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# Net uptake of orthophosphate in Ehrlich ascites tumor cells in the presence of purine riboside may be rate limiting for the expansion of the pool of ribonucleotides

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**Abstract**

Incubation with adenosine or with structural analogs thereof may in several cell types under some conditions result in the cellular accumulation of abundant amounts of the corresponding triphosphates. In the present work we have found that incubation of cells at high concentrations of orthophosphate ($P_i$) results in increased intracellular levels thereof, although they become not as high as the extracellular concentration. In the presence of purine riboside (nebularine, Pr) and high concentration of $P_i$ the intracellular $P_i$ is, however, kept at a low steady-state level, probably because it immediately upon uptake is being trapped primarily as the triphosphate of purine riboside. The latter compound accumulates at a constant rate for at least 1 h. The rate of accumulation of the sum of phosphate residues present in $P_i$, adenosine phosphates and purine riboside phosphates appears to be proportional to the extracellular concentration of $P_i$ and to be highly dependent on pH (6.5 and 7.0 being optimal and 7.9 nonpermissible) but it is unaffected by substitution of $Na^+$ by choline.

*Key words:* Inorganic phosphate uptake; Intracellular inorganic phosphate; Nucleotide catabolism; Purine riboside; Nucleotide pool

## 1. Introduction

It has been known for a while that a number of both synthetic and naturally occurring structural analogs of adenosine become phosphorylated and accumulate mainly as the corresponding triphosphates in several cell types. These processes may be accompanied by a partial or a complete depletion of the cellular pool of adenine ribonucleotides [1–6]. Under aerobic conditions this depletion depends on the activation of AMP deaminase and of an IMP specific 5′-nucleotidase resulting in conversion of AMP to inosine with IMP as an intermediate. For adenosine similar phenomena may apply. Experiments with mouse fibroblasts and Ehrlich ascites tumor cells have shown that in the presence of 3′-deoxyadenosine a high extracellular concentration of $P_i$ may lead to accumulation of abundant amounts of 3′-deoxy ATP without a concomitant reduction of the pool of adenosine phosphates.

In the present work we have studied the mechanism by which extracellular $P_i$ affects the intracellular $P_i$-concentration and the rate of phosphorylation of the adenosine analog purine riboside.

## 2. Materials and methods

Purine riboside and silicone oil (density 1.05 g/ml) were obtained from Sigma (USA), and paraffin highly liquid was obtained from Merck (Germany). [6-$^3$H]Purine riboside was obtained from Moravek Biochemicals, CA (USA) and [*methoxy*-$^{14}$C]inulin and [$^3$H]$H_2O$ from NEN (Germany). Ehrlich ascites tumor cells, strain ELT, kindly supplied by Dr. G. Klein, Karolinska Instituttet, Stockholm (Sweden), were maintained in female mice (NMRI) by intraperitoneal

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growth and harvested 6 days after injection of about $15 \cdot 10^6$ cells. Cells were washed once in 150 mM NaCl, suspended in the same solution and kept on ice for less than 15 min. After sedimentation the cells were suspended and preincubated for 5 min at 37°C in a buffer, containing 142 mM NaCl, 0.5 mM $MgSO_4$, and 25 mM Hepes adjusted with NaOH to the desired pH. The experiments were initiated by addition of aliquots of the cell suspension to constant volumes of isotonic solutions with varying ratios of NaCl and sodium phosphate at the appropriate pH. These solutions contained in addition sodium lactate to give a final concentration of 15 mM. In experiments designed to study the effect of extracellular $Na^+$ the concentration of this ion was altered by replacement with choline ions.

Experiments were performed at 37°C. Cells were kept in suspension by gentle shaking at a density of approximately $30 \cdot 10^6$ cells/ml. At appropriate times aliquots (300 $\mu$l) were transferred to Eppendorf tubes on ice containing 0.3 ml of silicone oil/liquid paraffine mixture (final density 1.034 g/ml) overlayered with 0.9 ml of isotonic NaCl, and immediately centrifuged for 2 min at $15000 \times g$. After centrifugation the supernatant was sucked off, and the tubes were washed four times with 900 $\mu$l ice-cold isotonic NaCl before the oil layer was aspirated. The cell pellet was extracted for 10 min with 225 $\mu$l of 0.4 M perchloric acid (PCA), and the resulting extract was neutralized with KOH, and analyzed for orthophosphate [9] and nucleotides. The nucleotides were separated and quantitated by HPLC using a strong anion exchange column (Partisil-10 Sax, 2 mm $\varnothing \times 200$ mm, Whatman, Clifton, NJ). The column was eluted for 3 min with 0.005 M $KH_2PO_4$ (pH 3.5), followed by a linear gradient for 14 min to 0.4 M $KH_2PO_4$, 0.8 M NaCl (pH 3.5); an isocratic period was allowed for 20 min, and the column was reequilibrated with the starting buffer before a new sample injection was started. The flow rate was 0.7 ml/min. The absorbancy at 254 nm was recorded, and the peaks were automatically integrated.

Cellular total phosphate was determined according to Ames and Dubin [10] as follows. Samples of cell suspensions containing $(1–2) \cdot 10^6$ cells were diluted 200-fold with ice-cold 150 mM NaCl in $13 \times 100$ mm pyrex test tubes and centrifuged for 7 min at $3000 \times g$ at 2°C. After centrifugation the diluted medium was removed. The centrifugation was repeated and residual medium was completely aspirated. The cell pellet was suspended in 0.1 ml of 150 mM NaCl, and 0.1 ml of 0.4 M $Mg(NO_3)_2 \cdot 6H_2O$ in ethanol was added. The mixture was evaporated to dryness over a strong flame with rapid shaking and further heated in the flame until the brown fumes had disappeared. After the tubes had cooled 1.0 ml of 1.0 M HCl was added. The tubes was capped with a drip-catcher and heated in a boiling water bath for 15 min to hydrolyze any pyrophosphate formed in the ashing procedure. Total cellular phosphate was determined by measuring the content of inorganic phosphate in the sample [10].

Cellular water content was determined in experiments performed in the presence of tritiated water from the $^3H$ content of the medium and that of the cells isolated by centrifugation through oil. The value obtained was corrected for extracellular water based on experiments with [*methoxy*-$^{14}$C]inulin and determined from the radioactivity of the medium and that of the isolated cells. The values for $10^6$ cells were $1.10 \pm 0.09$ $\mu$l (means ± S.D. [17]).

### 2.1. Experimental setup

Like several other structural analogs of adenosine also purine riboside may be taken up by cells and accumulate as phosphorylated derivatives, primarily as the triphosphate [11,12]. Using (6-$^3$H)-labeled purine riboside we have found that in Ehrlich ascites tumor cells more than 96% of all of the phosphorylated derivatives may be accounted for as the mono-, di- and triphosphate (PrMP, PrDP and PrTP, respectively), (data not shown). The determination of these three compounds in addition to that of AMP, ADP, ATP and $P_i$ permits a quantitation of the main amounts of the acid-soluble phosphorylated compounds. When purine riboside was added its amount was in several fold excess over the phosphorylated derivatives which may accumulate in the cells and its concentration more than 10-fold the apparent $K_m$ value (12.5 $\mu$M) for its intracellular accumulation as phosphorylated derivatives. Since glucose affects the cellular content of adenosine phosphates in Ehrlich cells [13] we have used lactate (15 mM) as carbon source. The ATP/ADP ratio in control cells was constant over 1 h under such conditions.

## 3. Results

### 3.1. Experiment with varying concentrations of $P_i$ at pH 6.5

*Experiment in the absence of purine riboside*

The relationship between extracellular $P_i$ concentration and the $P_i$ content of the cells as function of time has been determined. It appears from Fig. 1 that in the absence of $P_i$ in the medium the amount and the concentrations of intracellular $P_i$ decreases to a low value while it at 12 mM extracellular $P_i$ is almost constant at 5 mM. At 25 mM and even more so at 45 mM extracellular $P_i$ its intracellular content and concentration initially increases but levels off after about 20 min at concentrations (approx. 10 mM and 18 mM) that are considerably below the extracellular value.

The nucleotide content was unchanged at all $P_i$ concentrations in the experimental period (results not shown).

These results are similar to those obtained by Barankiewicz et al. [13] with the same type of cells in a glucose containing medium. They suggested that the fact that the intracellular concentration of $P_i$ does not approach that of the medium at high concentrations might be explained by a rapid esterification of intracellular $P_i$ and hence not included in the results obtained by the determination performed. If this is the case one should expect to find a continuous increase in the total amount of phosphate residues in cells incubated with high concentration of $P_i$. According to Fig. 1 the increase should be at least 120 $\mu$mol per $10^6$ cells in 2 h at 45 mM $P_i$. We have made such determinations in cells incubated with 45 mM $P_i$ and the results showed no significant increase over 2 h in the total content of phosphate derivatives. This is in contrast to cells incubated with both 45 mM $P_i$ and purine riboside showing a considerable increase in total phosphate (Fig. 2). We would, therefore, rather suggest that intracellular $P_i$ inhibits its own net uptake over the cell membrane in a concentration dependent manner. This may partially be explained by the electrochemical properties of the cells.

*Experiments in the presence of purine riboside*

At 45 mM $P_i$, PrTP accumulated in the cells at an almost constant rate for 1 h. Only small amounts of PrDP of PrMP were formed while the content of the adenosine phosphates and of $P_i$ were almost unchanged (Fig. 3). The utilization of intracellular $P_i$ for accumulation of PrTP apparently prevents its content to increase and it may at the same time also prevent its presumed inhibitory effect on its own net uptake.

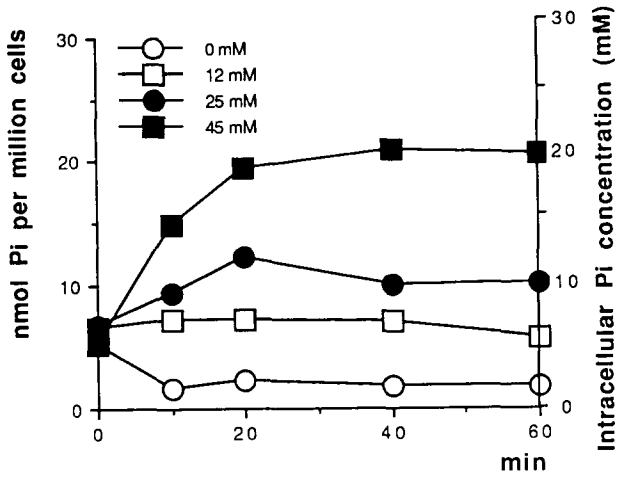


Fig. 1. Time-dependent effect of different extracellular $P_i$ concentrations on its own cellular accumulation. At zero time cells suspended in 140 mM of NaCl, 0.5 mM of $MgSO_4$ and 25 mM of Hepes of pH 6.5 were transferred to constant volumes of isotonic mixtures of NaCl and sodium phosphate (pH 6.5) to give final phosphate concentrations of 0, 12, 25 and 45 mM. Sodium lactate was added to a final concentration of 15 mM. At the indicated times cell pellets were obtained by centrifugation through oil and extracted with PCA, and $P_i$ content determined.

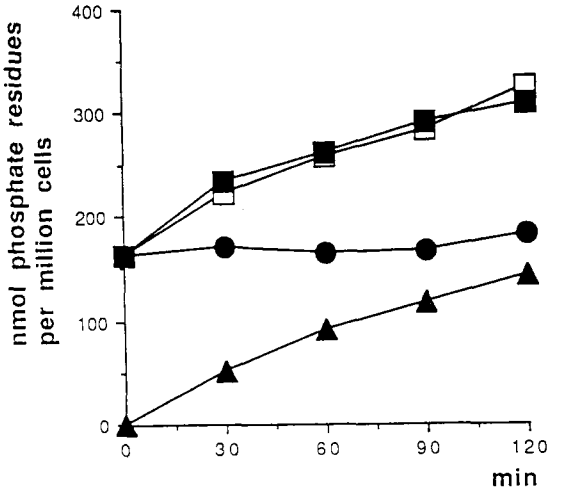


Fig. 2. Amounts of total phosphate residues present in cells incubated at 45 mM $P_i$ in the presence or in the absence of purine riboside. At zero time isotonic sodium phosphate buffer (pH 6.5) and lactate were added to a cell suspension to give a concentration of $P_i$ of 45 mM 15 mM, respectively, and that of purine riboside when added of 2.5 mM. After various times of incubation two samples were taken. In one sample cells were separated from the medium by centrifugation through a layer of oil followed by an extraction of the cells by PCA. The neutralized extract was used for nucleotide analysis. In the second sample the cells were decomposed by an ashing procedure for determination of the amount of total phosphate residues in the cells. Content of total phosphate residues in control cells incubation in the absence of purine riboside (●); sum of total phosphate residues in experimental cells incubated with purine riboside (□); content of phosphate residues present in purine riboside phosphates accumulated in experimental cells (▲); sum of phosphate residues present in purine riboside phosphates accumulated in experimental cells plus total phosphate residues present in control cells (■). The curves represent one of two identical experiments.

Experiments like that in Fig. 3 but with lower extracellular concentration of $P_i$ showed that the sum of the intracellular phosphate residues as present in $P_i$ and in adenosine- and purine riboside phosphates plotted against time gave almost linear rates at all five $P_i$ concentrations used (see Fig. 4). Linear regression analyses of these results showed that the rate of accumulation of phosphate compounds increased linearly with the extracellular concentration of $P_i$ at least up to 45 mM (see inset in Fig. 4). The experiments showed in addition that the intracellular $P_i$ content was lower at lower extracellular $P_i$ concentration and that the pool of adenosine phosphates was exhausted at rates that increased as the extracellular $P_i$ concentration decreased (Table 1). The assumption that it is the extracellular $P_i$ solely and not some pool of intracellular phosphate compound (e.g., polyphosphates) that is the source of the phosphate that accumulates in the cells as purine riboside phosphates was supported by experi-

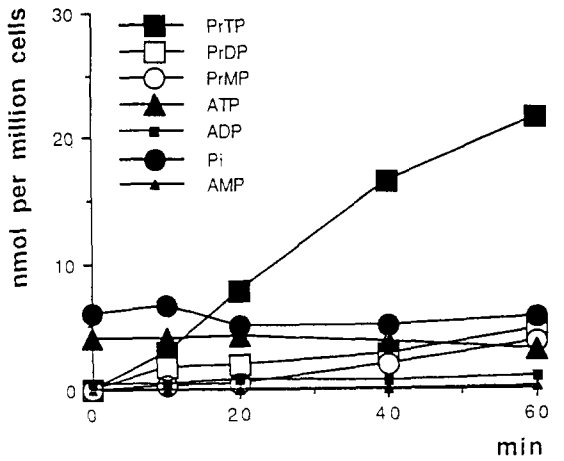


Fig. 3. Effect of purine riboside on cellular content of $P_i$, purine riboside phosphates and adenosine phosphates. At zero time a cell suspension was transferred to an isotonic mixture of NaCl and sodium phosphate (pH 6.5) to give a final concentrations of 45 mM phosphate. Lactate and purine riboside were added to give final concentration of 15 mM and 3 mM, respectively. Aliquots were treated as in Fig. 1 at the indicated times and analyzed for $P_i$ and nucleotides. The curves represent one out of three identical experiments. Ordinate: intracellular content of phosphate compounds.

ments which showed that the presence of purine riboside causes a decrease at an almost constant rate in the $P_i$ content of the medium for about 2 h and that the sum of phosphate residues present in adenosine- and purine riboside phosphates in the cells increases by the same rate (data not shown). In addition, the increase in

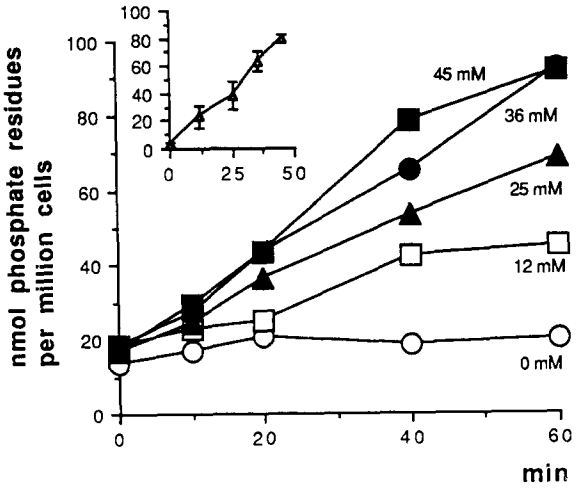


Fig. 4. Effect of different extracellular concentrations of $P_i$ on accumulation of cellular phosphate residues present in $P_i$, purine riboside phosphates and adenosine phosphates in cells incubated with purine riboside. Experiments were performed as in Fig. 3 except for extracellular concentration of $P_i$ which varied as indicated. The curves represent one out of three identical experiments. Ordinate: sum of phosphate residues present in $P_i$, purine riboside phosphates and adenosine phosphates. (Inset) Rates (nmol phosphate residues per $10^6$ cells per h) as obtained by linear regression analyses of accumulation of phosphate residues as function of extracellular concentrtion of $P_i$ (mM). Values are means ± S.D. ($n = 3$).

Table 1
Steady-state concentrations of $P_i$ and exhaustion of the pool of adenosine phosphates in cells incubated with purine riboside and various concentrations of $P_i$

| Extracellular $P_i$ (mM) | Intracellular $P_i$ (mM) [a] | Decrease in the sum of ATP, ADP and AMP [b] | |
|---|---|---|---|
| | | mM | per cent |
| 0 | 0.2 ± 0.02 | 2.4 ± 0.4 | 70 ± 12 |
| 25 | 1.7 ± 0.9 | 1.5 ± 0.3 | 44 ± 9 |
| 45 | 4.6 ± 0.9 | 0.8 ± 0.1 | 24 ± 3 |

Cells were incubated as described for Fig. 4.
[a] Means ± S.D. ($n = 9$) of values at 20, 40 and 60 min for three independent experiments.
[b] Decrease in 1 h from the content at 0 min (3.4 ± 0.5 mM). Values are means ± S.D. for three independent experiments.

total phosphate residues in cells incubated at 45 mM $P_i$ and purine riboside could completely be accounted for by the increase in the sum of phosphate residues present in $P_i$ and in adenosine- and purine riboside phosphates (Fig. 2). The rate of cellular net uptake of $P_i$ may, therefore, be determined by the rate of intracellular increase in the sum of these phosphate residues.

### 3.2. Experiments at varying pH and at 45 mM $P_i$

*Experiments in the absence of purine riboside*

At pH 7.0 the initial rate of increase of cellular $P_i$ content and the final level were similar to that at pH 6.5 (Fig. 1). At pH 7.9 very modest uptake of $P_i$ was observed and at pH 7.5 intermediary values were obtained (data not shown).

*Experiments in the presence of purine riboside*

The changes in cellular content of $P_i$ and of adenosine-and purine riboside phosphates at pH 7.0 were very similar to those at pH 6.5 (see Fig. 3). At pH 7.5 and pH 7.9, however, the cellular content of $P_i$ decreased immediately and the rate of accumulation of purine riboside phosphates was considerably lower at pH 7.5 and it was almost zero at pH 7.9. At the latter pH there was in addition a pronounced exhaustion of the pool of adenosine phosphates (data not shown). The sum of the intracellular phosphate residues present in $P_i$ and in the adenosine- and purine riboside phosphates plotted against time gave almost linear rates at all four pH values (Fig. 5). The rates as obtained by linear regression analyses of these data were plotted against pH and showed a dramatic decrease between pH 7.0 and 7.9 (see inset in Fig. 5).

### 3.3. Lack of effect of extracellular $Na^+$

Determination of steady-state $P_i$ exchange flux has revealed that two transport systems exist in Ehrlich

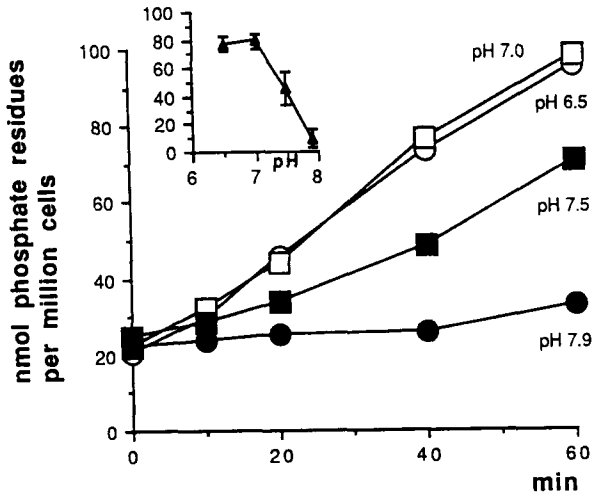


Fig. 5. Effect of extracellular pH on accumulation of the sum of phosphate residues present in $P_i$, purine riboside phosphates and adenosine phosphates in cells incubated in the presence of purine riboside. Experiments were performed as in Fig. 3 at an extracellular $P_i$ concentration of 45 mM in the presence of purine riboside (3 mM) and at extracellular pH-values as indicated. The curves represent one out of three identical experiments. Ordinate: sum of intracellular phosphate residues present in $P_i$, purine riboside phosphates and adenosine phosphates. (Inset) Rates (nmol of phosphate residues per $10^6$ cells per h) as obtained by linear regression analyses of accumulation of phosphate residues as function of extracellular pH. The values are means ± S.D. ($n = 3$).

ascites tumor cells; a $Na^+$ dependent system that shows saturation kinetics and that has $H_2PO_4^-$ as substrate [13], and a $Na^+$-independent system for which the flux is proportional to the extracellular $P_i$ concentration at least up to 30 mM [14]. We have found that at 45 mM $P_i$ the net uptake of $P_i$ in the absence of nucleosides is unchanged when $Na^+$ in the medium is substituted for by choline (data not shown). In the presence of purine riboside but under otherwise identical conditions a partial or a complete substitution of $Na^+$ by choline had also no effect on the rate of increased intracellular phosphate given as the sum of phosphate residues present in $P_i$ and in adenosine-and purine riboside phosphates (Fig. 6). It is, therefore, concluded that the net uptake of $P_i$ under these conditions takes place by the $Na^+$-independent transport system for which the rate is proportional to the $P_i$ concentration.

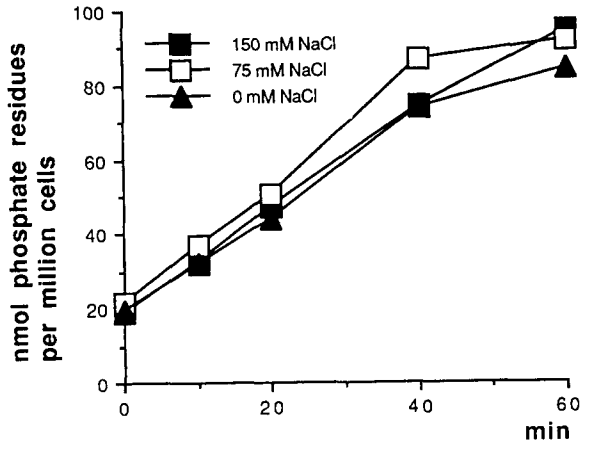


Fig. 6. Effect of extracellular $Na^+$-concentration on intracellular accumulation of the sum of phosphate residues present in $P_i$, purine riboside phosphates and adenosine phosphates. The experiment was performed as described for Fig. 3 except that $Na^+$ was partially or completely replaced by choline ions, the sum of the two being kept constant at 150 mM. Ordinate: accumulation of the sum of phosphate residues present in $P_i$, purine riboside phosphates and adenosine phosphates.

## 4. Discussion

It has been found under a number of conditions that in the presence of purine riboside the cellular steady-state content of $P_i$ is low compared to that seen in the absence of this nucleoside. Furthermore, conditions like low extracellular concentrations of $P_i$ or high pH that both limit $P_i$ uptake also, in the presence of purine riboside, limit the steady-state concentration of intracellular $P_i$ and the expansion of the pool of ribonucleotides. This shows that immediately upon entry $P_i$ is being incorporated in nucleotides leading to accumulation of the triphosphate of purine riboside. These findings indicate that it is the rate of net cellular uptake of $P_i$ and not that of its esterification, or of the uptake of purine riboside, or the phosphorylation of this compound that is rate limiting for the expansion of the pool of ribonucleotides.

When the cells were incubated at pH 6.5 in the presence of purine riboside and 45 mM $P_i$ the intracellular $P_i$ content was unchanged for 1 h at about 5 nmol/$10^6$ cells which corresponds to an intracellular concentration of approx. 5 mM (Fig. 3). Incubation of cells at lower $P_i$ concentrations resulted in lower cellular concentration of $P_i$ and in exhaustion of the pool of adenosine phosphates at rates that were faster the lower the extracellular $P_i$ concentration was (Table 1). These observations suggest that the intracellular concentration of $P_i$ may play a major role for the stability of the pool of adenosine phosphates. Under aerobic conditions this pool may be catabolized by deamination of AMP to IMP followed by dephosphorylation of the latter compound to inosine. $P_i$ is known to be an effective inhibitor of both AMP deaminase and 5'-nucleotidase [17], and the present findings are in agreement with the possibility that at 5 mM $P_i$ or higher concentrations the intracellular activity of these two enzymes is greatly inhibited, while they may be active at lower intracellular concentrations of $P_i$.

Under physiological conditions the extracellular

concentration of $P_i$ is usually relatively low. In human blood serum it is 1 to 2 mM and in the RPMI and DME media it is 5.6 mM and 0.9 mM, respectively. We have previously found that in 3T3 mouse fibroblats grown in the latter medium adenosine gives rise to relatively rapid catabolism of adenine ribonucleotides (approx. 30 nmol of hypoxanthine plus inosine formed per $10^6$ cells per h). This process was slightly overcompensated by phosphorylation of adenosine to AMP resulting in a rather slow expansion of the ATP pool (ab 5 nmol per $10^6$ cells per h) [7]. Under similar conditions 3'-deoxyadenosine gave rise to exhaustion of the pool of adenosine phosphates in less than 1 h and to accumulation of 3'-deoxyATP [8]. These findings resemble the results obtained by treatment of humans with the adenosine deaminase inhibitor 2'-deoxycoformycin [18] and they also resemble the state which prevail in humans suffering of adenosine deaminase deficiency [19]. Under both conditions the degradation of DNA in the cells that are precursors to erythrocytes gives rise to accumulation of 2'-deoxyadenosine because its deamination is prevented. This leads to the presence of relatively very large amounts of 2'-deoxyATP and low content of ATP in blood cells. All of these observations are in accordance with the present findings that purine riboside activates the catabolism of adenine ribonucleotides most efficiently when the concentration of $P_i$ is low and that purine riboside triphosphate accumulates only slowly under these conditions due to slow net uptake of $P_i$. This mechanism of effect may, thus, be valid for a number of analogs of adenosine that are subject to intracellular phosphorylation.

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