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S-ADENOSYLMETHIONINE INCREASES ERYTHROCYTE ATP *IN VITRO* BY A ROUTE INDEPENDENT OF ADENOSINE KINASE

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Abstract—The mechanism by which *S*-adenosylmethionine (SAM) and adenosine (Ado) increase ATP levels in intact human erythrocytes *in vitro* has been compared. The use of erythrocytes from healthy controls and from subjects totally deficient in adenine phosphoribosyltransferase (APRT), plus inhibitors of adenosine kinase (AK) and adenosine deaminase (ADA) separately and together, has enabled us to demonstrate that this increment in ATP levels occurred via totally different metabolic routes. The results show that: (i) whilst the Ado-induced increment in ATP was AK dependent, that produced by SAM was independent of AK; and (ii) the SAM-induced increment in ATP was totally dependent on APRT and that some of the increment produced by Ado might also be APRT dependent. The above data are consistent with the metabolism of SAM to ATP by a route recently identified by us whereby ATP is formed from deoxyadenosine: namely binding to the enzyme *S*-adenosylhomocysteine hydrolase with subsequent release of adenine and further conversion to ATP via APRT.

The mechanism by which the human erythrocyte is able to sustain its ATP levels is different from that in nucleated cells, the erythrocyte effectively lacking two key enzymes essential to the *de novo* synthetic and salvage pathways of purine metabolism (Fig. 1) [1]. The nucleoside adenosine (Ado‡) has long been considered the normal ATP precursor; ATP formation being facilitated by the favourable K_m for Ado with adenosine kinase (AK:EC2.7.1.20) relative to adenosine deaminase (ADA:EC 3.5.4.4) at the physiological substrate levels (below 5 μM) pertaining *in vivo* [1–3]. Ado in excess of these levels is degraded rapidly by ADA to hypoxanthine which can be metabolized to IMP, but no further by the erythrocyte [1, 2].

The Ado analogue, *S*-adenosylmethionine (SAM) has also been found to elevate erythrocyte ATP levels at high substrate concentrations (1.25 mM), but the authors could only speculate as to the mechanism involved [4]. Recently we reported ATP formation *in vitro* from the Ado analogue deoxyadenosine (dAdo) in the human erythrocyte which, because of its unique metabolism, could not have occurred by any known route [5]. A novel pathway, involving binding of dAdo to of *S*-adenosylhomocysteine (SAH) hydrolase (EC 3.3.1.1) with subsequent release of adenine, which was further metabolized to ATP via adenine phosphoribosyltransferase (APRT:EC2.4.2.7), was proposed [5].

The present study was instigated to determine whether the reported effect of SAM on erythrocyte ATP levels could be mediated by this novel route [5]. The results not only support this concept, but also imply that Ado might likewise produce some increment in ATP via this route under appropriate circumstances.

MATERIALS AND METHODS

Controls and patients. Fresh venous blood from healthy volunteers was collected into lithium heparin and centrifuged immediately. Heparinized blood was also obtained from two patients who originally presented with 2-8,dihydroxyadenine lithiasis associated with a complete deficiency of APRT as reported previously [3], during their annual clinical evaluation. The buffy coat and top fifth layer of cells were discarded and the cells washed twice with isotonic saline and then once with the incubation buffer—Earls balanced salt solution containing 5.6 mM glucose and 18 mM P_i (PP-ribose-P generating conditions [2]) adjusted to pH 7.4—and diluted in this buffer to a haematocrit of 40%.

Materials. Ado, SAM chloride, SAM paratoluenesulfonate, adenine and 2′-deoxycoformycin (dCF), were of the highest quality available and were obtained from the Sigma Chemical Co. (Poole, U.K.). 5-Iodotubercidin (ITu) was from Research Biochemicals Ltd (St Albans, Herts, U.K.). The other materials used were analytical grade.

Methods. The final concentration of Ado and SAM employed was 1.25 mM, as used by Maeda *et al.* [4], both substrates being made up fresh daily in the incubation buffer and checked for purity by

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‡ Abbreviations: Ado, adenosine; SAM, *S*-adenosylmethionine; SAH, *S*-adenosylhomocysteine; dAdo, deoxyadenosine; MTAdo, methyl-thioadenosine; dCF, deoxycoformycin; ITu, iodotubercidin; APRT, adenine phosphoribosyltransferase; ADA, adenosine deaminase; AK, adenosine kinase; P_i, inorganic phosphate; PP-ribose-P, phosphoribosylpyrophosphate; TCA, trichloroacetic acid; ACN, acetonitrile; HPLC, high performance liquid chromatography.

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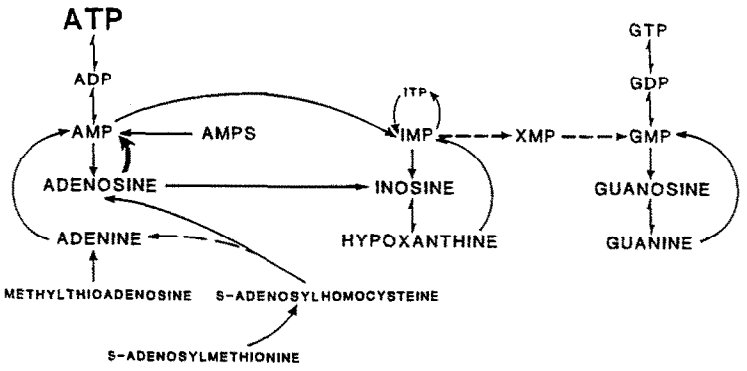

Fig. 1. Potential pathways for ATP synthesis in the human erythrocyte showing the principal route of formation from adenosine (Ado) mediated by adenosine kinase (AK), as well as the only alternative route involving conversion of adenine to AMP via adenine phosphoribosyltransferase (APRT). The novel route from *S*-adenosylmethionine (SAM) requiring prior binding of *S*-adenosylhomocysteine (SAH), or Ado analogues, to SAH hydrolase, with subsequent release of adenine (broken arrow) which is then converted to AMP/ATP via APRT [5], is indicated. Note that although the conversion of methylthioadenosine to adenine has been demonstrated in human erythrocytes [3], pathways for its formation from SAM appear to be lacking [4, 13, 14, 15].

HPLC. These high unphysiological substrate levels were reproduced in order to enable direct comparison with the previous studies [4]. The inhibitors of ADA and AK, dCF and ITu, were used at a concentration of 10 and 20 μM, respectively, concentrations slightly higher than those used by others [6] to ensure effective inhibition at these high substrate levels [5]. In the experiments involving ADA inhibition, erythrocytes were diluted in buffer containing dCF (10 μM) and preincubated for 20 min. The reaction was started by the addition of 100 μL of this suspension to the buffer substrate mixture as described below. For incubations incorporating ITu (20 μM), the inhibitor was added together with the substrate to the incubation buffer at the commencement of the reaction.

Incubations. All experiments were commenced by the addition of 100 μL of erythrocytes (40% haematocrit) to 150 μL of the incubation buffer containing the appropriate substrate. Controls consisted of erythrocytes diluted in incubation buffer alone. All tubes were then incubated for 3 hr at 37° in a shaking water bath. Incubations were generally terminated by adding 50 μL of 40% trichloroacetic acid (TCA).

Extraction procedures. Precipitated protein was removed by centrifugation in a microfuge for 3 min and the TCA was immediately extracted from the supernatant with water-saturated diethyl-ether to a pH above 5 (4–5 extractions). Basal nucleotide levels were also measured in each set of experiments by stopping the reaction at zero time. Nucleotide extracts prepared in this way were analysed immediately (see below).

Separation of cells and medium. The potential for non-enzymatic degradation of either SAM or Ado to adenine under the acidic conditions pertaining, using TCA [5], was investigated in a few experiments using both control and APRT deficient cells. Cells and medium were separated by centrifuging for 30 sec at the end of the 3 hr incubation. The reaction was then stopped in separated cells and medium by adding 200 μL of acetonitrile (ACN). The supernatants from both medium and cells were then extracted using diethyl-ether. Blanks used for both media and cells consisted of separating cells and medium at zero time and stopping the reaction as above. Samples were either analysed immediately or stored at −20° for less than 24 hr.

Analytical methods. Erythrocyte nucleotides were separated and quantified by high performance liquid chromatography (HPLC), using a fully-automated System (Millipore Waters) as described [7]. Extract (25 μL) was injected onto an anion exchange column (5μ APS-Hypersil 250 × 4.9 mm) and the nucleotides separated using a linear phosphate buffer gradient elution system (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 detection at 254 and 280 nm.

Reversed-phase separations of nucleosides and bases in the medium were carried out on a Hichrom Spherisorb 5μ ODS 250 ×4.9 mm analytical column using linear gradient elution and a flow rate of 1 mL/min, with dual wavelength analysis. The gradient ran from 100% A (40 mM ammonium acetate buffer, pH 5.0) to 30% B (80% methanol:10% acetonitrile:10% tetrahydrofuran, by vol.) over 30 min and included a 10 min equilibration period [8].

Because of the known instability of SAM, SAM and Ado used as substrates in these experiments were not only tested for their purity by HPLC, the incubations stopped at zero time were also checked by HPLC and were found to contain no evidence of contamination by either adenine or methylthioadenosine (MTAdo). The adenine used likewise did not contain any contaminating Ado.

RESULTS

Studies in erythrocytes from healthy controls

The mean increments in ATP levels for eight

Table 1. Effect of different substrates and inhibitors on adenine nucleotide levels in erythrocytes from healthy controls (μmol/L packed cells)

	ATP	ADP	AMP	ATP	ADP	AMP	ATP	ADP	AMP
(A)	Ado			Ado + dCF			Ado + dCF + ITu		
Zero time	1370 (1344–1395)	113 (100–126)	11 (6–16)	1369 (1357–1381)	114 (113–115)	15 (13–16)	1318 (1291–1344)	104 (88–120)	10 (10–10)
+180 min	1728 (1678–1779)	118 (107–128)	19 (11–26)	2579 (2487–2670)	213 (106–320)	36 (15–57)	2014 (1990–2037)	144 (111–176)	24 (16–31)
Increment	+358	+5	+8	+1210	+99	+21	+696	+40	+14
(B)	SAM			SAM + dCF			SAM + dCF + ITu		
Zero time	1413 (1344–1482)	121 (115–126)	10 (4–16)	1360 (1357–1376)	111 (96–115)	13 (10–16)	1366 (1344–1387)	108 (105–120)	12 (10–14)
+180 min	1967 (1944–1990)	179 (108–249)	29 (6–51)	1911 (1806–2016)	153 (105–200)	29 (14–44)	1860 (1741–1978)	159 (119–198)	26 (15–37)
Increment	+554	+58	+19	+551	+42	+26	+494	+51	+14

ATP, ADP and AMP levels (mean plus range) in erythrocytes from healthy controls incubated in duplicate for 3 hr with either (A) Ado, or (B) SAM (both 1.25 mM) alone, or after pre-incubation with dCF (10 μM) to inhibit ADA, or dCF plus the AK inhibitor ITu (20 μM), as described in Materials and Methods.

different experiments performed in duplicate following incubation of erythrocytes with SAM and Ado, with and without the different inhibitors, are listed in Table 1. Absolute values (mean plus range) for all three adenylates, ATP, ADP and AMP in control erythrocytes at zero time and following incubation for 3 hr with Ado or SAM (alone or together with dCF, or dCF plus ITu) are also listed.

The effect of SAM on the generation of ATP in the erythrocytes was studied separately with two different salts: SAM chloride and SAM paratoluenesulfonate. Since there was no difference in nucleotide formation under any of the different incubation conditions, the results were combined.

The results confirm that the principal effect of SAM or Ado was on ATP levels and that neither substrate altered ADP or AMP to any great degree under any of the different experimental conditions used. Total adenylate levels in cells incubated in buffer alone for 3 hr also showed little change compared with zero time (data not shown), indicating that the increments in ATP with Ado and SAM were the result of net synthesis, not regeneration. Apart from IMP, which increased only in the studies with Ado alone, the other erythrocyte nucleotides also showed no change (not shown). The results demonstrated that SAM and Ado produced their effects on ATP levels by markedly differing routes as follows:

Incubations with Ado. Incubations with Ado alone in control cells produced a mean increment of around 25% in ATP levels, which further increased up to three-fold in cells incubated with Ado plus dCF to inhibit ADA, resulting in a virtual doubling in ATP levels (Table 1). This increment in ATP was reduced (up to 50%), but not completely inhibited, when Ado was incubated with dCF plus the AK inhibitor ITu at 20 μM. No further reduction was produced in a few experiments where 80 μM ITu was employed (data not shown).

Incubations with SAM. Incubations with SAM alone (Table 1), produced a mean increment in ATP levels of approximately 40% in control cells, higher than the increment with Ado alone and, again in contrast to the Ado studies, this was not altered by incubation with dCF, or dCF plus ITu, demonstrating that this increment in ATP was independent of either ADA or AK, as confirmed by the absence of any deamination products in the medium (see below) or increment in IMP in the cells under any conditions.

Nucleoside and base levels. Nucleoside and base levels were investigated in the medium of the few experiments where cells and medium were separated. No adenine or MTAdo were detected in any reactions stopped at zero time, whether terminated with TCA or ACN, thereby excluding the possibility of non-enzymatic degradation of substrate under the experimental conditions employed. Cells incubated with Ado alone revealed no unmetabolized Ado, Ado not converted to ATP being completely metabolized to inosine and hypoxanthine (and IMP). The presence of these Ado degradation products in the medium and IMP in the cells in the studies with Ado alone, and their absence in the studies with dCF, confirmed that the principal route of Ado metabolism at the high concentrations (1.25 mM) used was degradation by ADA [2]. Because accurate quantification would have required collection and evaluation of nucleosides and bases eluted in the front from the cell extracts run by HPLC on the anion exchange system, it is not possible to present the results in tabular form.

By contrast, in the experiments using SAM alone, no inosine or hypoxanthine were found in the medium under any conditions, although low levels of adenine were present at the end of the incubation period. (Adenine was absent at zero time; in a few studies stopped at 40 min as compared with 180 min, detectable levels were present, which increased with time.)

The above results in control cells excluded

Table 2. Effect of different substrates and inhibitors on adenine nucleotide levels in erythrocytes from patients with adenine phosphoribosyltransferase deficiency (μmol/L packed cells)

	ATP	ADP	AMP	ATP	ADP	AMP	ATP	ADP	AMP
(A)	Ado			Ado + dCF			Ado + dCF + ITu		
Zero time	1273 (1208–1338)	81 (72–90)	7 (7–8)	1264 (1203–1324)	87 (84–90)	8 (7–8)	1259 (1177–1341)	90 (89–90)	11 (10–12)
+180 min	1470 (1416–1523)	69 (65–72)	17 (16–17)	1968 (1644–2291)	115 (88–141)	23 (21–25)	1314 (1156–1472)	68 (44–81)	17 (13–24)
Increment	+197	−8	+9	+704	+28	+17	+55	−22	+6
(B)	SAM			SAM + dCF			SAM + dCF + ITu		
Zero time	1287 (1208–1366)	87 (83–90)	9 (9–8)	1280 (1203–1357)	91 (90–91)	10 (8–12)	1259 (1177–1341)	93 (89–97)	9 (6–12)
+180 min	1303 (1247–1359)	77 (63–85)	22 (8–30)	1251 (1130–1371)	69 (53–85)	14 (10–18)	1195 (1064–1325)	67 (49–85)	23 (17–29)
Increment	+16	−10	+13	−29	−22	+4	−64	−26	+14

Effect of SAM and Ado (1.25 mM) on ATP, ADP and AMP levels in erythrocytes from patients completely deficient in adenine phosphoribosyltransferase (APRT) after incubation for 3 hr under identical conditions to Table 1.

involvement of both ADA or AK in the SAM-induced increment in ATP levels.

Studies with APRT deficient erythrocytes

The results (mean plus range) of identical studies with SAM and Ado carried out in duplicate in erythrocytes from two APRT deficient subjects are listed in Table 2. The mean increment in ATP levels in APRT deficient cells is compared in Fig. 2 with the values found in control cells. Incubations were also performed with adenine (1.25 mM), both alone, with dCF, or dCF plus ITu over 3 hr using APRT deficient cells, compared with cells from two control subjects. The results are summarized in Fig. 2.

Incubations with adenine. Incubations with adenine confirmed the complete deficiency of APRT demonstrated and reported previously in intact and lysed cells from these patients [3], with no increment in ATP levels being noted under any conditions (Fig. 2). Control cells in the presence of either inhibitor showed a similar increment in ATP (approx. 40%) to that produced by adenine alone. This was the same pattern of ATP production as noted above with SAM in control cells (Fig. 2).

Incubations with Ado. Incubations with Ado were the only studies in these APRT deficient erythrocytes to show any increment in ATP levels. Although this increment was of smaller magnitude than that found in control cells, it was likewise trebled using Ado together with dCF. However, when using Ado together with ITu and dCF, no measurable increment in ATP was noted in these APRT deficient cells, the levels varying only within the range of experimental error, indicating complete inhibition of AK by 20 μM ITu. This finding was in marked contrast to the studies with dCF plus ITu in control cells (Fig. 2).

Incubations with SAM. Incubations with SAM likewise showed a complete contrast to the results in control cells. SAM, either alone or with inhibitors, produced no measurable increment in ATP levels under any conditions in these APRT deficient erythrocytes (Fig. 2). This demonstrated conclusively the essential involvement of APRT in the SAM-induced increment in ATP levels in control cells.

The virtual absence of any increment in ATP in the APRT deficient erythrocytes when using Ado with 20 μM ITu plus 10 μM dCF, was equally noteworthy and suggested that AK must have been effectively inhibited by 20 μM ITu in control cells. Thus, some ATP must have been produced from Ado by the same APRT-dependent route demonstrated for SAM (Fig. 2). It is also evident from Fig. 2 that the increment in ATP levels produced by Ado with ITu plus dCF in control cells was of similar magnitude to that produced by SAM. This observation together with the similarity of the increment in ATP levels in control cells when using either adenine or SAM (Fig. 2), with or without inhibitors, provides additional support for the hypothesis that ATP formation from SAM occurred via the novel route involving adenine and APRT previously described by us [5].

Nucleoside and base levels. Nucleoside and base levels in the medium following incubation of SAM or Ado alone with these APRT deficient cells were similar to control incubations, the studies with Ado alone again showing complete metabolism of Ado to inosine and hypoxanthine (and IMP). No inosine or hypoxanthine were found in the medium at the end of the incubation period with Ado plus dCF, or dCF plus ITu, or with SAM under any conditions. Unmetabolized substrate was present in the medium at the end of the incubation period with Ado plus dCF or dCF plus ITu, or with SAM with or without inhibitors, together with some adenine, as noted above in control cells. The latter showed a proportional increment in the studies using APRT deficient erythrocytes (but likewise could not be evaluated accurately without measurement of the intracellular levels).

The finding that the amount of ATP formed using 1.25 mM adenine was comparable to that formed

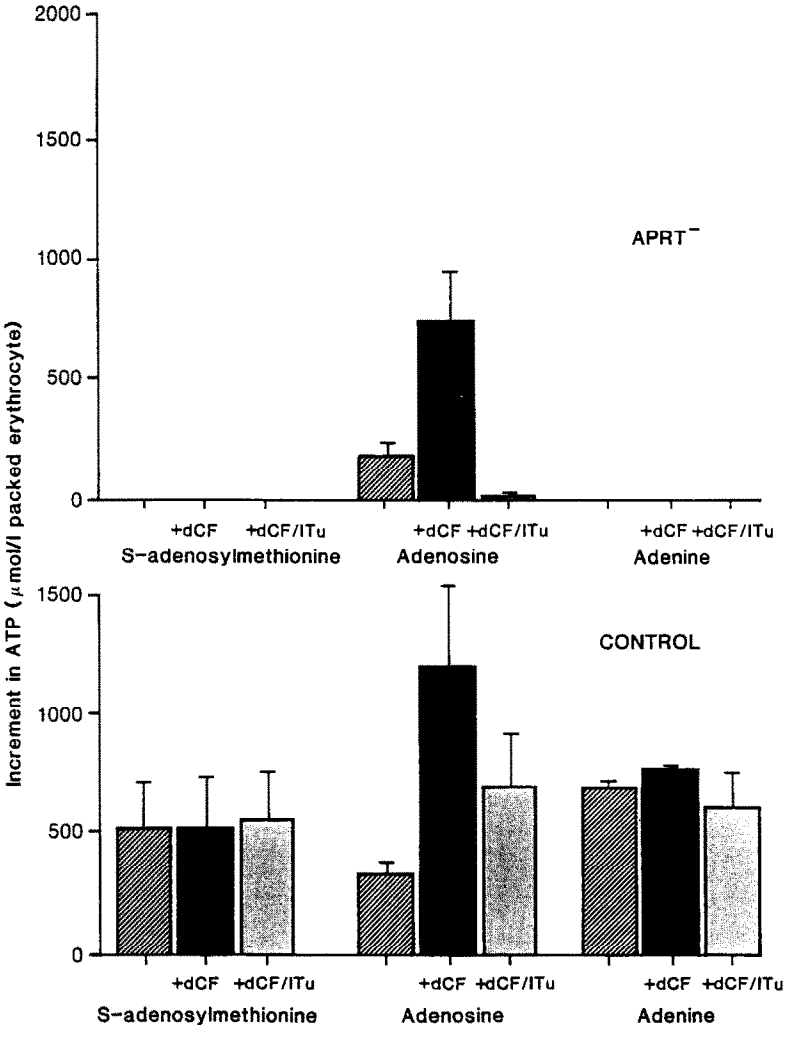

Fig. 2. Histograms demonstrating the mean increment in ATP levels produced following the incubation of erythrocytes from APRT deficient subjects (upper panel) compared with healthy controls (lower panel) for 3 hr with 1.25 mM SAM (left), 1.25 mM Ado (centre) and 1.25 mM adenine (right): (a) alone, (b) with 10 μM dCF and (c) with dCF plus 20 μM ITu, respectively.

with SAM (Fig. 2), despite the presence of high levels of unmetabolized adenine in the medium, indicates that the rate-limiting step for adenylate formation from adenine at these high unphysiological substrate levels in all the different studies was the activity of APRT, coupled with the availability of PP-ribose-P [2, 3].

DISCUSSION

SAM is a ubiquitous compound found in all living cells, participating not only in the numerous transmethylation reactions which require the sequential action of SAH hydrolase for their continuance, but also in sulphur metabolism and polyamine synthesis [9–12]. The only metabolic role hitherto attributed to SAM in the mature human erythrocyte has been its action as a substrate for the carboxyl methylation of membrane and cytosolic proteins [13], entering into the intact erythrocyte through the membrane [4]. The studies in this paper not only confirm that SAM is even more effective than Ado in increasing erythrocyte ATP levels *in vitro* as recently reported by Maeda *et al.* [4], they also demonstrate conclusively that this occurs via a different route, i.e. one not involving AK. Although the magnitude of the ATP increment with SAM was lower in our studies this may relate to differences in the experimental design of Maeda *et al.* [4] (lower haematocrit, stored cells, higher P_i).

The significant observation in our study was that while the Ado-induced increment in ATP, as anticipated, was virtually trebled when Ado catabolism was prevented with dCF and reduced significantly when AK was inhibited with ITu, neither inhibition of AK or ADA had any effect on the SAM-induced increment in ATP. The increment in ATP induced by SAM was the same under all

experimental conditions. Moreover, the studies in the APRT deficient erythrocytes demonstrated an absolute dependence of this SAM-induced increment in ATP on functional APRT activity. The proposal that the SAM-induced increment in ATP might be caused by activation of AK and inhibition of ADA [4] could thus be discounted, raising the important question as to the mechanism by which this SAM-induced increment in ATP is actually mediated and the need for alternative explanations.

The first possibility involves the enzyme SAH hydrolase. The catabolism of SAH, the toxic by-product of cellular methylation reactions involving SAM, to homocysteine and Ado is considered to be the principal function of SAH hydrolase in human cells [9]. Degradation of SAH is favoured by the further metabolism of Ado by either AK or ADA (Fig. 1). However, although increases in ATP levels could occur via this route it would involve AK, inhibition of which had no effect on the SAM-induced increment in ATP in these studies. Likewise, the absence of inosine or hypoxanthine from the medium in any of the SAM studies, in contrast to the studies using Ado alone, demonstrated that SAM or its metabolites were clearly not ADA substrates. It would thus appear that Ado is not a product of SAH hydrolase activity following incubation of SAM with intact erythrocytes, at least at the high concentrations employed.

Alternatively, SAM could be metabolized by a pathway other than the route involving SAH hydrolase, either the polyamine pathway of which adenine is a metabolic by-product [3], or by a non-enzymatic route to MTAdo, which could then be converted to adenine in the erythrocyte [3]. Although no MTAdo was detectable in the medium at any time, the possibility that some MTAdo could have been generated intracellularly during the incubation and metabolized immediately to adenine cannot be discounted. However, this would not explain the Ado results, nor does the polyamine pathway appear to be operative (Fig. 1) in the human erythrocyte [4, 13, 14], findings in accordance with the results of Stramentinoli *et al.* [15] in rat erythrocytes.

The most likely possibility is that the high SAM concentrations used could favour accumulation of the unstable 3′-keto-adenosine intermediate formed from SAH following cleavage of homocysteine in the normal catalytic cycle proposed by Palmer and Abeles [16]. If not immediately reduced to adenosine this unstable intermediate is known to break down rapidly with the release of adenine [5, 14, 17]. Obviously, our establishment of the involvement of APRT in the SAM-induced increment in erythrocyte ATP levels was crucial to the understanding of the differing effects of SAM and Ado. The results support our original hypothesis that ATP formation from SAM might be mediated by the novel route previously demonstrated for dAdo, which involved prior binding to SAH hydrolase, followed by the release of adenine and sequential action of APRT [5]. Moreover, the lower increment in ATP produced by Ado in the APRT deficient cells, alone or with dCF, coupled with the virtual absence of any increment in ATP when both ADA and AK were inhibited, suggests that some Ado (the natural substrate for the reverse SAH hydrolase reaction) must also have been metabolized to ATP via the same novel route proposed for SAM.

SAM has recently attracted attention in the clinical field because of its reputed anti-inflammatory, analgesic and antidepressive effects [10–12, 18]. Our results suggest that SAM, under appropriate conditions, could have another important metabolic function in the human erythrocyte, and possibly other cells. The present findings could provide an additional metabolic basis for at least some of the beneficial clinical effects of SAM reported *in vivo*, which have been poorly understood hitherto.

The combined findings in this paper demonstrate that SAM, unlike Ado, increases erythrocyte ATP levels *in vitro* by a route independent of AK and ADA, and support the hypothesis that this is mediated by a novel pathway requiring the sequential involvement of SAH hydrolase and APRT. The potential for this route exists in other cells.

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