Biochemical Pharmacology, Vol. 42, No. 9, pp. 1767–1773, 1991.
Printed in Great Britain.

0006-2952/91 $3.00 + 0.00

EFFECTS OF ADENOSINE ANALOGUES ON ATP CONCENTRATIONS IN HUMAN ERYTHROCYTES

FURTHER EVIDENCE FOR A ROUTE INDEPENDENT OF ADENOSINE KINASE

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(*Received* 20 *May* 1991; *accepted* 14 *June* 1991)

Abstract—Adenosine derivatives are frequently used in chemotherapy because of their potent antitumor, antiviral and antiparasitic activity. We investigated the metabolism of some adenosine analogues in adenosine deaminase inhibited normal and adenine phosphoribosyltransferase (APRT) deficient human erythrocytes. The ATP and GTP concentrations and the formation of unusual nucleotides were measured. Some of the analogues studied (tubercidin, 9β-D-arabinofuranosyladenine, 2′-deoxyadenosine, 2-chloroadenosine, neplanocin A) were phosphorylated to the corresponding nucleoside triphosphates and this process was abolished by iodotubercidin—an adenosine kinase inhibitor. With the exception of 2′-deoxyadenosine, nucleotide analogue formation was accompanied by ATP depletion. ATP decrease was not observed after adenosine kinase inhibition and ATP concentration even increased in the presence of 2′-deoxyadenosine, neplanocin A and 5′-iodo-5′-deoxyadenosine. However, the latter increment was not observed in APRT deficient erythrocytes. Bredinin, *S*-adenosylhomocysteine, deoxycoformycin and adenosine dialdehyde did not form nucleotide derivatives or exert any effects on ATP concentration. It is concluded that adenosine analogues can either enter the nucleotide pool via phosphorylation mechanisms, or may be converted to ATP by the pathways involving the intermediate formation of adenine.

Adenosine salvage via adenosine kinase (AK§, EC 2.7.1.20) is believed to be the sole means by which this nucleoside and its analogues can enter the adenine nucleotide pool in the human erythrocyte [1]. ATP could be synthesized in this cell also from adenine by adenine phosphoribosyltransferase (APRT, EC 2.4.2.7), but not via *de novo* synthesis or hypoxanthine salvage because mature erythrocytes lack one of the enzymes converting IMP to AMP [2]. In contrast to other purine nucleosides, adenosine and its analogues cannot be subjected to direct phosphorolysis by purine nucleoside phosphorylase (PNP, EC 2.4.2.1) to produce the corresponding base [3]. However, studies on purified *S*-adenosylhomocysteine hydrolase (SAHH, EC 3.3.1.1) revealed formation of the unstable 2-ketoadenosine intermediate during the normal reaction catalysed by this enzyme [4]. This intermediate could decompose with the release of adenine. We have suggested previously that a hitherto unidentified pathway of adenine nucleotide synthesis may operate in the erythrocytes which would include adenine release following nucleoside binding to SAHH, with subsequent incorporation of adenine into AMP via APRT [2, 5]. Alternatively, adenine could be released from some adenosine analogues by methylthioadenosine phosphorylase (MTAP, EC 2.4.2.28), an enzyme which is also present in erythrocytes [6].

A number of synthetic adenosine analogues express potent antineoplastic, antiviral and antiparasitic activity [7–9]. Understanding of the pathways involved in the metabolism of these compounds is essential for evaluation of their mechanism of action, therapeutic efficacy and possible side effects, as well as for development of more efficient analogues. Depending on the specificity of the enzymes involved in nucleoside metabolism, these compounds could be deaminated, subjected to phosphorolysis or phosphorylated [10, 11]. Some adenosine analogues may also influence the physiological concentration of normal nucleotides including ATP, leading to either an increase or decrease of ATP concentration [12, 13]. Several adenosine analogues could be either substrates, or inhibitors of SAHH [3, 14–19]. Some have been shown to release adenine during interaction with this enzyme. However quantitative changes of ATP concentration in intact cells resulting from this interaction are not known.

The present research was undertaken to study metabolic pathways in the erythrocyte involved in nucleotide formation using several adenosine

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§ Abbreviations: dCf, 2′-deoxycoformycin; ITu, 5′-iodotubercidin; APRT, adenine phosphoribosyltransferase; ADA, adenosine deaminase; AK, adenosine kinase; SAHH, *S*-adenosylhomocysteine hydrolase; MTAP; methylthioadenosine phosphorylase; PPRP, phosphoribosylpyrophosphate.

analogues. These studies included evaluation of their effect on the ATP level. We found that in addition to the accepted adenosine kinase mediated process, some nucleotide derivatives could be formed via alternative pathways. Nucleotide analogue synthesis via adenosine kinase was associated with ATP depletion, in some but not all cases. Adenine mediated adenylate formation from some adenine nucleosides was also proven to be operative.

MATERIALS AND METHODS

Chemicals. Adenosine analogues were purchased from the Sigma Chemical Co. (Poole, U.K.) with the exception of neplanocin A and bredinin which were generous gifts from Dr T. Saito (Toyo Jozo Co. Ltd, Japan). 5′-Iodotubercidin (Itu) was purchased from Research Biochemicals Ltd (St Albans, U.K.). All other reagents were of analytical grade from BDH-Merck (Poole, U.K.).

Erythrocytes. Fresh blood from healthy laboratory personnel and from an APRT deficient patient, collected into lithium heparin, was used for the experiments which were performed immediately following venipuncture. As described previously [5] the blood was centrifuged, the buffy coat and top fifth layer of erythrocytes were discarded and the cells were washed twice with isotonic sodium chloride and once with incubation buffer (Earl's balanced salt solution containing 5.6 mM glucose and 18 mM orthophosphate).

Incubation. All adenosine analogues were used at the final concentration of 1.25 mM. 2′-Deoxycoformycin (dCf), an adenosine deaminase inhibitor, was present in all incubations at 10 μM concentration. 5′-Iodotubercidin (Itu), an adenosine kinase inhibitor, was used where indicated at 20 μM concentration. Erythrocytes were diluted with incubation buffer to achieve a final hematocrit of 40% before commencement of the incubation, dCf was added simultaneously, and the erythrocytes were preincubated for 30 min. Incubations were started by the addition of 100 μL of 40% erythrocyte suspension to 150 μL of the incubation buffer containing adenosine analogues and inhibitors. Incubations were carried out for 3 hr at 37° in a shaking water bath and were terminated by the addition of 50 μL of 40% trichloracetic acid, with the exception of 2′-deoxyadenosine which is known to decompose in acidic conditions. In this case the incubation was terminated by the addition of 200 μL of acetonitrile. After removal of protein precipitate by centrifugation, trichloracetic acid or acetonitrile were extracted by water saturated diethyl ether until a pH > 5 was achieved.

Analytical methods. Erythrocyte nucleotides were separated and quantified by high performance liquid chromatography (HPLC), using a fully-automated system (Millipore Waters) as described [20]. Erythrocyte extract (25 μL) was injected onto an anion exchange column (5 μ APS-Hypersil 250 × 4.9 mm) and the nucleotides separated using a linear gradient (Buffer A; 5 mM KH_2PO_4 pH 2.65; Buffer B: 0.5 M KH_2PO_4/1.0 M KCl pH 3.5) at a flow rate of 1 mL/min with dual wavelength analysis at 254 and 280 nm. Since nucleotide derivatives of the analogues studied were not commercially available (with the exception of 2′-deoxy-ATP) the identification of these compounds in erythrocyte extracts was based on the chromatographic behavior on the anion exchange system used, which is typical for mono-, di-, and triphosphates. The similarity of the purine ring in these phosphate metabolites with that of their nucleoside analogue precursor was confirmed by the identical UV spectra obtained using a Millipore Waters 990 photodiode array detector. Additional confirmation of the nature of these metabolites was achieved after treatment of the peaks corresponding to triphosphates on the anion exchange systems with alkaline phosphatase followed by analysis on a reversed phase nucleoside and base system [21]. In all cases corresponding nucleosides were recovered. In addition, where increase of ATP concentration was observed purity of the ATP peak was confirmed by both the above methods. Another proof of identity is based on the experiments with the adenosine kinase inhibitor, where complete or almost complete inhibition of the formation of corresponding nucleotide analogues was observed. In all cases separation of the peaks of triphosphate analogue derivatives from ATP or GTP was sufficient for quantitative analysis. The concentrations of triphosphate analogues were calculated initially as ATP and corrected according to the ratio of nucleoside analogue response factor to that of adenosine. The purity of the adenosine analogues was checked and analyses of the incubation medium were performed using a reversed phase HPLC system for nucleosides and bases described previously [21].

RESULTS

The influence of nucleoside analogues on ATP concentration of ADA-inhibited erythrocytes from healthy subjects or from an APRT deficient patient is summarized in Fig. 1. In normal erythrocytes, in the absence of the AK inhibitor (Itu), some analogues caused a decrease of ATP, varying from slight (9β-D-arabinofuranosyladenine, 2-chloroadenosine, neplanocin A) to profound (tubercidin). An increase in ATP was observed with 5′-iodo-5′-deoxyadenosine. However, *S*-adenosylhomocysteine, adenosine dialdehyde, 2′-deoxycoformycin, 2′-deoxyadenosine and brednin did not cause any significant change in ATP. In the studies incorporating Itu an increase in ATP was still observed in the presence of 5′-iodo-5′-deoxyadenosine, 2′-deoxyadenosine and also neplanocin A, while no change of ATP was observed in the presence of any other nucleoside. NAD and NADP concentrations were constant and only minimal changes in GTP concentration were observed (not shown). In APRT-deficient erythrocytes a decrease in ATP was observed in the presence of tubercidin without Itu, but in contrast to normal erythrocytes no evident change in ATP was observed under any other condition.

Table 1 lists the concentrations of nucleotide analogues formed from some of the nucleosides. In normal erythrocytes, without Itu, nucleoside triphosphate concentration, comparable with or

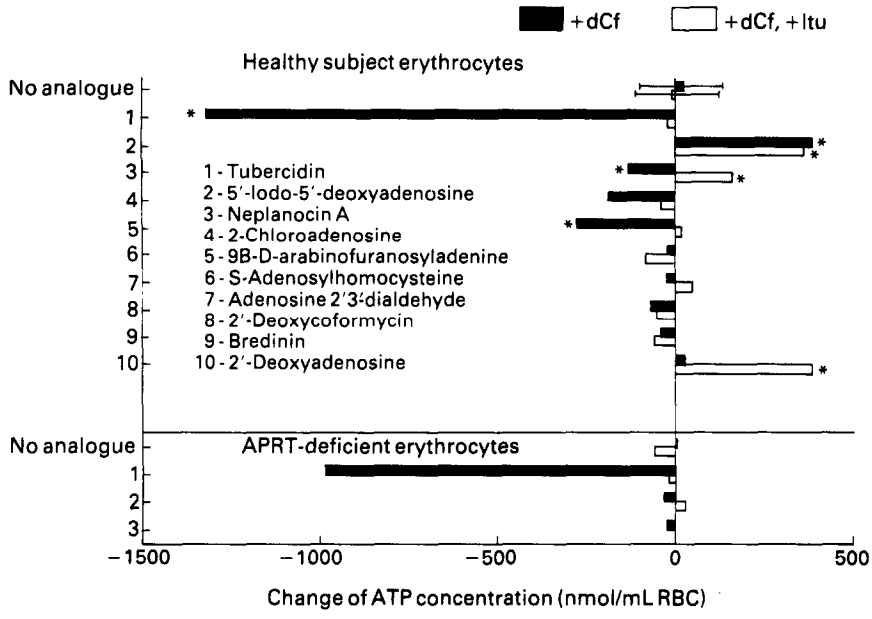

Fig. 1. Change of ATP concentration in human erythrocytes after incubation in the presence of adenosine analogues. Incubations were carried out at 16% hematocrit in Earl's balanced salt solution containing 18 mM phosphate and 5.6 mM glucose in the presence of 10 μM 2'-deoxycoformycin (dCf). The temperature was 37° and the incubation time was 3 hr. Adenosine analogues were present at 1.25 mM concentration. 5'-Iodotubercidin (Itu) was present where indicated at 20 μM concentration. Initial ATP concentration was within the range 1300–1800 nmol/mL RBC. In healthy subjects, values represent the mean of 2–3 incubations with analogues or the mean of 11 control incubations (with the range). Results from APRT-deficient erythrocytes are from a single experiment. The asterisks indicate $P < 0.05$ as compared with control using Mann–Whitney U-test.

exceeding the ATP concentration was observed in the presence of 9β-D-arabinofuranosyladenine, neplanocin A, tubercidin and 2'-deoxyadenosine. A small accumulation of the triphosphate derivative of 2-chloroadenosine was also observed. The addition of Itu caused a marked inhibition of nucleotide derivative formation, varying from 85 to 98% (2-chloroadenosine, neplanocin A, 2'-deoxyadenosine) to 100% (9β-D-arabinofuranosyladenine). In APRT-deficient erythrocytes formation of triphosphate derivatives of tubercidin and neoplanocin A proceeded at a comparable rate to the erythrocytes of healthy subjects.

Figures 2 and 3 illustrate the relationship between the concentration of the natural nucleotides and the unphysiological nucleotides of tubercidin and

Table 1. Formation of nucleotide triphosphate analogues in the presence of nucleoside analogues

Nucleoside analogue	Analogue nucleotide triphosphate	
	+dCf	+dCf, +Itu
	(nmol/mL RBC)	
Healthy subjects		
9β-D-Arabinofuranosyladenine	731	<5
2-Chloroadenosine	240	54
Neplanocin A	1817	99
Tubercidin	3622	46
2'-Deoxyadenosine	2201	36
APRT-deficient erythrocytes		
Neplanocin A	1346	168
Tubercidin	3808	125

Values represent the mean of 2–3 experiments (healthy subjects) or the results of a single experiment with APRT-deficient erythrocytes. Incubation conditions are described in Fig. 1.

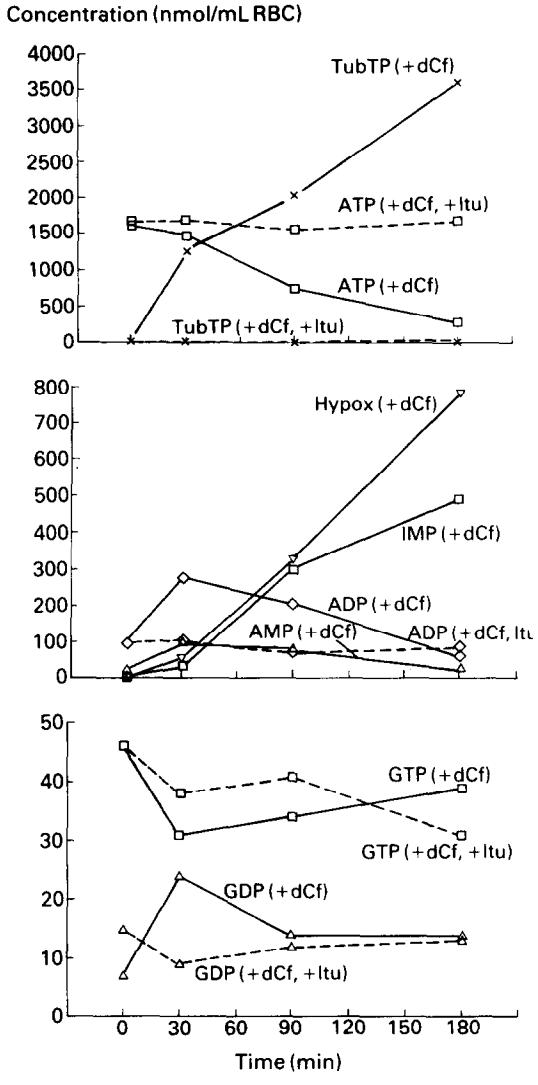

Fig. 2. Concentrations of nucleotides and hypoxanthine during incubation of erythrocytes as described in Table 1 with 1.25 mM tubercidin in the presence of dCf with (broken line) or without 20 μM iodotubercidin. Initial concentration of AMP, IMP or hypoxanthine did not change in the presence of dCf and Itu.

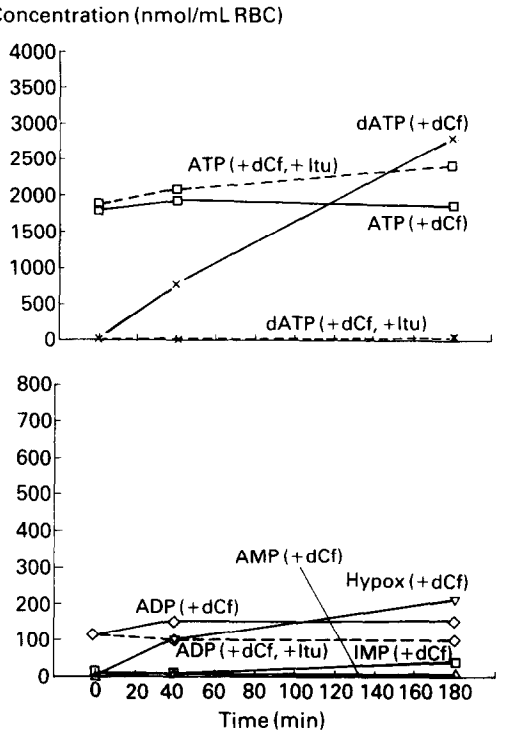

Fig. 3. Concentration of nucleotides and hypoxanthine during incubation of erythrocytes as described in Table 1 with 1.25 mM 2′-deoxyadenosine in the presence of dCf with (broken line) or without Itu. Initial concentration of AMP, IMP or hypoxanthine did not change in the presence of dCf and Itu

2′-deoxyadenosine in incubated erythrocytes. In the absence of Itu, formation of tubercidin triphosphate was accompanied by the profound depletion of ATP, transient elevation of AMP and ADP concentrations and sustained increase of IMP and hypoxanthine. Formation of mono- and diphosphates of tubercidin was also demonstrated (not shown). Interestingly, only minor changes of GTP concentration were observed, even when profound depletion of ATP occurred. The observed changes were abolished almost completely in the presence of Itu. Formation of 2′-deoxy-ATP proceeded at a rate comparable to tubercidin triphosphate formation, but in contrast, no decrease of ATP was observed and accumulation of IMP or hypoxanthine was much less. In the presence of Itu only minimal formation of 2′-deoxy-ATP was observed and a substantial increase of ATP concentration was demonstrated.

DISCUSSION

The major finding of this study was the demonstration of the possible involvement of a novel pathway for adenine nucleotide synthesis using a variety of adenosine analogues. This pathway necessitates purine base release with its subsequent incorporation into nucleotides (Fig. 4). Consequently, either the intact nucleoside or nucleoside-derived base may be incorporated into the nucleotide pool. Involvement of different pathways in the metabolism of particular analogues may differ greatly according to the substrate specificity of the enzymes involved in these pathways.

Nucleotide derivatives of 9β-D-arabinofuranosyladenine were formed efficiently via AK but not via any other pathway as demonstrated by complete inhibition of the corresponding nucleoside triphosphate formation by Itu and the lack of any increase in ATP. 9β-D-Arabinofuranosyladenine can release adenine during interaction with purified SAHH [3], but this probably did not occur in intact erythrocytes. Formation of nucleotide derivatives from this nucleoside has been demonstrated also by

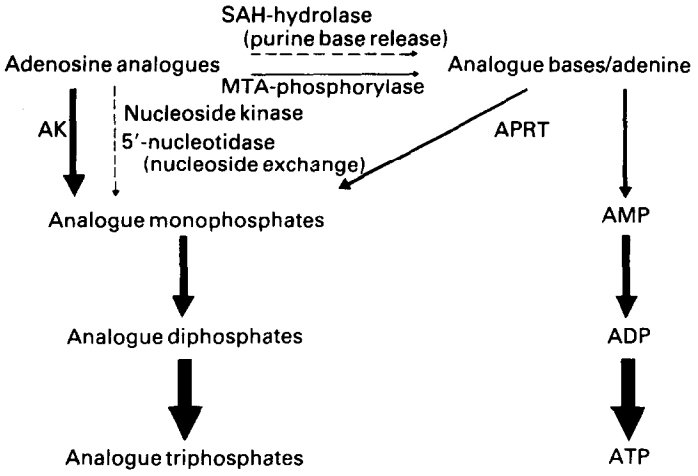

Fig. 4. Pathways of nucleotide synthesis from adenosine analogues. Nucleoside phosphorylation by adenosine kinase (AK) is the primary route in erythrocytes. Some nucleosides can enter nucleotide pool by AK independent processes, either via other nucleoside kinases, or as the result of nucleoside exchange mediated by a soluble 5′-nucleotidase. Adenosine analogues can also release adenine (or analogues) via interaction with *S*-adenosylhomocysteine (SAH) hydrolase or methylthioadenosine (MTA) phosphorylase which is subsequently incorporated into AMP by adenine phosphoribosyltransferase (APRT).

some [23] but not all investigators [22], most likely because of rapid deamination of this compound by ADA, which was prevented in our studies by the presence of dCf [23]. In contrast, 2′-deoxyadenosine appeared to be phosphorylated via both adenosine kinase dependent and independent processes as evidenced by the incomplete inhibition of 2′-deoxy-ATP formation by Itu. An involvement of deoxycytidine kinase could be considered but there does not appear to be any reports of its existence in the mature erythrocytes.

A possible objection to the interpretation of our results may concern the efficiency of inhibition of AK, but this enzyme seemed to be fully inhibited by the very high concentration of Itu used. This was evidenced by complete inhibition of 9β-D-arabinofuransoyladenine triphosphate formation. It is possible that a mechanism involving nucleoside exchange demonstrated using purified cytosolic 5′-nucleotidase [24], or activity of other nucleoside kinases (deoxycytidine kinase), were involved in this process.

An important finding was an increment in ATP concentration observed with 2′-deoxyadenosine plus Itu. We have demonstrated previously that this process is mediated by adenine formation [2] and it was hypothesized that this occurs via interaction with SAHH (a release of adenine was demonstrated using purified enzyme [4]). The possibility of any involvement of MTAP in this process can be excluded because 2′-deoxyadenosine is not a substrate for this enzyme, even at very high concentration (results not shown).

Although neplanocin A tends to be metabolized by the same pathways as 2′-deoxyadenosine, the origin of the adenine is less certain. This compound can release adenine via interaction with purified SAHH [19], but to our knowledge there is no data on possible phosphorolysis by MTAP. In support of the involvement of adenine in the ATP increment observed, no change in ATP was noted in APRT deficient erythrocytes (Fig. 1). Formation of phosphate derivatives of neplanocin A have been demonstrated previously [25].

2-Chloroadenosine was slowly phosphorylated by AK, confirming that this compound is a poor substrate for this enzyme. Interestingly, relative attenuation of its triphosphate derivative formation by Itu was less than with the other analogues. The possibility thus exists that 2-chloroadenine was released by interaction with SAHH [2] with its subsequent incorporation via APRT. A nucleoside exchange or phosphorylation by other nucleoside kinases as discussed above, is also possible.

5′-Iodo-5′-deoxyadenosine caused an increase in ATP concentration (Fig. 1), but like all nucleosides lacking a 5′-hydroxymethyl group it cannot be a substrate for any nucleoside kinase [26]. We can exclude likewise, formation of a triphosphate derivative co-eluting with ATP. Consequently, an increase in ATP must have occurred by intermediate adenine formation. Further support for the adenine and APRT involvement is given by the data obtained using APRT-deficient erythrocytes, where no change in ATP has been observed. Generation of adenine via MTAP is a possible source since 5′-iodo-5′-deoxyadenosine is a good substrate for MATP [27]. However, the release of adenine due to interaction with SAHH is equally possible [3]. Exceptionally, in the case of this compound, among all those used in the study, some slight adenosine contamination was found which disappeared during incubation. However, this could account for no more than 10% of the observed ATP increase. Adenine

contamination was absent in all analogues studied. Lack of ATP elevation during incubation with *S*-adenosylhomocysteine, which might have occurred with sequential action of SAHH and AK, confirms that this compound cannot cross the erythrocyte membrane [23].

An interesting aspect of this study was the decrease in ATP caused by some of the analogues. In this respect our study closely resembled experiments described recently by Bontemps and Van den Berghe [12] who analysed the mechanism of adenylate degradation in erythrocytes using various adenosine analogues. The major difference in our results is the lack of decrease in ATP concentration during incubation with 2′-deoxyadenosine (Figs 1 and 3). This difference may relate to the higher phosphate concentration in our incubation medium which would have increased intracellular phosphate concentration in the erythrocytes. This in turn could have inhibited AMP-deaminase [28], stimulated glycolysis, and elevated PRPP concentrations [29]. Bontemps and Van den Berghe [12] mentioned also the critical role of phosphate concentration for induction of erythrocyte ATP depletion by nucleosides. Our results support a general relationship between AMP concentration and the rate of adenine nucleotide catabolism [12], since the stimulation of adenylate degradation caused by tubercidin was accompanied by an elevation of AMP (Fig. 2). However, hypoxanthine production during incubation with 2′-deoxyadenosine was not associated with a corresponding increase of AMP (Fig. 3). This implies activation of AMP-deaminase, most likely by triphosphate analogues formed, since the possibility of deamination and phosphorolysis of 2′-deoxyadenosine can be excluded because of the presence of dCf during incubation.

Therefore, when considering the regulation of erythrocyte adenylate catabolism, modulation of AMP-deaminase activity cannot be neglected in the final balance of adenine nucleotide degradation, at least under specific metabolic conditions. The importance of this mechanism has been demonstrated also by other authors [30]. It should be noted that erythrocytes in various parts of the circulatory system are subjected to a changing environment, including phosphate concentration [31]. It is thus inadvisable to assign one particular set of *in vitro* conditions to the purely "physiological" *in vivo* situation.

A discrepancy between the rate of nucleotide analogue synthesis and the rate of ATP degradation is striking (Figs 1 and 3). The triphosphates of tubercidin, 2′-deoxyadenosine and neplanocin A were generated at compatible rates, but analogue nucleotide formation was not accompanied by a corresponding ATP decrease or catabolite accumulation. This raises the question as to whether phosphorylation of nucleoside analogues may be a primary cause of energy disturbance leading to accelerated nucleotide degradation, as previously suggested [12]. In our opinion, tubercidin or its metabolites must disturb energy metabolism in the erythrocyte in some other way; direct interference by tubercidin or its metabolites with ATP-generating enzymes [32] seems to be likely. As the depletion of intracellular phosphate at its very high extracellular concentration is unlikely, inhibition of ATP synthesis by tubercidin diphosphate could be taken into consideration but definite proof of this will require further studies. An interesting fact is that tubercidin did not cause GTP depletion in the erythrocyte (Fig. 2). However, some alterations of the GTP/GDP ratio have been observed in the initial phase, accompanied by probable GMP elevation. This suggests that the enzymes of guanine nucleotide degradation are less active in the erythrocyte, but again activation of AMP-deaminase by phosphate derivatives of tubercidin could be the reason for this discrepancy.

This study has demonstrated that adenosine analogues can enter the nucleotide pool directly via adenosine kinase or may be converted to ATP by pathways involving adenine as a metabolic intermediate. It has also confirmed that a number of these analogues can significantly decrease ATP concentrations.

Acknowledgements—These studies were supported by the Arthritis and Rheumatism Council, the Special Trustees of Guy's Hospital and the Ministerio de Educacion y Ciencia, Madrid. R.T.S. is recipient of a fellowship from the Smith and Nephew Foundation.

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