Almost all cells contain actin, which in its polymerized form, F-actin, binds 1 molecule of ADP/monomer. Little is known about the availability to metabolism of this bound ADP. A comparison was therefore made between perchloric acid and EDTA/ethanol extracts of human blood platelets. When the cells were extracted under conditions where the ATPase activity was negligible, the ethanol extracts had a 75% higher ATP/ADP ratio and a higher adenylate energy charge than perchloric acid extracts. The methods differed in that a considerable portion of protein-bound ADP was not extracted by ethanol. This bound ADP behaved as though it were unavailable to energy metabolism and should thus be considered as a compartment separate from the bulk metabolic pool of extragranular platelet adenine nucleotides. These results suggest that the level of ADP obtained with the common acid extraction overestimates the level available to participation in metabolism.

Adenine nucleotides play a central role in the energy metabolism of most cells. Complete understanding of the role of adenine nucleotides in the regulation of various enzymatic reactions and as an energy source requires accurate information about their exact cellular concentrations in various metabolic states. The fact that cells contain various nucleotide compartments is often overlooked.

Two major pools of platelet adenine nucleotides have been previously described (1, 2): the granular pool, which is secreted when platelets are stimulated, and the metabolic or extragranular pool, which is radiolabeled by incubation of the cells with certain radioactive precursors. The exact measurement of metabolically active adenine nucleotides, especially ADP, is complicated by this compartmentalization. In human platelets, there is about 6 times more granule-stored ADP than extragranular ADP (1, 2). This problem can be overcome since radiolabeled adenine is only incorporated into the nucleotides of the extragranular pool during the period of a few hours (3) in which most experiments are performed. The measured radioactivity of ATP, ADP, AMP, and IMP in platelet extracts is directly proportional to their concentration in the extragranular pool. A second factor which complicates measurement of metabolically active ADP in platelets is the presence of large amounts of actin (4). In the polymerized form, F-actin, this protein binds ADP in a manner that makes it inaccessible to other enzyme systems (5, 6). Up to 50% of the radioactive ADP in platelets is not extracted by 43% ethanol. It can be extracted from the ethanol-insoluble residue with HClO₄ (7) and has been shown to be associated with the actomyosin complex (8). More specifically, the ethanol-insoluble ADP is associated mainly with platelet actin. In resting platelets, we found that the actin-bound ADP is exchangeable with the bulk extragranular pool with a turnover time of about 10 s and, therefore, is included in the radioactive measurement when HClO₄ extraction is used (9). Since the actin-ADP pool constitutes up to 50% of the total metabolic ADP, the use of HClO₄ extraction may cause overestimation of the ADP concentration which is directly available to cellular enzyme systems. This problem has previously been considered for the case of skeletal muscle, a tissue in which the presence of actin-bound ADP has been long recognized (10). It has not yet been considered for nonmuscle cells and may be of general importance since actin is a major protein in many cells and has been found in almost all cells where its presence has been investigated (11).

In this paper we show that ethanol extraction, when properly controlled for nucleotide breakdown, can distinguish between the metabolically available and unavailable extragranular ADP in platelets and thus provides a method to separate the actin-bound ADP from the rest of the extragranular pool of adenine nucleotides.

**MATERIALS AND METHODS**

5'-Adenylic acid deaminase (AMP deaminase, E.C. 3.5.4.4) from rabbit muscle was purchased from Sigma Chemical Co. Hexokinase (E.C. 2.7.1.1) from yeast and myokinase (E.C. 2.7.4.3) from rabbit muscle were obtained from Boehringer-Mannheim Biochemicals.

Human platelet-rich plasma was prepared, and the extragranular (metabolic) pool of adenine nucleotides was labeled as previously described (12) except that [U-¹⁴C]adenine from Amersham/Searle (Arlington Heights, Ill., Code CFA 436) was used [Adenine-¹¹C]. Adenosine-5'-triphosphate, ammonium salt, was also from Amersham/Searle (Code CFA 441).

The labeled platelets were separated from plasma proteins by gel filtration with the use of a Ca²⁺-free Tyrode's elution fluid containing 0.2% bovine serum albumin in which SrCl₂ (0.2 mM) was substituted for MgCl₂ (13). MgCl₂ to a final concentration of 2 mM was added to the suspension of gel-filtered platelets (3 x 10⁶ cells/ml) immediately after collection.

Concentrated platelet suspensions were prepared by differential centrifugation. Platelet-rich plasma was diluted with 1 volume of buffer (0.12 mM NaCl, 3 mM EDTA, 5 mM glucose, 30 mM Tris-HCl, pH 7.4, 0°C) and centrifuged at 1000 x g for 20 min at 4°C. The platelets were resuspended in a small volume of buffer and centrifuged once again. The pellets were resuspended in cold buffer containing 7 mM MgCl₂ (the pH was readjusted to 7.3 with NaOH) at a concentration of 2 x 10⁶ cells/ml.

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§ Recipient of an Established Investigatorship Award from the American Heart Association.

1 J. L. Daniel, I. Molish, and H. Holmsen, unpublished observations.
Platelet counts were determined in a ZB, Coulter counter with a 60-μ aperture.

**Extraction with Perchloric Acid**

Platelet suspensions maintained at 37°C were added to an equal volume of 1.2 N HClO₄ on ice. The insoluble material was separated in an Eppendorf model 5400 centrifuge (12,000 × g for 2 min). The supernatant was neutralized by addition of an appropriate volume of 2 M K₂CO₃. The precipitate of KClO₄ was removed by centrifugation. This will be referred to as “direct perchloric extraction.”

**Extraction with EDTA/Ethanol**

The EDTA/ethanol was freshly prepared by mixing 9 volumes of 95% ethanol with 1 volume of 77 mM EDTA, pH 7.4 (14).

**Procedure A**—One volume of platelet suspension (37°C) was added to 1 volume of EDTA/ethanol in an ice bath. The insoluble material was pelleted by centrifuging in an Eppendorf centrifuge at room temperature for 2 min. The supernatant, ethanol-soluble extract, was saved and the pellet treated as described below.

**Procedure B**—One volume of platelet suspension (37°C) was added to 2 volumes of EDTA/ethanol which was equilibrated with a bath containing 40% methanol in water that had been kept overnight in a freezer at -60°C. The mixture was centrifuged in a Dupont Sorvall RC-2B centrifuge at 17,000 × g for 10 min at 4°C. The supernatant was saved and the pellet treated as described below.

The pellets from Procedures A and B were washed twice with a 1:1 mixture of ice-cold 0.15 M NaCl/EDTA/ethanol by two successive centrifugation/resuspension cycles in a Sorvall RC-2B centrifuge as above. They were then extracted with ice-cold 0.8 M HClO₄ and centrifuged, and the supernatant was neutralized with K₂CO₃, as above. These extracts are referred to as “ethanol-insoluble extracts.” Less than 5% of total radioactivity was found in these washes, most likely due to nonspecific binding by plasma proteins (7). Thus, when a plasma system is used, nonspecific binding must be considered.

**Analysis of Extracts**

The radioactive adenine nucleotides and metabolites were separated by high voltage paper electrophoresis (50 ml of each extract) as previously described (12). The spots were cut from the paper strips and added to 15 ml of toluene containing 0.5% of 2,5-diphenyloxazole. Radioactivity was measured in a Beckman LS-300 liquid scintillation spectrometer (10 min). ATP and ADP were measured by a firefly luciferase assay (14).

**Calculations**

The radioactivity of each metabolite is expressed as a percentage of the sum of the radioactivity of all metabolites. This sum was constant in these experiments. The adenylate energy charge was calculated according to Atkinson and Walton (15) using per cent radioactivity of the adenylates instead of their concentrations (16).

**RESULTS**

A comparison of extraction of [¹⁴C]adenine-labeled gel-filtered platelets by HClO₄ or EDTA/ethanol (Procedure A) is shown in Table I, Method A. Initial comparison of the nucleotide radioactivity solubilized in HClO₄ and EDTA/ethanol suggests that the two methods produce comparable results; i.e. the ATP/ADP ratio and the adenylate energy charge obtained are almost identical. However, when the adenosine nucleotide fraction which is not extractable by ethanol is taken into account, the correlation no longer exists. Table I, Method A, shows that a fraction consisting mainly of ADP is not extracted by ethanol, and that it comprises 6% of the total platelet metabolic adenine nucleotides and thus about 30% of the ADP. When this fraction is added to the ethanol-extractable nucleotides, a new ATP/ADP ratio and adenylate energy charge can be calculated which are significantly lower than those obtained with HClO₄ extraction. The lower adenylate energy charge occurs because there is a 5% decrease in the percentage of radioactivity in ethanol-soluble ATP with a corresponding increase in the percentage of radioactivity in AMP in the EDTA/ethanol extracts (Table I, Method A).

The fact that the ethanol sum is not the same as the perchloric acid extract suggests that ATP is hydrolyzed during ethanol extraction by Procedure A. This hypothesis is further supported by the experiment shown in Fig 1. Here, [¹⁴C]ATP (containing 10% [¹⁴C]ADP) was added to EDTA/ethanol at 0°C. Dilutions of a concentrated platelet suspension were added in a 1:1 (v/v) ratio to the [¹⁴C]ATP/EDTA/ethanol mixture, and the distribution of [¹⁴C] in ATP and ADP was determined. Over 20% of the ATP was hydrolyzed, mainly to ADP, at the highest cell concentration. The degree of hydrolysis was almost linear with respect to the cell concentration. This reaction appeared to be very rapid, since the amount of ATP recovered was independent of time in the range of a few seconds to several minutes. Obviously, addition of platelet suspensions to EDTA/ethanol in a 1:1 (v/v) ratio (Procedure A) did not instantaneously inactivate ATPases present in these cells.

We have attempted several modifications of the extraction procedure to prevent ATP hydrolysis. The results obtained...

**Table I**

Comparison of nucleotide composition of HClO₄ extracts to both of those made by EDTA/ethanol extraction at 0°C and at -40°C

<table>
<thead>
<tr>
<th>Extract</th>
<th>% of total radioactivity</th>
<th>ATP/ADP</th>
<th>AEC*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Perchloric acid</td>
<td>78.5 ± 0.7</td>
<td>16.3 ± 0.6</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>Method A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethanol-soluble</td>
<td>73.5 ± 2.6</td>
<td>14.2 ± 1.8</td>
<td>0.9 ± 0.4</td>
</tr>
<tr>
<td>Ethanol-insoluble</td>
<td>0.2 ± 0.06</td>
<td>5.7 ± 0.5</td>
<td>0.2 ± 0.01</td>
</tr>
<tr>
<td>Sum, ethanol</td>
<td>73.7 ± 2.6</td>
<td>19.8 ± 2.0</td>
<td>1.2 ± 0.4</td>
</tr>
<tr>
<td>Method B</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethanol-soluble</td>
<td>77.8 ± 1.6</td>
<td>9.1 ± 1.6</td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td>Ethanol-insoluble</td>
<td>0.7 ± 0.2</td>
<td>6.2 ± 1.2</td>
<td>0.4 ± 1.2</td>
</tr>
<tr>
<td>Sum, ethanol</td>
<td>78.5 ± 1.5</td>
<td>15.4 ± 0.8</td>
<td>0.9 ± 0.3</td>
</tr>
</tbody>
</table>

*Adenylate energy charge.
with the best procedure (Procedure B) are given in Table I, Method B. The ATP/ADP ratio and adenylate energy charge for the sum of the ethanol-soluble and -insoluble nucleotides are comparable to those obtained by direct HClO₄ extraction. The ATP/ADP ratio in the ethanol-soluble fraction was 77% higher than that of the direct perchloric extract. Forty-four per cent of the radioactive ADP was not extracted by EDTA/ethanol, which accounted for the increase in ATP/ADP and adenylate energy charge.

If the ethanol-insoluble (actin-bound) ADP is not available for metabolic processes, the amount of insoluble ADP should not be altered when the metabolic state of the platelet is drastically altered; furthermore, actin-bound ADP should not be accessible to added enzymes which catalyze reactions which consume ADP. The first point is illustrated in Table II. Platelets were treated with antimycin A, 2-deoxy-D-glucose, and glucose-δ-lactone, which produces a rapid breakdown of cytoplasmic (radioactive) ATP and ADP (17). Even though levels of ethanol-soluble ATP were reduced 40-fold and ADP levels were reduced 8-fold, no significant changes in the ethanol-insoluble ADP took place. The second point is illustrated in Table III. Here, the enzymes hexokinase, adenylate kinase, and AMP deaminase were added to lysed platelets. Again, dramatic changes in the levels of ethanol-soluble ATP and ADP were found with only a very small change in ethanol-insoluble ADP. Similar experiments with pyruvate kinase and creatine phosphokinase gave similar results.

**DISCUSSION**

Ethanol extraction, which excludes actin-ADP, clearly reflects better the freely available ADP concentration in platelets than does perchloric acid extraction. These studies accord with those of Seraydarian et al. (10) on skeletal muscle, in which a substantial portion of muscle ADP was not extracted by ethanol and was regarded to be bound to actin. The ADP bound to actin has been shown to be unavailable to enzymes such as creatine kinase (6). We have shown, both in intact cells by addition of metabolic inhibitors and in lysed cells by addition of adenine nucleotide-metabolizing enzyme systems, that the amount of ethanol-insoluble ADP remained constant while the ethanol-soluble adenine nucleotides varied over a wide range. This is most likely due to the fact that the affinity of actin for nucleotides is so high (11) that it is always saturated in the living cell.

Recent 31P NMR studies on intact human platelets have demonstrated that the granular pool of ATP and ADP is not detected (18). Therefore, in the NMR spectra the adenine nucleotide resonances originate exclusively from the extragranular pool. When these spectra are compared to the levels of the [14C]adenine-labeled nucleotides in the perchloric extracts (present paper), the data suggest that not all the extragranular ADP is detected by 31P NMR, which most likely monitors free cellular nucleotide levels.

The evidence presented here strongly suggests that the actin-bound (ethanol-insoluble) ADP in platelets does not participate directly in the metabolic processes in which adenine nucleotides participate and which they regulate in spite of the fact that actin-bound ADP exchanges rapidly with the bulk pool of extragranular adenine nucleotides (9). This fraction of ADP, which is included in the usual used acid extract, should therefore not be included in calculations of ATP/ADP adenylate energy charge, or the concentration of metabolically available ADP. While the difference in the adenylate energy charge using ethanol extraction as opposed to HClO₄ is small, some platelet functions are quite sensitive to small changes of energy charge in this range (19). This difference relates only to the measured amount of ADP, which can vary almost by

---

**Table II**

The effect of metabolic inhibitors on the ethanol-soluble and -insoluble nucleotides of intact platelets

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Insoluble ADP</th>
<th>Soluble ATP</th>
<th>ADP</th>
<th>AMP</th>
<th>IMP</th>
<th>Hypoxanthine</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>5.8</td>
<td>62.7</td>
<td>21.7</td>
<td>3.0</td>
<td>4.8</td>
<td>0.7</td>
</tr>
<tr>
<td>1</td>
<td>5.3</td>
<td>20.1</td>
<td>19.5</td>
<td>22.5</td>
<td>30.4</td>
<td>2.2</td>
</tr>
<tr>
<td>2</td>
<td>5.2</td>
<td>10.8</td>
<td>13.6</td>
<td>28.5</td>
<td>37.8</td>
<td>3.6</td>
</tr>
<tr>
<td>5</td>
<td>6.4</td>
<td>4.0</td>
<td>8.1</td>
<td>33.0</td>
<td>39.9</td>
<td>7.8</td>
</tr>
<tr>
<td>20</td>
<td>6.5</td>
<td>3.1</td>
<td>8.0</td>
<td>23.9</td>
<td>28.8</td>
<td>31.6</td>
</tr>
<tr>
<td>75</td>
<td>5.6</td>
<td>1.6</td>
<td>2.8</td>
<td>8.1</td>
<td>9.3</td>
<td>72.3</td>
</tr>
</tbody>
</table>

**Table III**

Effect of added enzymes on the adenine nucleotide of a platelet lysate

Washed platelets were suspended (1 × 10⁹ cells/ml) in a medium containing 60 mM NaCl, 50 mM KCl, 2.5 mM MgCl₂, 1 mM ethylenebis(oxyethylenenitriilo)tetraacetic acid 10 mM sodium phosphate, and 15 mM Tris-HCl pH 7.6. The platelets were lysed by the addition of digitonin to a final concentration of 2 mM. At time zero, glucose, hexokinase, myokinase, and AMP deaminase were added to final concentrations of 4 mM, 80 μg/ml, 16 μg/ml, and 0.24 units/ml, respectively. (Note: The difference in ATP/ADP ratio in this table and that of Table I is due to two factors: (a) ATP and ADP hydrolysis occurs during the lysis procedure; (b) lys of the cell allows mixing of the cytoplasmic and granule pools. Table I presents radiochemical data of the intact cell, where the two pools are separate.)

**Figure 1.** Breakdown of ATP after addition of concentrated platelet suspensions to EDTA/ethanol containing [14C]ATP. A concentrated platelet suspension was diluted to the indicated concentrations by adding of suspending buffer. Each dilution was equilibrated to 37°C, and a 250-μl aliquot was added to 250 μl of EDTA/ethanol containing 0.25 μC of [14C]ATP (specific activity was 268 mCi/mmol). The extracts were analyzed by high voltage paper electrophoresis. ATP (O) and ADP (∇) are expressed as percentages of total radioactivity (approximately 17,000 cpm).
a factor of two between the two procedures, and hence a 70-
to 80% difference in the ATP/ADP ratio. Therefore, we feel
that ethanol extraction may be more appropriate in studies
where the fine tuning of metabolic processes influenced by
ADP levels are under investigation. This is especially impor-
tant in light of the recent controversy concerning whether the
[ATP]/[ADP] or the ratio of the components of the
phosphorylation reaction, [ATP]/([P.] [ADP]), determines
the rate of ADP transport into mitochondria and of mito-
chondrial respiration. Experiments of Erecinska et al. (20), in
which the phosphate concentration inside intact cells was
altered, suggested that the ratio [ATP]/([ADP][P.]) regulates
the respiration rate of a cell. In contrast, experiments of Davis
and Davies-vanThienen (21), using isolated mitochondrial
preparations, have shown that respiration rate was independ-
ent of the phosphate concentration in the physiological range
and more closely related to [ATP]/[ADP]. These authors
suggested, as do we, that compartmentalization of nucleotides
within intact cells might explain this discrepancy.

Therefore, since actin is a major protein in many cells (11),
we feel our studies on platelets are of general importance and
raise the question of compartmentalization of ADP as one to
be seriously considered in future studies in all cell types.

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