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ADENOSINE METABOLISM IN HUMAN ERYTHROCYTES

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SUMMARY

1. The metabolism of [8-¹⁴C]adenosine was examined in human erythrocytes and erythrocyte lysates.

2. The metabolism of [8-¹⁴C]adenosine in erythrocytes is largely dependent upon its concentration.

3. Adenosine deaminase and adenosine kinase activities were assayed in crude erythrocyte lysates. No adenosine phosphorylase activity was detected.

4. Adenosine deaminase exhibited a K_m for adenosine of $4 \cdot 10^{-5}$ M. Substrate inhibition of the enzyme was exhibited at concentrations of adenosine greater than $2 \cdot 10^{-4}$ M. No inhibition of deaminase activity by equimolar or greater concentration of AMP, ADP, ATP, GDP, GTP, P_i, guanylic acid or 2,3-diphosphoglycerate was noted, but 20 % inhibition by high concentrations of adenine and inosine was observed.

5. Adenosine kinase required ATP ($K_m = 4 \cdot 10^{-4}$ M), adenosine ($K_m = 1.9 \cdot 10^{-6}$ M), and Mg²⁺ for activity. No substrate inhibition by adenosine occurred at concentrations 20 times its K_m . Adenosine 5'-triphosphate and Mg²⁺ were inhibitory at concentrations greater than 1.25 mM and 0.50 mM, respectively. The reaction of ATP with adenosine kinase was competitively inhibited by AMP, ADP, guanylic acid, GDP, IMP, and adenine. Adenosine 5'-triphosphate was variably replaceable as the phosphate donor by a wide range of triphosphates.

INTRODUCTION

Adenosine occupies an interesting central position in purine metabolism. Although largely derived from the dephosphorylation of adenine nucleotides, this nucleoside is subject to three metabolic fates: phosphorylation to the nucleotide level, deamination to inosine, or conversion to the base level by adenosine phosphorylase (EC 2.4.2.1). Purified preparations of the enzyme adenosine kinase (EC 2.7.1.20) have been studied extensively¹⁻⁶, and adenosine deaminase (EC 3.5.4.4) has been examined in selected systems⁷⁻¹¹. Adenosine phosphorylase has been found only in bacterial systems¹²⁻¹⁴. However, most studies of enzymes involved in adenosine metabolism have been directed toward investigation of the metabolism of purine analogues. Only recently have attempts been made to study the intermediates formed during the metabolism of adenosine and the factors influencing the meta-

bolism of adenosine itself^{6,15-18}. This communication describes the metabolism of adenosine in human erythrocytes and erythrocyte lysates. Special attention is directed toward elucidating the factors controlling adenosine metabolism.

MATERIALS AND METHODS

Preparation of erythrocytes and erythrocyte lysates

Whole blood was obtained by venipuncture and collected in heparinized tubes. The blood was centrifuged at $2000 \times g$ for 10 min and the plasma and buffy coat discarded. The packed erythrocytes were washed twice with 0.9 % saline and centrifuged with an equal volume of 0.9 % saline at $1000 \times g$ for 10 min. This erythrocyte suspension was used for all whole cell studies.

Erythrocyte lysates were prepared by freeze-thawing the erythrocyte suspension twice. The lysate was dialyzed for 2 h against distilled water and the dialysate centrifuged at $2000 \times g$ for 10 min. The dialysate supernatant was used as the source of adenosine deaminase and adenosine kinase. All procedures were conducted at 4° .

Whole cell assay

The standard assay was conducted by incubating $50 \mu\text{l}$ of the erythrocyte suspension with $100 \mu\text{l}$ of isotonic phosphate buffer (50 mM, pH 7.4), supplemented with 1.5 mM dextrose, and $100 \mu\text{l}$ of 0.54 mM $[8\text{-}^{14}\text{C}]$ adenosine ($53 \mu\text{C}/\mu\text{mole}$) for 1.0 h at 37° . Upon completion of the incubation, $25 \mu\text{l}$ of 1 M HCl was added.

Lysate assays

Adenosine deaminase. The standard assay was conducted by incubating 0.25 mg of protein with $50 \mu\text{moles}$ of acetate buffer (pH 5.8) and $0.0135 \mu\text{mole}$ of $[8\text{-}^{14}\text{C}]$ adenosine for 5 min at 37° . The final volume of the incubation mixture was 0.125 ml. The reaction was stopped by rapid freezing.

Adenosine kinase. The standard assay was conducted by incubating $50 \mu\text{moles}$ of acetate buffer (pH 5.7), $0.25 \mu\text{mole}$ of ATP, $1.25 \mu\text{moles}$ of MgCl_2 , $0.001 \mu\text{mole}$ of $[8\text{-}^{14}\text{C}]$ adenosine ($5 \cdot 10^{-6} \text{ M}$) with lysate (0.5 mg protein) for 15 min at 37° . The final volume of the incubation mixture was 0.2 ml. The reaction was stopped by rapid freezing. Protein was measured by the method of LOWRY *et al.*¹⁹.

Separation of adenosine metabolites

Neutralized ethylenediaminetetraacetic acid (EDTA), $5 \mu\text{moles}$, was added to the reaction mixture to prevent further adenosine metabolism during electrophoresis. $25 \mu\text{l}$ of this mixture was spotted onto Whatman No. 3 paper, which had been spotted previously with 10 ng of each purine. The purines were separated by electrophoresis (3000 V, 90 min) in a Savant Electrophorator, using a borate buffer system, 0.05 M, pH 9.0. The electrophoretogram was dried, and the purine spots were visualized with ultra-violet light, cut out, and counted in a toluene-based scintillation fluid with an efficiency of 55 %. In lysate experiments in which ATP was not present, the only labeled purines present after incubation were adenosine, inosine, adenine, and hypoxanthine.

In lysate experiments in which ATP was present, labeled ATP, ADP, AMP, and IMP were also present after incubation. The reaction mixture was treated as just described except the purines were separated by ascending chromatography in a butanol-acetic acid-water system (2 : 1 : 1, by vol.) for 24 h. Adenosine 5'-phosphate and IMP ran together as one spot as did hypoxanthine and adenosine. The purines in these two spots were eluted separately with 250 μ l of 0.1 M HCl and spotted quantitatively on Whatman No. 3 paper for further separation. The mononucleotides were separated by electrophoresis (1600 V, 2 h) in a Gilson electrophorator, using a formate buffer system (pH 3.5). Hypoxanthine and adenosine were separated by electrophoresis in the borate buffer system just described.

In whole cell experiments small amounts of xanthylic acid (XMP) and guanylic acid were also formed. These nucleotides ran together with IMP and AMP in the borate chromatography system. They were separated from AMP in the formate buffer system. Counts found in XMP and guanylic acid are included in the IMP counts in the data presented.

Chemicals and radiochemicals

The nonradioactive nucleotides, nucleosides, and purines were purchased from Sigma Corporation. [8- 14 C]Adenosine, dissolved in 50 % ethanol was purchased from CalAtomic.

RESULTS

Metabolism of adenosine in intact erythrocytes

Effect of time

[8- 14 C]Adenosine incubated with the isotonic phosphate buffer and erythrocyte suspension had a half-life of 30 min, but after 2 h of incubation 15 % of the labeled adenosine remained unmetabolized (Fig. 1). The low level of labeled inosine recovered indicates that after its deamination adenosine is rapidly converted to hypoxanthine and IMP. It is of interest that even after 15 min labeled IMP represented a greater proportion of the recovered labeled purines than did hypoxanthine. This observation suggests that inosine may be converted directly to the nucleotide level. Labeled adenine nucleotides were slowly formed and at the end of 2 h represented 20 % of the labeled purines. Less than 0.50 % of the total radioactivity was recovered in the adenine spot.

Effect of adenosine concentration

The concentration of [8- 14 C]adenosine in the erythrocyte suspension had a marked effect on the distribution of 14 C recovered after incubation (Table I). The percentage of 14 C recovered in adenosine and hypoxanthine rose from 4 and 6 % to 24 and 53 %, respectively, as the concentration of [8- 14 C]adenosine was increased from 0.02 to 2.0 mM. However, the percentage of 14 C recovered in IMP fell from 68 to 0.7 %. The marked rise in the percent of 14 C recovered in inosine as the adenosine concentration was increased beyond 1 mM in addition to the similar percentage of 14 C found in inosine+hypoxanthine at adenosine concentrations of 1 and 2 mM suggests that hypoxanthine may be converted preferentially to inosine at the higher concentrations of adenosine. Although the total amount of adenosine being converted

Fig. 1. Distribution of ^{14}C in erythrocytes with time (% of total counts/min in assay): ●—●, adenosine; △—△, inosine; ○—○, hypoxanthine; ◇—◇, IMP; and ▲—▲, adenine nucleotides. $[8\text{-}^{14}\text{C}]\text{Adenosine}$ (0.208 mM) was incubated with erythrocytes as described in MATERIALS AND METHODS.

TABLE I

EFFECT OF ADENOSINE CONCENTRATION ON ITS METABOLISM IN ERYTHROCYTES

The whole cell assay was used (see MATERIALS AND METHODS). Adenosine was adjusted to the concentration stated in the Table. Figures in parentheses represent the amount of compound formed in nmoles.

Adenosine (mM)	% of radioactivity					
	Adenine	Adenosine	Hypoxanthine	Inosine	IMP	Adenine nucleotides
0.01	< 1	3 (0.15)	5 (0.25)	< 1	66 (3.30)	25 (1.25)
0.02	< 1	5 (0.37)	17 (1.27)	< 1	61 (4.55)	16 (1.20)
0.12	< 1	22 (5.70)	17 (4.40)	< 1	45 (11.7)	15 (3.90)
1.02	< 1	22 (55.0)	65 (162)	3 (7.50)	6 (15.0)	3.8 (9.50)
2.02	1.6 (7.62)	23 (109)	51 (242)	20 (95.5)	0.7 (3.30)	4.2 (20.0)

to adenine nucleotides increased 16-fold (1.25 to 20.0 nmoles) with a 100-fold increase in adenosine concentration (0.02 to 2.02 mM), the percentage of labeled $[8\text{-}^{14}\text{C}]\text{adenosine}$ entering into the adenine nucleotides decreased from 26 to 4 %. These various changes in ^{14}C label recovered at different concentrations of adenosine suggest that at low adenosine concentrations the nucleoside is converted directly to the nucleotide level or metabolized rapidly to IMP, whereas at higher concentrations adenosine remains partly unmetabolized and converted largely to its deaminated nucleoside and deaminated base. These concentration-dependent effects explain the apparently conflicting reports of others²⁰⁻²³.

Metabolism in erythrocyte lysates

Adenosine deaminase reaction

Effect of adenosine concentration: $[8-^{14}\text{C}]$ adenosine at a concentration of 0.104 mM was rapidly (half-life 3 min) deaminated to inosine by the erythrocyte lysate (Fig. 2) although even after 20 min a small (15 %) portion of the adenosine seemed unavailable for metabolism. The activity of adenosine deaminase was calculated to be approximately 250 nmoles per mg protein per hour in crude erythrocyte lysate preparations. The velocity of the adenosine deaminase reaction was markedly dependent on the adenosine concentration with an optimum of 0.1 to 0.16 mM (Fig. 3). A K_m for adenosine of $4 \cdot 10^{-5} \text{ M}$ was calculated from the half-maximal rate, a value which agreed well with an independent calculation of the K_m using a Lineweaver-Burk plot. 50 % inhibition occurred at a concentration of adenosine 10 times its K_m (Fig. 3).

Fig. 2. Deamination of adenosine (Ado) to inosine in erythrocyte lysates with time. The deamination of adenosine (0.104 mM) was determined as described in MATERIALS AND METHODS. 50 % ($6.9 \mu\text{moles}$) of adenosine was deaminated in 3 min. Each point represents mean $\pm$ S.D.

Fig. 3. Effect of adenosine (Ado) concentration on adenosine kinase and adenosine deaminase activity in erythrocyte lysates. The kinase (Δ - Δ) and deaminase ($\bullet$ - $\bullet$) activities were determined by the standard assays as described in MATERIALS AND METHODS. Maximal activity for the kinase and deaminase were, respectively, 231 pmoles of adenosine phosphorylated per 15 min and $6.9 \mu\text{moles}$ of adenosine deaminated per 5 min. Units of adenosine = $100 \mu\text{M}$ for adenosine deaminase, $10 \mu\text{M}$ for adenosine kinase.

Inhibitors. Various compounds were tested as inhibitors of the adenosine deaminase reaction. No inhibition was noted with equimolar or greater concentrations of 2,3-diphosphoglycerate, AMP, ADP, ATP, GDP, GTP, P_i , and guanylic acid. At a concentration of 10 mM P_i , 98 % of the ^{14}C was found in hypoxanthine. At high concentrations of inosine (0.80 mM) and adenosine (0.50 mM) 20 % inhibition of the adenosine deaminase reaction occurred. The deamination of adenosine seemed to be independent of pH from 5.5 to 8.0 (Fig. 4).

Adenosine kinase reaction

Effect of adenosine concentration. The phosphorylation of adenosine to adenine nucleotides was studied at an adenosine concentration of $5 \cdot 10^{-6} \text{ M}$. At this concen-

Fig. 4. Effect of pH on adenosine kinase and adenosine deaminase in erythrocyte lysates. Conditions for assay are described in MATERIALS AND METHODS, and symbols are the same as those for Fig. 3. Maximal activity (100 %) represents 0.0103 μ moles adenosine deaminated per 5 min for the adenosine deaminase reaction and 0.0002 μ mole adenosine phosphorylated per 15 min for the adenosine kinase reaction. The following buffers were used: 40 μ M sodium acetate (pH 5.5 to 6.5), 20 μ M potassium phosphate (pH 6.5 to 7.5), and 20 μ M glycine (pH 7.5 to 8.0).

Fig. 5. Effect of adenosine (Ado) concentration on the percentage (Δ - Δ) and amount ($\bullet$ - $\bullet$) of adenosine phosphorylated to the nucleotide level by erythrocyte lysates. Conditions same as Fig. 3.

tration 15 % of the $[8-^{14}\text{C}]$ adenosine was incorporated into the adenine nucleotides. At higher concentrations of adenosine a progressively greater percentage of adenosine was deaminated (Fig. 5). The velocity of the adenosine kinase reaction was not inhibited by concentrations of adenosine that were at least ten times its K_m (Fig. 3.) Half-maximal velocity was noted at an adenosine concentration of $1.9 \cdot 10^{-6}$ M (Fig. 3.)

Effect of adenosine 5'-triphosphate and Mg^{2+} concentrations. The adenosine kinase reaction was markedly dependent on both ATP and Mg^{2+} concentrations. At a Mg^{2+} concentration of 0.30 mM the K_m for ATP was $4 \cdot 10^{-4}$ M (Fig. 6). Concentrations of ATP greater than 1.25 mM were inhibitory. At a fixed concentration of ATP

Fig. 6. Effect of ATP concentration on the activity of adenosine kinase. The standard assay as described in MATERIALS AND METHODS was used with a Mg^{2+} concentration of 0.30 μ M. Maximal activity (100 %) of adenosine kinase ranged from 190 to 270 pmoles of adenosine phosphorylated per 15 min.

Fig. 7. Effect of Mg^{2+} concentration on the activity of adenosine kinase. Standard assay was used with an ATP concentration of 1 mM. The range of maximal activity was the same as in Fig. 6.

(1.0 mM) the adenosine kinase reaction exhibited maximal activity at Mg^{2+} concentrations from 0.25 to 0.50 mM with 50 % inhibition occurring at 1.0 mM (Fig. 7).

Phosphate donor. The triphosphate acting as the phosphate donor for the adenosine kinase reaction was found to be relatively nonspecific. The following relative rates were observed with a range of triphosphate donors: ATP, 100; GTP, 100; CTP, 65; UTP, 45; and TTP, 21.

Inhibitors. Inhibition of the adenosine kinase reaction by various compounds was tested under standard conditions. Adenosine 5'-phosphate, ADP, GDP, IMP, adenine and guanylic acid were competitive inhibitors of ATP in the adenosine kinase reaction, and the values of K_i found were, respectively, 0.4, 0.4, 1.4, 2.0, 8.0, and 2.0 mM. No inhibition was found with concentrations of 2,3-diphosphoglycerate twenty times the ATP concentration.

The phosphorylation of adenosine was maximal in acetate buffer at pH 5.5 to 6.2, decreasing rapidly at higher pH levels (Fig. 4).

Effect of protein concentration. Adenosine deaminase activity in the presence of ATP and Mg^{2+} was related to the amount of protein and the concentration of adenosine in the assay system (Fig. 8): (1) As the amount of protein was increased, the apparent activity of adenosine deaminase decreased at all concentrations of adenosine tested, and (2) The relative activity of adenosine kinase decreased markedly as the adenosine concentration was increased.

Fig. 8. Effect of protein concentration on the relative adenosine (Ado) kinase and adenosine (Ado) deaminase activities in crude erythrocyte lysates at different adenosine concentrations. Adenosine kinase (---) and adenosine deaminase (—) activities were assayed simultaneously using the standard assay as described in MATERIALS AND METHODS for adenosine kinase at adenosine concentrations of $2.5 \cdot 10^{-6}$ M (Δ - Δ , $\blacktriangle$ - $\blacktriangle$); $2.5 \cdot 10^{-5}$ M ($\circ$ - $\circ$, $\bullet$ - $\bullet$); and $5 \cdot 10^{-5}$ M ($\square$ - $\square$, $\blacksquare$ - $\blacksquare$). Adenosine deaminase was also assayed in the absence of ATP and Mg^{2+} ($\rightarrow$ - $\rightarrow$). The activity of adenosine deaminase is expressed as a percentage of the maximal rate of deamination. The maximal rate of deamination and phosphorylation were, respectively, 905 pmoles of adenosine deaminated and 250 pmoles of adenosine phosphorylated per 15 min. The activity of the adenosine kinase is expressed as a percentage of the maximal percentage of adenosine phosphorylated.

Adenosine phosphorylase reaction

At no time was ^{14}C detected in adenine. Incubation of the reaction mixtures with high concentrations of P_i (which is known to stimulate purine nucleoside phosphorylase activity²⁴⁻²⁶) resulted in conversion of 98 % of the original $[8\text{-}^{14}\text{C}]\text{adenosine}$ to $[^{14}\text{C}]\text{hypoxanthine}$.

DISCUSSION

The metabolism of adenosine in human erythrocytes is directed along two major pathways. The larger proportion is deaminated to inosine while a smaller amount is phosphorylated to its nucleotide form. Similar findings in human platelets have been reported although the pattern of labeled purine recovered after incubation was markedly different^{6,15}. Several investigators have demonstrated indirectly that in erythrocytes the adenine nucleotides recovered after incubation with isotopic adenosine are the result of direct phosphorylation^{18,20-22}. The indirect demonstration in erythrocytes of the absence of adenosine phosphorylase^{18,20} and adenylic synthetase²³ suggests that the labeled adenine nucleotides are not formed via either adenine or IMP. Although a hypoxanthine $\rightarrow$ adenine $\rightarrow$ AMP pathway has not been excluded the fact that $[^{14}\text{C}]\text{hypoxanthine}$ incubated with erythrocytes did not appear in the adenine nucleotides seems to exclude this pathway²³. The failure of purified erythrocyte purine nucleoside phosphorylase to demonstrate affinity for adenosine or adenine lends support to this supposition²⁴⁻²⁶. Our finding that $[^{14}\text{C}]\text{adenine}$ represents less than 0.5 % of the recovered label after incubation with 0.12 mM $[8\text{-}^{14}\text{C}]\text{adenosine}$ supports the concept that adenosine phosphorylase and an enzyme converting hypoxanthine to adenine are absent in erythrocytes. Since adenyate synthetase is absent in human erythrocytes²³ the relative importance of the adenosine kinase pathway for adenine nucleotide production assumes added significance.

The metabolism of $[8\text{-}^{14}\text{C}]\text{adenosine}$ in erythrocytes (Table I) suggests that the concentration of adenosine is an important controlling factor in its metabolism. Because of the possibility of enzyme inactivation with long periods of incubation and the effects of other enzymes earlier in the metabolic sequences, interpretation of the data obtained in the whole erythrocyte studies is difficult. The role of adenosine in its own metabolism and in overall purine metabolism is elucidated more clearly by the study of adenosine deaminase and adenosine kinase activities in crude erythrocyte lysates.

The rapid deamination of adenosine observed in erythrocyte lysates (Fig. 2) is similar to that observed by others in crude erythrocyte¹⁷ and platelet lysates¹⁶. The failure of a wide range of compounds (2,3-diphosphoglycerate, AMP, ADP, ATP, GDP, GTP, and P_i) to inhibit the adenosine deaminase reaction in erythrocyte lysates seems to be a property of adenosine deaminase shared by other systems^{7-11,16,17}. The failure of 2,3-diphosphoglycerate to inhibit adenosine deaminase is especially intriguing since 2,3-diphosphoglycerate has been shown to have a marked inhibitory effect on erythrocyte adenyate deaminase²⁷.

The low K_m of adenosine ($1.9 \cdot 10^{-6} \text{ M}$), and the failure of high concentrations of adenosine to inhibit the adenosine kinase reaction (Fig. 3), has been observed in other tissues^{2,4,6}, but its potential relationship to the higher K_m of adenosine ($4 \cdot 10^{-5} \text{ M}$), and the substrate inhibition of adenosine at higher concentrations ($2 \cdot 10^{-4} \text{ M}$) has not been pointed out previously.

The importance of adenosine concentration in adenosine metabolism is further suggested by the following observations: (1) the percentage of adenosine phosphorylated increased as the adenosine concentration approached the K_m for adenosine kinase (Figs. 5 and 8), and (2) the activity of adenosine deaminase in the presence of ATP and Mg^{2+} decreased as protein concentration increased and adenosine kinase activity remained constant, especially notable as the concentration of adenosine approached the K_m of adenosine kinase (Fig. 8). The twenty-fold difference in K_m suggests a unique method for the competitive handling of a common substrate by a degradative and synthetic enzyme. The difference in K_m for adenosine would assure that the intracellular concentration of adenine nucleotides would not fall secondary to rapid coupled phosphatase and deaminase action. The relative K_m values of the two enzyme activities for adenosine would allow the levels of adenine nucleotides to be modulated effectively by the adenosine concentration itself.

The K_m of ATP for the erythrocyte adenosine kinase reaction is $4 \cdot 10^{-4}$ M. The concentration of ATP in erythrocytes has been shown to be approximately 1.3 mM^{28,29}. This high level of ATP would be conducive to maximum adenosine kinase expression were it not for (1) multiple feedback inhibition observed (AMP, ADP, GDP, IMP, guanylic acid) and (2) the self-regulatory role of ATP. In other tissues many other nucleotides have been observed to exert feedback inhibition on adenosine kinase¹⁻⁶. Adenosine 5'-triphosphate becomes inhibitory for the erythrocyte adenosine kinase reaction at a concentration of approximately 1.25 mM (Fig. 6), a value in general agreement with that noted in other studies¹⁻⁶. Strict comparisons between tissues of the effect of ATP were not possible as the effect of ATP concentration seems in turn to be dependent on Mg^{2+} concentration (Fig. 7)¹⁻⁶.

The complex and rigid control of adenosine kinase activity suggests that this enzyme is important in the regulation of intracellular adenine nucleotide levels (Fig. 9). Any loss of adenosine kinase control would open a readily available pathway (phosphatase and deaminase) by which ATP levels could be severely compromised.

Fig. 9. Pathways of adenosine metabolism. →, pathways known to be present in erythrocytes ---→, pathways known to be present in bacterial systems but absent in erythrocytes and presumed (indirect demonstration) to be present in mammalian systems. PRPP, 5'-phosphoribosyl-pyrophosphate; R-1-P, ribose-1-phosphate. Numbers identify the following enzymes; 1, adenylylase; 2, adenylyl phosphatase; 3, adenosine kinase; 4, adenine phosphoribosyl transferase; 5, adenosine deaminase; 6, inosine phosphoribosyl transferase; 7, inosinate phosphatase; 8, hypoxanthine-guanine phosphoribosyl transferase.

In tissues that have an active synthetic pathway for AMP directly from IMP, feedback control of *de novo* purine synthesis would tend to maintain constant AMP levels^{30,31}. The relative lack of importance of adenine phosphoribosyl transferase in controlling intracellular AMP levels is indicated by the normal uric acid production in patients who lack this enzyme³². This important observation increases the likelihood that the fine regulation of adenine nucleotide levels is in large part controlled by adenosine kinase, especially in erythrocytes where a *de novo* pathway for adenine nucleotides is absent.

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