Effect of Energy-Rich Compounds on Release of Intracellular Enzymes from Human Leukocytes and Rat Lymphocytes

J. Henry Wilkinson and Jean M. Robinson

Experiments have been performed to determine some of the factors affecting the release of intracellular enzymes, in the hope of improving our understanding of the mechanisms whereby tissue enzymes reach the circulation in disease processes. The discharge of intracellular enzymes into the medium during prolonged incubation of human leukocytes and rat lymphocytes has been shown to be inversely related to their ATP contents. Incorporation of ATP into the medium has a marked protective effect against enzyme loss. Other high-energy phosphates, such as uridine triphosphate and phosphoenolpyruvate, which can readily be converted into ATP, also exert a protective effect, but creatine phosphate, which cannot be so converted owing to the low activity of creatine kinase in the cells, exhibits no such action. Glucose and certain intermediates of the glycolytic pathway also reduce the leakage of intracellular enzymes, an effect which parallels their concentrations in the media. ATP also protects rat lymphocytes against enzyme loss provoked by high potassium concentrations. It is suggested that the integrity of the cell membrane, as assessed by its ability to prevent the leakage of enzymes, depends on the energy content of the cell, a decrease of which may be a common factor in clinical situations associated with elevated enzyme activities in the serum.

Although serum enzyme measurements have been extensively used in diagnosis for more than two decades, the mechanisms by which intracellular enzymes are released into the circulation from damaged cells remain obscure. Complete disruption of the cell in necrosis leads to the discharge of its contents, but little is known of the cause of the increased membrane permeability in reversible inflammatory states. Zierler suggested that membrane permeability is linked to cellular metabolism when he demonstrated that the efflux of aldolase from intact rat muscles into the medium was increased by anoxia, glucose deprivation, exposure to excessive potassium ion concentrations, or treatment with metabolic inhibitors (1, 2).

In a previous paper (3) we described rat lymphocytes as a model for the study of the release of intracellular enzymes in response to external stimuli. The rate of enzyme release was greatly enhanced by treatment with phospholipases A and C, the effect being more pronounced in the case of cytosolic enzymes than with mitochondrial enzymes. Subsequently we demonstrated that adenosine triphosphate (ATP) exerted a marked protective effect on rat lymphocytes against the action of phospholipases and also that ATP substantially reduced the efflux of lactate dehydrogenase from similar cell preparations incubated at 37 °C for prolonged periods (4).

The present paper describes experiments designed to test the hypothesis that increased membrane permeability leading to enzyme release is inversely related to the energy content of the cell. For this purpose the ATP concentrations of human leukocytes and rat lymphocytes were measured at various time intervals during prolonged incubation and the results were compared with the adenylate kinase (EC 2.7.4.3), aspartate aminotransferase (EC 2.6.1.1), lactate dehydrogenase (EC 1.1.1.27), and malate dehydrogenase (EC 1.1.1.37) activities released into the medium. We assessed the effects on enzyme release of incorporating ATP and other "high-energy" compounds into the medium.

If the hypothesis is sound, incubated cells should be able to protect themselves against excessive loss of intracellular enzymes by synthesizing ATP from glucose and various intermediates of the glycolytic cycle. Accordingly, enzyme discharge was measured in preparations treated with glucose, glucose 6-phosphate, and other substances likely to lead to enhanced ATP production. Finally, the effect of exposure to high potassium concentrations was measured in the presence or absence of added ATP.

Materials and Methods

Chemicals

ATP (adenosine-5'-triphosphoric acid, disodium trihydrogen salt), ADP (adenosine-5'-diphosphoric acid, trisodium salt), AMP (adenosine-5'-phosphoric acid, disodium salt), UTP (uridine-5'-triphosphoric acid, trisodium salt) and creatine phosphate (creatin-phosphoric acid, sodium salt) were purchased from BDH Chemicals Ltd., Poole, Dorset, BH12 4NN.

PEP (phosphoenolpyruvate, monosodium salt) and AMP-PNP (adenylylimidodiphosphate, tetralithium salt) were purchased from the Boehringer Corp. (London) Ltd., London, W5 2TZ.

Solutions were freshly prepared in Krebs–Ringer glucose buffer and were adjusted to pH 7.4 by addition of sodium hydroxide.
Krebs–Ringer glucose buffer, pH 7.4, was prepared by dissolving NaCl (117 mmol), KCl (3.5 mmol), CaCl₂ (2.5 mmol), KH₂PO₄ (1.2 mmol), MgSO₄ (1.2 mmol), NaHCO₃ (28 mmol), and glucose (8.3 mmol) in distilled water and adjusting the volume to 1 liter.

Determination of Enzyme Activities

Enzyme activities were measured at 25 °C by methods involving the reduction of NAD⁺ or NADP⁺ or the oxidation of the corresponding dihydro compounds. The change in absorbance at 340 nm with time was recorded on a Unicam SP 800 spectrophotometer fitted with a scale expansion unit. Chemicals of reagent grade were used throughout.

Lactate dehydrogenase activity was determined by the reduction of pyruvate (5), malate dehydrogenase by the reduction of oxaloacetate (6). Aspartate transaminase was also determined spectrophotometrically (7).

Adenylate kinase was measured by the method of Oliver (8), with use of reagent tablets (9) kindly provided by Smith Kline Instruments Inc., Palo Alto, Calif. 94304. The relatively high adenylate kinase activities encountered in the cell preparations precluded the use of the corresponding technique (8, 10) for creatine kinase (EC 2.7.3.2), which was determined by the method of Hess et al. (11), the reference cell containing a blank reaction mixture from which creatine phosphate was omitted.

Pyruvate kinase (EC 2.7.1.40) activity was measured by the method of Tanaka et al. (12).

Determination of ATP

The cell suspensions (0.2 ml) were mixed with 0.8 ml of ice-cold perchloric acid (0.6 mmol/liter) and the cells were disrupted in an M.S.E. ultrasonic disintegrator for 20 s. The mixture was set aside at room temperature for 10 min, then centrifuged in a Beckman 152 Microfuge for 2 min. The ATP concentration in the supernatant fluid was determined by the method of Büchler (13) by use of a kit purchased from Boehringer Corp. (London) Ltd.

Human Leukocyte and Rat Lymphocyte Suspensions

Human leukocyte suspensions were prepared from fresh whole blood, and rat lymphocyte suspensions were prepared from the axillary and mesenteric lymph nodes of a freshly killed rat, by procedures already described (3). The washed cells were suspended in Krebs–Ringer glucose buffer to give concentrations of about 10 000 human leukocytes per microliter or 60 000 rat lymphocytes per microliter.

Release of Enzymes from Cells

Freshly prepared rat lymphocyte or human leukocyte suspensions (1.5–2.5 ml) were incubated at 37 °C for 4 h in the presence or absence of the agents under investigation. At hourly intervals, 0.4-ml samples were withdrawn and centrifuged for 2 min in a Beckman 152 microcentrifuge. The supernatant fluids were separated and kept at 4 °C until enzyme determinations were made, usually within 3 h but never later than 5 h after separation. We saw no significant loss of enzyme activity during this period.

During each experiment a second sample of the cell suspension was homogenized at 0–5 °C in an M.S.E. ultrasonic disintegrator for 20 s. After centrifugation for 4 min in the Beckman 152 microcentrifuge, the enzyme activities in the supernates were determined. Because no more than traces of additional activity were obtained by re-extracting the cell debris, we regarded the values observed as the total activity in the suspension. The amounts released during incubation were expressed as a percentage of the total activity.

Results

Although we saw considerable variation in the amount of enzyme released in control experiments from the cells of different rats, the amounts liberated from cells from the same animal were remarkably constant. In different experiments, rat lymphocytes lost 40–80% of their lactate dehydrogenase during incubation in Krebs–Ringer solution for 4 h, but replicate incubations with cells from the same rat gave a coefficient of variation of 2–4% for the release of lactate dehydrogenase into the medium. In all experiments the same cell populations were used in control and test incubations.

The loss of intracellular enzymes during prolonged incubation in Krebs–Ringer solution is markedly diminished by the incorporation of ATP into the medium. This is illustrated in Table 1, which shows that the release of lactate dehydrogenase up to 2–3 h from rat lymphocytes is slightly but significantly reduced by ATP concentrations (5 mmol/liter) in the physiological range, while greater concentrations produce highly significant decreases in the amount of enzyme diffusing out of the cells. Similar effects are observed in comparable experiments with human leukocytes. Figure 1 shows that release of lactate dehydrogenase

<table>
<thead>
<tr>
<th>Period of incubation at 37 °C, h</th>
<th>Enzyme release in controls (Percentage of total activity — mean (±SD))</th>
<th>Enzyme release in presence of ATP (mmol/liter) (Percentage of control mean — mean (±SD))</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>47 ± 19, 79 ± 17, 68 ± 16, 45 ± 24</td>
<td>5 10 50</td>
</tr>
<tr>
<td>3</td>
<td>66 ± 18, 85 ± 23, 74 ± 22, 32 ± 19</td>
<td>P &lt; 0.01, P &lt; 0.001, P &lt; 0.001</td>
</tr>
<tr>
<td>4</td>
<td>86 ± 13, 95 ± 18, 85 ± 13, 71 ± 17</td>
<td>N.S. P &lt; 0.01, P &lt; 0.001</td>
</tr>
</tbody>
</table>

*Cells from each of six different rats were used in each experiment. They were incubated at pH 7.4 in Krebs–Ringer solution.
and malate dehydrogenase is markedly decreased in the presence of 25 mmol of ATP per liter.

Figure 2 shows the results obtained when similar experiments were performed with other high-energy phosphates. Release of lactate dehydrogenase was markedly decreased in the presence of phosphoenolpyruvate and uridine triphosphate, but creatine phosphate exerted virtually no protective effect. It was observed that rat lymphocytes contain very little creatine kinase activity (<0.5 U/10^7 cells), but the cells are relatively rich in pyruvate kinase (7.5 U/10^7 cells).

AMP-PNP, a compound reported to be a potent competitive inhibitor of heavy meromysin ATPase (14), and to be unable to be utilized by hexokinase and myokinase (15), was found to have little effect on the release of lactate dehydrogenase from rat lymphocytes, though a slight ATP-like effect was observed after 2 h of incubation. ADP also exhibited a slight but significant protective effect after 2 and 3 h of incubation (Table 2).

In an attempt to establish a relationship between the rate of enzyme release and the intracellular ATP content, we did an experiment in which rat lymphocytes were incubated in Krebs–Ringer solution in the absence of glucose. Samples of the cell suspension were removed at intervals and the ATP content of the centrifuged cells and the lactate dehydrogenase activity of the supernatant fluid were measured. The results are shown in Figure 3. Little enzyme release occurs until the ATP concentration is reduced to negligible levels.

Because the ATP concentration appears to be an important factor in maintaining the integrity of the cell membrane as judged by its ability to retain intracellular enzymes, and because the metabolism of glucose is normally the principal source of ATP synthesis, it seemed that the incorporation of glucose in the medium should also reduce the efflux of enzymes from the cells. The effects of increasing the glucose concentration in the medium on the release of lactate dehydrogenase from rat lymphocytes are shown in Table 3. No significant effect was observed in the 1-h and 2-h samples, but in the 3-h sample, high concentrations of glucose produced a highly significant decrease in the enzyme activity released into the medium, and in the 4-h sample concentrations of glucose as low as 1.1 mmol/liter significantly decreased enzyme efflux.

That the catabolism of glucose was responsible for these protective effects was shown by carrying out experiments in the presence of iodoacetate. No effect

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Table 2. Release of Lactate Dehydrogenase from Rat Lymphocytes Incubated with AMP, ADP, ATP, and AMP-PNP

<table>
<thead>
<tr>
<th>Period of incubation at 37 °C, h</th>
<th>Percentage of total enzyme released (mean ± SD)* when cells treated with</th>
<th>AMP</th>
<th>ADP</th>
<th>ATP</th>
<th>AMP-PNP</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td>28 ± 0.4</td>
<td>25 ± 1.0</td>
<td>22 ± 0</td>
<td>26 ± 0.6</td>
</tr>
<tr>
<td>N.S.</td>
<td></td>
<td>N.S.</td>
<td>N.S.</td>
<td>P &lt; 0.001</td>
<td>N.S.</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>31 ± 1.4</td>
<td>26 ± 0.9</td>
<td>23 ± 0.5</td>
<td>26 ± 0.8</td>
</tr>
<tr>
<td>N.S.</td>
<td></td>
<td>P &lt; 0.001</td>
<td>N.S.</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.02</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>50 ± 1.1</td>
<td>34 ± 1.4</td>
<td>26 ± 0.8</td>
<td>36 ± 0.05</td>
</tr>
<tr>
<td>P &lt; 0.001</td>
<td></td>
<td>P &lt; 0.01</td>
<td>P &lt; 0.001</td>
<td>N.S.</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>60 ± 0.2</td>
<td>38 ± 0.6</td>
<td>26 ± 0.8</td>
<td>37 ± 0.5</td>
</tr>
<tr>
<td>P &lt; 0.001</td>
<td></td>
<td>P &lt; 0.001</td>
<td>N.S.</td>
<td>N.S.</td>
<td></td>
</tr>
</tbody>
</table>

* In each experiment 5 cell suspensions were incubated at pH 7.4 in Krebs–Ringer solution containing the substances stated. 10 mmol/liter concentrations of compounds used in each case.
Table 3. Effects of Increasing Glucose Concentration on Release of Lactate Dehydrogenase from Rat Lymphocytes

<table>
<thead>
<tr>
<th>Period of incubation at 37 °C, h</th>
<th>Percentage of total enzyme released (mean ± SD)* Glucose concn (mmol/liter)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>26 ± 0.7</td>
</tr>
<tr>
<td>2</td>
<td>27 ± 1.3</td>
</tr>
<tr>
<td>3</td>
<td>40 ± 1.3</td>
</tr>
<tr>
<td>4</td>
<td>59 ± 2.2</td>
</tr>
</tbody>
</table>

* In each experiment, five cell suspensions were incubated at pH 7.4 in Krebs–Ringer solution containing the stated concentration of glucose.

was observed until the cells had been incubated for 4 h, when 33.4% of the total lactate dehydrogenase activity was released in the presence of 2.8 mmol of glucose per liter, and 61.2% in the presence of 2.8 mmol of glucose and 10 mmol of iodoacetate per liter.

Agents that uncouple oxidative phosphorylation, such as 2,4-dinitrophenol, also slightly decreased the protective effect of glucose. In the presence of 8.7 mmol of glucose per liter, cells incubated for 4 h liberated 31.6% of the total lactate dehydrogenase, but 41.6% and 44.5% when the medium also contained $0.25 \times 10^{-5}$ and $5 \times 10^{-5}$ moles of dinitrophenol per liter, respectively.

High concentrations of glucose also decreased the release of intracellular adenylate kinase, malate dehydrogenase, and aspartate transaminase into the medium (Table 4) and diminished the rate at which intracellular ATP disappeared (Table 5). Glucose 6-phosphate also decreased the rate at which lactate dehydrogenase was released into the medium, but its effect was somewhat less than that of glucose (Figure 4). Fructose, however, appears to exert little if any protective action, although fructose 6-phosphate acts similar to glucose 6-phosphate (Figure 5).

Figure 6 shows that increased K⁺ concentration causes increased leakage of lactate dehydrogenase from rat lymphocytes incubated at 37 °C in Krebs–Ringer solution containing 1.1 mmol of glucose per liter. This effect is decreased when higher concentrations of glucose are incorporated into the medium, and is abolished altogether in the presence of excess ATP.

Discussion

The results described in this paper (Table 1 and Figure 1) establish that inclusion of ATP into the external medium decreases the leakage of a number of intracellular enzymes from human leukocytes and rat lymphocytes. This protective effect is related to the ATP concentration, because at physiological concentrations the action is slight and relatively short-lived, whereas at higher concentrations it is more pronounced and persists for a longer period.
membrane without entering the cell, these findings, together with our demonstration of an inverse relationship between the intracellular ATP content and enzyme leakage, suggest that the membrane may be permeable to ATP. The demonstration by Chaudry and Gould (18) of the transport of ATP into muscle cells and that by Petkau and Chelack (19) of its diffusion through lipid membranes are of interest in this connection.

Whether the effect of ATP is mediated within the cell or on the external surface of the membrane does not affect our hypothesis that enzyme leakage is associated with a decrease in the intracellular energy content. The protective effects of glucose, glucose-6-phosphate, and fructose-6-phosphate (Figure 4) support our contention, because their metabolism leads directly to ATP production. Further evidence is provided by the actions of agents that interfere with this process. Iodoacacetate, which inhibits glycolysis, and 2,4-dinitrophenol, an uncoupler of oxidative phosphorylation, were both shown to diminish the protective effect of glucose. The more marked effect we showed for glucose, as compared with that of glucose 6-phosphate and fructose 6-phosphate, may be due to the relative ease with which the unphosphorylated compound can enter the cell. The failure of fructose to exert any protective effect may be attributed to the loss of adenine nucleotides that accompanies the formation of fructose 1-phosphate (27). The protective effect of phosphoenolpyruvate is probably attributable to the fact that rat lymphocytes are rich in pyruvate kinase, which forms ATP directly from this substrate. Creatine phosphate, however, does not decrease enzyme efflux, presumably because the cells do not contain demonstrable creatine kinase and hence are unable to convert this energy-rich phosphate into ATP.

The slight protective effect of ADP (Table 2) appears to be due to the action of adenylate kinase,
high activities of which were demonstrated in the cells (63 U/10^12 cells). It seems that the action of ADP is due to the formation of ATP:

\[ 2 \text{ADP} \rightleftharpoons \text{AMP} + \text{ATP} \]

because AMP was found to accelerate enzyme release. This appears to be due to displacement of the equilibrium of the adenylate kinase reaction leading to loss of ATP.

Increased enzyme efflux from rat lymphocytes exposed to high K+ concentrations (Figure 6) may also be attributed to loss of ATP. The Na+/K+ pump mechanism is dependent upon energy released by ATPase-mediated hydrolysis of ATP (20) and the additional demands upon this process caused by the high extracellular K+ concentration would be expected to lead to a decrease in the availability of ATP.

Tissue damage due to virus infection, chemical or mechanical trauma, hypoxia, etc., frequently leads to the release of intracellular enzymes into the plasma, and we have proposed (4) that a common factor in such clinical conditions may be failure to synthesize ATP in adequate amounts. The results described in the present work are consistent with this view. A similar suggestion has been made by Sweetin and Thomson (21) to account for their finding that significant loss of enzymes from human erythrocytes did not occur until the glucose content of the medium was exhausted. Also in agreement with the results of the present investigation is the observation that reduction in intracellular ATP concentrations in isolated human leukocytes is related to the amount of lactate dehydrogenase released into the incubating medium (22). It has been reported (23) that adenosine nucleotides protect human erythrocytes against osmotic lysis when incubated in vitro, and Mohler (24) has described an association between increased fragility and a faster disappearance rate for ATP in erythrocytes from patients with hereditary spherocytosis.

ATP is required for the incorporation of added fatty acids into phosphatidyl choline in rat and human erythrocytes in vitro (25). It also plays a vital role in the biosynthesis of phosphatidic acids (26) required for the synthesis of membrane phospholipids and deficiencies in the ATP content of cells may lead to impairment of the processes responsible for maintenance of membrane integrity, allowing discharge of intracellular enzymes.

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References