium to cause the release of a labile agent that relaxes subjacent smooth muscle.4-6 ADP is a powerful platelet stimulant, whereas ATP competitively inhibits ADP-induced platelet aggregation. By increasing platelet cyclic AMP levels, adenosine inhibits stimulation by any agonist.7

Circulating levels of adenosine under normal conditions are too low (<10^{-7} M) to have significant biological effects,8,9 but the concentration rises to active levels (>10^{-6} M) during exercise or ischemia, and it is generally agreed that adenosine is an important mediator of the reactive hyperemia following these events.10 Attempts at measurement of levels of ATP circulating in vivo have been less successful. Concentrations of greater than 10^{-8} M were recently found in conventionally collected plasma samples,11 but these values are likely to be artifactually high. They imply that biologically active levels of ATP circulate in vivo; however, as little as 0.1% hemolysis during sampling would produce this level in plasma ex vivo; in addition, the anticoagulant and antiaggregant used (EDTA) can itself induce selective nucleotide release from cells.12 In a careful study, Forrester13 measured basal levels of less than 2 x 10^{-8} M ATP in human venous plasma and was able to detect increases of more than 50-fold during partial arterial occlusion and exercise. Biologically active concentrations of ATP are also generated in
plasma during thrombus formation (human platelets release approximately equal amounts of ATP and ADP when stimulated\(^{14}\)); can be detected after transection of small blood vessels, where at least part of the source of ATP is the damaged vessel wall\(^{15}\); and have been demonstrated to be a major inducer of shock after physical trauma.\(^{16}\)

Mechanisms for inactivation of circulating adenine nucleotides are therefore important in the control of their vascular actions. We have characterized ectonucleotidase enzymes on porcine vascular endothelial cells\(^{17}\) and suggested that such enzymes are regulators of plasma nucleotide levels, particularly in the microcirculation, where the area of endothelial cell surface exposed to unit volume of blood is very high. However, there are several circumstances (e.g., within a forming thrombus) in which intravascular regulation of nucleotide levels may be significant.

The breakdown of ATP and ADP in plasma was first reported over 20 years ago,\(^{18,19}\) and an ADPase has been partially purified.\(^{20}\) More recently, the potential participation of cellular elements of the blood in extracellular nucleotide metabolism has been studied,\(^{21-24}\) but there has been little attempt to investigate the metabolic routes of adenine nucleotide catabolism in blood and plasma or to compare the relative contributions of plasma and cellular enzymes to this process. In this study, we have measured the patterns and rates of breakdown of a range of concentrations of adenine nucleotides in human plasma and whole blood, and compared the results with those obtained using human endothelial cells cultured in vitro.

### Materials and Methods

#### Preparation of Plasma and Cells

Blood was taken from healthy volunteers by venipuncture with a 19-gauge needle into a syringe. The needle was removed, and the blood was transferred to siliconized glass tubes containing heparin and gently inverted to give a final heparin concentration of 5 units/ml. Platelet-free plasma was prepared by centrifugation at 1,800g for 60 minutes at 4°C; in some experiments, platelet-rich plasma was prepared by centrifugation at 400g for 20 minutes. The contribution of leukocytes to nucleotide metabolism in blood was assessed in two ways: either by removal of theuffy coat after centrifugation and remixing of packed erythrocytes and plasma, or by isolation of lymphocytes and granulocytes from whole blood by dextran sedimentation followed by centrifugation over Ficoll-paque.\(^{25,26}\)

Human umbilical vein endothelial cells were isolated by brief collagenase treatment,\(^{27}\) cultured in Dulbecco's medium containing 10% fetal calf serum and 10% newborn calf serum, and used for experiments in 24-well culture trays (Falcon, Becton Dickinson UK, Oxford, UK) after two to four passages.

### Experiments

\(^{2-3}\)H-labeled adenine nucleotides (Amersham International, Amersham, Bucks, UK) at final concentrations of 1–100 \(\mu\)M (3 \(\mu\)Ci/ml) were incubated with samples for up to 3 hours at 37°C, and aliquots were taken at timed intervals. Nucleotides were added directly to whole blood or plasma immediately after sample preparation, or were incubated with isolated leukocytes or cultured endothelial cells in phosphate-buffered saline (PBS) containing 1.8 mM Ca\(^{2+}\) and 0.8 mM Mg\(^{2+}\). Blood cells were kept in suspension by continuous orbital mixing. Aliquots of whole blood were immediately centrifuged (10,000g for 20 seconds), and the supernatant plasma was added to 2 volumes of 14% trichloroacetic acid (TCA) at 4°C. Aliquots of plasma were added directly to TCA. Precipitated proteins were removed by centrifugation (10,000g for 120 seconds), the supernatants neutralized by the addition of 5 M K\(_2\)CO\(_3\), and the aliquots stored at -20°C for analysis by thin-layer chromatography (TLC) or high-performance liquid chromatography (HPLC) within 14 days. Cell-free aliquots in PBS were frozen at -20°C without TCA extraction.

#### Nucleotide Estimations

ATP, ADP, AMP, inosine, and adenosine were separated by TLC on glass-backed silica gel plates (Camlab, Cambridge, UK) by use of the solvent system of Norman et al.\(^{28}\) and radioactivity was quantified with a Berthold Model LB284 linear analyzer (Laboratory Impex, Teddington, Middlesex, UK). Selected samples were analyzed by reversed-phase HPLC by use of a 250 x 5 mm ODS Hypersil (Shandon Southern Products, Runcorn, Cheshire, UK) 5-\(\mu\)m column. ATP, ADP, and AMP were separated with 0.05 M NH\(_4\)H\(_2\)PO\(_4\) (pH 6.5) at a flow rate of 1 ml/min; after 10 minutes, a linear gradient of 0–30% methanol in the same buffer for 15 minutes was used to separate inosine and adenosine.

#### Chemicals

Dipyridamole (Persantin Injection) was from Boehringer, (BCL, Lewes, Sussex, UK). Except for adenosine monophosphorothioate (AMPS), the nucleotides, nucleosides, and nucleotide analogues were from Sigma Chemical Company (Poole, Dorset, UK) or Boehringer.

### Results

Samples of plasma prepared as described above were analyzed by HPLC for nucleotides. Without the addition of exogenous nucleotides, barely detectable or undetectable levels of total nucleotides (<1 \(\mu\)M) were consistently found. Figure 1 shows the rates of catabolism of 1, 10, and 100 \(\mu\)M ATP, ADP, and AMP added to whole blood and plasma. Typical time courses obtained with a single blood sample are presented in each panel. These experiments
Adenine Nucleotide Metabolism in the Blood

have been repeated six to eight times with blood from different individuals; the results are summarized in Table 1.

In plasma, added ATP, ADP, and AMP were each metabolized with longer half-lives as the concentration was increased from 1 to 100 μM and with a tendency for an initial lag phase after addition of 100 μM nucleotides (Figure 1a). Metabolism of AMP was faster than that of either ATP or ADP, particularly at the 1 and 10 μM initial concentrations. When nucleotides (1–100 μM) were added to whole blood, the half-lives again increased with increasing substrate concentration (Figure 1b). ADP and AMP (1 and 10 μM) were metabolized faster than ATP, and at 100 μM, ADP was catabolized more rapidly than ATP or AMP. Table 1 also shows that the rates of metabolism of ADP at 1 and 10 μM and of ATP at all tested concentrations were significantly greater in whole blood than in plasma, but that this relation was not true of AMP at any concentration.

The contribution of cellular elements of the blood to nucleotide metabolism was studied by comparison of rates of metabolism in plasma with those in whole blood or in whole blood depleted of white blood cells by removal of the buffy coat after centrifugation (Figure 2). Experiments of this type demonstrated that white blood cells do contribute significantly to the catabolism of circulating ADP and ATP but that red blood cells do not. Also, platelets do not contribute since the rates of nucleotide metabolism in platelet-rich and platelet-poor

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**TABLE 1. Catabolism of Exogenous Nucleotides by Whole Blood or Cell-Free Plasma**

<table>
<thead>
<tr>
<th>Substrate [μM]</th>
<th>Cell-free plasma</th>
<th>Whole blood</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP 1</td>
<td>0.07±0.01*</td>
<td>0.12±0.01</td>
</tr>
<tr>
<td></td>
<td>(8)</td>
<td>(8)</td>
</tr>
<tr>
<td>ATP 10</td>
<td>0.33±0.04*</td>
<td>0.51±0.07†</td>
</tr>
<tr>
<td></td>
<td>(6)</td>
<td>(6)</td>
</tr>
<tr>
<td>ATP 100</td>
<td>1.10±0.11‡</td>
<td>1.90±0.13</td>
</tr>
<tr>
<td></td>
<td>(6)</td>
<td>(6)</td>
</tr>
<tr>
<td>ADP 1</td>
<td>0.08±0.01*</td>
<td>0.23±0.06</td>
</tr>
<tr>
<td></td>
<td>(7)</td>
<td>(6)</td>
</tr>
<tr>
<td>ADP 10</td>
<td>0.25±0.04‡</td>
<td>1.07±0.15</td>
</tr>
<tr>
<td></td>
<td>(7)</td>
<td>(7)</td>
</tr>
<tr>
<td>ADP 100</td>
<td>3.17±0.44</td>
<td>4.68±0.82§</td>
</tr>
<tr>
<td></td>
<td>(6)</td>
<td>(6)</td>
</tr>
<tr>
<td>AMP 1</td>
<td>0.32±0.05</td>
<td>0.28±0.05</td>
</tr>
<tr>
<td></td>
<td>(6)</td>
<td>(6)</td>
</tr>
<tr>
<td>AMP 10</td>
<td>1.28±0.28</td>
<td>1.80±0.16</td>
</tr>
<tr>
<td></td>
<td>(6)</td>
<td>(6)</td>
</tr>
<tr>
<td>AMP 100</td>
<td>3.60±0.62</td>
<td>2.31±0.42</td>
</tr>
<tr>
<td></td>
<td>(6)</td>
<td>(6)</td>
</tr>
</tbody>
</table>

Values are mean±SEM of n experiments (parentheses). Significant differences between means were determined by unpaired t tests on nontransformed data.

*Less than rate in whole blood, p<0.01.
†Less than rate for ADP or AMP (10 μM) in whole blood, p<0.01.
‡Less than rate for ADP or AMP (100 μM) in cell-free plasma, p<0.01.
§Less than rate in whole blood, p<0.05.
¶Greater than rate for ATP or AMP (100 μM) in whole blood, p<0.01.
*Greater than rate for ATP or ADP (1 and 10 μM) in cell-free plasma, p<0.01.
whole blood was approximately twice that found in plasma (panel a) or whole blood (panel b), and rates of formation of ADP (a), AMP (b), and total nucleosides (c) were measured by thin-layer chromatography.

Further analyses were carried out for determination of the pattern of nucleotide catabolism in these experiments and the relative concentrations of products formed. In our preliminary experiments, we found a loss of $^3$H at later times in experiments with whole blood. This $^3$H was found in blood cells (predominantly erythrocytes), and its accumulation was prevented by the presence of dipyridamole (10 $\mu$M), a potent inhibitor of nucleoside transport.

Evidence for the presence of distinct nucleotidases in plasma and on white blood cells was sought by use of nucleotide analogues to inhibit catabolism. In plasma, the catabolism of 1 $\mu$M $\alpha,\beta$-methylene-ATP (APPNP) or 100 $\mu$M $\alpha,\beta$-methylene-ATP (APCP) was inhibited more than 80% by 100 $\mu$M $\beta,y$-imido-ATP (AMPS). The latter compound did not inhibit AMP breakdown, but was an effective inhibitor of ATP catabolism. Breakdown of 1 $\mu$M AMP in plasma was inhibited more than 95% by 100 $\mu$M $\beta,y$-methylene-ATP (APPNP) or 100 $\mu$M $\beta,y$-methylene-ATP (APCP). The latter compound did not inhibit ATP or ADP catabolism and AMPS only inhibited poorly. These data indicate that AMP catabolism is due to a distinct enzyme, but we have no evidence that ATPase and ADPase in plasma are separate enzymes.

In white cells, the same range of analogues was used to study ATP and ADP catabolism. APCP and AMPS did not inhibit ATP or ADP breakdown. APCP (100 $\mu$M) blocked 1 $\mu$M ADP catabolism without having any effect on 1 $\mu$M ATP breakdown.
Thus, the leukocyte ATPase and ADPase ectoenzymes are apparently distinct.

We compared the rates of nucleotide metabolism in whole blood with those achieved by endothelial ectoenzymes. Figure 5a shows that the rates of catabolism of 1 μM ATP and ADP were similar, ≈200 nmol/min/10^6 cells, when exposed to cultures of human umbilical vein endothelial cells; 1 μM AMP was converted to adenosine at two to three times this rate. Figure 5b demonstrates the pattern of ATP catabolism. In the presence of excess unlabeled ADP, [3H]ATP was predominately converted to [3H]ADP (not shown), again indicating that sequential dephosphorylation occurs. Catabolism of 1 μM AMP was specifically blocked by 100 μM APCP or AMPS; however, APPCP and APPNP were poor inhibitors of ADP catabolism.

Discussion

Our results demonstrate that human cell-free plasma catabolizes adenine nucleotides at rates varying from 0.07–0.32 nmol/min/ml with 1 μM substrates to 1.1–3.6 nmol/min/ml with 100 μM substrates. This range of concentrations is of pathophysiological relevance: micromolar concentrations of extracellular ATP are generated after local trauma and release of cytoplasmic ATP from red cells or cells of the vessel wall, and aggregating platelets discharge ATP and ADP from storage granules (where their concentration is ≈1 M), leading to local extracellular nucleotide concentrations that temporarily exceed 100 μM.

The pattern of degradation of each nucleotide in whole blood and in plasma suggests that sequential dephosphorylation from ATP→ADP→AMP→adenosine occurs. This conclusion is supported by inhibitor studies. The addition of excess unlabeled nucleotides also demonstrated that breakdown was at least predominantly by dephosphorylation; we obtained no evidence of pyrophosphatase or AMP deaminase activity.

In whole blood, the rates of breakdown of ATP and ADP (but not those of AMP) were greater than in plasma, indicating that cellular elements of the blood contributed to nucleotide metabolism. Neither red cells nor platelets were responsible for this activity. Purified granulocytes and lymphocytes in PBS each exhibited ecto-ATPase and ecto-ADPase activity of about 250 pmol/min/10^6 cells with 1 μM substrates. Assuming a leukocyte count of ≈8×10^9/l, this rate of metabolism is more than sufficient to account for the acceleration in ATP and ADP catabolism in whole blood as compared with plasma. From the results with 100 μM ATP or ADP, it appears that leukocytes contribute about one half the ATPase and more than 80% of the ADPase in blood at this substrate concentration. The similar catabolic rates of AMP in plasma and blood are consistent with our finding of insignificant activity degrading AMP (1–100 μM) on purified white cells. Although at least a subpopulation of human lymphocytes possesses ecto-5'-nucleotidase, its reported maximal activity is substantially lower than that of ecto-ATPase or ecto-ADPase on white cells, and it is clear from our results that its role cannot relate to clearance of circulating AMP, which, in whole blood, is completely accounted for by soluble 5'-nucleotidase activity. Similarly, although cytochemical evidence for human granulocyte ecto-5'-nucleotidase has been presented, levels of activity found biochemically have been virtually undetectable.

We used a variety of inhibitors, including nucleotide analogues, to investigate the nature of the phosphatases that degrade nucleotides in blood. The lack of effect of a large excess of an alternative phosphorylated substrate (β-glycerophosphate) or of conventional inhibitors of nonspecific phosphatases (fluoride and levamisole) indicates that such enzymes do not contribute to the catabolism of circulating nucleotides at neutral pH. This finding is substantiated by the relatively high affinity for each nucleotide substrate: rates of dephosphorylation were only 10–20 times higher with 100 μM substrates than with 1 μM substrates, indicating significant saturation of phosphatase activity at the higher concentration.

In addition, plasma AMP catabolism was selectively blocked by APCP, well characterized as a 5'-nucleotidase inhibitor, and by AMPS, known to block 5'-nucleotidase in endothelial smooth muscle cells but not to interact with nonspecific phosphatase. We found no evidence to distinguish ADPase from ATPase in plasma, but in leukocytes, APPCP and APPNP selectively inhibited ADP rather than ATP breakdown. In the absence of a selective inhibitor of ATP breakdown, however, and noting that ATP catabolism was somewhat slower in the presence of ADP (Figures 4a and 4b), further work for purification of the ADPase, as with the endothelial enzyme, is necessary for confirmation that it is separable from ATPase.
Thus, our results characterize the nature of nucleotide catabolism in blood and the contribution of leukocytes to this process. Nonetheless, it is relevant to conclude with a comparison of these findings with ectonucleotidase activity on endothelial cells. The rate of dephosphorylation of adenine nucleotides at the surface of human umbilical vein endothelial cells was $\approx 200$ pmol/min/10$^6$ cells for ATP or ADP and $\approx 500$ pmol/min/10$^6$ cells for AMP with 1 $\mu$M substrates (Figure 5a). Therefore, on a per-cell basis, ecto-ATPase and ecto-ADPase activities were similar to those on white cells, while 5'-nucleotidase activity per 10$^6$ endothelial cells was comparable with the soluble activity in 1 ml of blood. There is good evidence that large- and microvascular endothelium both possess active ectonucleotidases (references cited in Pearson46). If the rates we have found here are taken as a guideline, they imply that within large vessels, the capacity to catabolize adenine nucleotides resides approximately equally at the luminal surface of the vessel wall and in the flowing blood. In microvessels, where flow is much slower and the endothelial cell surface area exposed to unit volume of blood is increased by more than two orders of magnitude, endothelial catabolism of circulating nucleotides will predominate. This is consistent with measurements of the half-life of infused ATP or ADP in vivo (or in blood-free perfused organs) of seconds or less in contrast to the half-life of several minutes found in blood ex vivo.42 However, leukocyte ecto-ATPase and ecto-ADPase with soluble 5'-nucleotidase activity may be important in the regulation of the concentrations of vasoactive and platelet-active nucleotides, particularly in areas of impaired blood flow such as those within a forming thrombus.

Acknowledgment

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Adenine Nucleotide Metabolism in the Blood

Coade and Pearson


KEY WORDS • ATP • ectonucleotidases • leukocytes • endothelium
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