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COMMENTARY

TRENDS IN THE BIOCHEMICAL PHARMACOLOGY OF 5′-DEOXY-5′-METHYLTHIOADENOSINE

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The biological occurrence of the sulfur-containing nucleoside 5′-deoxy-5′-methylthioadenosine (MTA) was recognized more than half a century ago. Crystals of a substance later characterized as MTA were prepared from yeast in 1912 [1] and its correct molecular structure was proposed a dozen years thereafter [2], close to the time that methionine was first isolated [3]. Several nutritional and pharmacologic studies on MTA reported over the next 30 years [4] cast little light on the physiological significance of this nucleoside. That methionine is the precursor of the sulfur atom and methyl group of MTA was demonstrated in Schlenk's laboratory [5, 6] shortly before Cantoni [7, 8] in 1952 announced his discovery of *S*-adenosyl-L-methionine (AdoMet) and its role as a methyl group donor in enzymatic methyltransferase reactions. Subsequent investigations uncovered various biochemical pathways for the formation of MTA in which either AdoMet or decarboxylated AdoMet (i.e. *S*-methyl-adenosylhomocysteamine) serves as an immediate precursor. Although these reactions are commonly looked upon as mechanisms for the synthesis of unrelated biomolecules, it is conceivable that under certain biological circumstances the production of MTA by some of these pathways is of important functional significance, even though MTA does not accumulate substantially in many higher animal tissues. For a recent rush of investigations have shown that MTA as such exerts some striking regulatory effects, and is rapidly degraded by cells to products that can perturb a variety of metabolic events [4, 9–15]. It is the purpose of this article to assess these developments, mainly in the contexts of relationships of MTA to the growth and functions of normal and malignant mammalian cells, and of the actions of some chemotherapeutic drugs that can be regarded as analogs of MTA [15–18].

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Biosynthesis of MTA

In many mammalian tissues, the spermidine synthase and spermine synthase reactions (Fig. 1) apparently represent the quantitatively most important pathways for MTA formation [10, 13]. These two polyamine synthases are distinct and separable enzymes that exhibit no cofactor or prosthetic group requirements. The equilibrium constants of the reactions catalyzed by these enzymes have not been determined precisely but it is likely that they greatly favor the respective formation of spermidine or spermine and MTA from decarboxylated AdoMet and putrescine or spermidine, so that MTA utilization via the reverse reactions is probably insignificant *in vivo* [13]. As considered in detail later, MTA is a powerful product inhibitor of spermine synthase and to a lesser extent of spermidine synthase in mammalian cells [13]. The homogeneous spermidine synthase (aminopropyl transferase, EC 2.5.1.16) of *Escherichia coli* (which has a very limited inherent capacity to catalyze the spermine synthase reaction under physiological conditions) is also subject to considerable product inhibition by MTA [19]. It is noteworthy that *S*-adenosylhomocysteine (AdoHcy), the product of all AdoMet-dependent methyltransferase reactions and which acts as a potent inhibitor of nearly all methyltransferases [20], does not influence ornithine decarboxylase [21] and has only feeble inhibitory effects on mammalian polyamine synthases [22].

AdoMet cyclotransferase (EC 2.5.1.4) converts AdoMet to MTA and homoserine lactone. This enzyme is present in mammalian liver [23–25], yeast [26] and certain bacteria [27]. Yeast AdoMet cyclotransferase is moderately inhibited by the MTA product [26]. Not nearly enough is known about the tissue distribution and functions of AdoMet cyclotransferase in mammalian organisms, or quantitative aspects of its contribution to MTA production in living cells.

MTA is a by-product of the enzymatic transfer of a 3-amino-3-carboxyl-propyl group from AdoMet to specific uridine residues of *E. coli* $tRNA^{Phe}$ [28]. Rat liver extracts also catalyze an AdoMet-dependent synthesis of the modified base $4abu^3U$ in tRNA molecules. These reactions presumably contribute only negligibly to the total production of MTA *in vivo*. In apple tissue, MTA is formed as a result of cyclization of AdoMet to yield 1-aminocyclopropane-1-

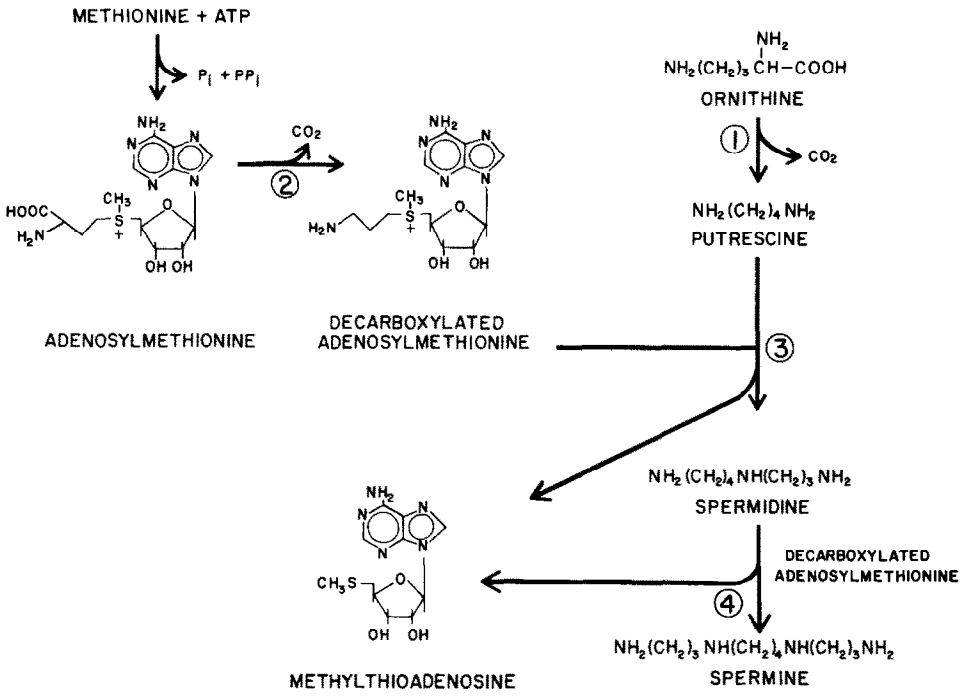

Fig. 1. The major pathway for spermidine and spermine biosynthesis in mammalian tissues. Reaction 1 is catalyzed by ornithine decarboxylase, reaction 2 by putrescine-activated AdoMet decarboxylase, reaction 3 by spermidine synthase, and reaction 4 by spermine synthase. MTA, AdoMet and decarboxylated AdoMet are depicted in the syn-conformation although they may exist predominantly in the anti-conformation under physiological circumstances.

carboxylic acid, the precursor of the fruit-ripening hormone ethylene [29]. It has been hypothesized that a different mode of cyclization of AdoMet is involved in the biosynthesis of azetidine-2-carboxylic acid in lily-of-the-valley [30, 31] with MTA as the other product, but other pathways for the formation of azetidine-2-carboxylic acid appear to predominate in several higher plant species [32–35]. As far as is known, biosynthesis of 1-aminocyclopropane-1-carboxylic acid or azetidine-2-carboxylic acid does not take place in any higher animal cells.

At mildly alkaline pH and 37°, MTA is formed by the non-enzymatic decomposition of *S*-adenosyl-2-oxo-4-methylthioketobutyrate. This α-keto derivative of AdoMet can be formed by transamination of AdoMet during the biosynthesis of 7,8-diaminopelargonic acid (the immediate precursor of desthiobiotin) in *E. coli* [36], and also by oxidative deamination of AdoMet by mammalian L-amino acid oxidase, which occurs more slowly than with AdoHcy [37]. Whether the non-enzymatic conversion of *S*-adenosyl-2-oxo-4-methylthioketobutyrate to MTA occurs in higher animal tissues is not known.

There is presumptive evidence for the formation of MTA during the biosynthesis of sym-norspermidine [$H_2N(CH_2)_3NH(CH_2)_3NH_2$] and sym-norspermine [$H_2N(CH_2)_3NH(CH_2)_3NH(CH_2)_3NH_2$] in thermophilic bacteria [13, 38]. These compounds have not been detected as yet in vertebrate animal cells, although they are present in certain unicellular and multicellular invertebrate organisms [13].

Disposition of MTA in mammalian cells

The concentrations of MTA in many mammalian cells are exceptionally low in comparison with those of AdoMet, ATP, and the polyamines spermidine and spermine. Early attempts to determine MTA levels in liver [6] and rat ventral prostate [39] were thwarted by insufficient sensitivity of analytical methods then available, but it was clear that the values were well below 0.2 μmole/g fresh weight. More recent investigations, based on either reversed phase high performance liquid chromatographic separations after prior removal of impurities on columns of phenylboronate-derivatized Affigel 601 [40], or on radioimmunossays [41], provided estimates of MTA concentrations of less than 3 nmoles/g in rat liver, heart, lung and kidney; in ventral prostate, an organ which contains and secretes strikingly high levels of spermidine and spermine [42, 43], MTA concentrations between 2 and 3 nmoles/g were found in younger adult animals and were about twice as high in ventral prostrates of elderly breeding males [40]. Even during the most active phase of ventral prostrate growth induced by testosterone in orchiectomized rats, at times when spermidine is accumulating in concentrations of several μmoles per g [42, 43] MTA did not accumulate at levels of more than 7 nmoles/g. These small amounts of MTA in the aforementioned organs are in sharp contrast to much higher AdoMet concentrations which span a range of roughly 25–80 nmoles/g in the same tissues

[44–47] but MTA levels are of the same order of magnitude as corresponding concentrations of decarboxylated AdoMet (0.9 to 2.5 nmoles/g) [44]. Conceivably the techniques employed for MTA analysis, despite the demonstration of excellent recoveries of exogenous MTA added to tissue specimens immediately frozen in liquid nitrogen [40], may have provided artefactually high numbers because of some degree of non-enzymatic conversion of endogenous AdoMet in the tissues to MTA during initial stages of fractionation of tissue extracts. However this may be, it is evident that only extremely small quantities of MTA accumulate in several types of normal cells. It is worth noting that the concentrations of MTA are about the same order of magnitude as those reported for adenosine in some tissues [48, 49].

The tiny concentrations of MTA in animal tissues suggest that this substance is either rapidly metabolized or is excreted from cells. A major pathway for MTA metabolism in a variety of eukaryotic organisms is its enzymatic splitting to form adenine and 5-methylthioribose-1-phosphate. An enzyme, now frequently designated as MTA phosphorylase, that catalyzes this reaction was discovered by Pegg and Williams-Ashman [50] as an outcome of their observations on lack of the expected stoichiometry between MTA and polyamine production during operation of the spermidine and spermine synthase reactions promoted by crude prostate and liver extracts, but not when more purified preparations of the polyamine synthases were employed [51, 52]. MTA phosphorylase has since been detected in many normal and some but not all malignant mammalian tissues [12, 14, 15, 53–62], in the insect *Drosophila melanogaster* [63], and in the thermophilic archebacterium *Caldariella acidophila* [64]. Two points are worthy of mention in this connection. One is that AdoHcy hydrolase, which cleaves its natural substrate to adenosine and homocysteine and is centrally involved in the degradation of AdoHcy in vertebrate animal cells and yeast [20], does not utilize MTA as a substrate [65, 66]. And the other is that certain bacteria contain enzymes that split MTA hydrolytically to yield free 5-methylthioribose and adenine and similarly also cleave AdoHcy to form adenine and *S*-ribosyl-L-homocysteine [67]. The latter type of hydrolase is apparently absent from mammalian cells.

Before turning to the characteristics and metabolic significance of MTA phosphorylase, other possible modes of MTA disposition in higher animal cells must be mentioned briefly. The non-specific adenosine deaminase of *Aspergillus oryzae* converts adenosyl thioethers such as MTA and AdoHcy (but not sulfonium compounds like AdoMet) to the corresponding inosine derivatives, a property which has been exploited as the basis of spectrophotometric methods for determination of purified methyltransferases [68] and aminopropyltransferases involved in polyamine biosynthesis [69]. However, mammalian adenosine deaminases do not appear to attack MTA at significant rates [18, 50, 62]. A close analog of MTA, 5′-deoxy-5′-isobutylthioadenosine (SIBA), is apparently converted appreciably to the corresponding inosine derivative by cultured mammalian neoplastic cells, perhaps as a result of direct deamination by 5′-AMP deaminase or a specific "heavy" form of adenosine deaminase [70]. But there is no evidence that direct enzymatic conversion of MTA to the corresponding inosine nucleoside occurs extensively in mammalian tissues. Although, as considered later, MTA is converted to methionine by both prokaryotic and eukaryotic organisms, this transformation requires an initial enzymatic cleavage of MTA to form adenine and 5-methylthioribose or 5-methylthioribose-1-phosphate, the latter pentose-1-phosphate serving as the precursor for methionine. Whether MTA can be excreted as such into extracellular fluids by normal mammalian cells *in vivo* is not known. But certain growing malignant tumor cells lines that are apparently devoid of MTA phosphorylase activity can secrete large amounts of MTA into the culture medium [60].

Properties of MTA phosphorylase and its metabolic and pharmacologic significance

MTA phosphorylases have been partially purified from several normal and neoplastic mammalian tissues [50, 53, 57, 62]. The enzyme exhibits an absolute requirement for inorganic ortho-phosphate which can be partially replaced at saturating concentrations of arsenate but not by many other inorganic anions [50, 53]. The optimal pH is about 7.5. No metal ions or other cofactors are required. Molecular weights in the range of 90,000–100,000 have been reported for human placental [54] and rat liver [56] MTA phosphorylase. The reaction products with MTA as substrate have been clearly identified as adenine and the α-anomer of 5-methylthioribose-1-phosphate (MTR-1-P) [15, 53, 55, 57]. From kinetic studies on the purified lung enzyme, Garbers [53] concluded that the enzyme reaction is an equilibrium-ordered one involving binding of MTA before that of inorganic phosphate and with release of the MTR-1-P product before that of adenine. This mechanism accords with observations that the reaction product adenine inhibits competitively with respect to both MTA and inorganic phosphate. Although higher concentrations of guanine and its 1-, 3-, 7- and 9-methylated derivatives are also inhibitory, MTA phosphorylase is unaffected by adenosine, guanosine, 3,5′-cyclic AMP, 3′,5′-cyclic GMP and many other natural nucleosides and nucleotides [53] and is also not inhibited by greater than 1 mM concentrations of putrescine, cadaverine [9], spermidine and spermine [12, 40]. Ribose-1-phosphate, 2-deoxyribose-1-phosphate and fructose-1-phosphate inhibit the enzyme at 2 mM concentrations, whereas glucose-1-phosphate, glucose-6-phosphate and ribose-5-phosphate do not. Investigations on MTA phosphorylases from other mammalian sources [15, 62] and *Caldariella acidophila* [64] have confirmed that the reaction mechanism is an equilibrium-ordered process subject to product inhibition by adenine. MTA phosphorylase and purine nucleoside phosphorylase act by analogous mechanisms, and both enzymes catalyze reversible reactions so that nucleosides are synthesized when appropriate purine bases and pentose-1-phosphates

are used as substrates [15]. (Accurate estimates of the equilibrium constants for any reactions catalyzed by MTA phosphorylase are lacking.) However, MTA phosphorylase is entirely distinct from purine nucleoside phosphorylase, as indicated by the substrate specificities and other properties of the two enzymes. Noteworthy in this regard is that adenosine, MTA and other purine nucleosides with an amino group at C(6) of the purine ring show hardly any or no activity as substrate for purine nucloside phosphorylase, which has, however, a definite, albeit weak, capacity to synthesize and phosphorolytically split 5′-methylthio-5′-deoxyinosine [15].

Before considering the nucleoside substrate specificity of MTA phosphorylase, it should be pointed out that certain bacteria can synthesize its reaction product, MTR-1-P, by other routes. *Enterobacter aerogenes* was shown by Ferro *et al.* [71] to contain a specific Mg^{2+}-dependent 5-methylthioribose kinase that catalyzes the phosphorylation by ATP of 5′-methylthioribose to form MTR-1-P (ribose and glucose were inactive as substrates). Thus, in some prokaryotes that lack MTA phosphorylase but contain enzymes that hydrolytically split MTA to 5-methylthioribose and adenine, the combined actions of such hydrolytic nucleosidases and 5-methylthioribose kinase form MTR-1-P from MTA in a two-step reaction whereas MTR-1-P synthesis is accomplished in a single step in cells that contain MTA phosphorylase. Mammalian cells contain phosphatases that convert MTR-1-P to free 5-methylthioribose [50] but this almost certainly represents a non-physiological side reaction, and there are no reports of the presence of 5-methylthioribose kinase in mammalian tissues.

MTA phosphorylase does not cleave AdoMet, AdoHcy or adenosine at the N-ribose bond. Parks, Savarese and coworkers [15, 18, 62] undertook penetrating studies on the nucleoside specificity of MTA phosphorylase from mouse Sarcoma 180 cells which, apart from their inherent enzymological interest, have important pharmacologic implications. Many 5′-deoxyribonucleosides of adenine are highly effective substrates for this enzyme. Although several 5′-deoxy-5′-thioalkyl derivatives of adenosine including MTA are active, the presence of a sulfur atom or thioether bond attached to C(5′) of 5′-deoxyadenosine is not mandatory for substrate activity. Thus, in terms of K_m and V_{max} values relative to those of MTA, the following compounds were also effective substrates: 5′-deoxyadenosine and its 5′-chloro- and 5′-iodo-derivatives as well as SIBA, whereas inosine, 2′-deoxyadenosine, 3′-deoxyadenosine, arabinosyladenine and xylosyladenine were essentially inert [62]. Also exhibiting high substrate activity was 5′-deoxy-5′-ethylthio-2-fluoroadenosine (5′-ETFAR) [18], a substance that differs from MTA in substituents on both the purine and the ribose portions of the molecule [17]. The activity of SIBA as a MTA phosphorylase substrate was also observed in Zappia's laboratory using the enzyme from human prostate [72] and *Caldariella acidophila* [64]. The aforementioned synthetic substrates are phosphorolytically cleaved to yield the free purine and the corresponding pentose-1-phosphate.

Predictably, the splitting of MTA by MTA phosphorylase is inhibited by drugs such as SIBA and 5′-deoxyadenosine (5′-dAdo) by virtue of these compounds acting as competitive substrates. Thus, one might expect that these synthetic substances could evoke a pile-up of MTA in cells that are rich in MTA phosphorylase. SIBA is well known to exert striking inhibitory effects on replication [73, 74] and cell transformation [75] by oncogenic viruses; mitogen-induced blastogenesis of human and rabbit lymphocytes [76]; and the growth of certain *Leishmania* species [77] and *Plasmodium falciparum* [78]. It has been postulated that inhibition of protein methylase I (AdoMet:protein arginine methyltransferase, EC 2.1.1.23) by SIBA [79] is related to the effects of this drug on the enzyme in normal (K_i SIBA, 635 μM; K_i AdoHcy, 8 μM; K_m AdoMet, 38 μM) and transformed (K_i SIBA, 320 μM; K_i AdoHcy, 6 μM; K_m AdoMet, 21 μM) fibroblasts [75]. But it is evident from the foregoing numbers that SIBA, which as a nucleoside thioether can be regarded as an analog of AdoHcy and MTA, is not a particularly potent inhibitor of the enzyme which methylates arginine residues in proteins. Conceivably SIBA could exert other effects on cells as a result of its cleavage to adenine and 5′-deoxy-5′-isobutylthioribose-1-phosphate via mechanisms germane to those of other active MTA phosphorylase substrates now to be discussed.

When Savarese *et al.* [18, 62] discovered that 5′-dAdo was a most effective substrate for MTA phosphorylase from Sarcoma 180 cells, they undertook a series of important investigations on the effects of 5′-dAdo and MTA on adenine nucleotide and 5-phosphoribosyl-1-pyrophosphate (PRPP) pools in intact tumor cells. Previously, Hunting and Henderson [80] reported that 5′-dAdo added to Ehrlich ascites tumor cells caused profound inhibition of PRPP levels, *de novo* purine biosynthesis, and glycolysis, with a "cross-over" at the phosphofructokinase step in the latter regard. These observations were puzzling because 5′-dAdo (which is a very poor substrate for mammalian adenosine deaminase) cannot be directly phosphorylated to any nucleotide derivatives. The phosphorolytic cleavage of 5′-dAdo and MTA by MTA phosphorylase yields adenine and the corresponding pentose-1-phosphate, and the liberated adenine can then react with PRPP to form 5′-AMP and inorganic pyrophosphate as catalyzed by the widely distributed enzyme APRT (adenine phosphoribosyltransferase, EC 2.4.2.7). Accordingly, 5′-AMP could accumulate and be further converted to ADP and ATP. Savarese *et al.* [62] indeed demonstrated that, when mouse Sarcoma 180 cells were incubated with 5′-dAdo or MTA at 500 μM, the intracellular concentrations of ATP and ADP increased 2- to 3-fold and that of 5′-AMP 4- to 5-fold within 3 hr at 37°, with a concomitant decline in PRPP levels to less than 10% of normal values (5′-dAdo does not directly inhibit PRPP synthetase). The metabolic consequences of the changes in adenine nucleotides and PRPP were profound, and include inhibitory actions of high ADP levels on PRPP synthetase, depressed *de novo* purine and pyrimidine nucleotide biosynthesis because of lack of PRPP, and possibly inhibition of phosphoribomutase by 5′-deoxyribose-1-P or MTR-1-P produced

by action of MTA phosphorylase on 5′-dAdo and MTA respectively.

Worthy of mention in this context is the proposal of Kessel [81] that 5′-dAdo may be used as a "non-metabolized" substrate for the study of adenine nucleoside transport system on the assumption that, since it is not phosphorylated enzymatically, 5′-dAdo is metabolically inert. This may be true in the L1210 leukemia cells that Kessel [81] employed for successful probing of 5′-dAdo transport because under certain culture conditions cells of this line are apparently devoid of MTA phosphorylase [59, 62], as considered shortly. However, 5′-dAdo is clearly metabolized rapidly in cells that are rich in this enzyme.

The cleavage of adenine from MTA, catalyzed by MTA phosphorylase, and the subsequent conversion of adenine to 5′-AMP by APRT obviously provide a pathway for metabolic salvage of the purine portion of ATP used for synthesis of AdoMet and hence of decarboxylated AdoMet utilized in the mammalian polyamine biosynthetic sequence. Since polyamine formation appears to be the major process by which MTA is synthesized in higher animal cells, MTA phosphorylase can for this reason be looked upon as an enzyme of polyamine biosynthesis. The action of MTA phosphorylase on MTA probably accounts for the output of small quantities of free adenine in urine [15] and the formation of 2,8-dihydroxyadenine (which is produced by the action of xanthine oxidase on adenine) in urinary stones in a patient with congenital deficiency of the enzyme APRT [82].

MTA phosphorylase substrates that differ from MTA in substituents on both the purine and ribose moieties may provide a means of delivery into cells of substituted adenine bases which might then be converted to the corresponding ribonucleotides by APRT-catalyzed reaction with PRPP. One such example is 5′-ETFAR mentioned above, which yields 2-fluoroadenine when it is cleaved by MTA phosphorylase. Montgomery *et al.* [17] indeed found that 5′-ETFAR was conspicuously cytotoxic to APRT-positive H. Ep.-2 cells in culture but not to H. Ep.-2/FA/FAR cells that lack APRT. In the same investigation, 5′-ETFAR was found not to affect the growth of L1210 leukemia *in vivo* but this probably reflects a deficiency of MTA phosphorylase in this tumor line. Similarly, cleavage by MTA phosphorylase is implicated in the cytostatic actions of 5′-ethylthio-2,6-diaminopurine riboside and 5′-methylthio-8-azaadenosine on cultured human tumor cells that are rich in MTA phosphorylase [83].

A few compounds have been prepared that are inactive as substrates for MTA phosphorylase but nonetheless are powerful competitive inhibitors of the enzyme. Coward *et al.* [16] showed that 5′-methylthiotubercidin (MTT), the 7-deaza analog of MTA which is devoid of substrate activity, inhibited ventral prostrate MTA phosphorylase (K_m MTA/K_i = 0.25). Savarese *et al.* [18] found that 5′-deoxy-5′-chloroformycin (7-amino-3-(5-deoxy-5-chloro-β-D-ribofuranosyl) pyrazolo [4,3-*d*]pyrimidine), a C-nucleoside that is not cleaved enzymatically, strongly inhibited mouse Sarcoma 180 MTA phosphorylase (K_m MTA/K_i = 30). Presumably both of these non-substrate inhibitors could elicit accumulation of MTA in cells that contain MTA phosphorylase.

Regulation of MTA phosphorylase activity in normal and malignant cells

MTA phosphorylase has been detected in all non-neoplastic cells of embryonic and adult mammals that have been studied, including non-nucleated erythrocytes. In every instance, the enzyme was found almost exclusively in the cytosol compartment and no isoenzymic variants have been described. The enzyme was undetectable in heparinized blood plasma of normal adult rats (although plentiful in non-hemolyzed plasmas at 24 hr after administration of the hepatotoxic agent carbon tetrachloride), but was weakly active in rat ventral and anterior prostate secretions [40] and also in fetal calf serum [60]. Ferro's laboratory has shown that MTA phosphorylase activities are increased when certain types of cells undergo proliferation. Thus, during transformation of human lymphocytes by phytohemagglutinin (PHA), MTA phosphorylase activity increased by 12, 81 and 108% at 12, 24 and 48 hr after application of the lectin [58]. It is noteworthy that MTA and the non-substrate inhibitor MTT both inhibit PHA- and concanavalin A-induced lymphocyte transformation in a dose-dependent fashion with more than 80% inhibition at 1 mM concentrations of these nucleosides [58, 84]; however, the effects of MTA were reversible whereas those of MTT were not overcome by washing the cells. According to Nicolette *et al.* [56], gonadectomy of adult rats of appropriate sexes at 34 days earlier resulted in a 95% reduction in rat ventral prostate MTA phosphorylase and when performed 16 days previously caused a 67% diminution in the uterine enzyme activity. Treatment with testosterone propionate for 4 days was stated to restore prostate MTA phosphorylase to more than 50% of the castrate levels. 17β-Estradiol stimulated the uterine enzyme to 35% above the castrate controls within 24 hr whilst 3 days of continuous estrogen administration increased the levels of the enzyme to 97% of the intact control levels. Neither castration nor 17β-estradiol treatment affected MTA phosphorylase in female rat liver or lung. The data of Nicolette *et al.* [56] are unfortunately expressed in terms of enzyme units per total organ, so that the actual changes in MTA phosphorylase activities are magnified as a result of multiplication of the values by wet weights of uterus or prostate, which are subject to marked decreases after castration and increases following subsequent sex hormone administration. Seidenfeld *et al.* [40] found that, when enzyme activities were expressed as units per g equivalent of tissue, MTA phosphorylase in rat ventral prostate decreased to 40% of normal control values at 7 days after orchiectomy and was virtually restored by testosterone administration over a subsequent 3-day period. The effects of androgen deprivation and treatment on MTA phosphorylase were much smaller than those on ornithine decarboxylase measured in the same experiments [40] and on AdoMet decarboxylase determined under similar conditions [42, 43]. The latter two polyamine biosynthetic enzymes are well known to turn over very fast in mammalian cells in which they are synthesized and degraded intracellularly with great rapidity and to exhibit apparent half-lives of

much less than 1 hr in prostate and several other organs [10, 13]. By contrast, the apparent half-life of MTA phosphorylase in rat liver and ventral prostate is clearly much greater than 12 hr [40]. Thus, changes in MTA phosphorylase activity resulting from alterations in androgenic status are much less dramatic than corresponding alterations in polyamine biosynthetic decarboxylases in the rat ventral prostrate. Androgenic influences on ventral prostate MTA phosphorylase might reflect little more than alterations in the relative proportion of secretory epithelial cells in the gland. No effects of androgens *in vivo* on MTA phosphorylase were seen in comparable studies on rat anterior prostate (coagulating) gland which does not secrete polyamines [40]. Kar and Pearson [85] noted that MTA phosphorylase activities of skeletal muscle biopsies were within the normal range in patients with various forms of muscular dystrophies, polymyosities, and certain denervating diseases, which represent disorders in which muscle polyamine levels are reported to be elevated [86].

Toohey [59, 87] was the first to notice that certain cultured malignant cell lines were devoid of detectable MTA phosphorylase. Working with various murine malignant hematopoietic cells, he found [87] that four cell lines, that apparently required, for their growth, an exogenous source of "methylthio" groups [88, 89] obtained by supplementation of the medium with small mixed disulfides of the type R—S—S—CH_3 (e.g. cysteine—(S)—S—CH_3), were devoid of MTA phosphorylase when grown on repeated passage in culture. Two of these enzyme negative cell lines (L1210 and AKS-SH) sometimes exhibited very weak MTA phosphorylase activity when grown *in vivo*. (Perhaps this could reflect MTA phosphorylase in the tumor's stroma cells of host origin.) By contrast, five other cell lines that exhibited no growth requirement for exogenous "methylthio" groups were replete with MTA phosphorylase. Experiments involving mixing of ultracentrifuged extracts from enzyme-positive and -negative cells suggested that those cells that apparently lacked MTA phosphorylase did not contain soluble inhibitors of the enzyme. The exogenous "methylthio" group-requiring cells without demonstrable MTA phosphorylase contained about the same amounts as the MTA phosphorylase-positive cells of an enzyme system, designated as "methylthiolase" that converted MTR-1-P to unidentified products that were soluble in diethyl ether, which Toohey [59, 87] believed to result from the splitting of MTR-1-P to a putative "CH_3—S"-containing product plus ribose-1-phosphate. Shortly we shall discuss other studies suggesting that a different pathway for MTR-1-P metabolism operates in certain mammalian cells.

Kamatani *et al.* [60, 61] have very recently reported that seven out of thirty-one (23%) human malignant tumor cell lines were completely deficient in MTA phosphorylase when assayed with 5'-deoxy-5'-chloroadenosine (CldAdo) as substrate. These enzyme-deficient malignant cell lines were derived from five leukemias, one melanoma and one breast carcinoma. By contrast none of sixteen cell lines of non-malignant origin, derived from lymphocytes, fibroblasts and epithelial cells, lacked MTA phosphorylase. Kamatani *et al.* [61] devised a clever autoradiographic technique for visualization of MTA phosphorylase based on incorporation of radioactivity into nucleic acids of intact cells of adenine derived from cleavage of Cl[2-^{3}H]dAdo. Using this procedure, enzyme-positive cultured cell lines, normal immature bone marrow cells, and four specimens of malignant tumors with demonstrable MTA phosphorylase activity readily incorporated the adenine moiety of CldAdo into nucleic acids, whereas enzyme-deficient malignant cell lines did not. When either enzyme-positive or -negative cells were cultured in a medium containing 0.4 μM methotrexate, 16 μM uridine and 16 μM thymidine (or alternatively in a medium to which 10 μM azaserine alone was added), none of the cells proliferated. But if MTA was added to the same media, only cells containing MTA phosphorylase divided, whereas most enzyme-deficient cells perished within 3 days. In other words, human malignant cells lines naturally deficient in MTA phosphorylase can be selectively killed when *de novo* purine synthesis is inhibited and MTA is the only exogenous source of purines. It was proposed [61] that, because the chemotherapeutic efficacy of methotrexate is limited by its toxicity to normal rapidly proliferating tissues such as bone marrow and intestinal mucosa, and in view of difficulties in rescuing normal cells from methotrexate poisoning by thymidine infusion, it might be possible in patients with malignant tumors lacking MTA phosphorylase to protect normal tissues by treatment with MTA (alone or in combination with thymidine) without influencing the killing of malignant cells by methotrexate.

Why a substantial number of human and animal malignant cells grown in culture apparently lose all MTA phosphorylase is mysterious. It remains to be worked out whether this might reflect a mutation in the gene for this enzyme, or alternatively some defect in the transcription, intranuclear processing, or translation of the normal mRNA for MTA phosphorylase. Kamatani and Carson [60] were able to maintain this enzyme-negative phenotype by passaging certain of the cell lines for up to 1 year in culture. In contrast to observations of Toohey [59, 87], none of the MTA phosphorylase-deficient lines studied by Kamatani and Carson [60] required a source of exogenous "methylthio" groups for their growth. The latter investigators found that, in contrast to enzyme-positive cells (which did not contain detectable quantities of MTA or excrete this nucleoside), malignant cells lacking MTA phosphorylase accumulated MTA and also excreted fairly large amounts of MTA into the culture medium. The accumulation of MTA in the medium of cultures of cells deficient in MTA phosphorylase was blocked progressively by increasing concentrations of methyl glyoxal bis(guanylhydrazone) (MGBG) with almost complete inhibition at 10 μM MGBG, a drug which Williams-Ashman and Schenone [90] earlier showed to be a potent and selective inhibitor of putrescine-activated AdoMet decarboxylase in normal and also malignant [91] cells. Furthermore, it was shown [60] that there was a strict parallelism between the inhibitory effects of MGBG on both

accumulation of MTA in the medium and the entry of radioactivity from [3-^{3}H]ornithine into intracellular spermidine in one MTA phosphorylase deficient cell line. These results strongly suggest that all MTA formation by the cells under these conditions is due to polyamine biosynthesis. Interestingly, exogenous MTA added at concentrations of 5–50 μM progressively elevated both the synthesis and accumulation of putrescine in enzyme-negative cells yet suppressed spermine production. As considered below, MTA is a strong product inhibitor of spermine synthase. Germane to this is evidence recently obtained in three laboratories [92–94] that spermidine excercises a negative control *in vivo* on the levels of AdoMet decarboxylase, which if decreased in activity might evoke putrescine accumulation because of diminished spermidine and spermine formation.

Metabolism of 5-methylthioribose-1-phosphate

Early studies on methionine auxotrophs of *Enterobacter aerogenes* [95, 96], the yeast *Candida utilis* [97] and the protozoan *Ochromonas malhamensis* [98] suggested that the methylthio group of MTA can be salvaged by its incorporation into methionine. Considerable evidence [71, 97, 98] hints that transformation of part of the MTA molecule into methionine involves an intermediary production of MTR-1-P that is formed in appropriate organisms either by the enzymatic hydrolysis of MTA to 5-methylthioribose followed by phosphorylation of this sugar to MTR-1-P by its specific phosphokinase [71] or by the action of MTA phosphorylase. Shapiro and Schlenk [99] recently observed that when 5′-methylthio[U-^{14}C]adenosine was incubated with *Candida utilis*, a substantial proportion of the radioactivity in the labeled nucleoside became incorporated into the 4-carbon chain of methionine, but none of the label in the pentose moiety was found in cellular AdoMet; it was concluded that certain pentose carbons, as well as the methylthio group of MTA, can be utilized for methionine synthesis. Backlund and Smith [100] subsequently reported experiments in which 5′-[*methyl*-^{14}C]MTA, 5′-[*methyl*-^{3}H]MTA, 5′-[^{35}S]MTA and 5′-[*adenosine*-U-^{14}C]MTA were incubated in the presence of Mg^{2+} with ultracentrifuged soluble extracts of rat liver, and the entry of radioactivity into methionine was estimated. Carbons from the ribose portion, carbons and hydrogens of the methyl group, and the sulfur of MTA were incorporated into methionine. The data were consistent with the view that this transformation entailed modification of the ribose moiety of MTA into the 2-aminobutyrate part of methionine with no loss of the methylthio group. Furthermore, the kinetics of methionine labeling concurred with an initial phosphorolytic cleavage of MTA to MTR-1-P followed by conversion of the pentose phosphate to methionine. Independent investigations in this laboratory by J. Seidenfeld and H. G. Williams-Ashman similarly demonstrated that MTR-1-P, formed *in situ* from 5′-[*methyl*-^{14}C]MTA by action of purified MTA phosphorylase preparations (which alone did not convert any MTA to methionine), was readily converted to methionine by soluble extracts of rat liver and ventral prostrate at 37° and pH 7.4 with Mg^{2+} supplementation. A hypothesis [12] that MTR-1-P could transfer its methylthio group to homoserine lactone, and with participation of water to yield methionine and ribose-1-phosphate, seemed unlikely to explain the formation of labeled methionine under these conditions because exogenous homoserine lactone (5–10 mM) only occasionally evoked very small (about 10%) increases in methionine production from labeled MTA. Thus, it seems likely that during its conversion to methionine, the 1-carbon of MTR-1-P is removed followed by extensive alteration and amination of the rest of the carbon chain of the pentose moiety with retention of the methylthio group. Presumably this is effected by a multienzyme complex in animal tissue cytosols that may contain many of the necessary cofactors in bound form, and further experimental insight into these reactions will be eagerly awaited. The combined action of MTA phosphorylase and of a system converting MTR-1-P to methionine would, of course, provide complete salvage as adenine and methionine of all regions of decarboxylated AdoMet molecules except the aminopropyl groups that are utilized for spermidine and spermine biosynthesis.

Whether MTR-1-P can undergo other types of metabolic transformations is a moot point. The relationship of the methionine-forming system to the earlier mentioned "methylthiolase" system described by Toohey [59, 87] is altogether foggy, since the products of the latter reaction(s) have not been rigourously identified. One can imagine that MTR-1-P could participate in enzymatic methylthio group transfers to specific macromolecules. It is also conceivable that MTR-1-P serves as a precursor for methanethiol which is known to be formed in mammalian organisms, especially in animals with liver damage [101]. Although it is has been shown that non-enzymatic decomposition at high temperatures of α-keto-γ-methylthiobutyrate can yield methanethiol [102], that incubation of methionine with mitochondria resulted in formation of methanethiol which then binds to proteins [103], and that methanethiol is produced in liver from 3-methylthiopropionate (a metabolite of methionine) [104], the eventuality of enzymatic production of methanethiol from MTR-1-P should be borne in mind.

Parenthetically, it may be added that MTA and 5-methylthioribose (and presumably MTR-1-P) can, in solution, undergo slow oxidation to the corresponding (±)-sulfoxides in solution [71, 105] and that hydrogen peroxide greatly accelerates these processes [106]. Perhaps an artefactual conversion of MTA or MTR-1-P to their (±)-sulfoxides could take place when crude tissue extracts are incubated with these substances under conditions where endogenous formation of hydrogen peroxide or superoxide radicals is catalyzed by flavoprotein or other appropriate enzymes.

Actions of MTA on isolated enzymes and intact cells

Various direct effects of MTA on enzymatic reactions must now be discussed in greater depth than hitherto. Inhibitions by MTA of brain histamine methyltransferase (EC 2.1.1.8), pineal *N*-acetylserotonin methyltransferase (EC 2.1.1.14) [107] and

tRNA (guanine-7) methyltransferase (EC 2.1.1.33) of *Salmonella typhimurium* [108] are feeble in comparison to those evoked by AdoHcy. As considered above, MTA is a much less effective inhibitor than AdoHcy of protein methylase I, which methylates arginyl residues [79]. And in the case of brain protein methylase II (AdoMet:protein-carboxyl methyltransferase, EC 2.1.1.24) which synthesizes methyl esters of glutamyl and aspartyl residues (K_m AdoMet, 2 μM), competitive inhibition by AdoHcy (K_i 0.65 μM) is much more potent in comparison with MTA (K_i 41 μM) [109]. By contrast, MTA at concentrations of 15–50 μM is more active than AdoHcy as an inhibitor of a DNA modification methylase [110] and of a restriction endonuclease [111] and its attendant ATPase activity [112] in *E. coli* strain B.

Inhibitory effects of MTA on aminopropyl group transfers promoted by mammalian spermidine and spermine synthases represent product inhibitions that are reminiscent of inhibition of AdoMet-dependent methyltransferases by AdoHcy. The effects of MTA on mammalian polyamine synthases seem to vary with the source of the enzyme. Pajula and coworkers [113, 114] studied the action of MTA on homogeneous bovine brain spermine synthase, a protein of 88,000 daltons composed of two subunits of equal size and exhibiting an apparent K_m for decarboxylated AdoMet of 0.6 μM and for spermidine of 60 μM. Inhibition by MTA (K_i 0.3 μM) was profound and competitive with respect to decarboxylated AdoMet. A curious observation was that adenosine in the range of 0.2 to 1 mM hardly affected spermine synthesis *per se* but partially reversed the inhibition produced by MTA. AdoMet (10–500 μM) and AdoHcy (10–100 μM) had no influence on the spermine synthase reaction and did not reverse the MTA-induced inhibition. Somewhat different results were obtained by Hibasami *et al.* [22] in their studies on partially purified spermidine and spermine synthases from rat ventral prostate. At saturating (42 μM) decarboxylated AdoMet concentration, 100 μM MTA evoked 98 and 82% inhibition of spermine and spermidine synthase respectively. MTT, a non-substrate inhibitor of MTA phosphorylase, was about as effective an inhibitor of both polyamine synthases as MTA. In contrast to bovine brain spermine synthase which is unaffected by 100 μM AdoMet or AdoHcy [113], the rat prostate spermidine and spermine synthases were inhibited by 100 μM AdoMet by 30 and 75%, respectively, whereas both enzymes were less sensitive to inhibition by 100 μM AdoHcy [22]. The MTA phosphorylase substrate SIBA at 0.5 mM also caused considerable inhibition of prostate spermine synthase with lesser effects on spermidine synthase [115]. Attempts to gauge the relevance of the foregoing results to inhibition of spermine and spermidine synthesis in intact mammalian cells must take into account: (a) the very low concentrations of decarboxylated AdoMet (1–2 nmoles/g) and MTA (< 3 nmoles/g) in many tissues (*vide supra*), and (b) the fact that certain synthetic substrates (e.g. SIBA) and non-substrate inhibitors (e.g. MTT) of MTA phosphorylase, which among other things might be expected to elicit a pile-up of MTA in cells, are also potent inhibitors of spermine synthase and to a lesser extent of spermidine synthase.

Another attribute of MTA is its ability to inactivate AdoHcy hydrolase when it is preincubated with the enzyme in the absence of its substrates. Hershfield [66] showed that several adenine nucleosides, notably 2'-deoxyadenosine, can irreversibly inactivate AdoHcy hydrolase, and mentioned that MTA exerts a similar action. The suicide-like inactivation of AdoHcy by 2-deoxyadenosine and MTA follows first-order kinetics for some 2–3 half-lives, is saturable by appropriate nucleosides, and is protected against by AdoHcy [116]. Very recently, Ferro *et al.* [117] reported that highly purified human erythrocyte AdoHcy hydrolase is inactivated at nearly the same rate by almost saturating concentrations (600 μM) of either 2'-deoxyadenosine or MTA at pH 7.6 with an apparent half-life of the enzyme of roughly 10 min. The concentration of MTA required for 50% of maximal rate of inactivation was 36 μM. Neither adenine nor 5-methylthioribose was effective. Hershfield *et al.* [116] and Abeles *et al.* [118] have discussed possible mechanisms of suicide inactivation of AdoHcy by active adenine-containing nucleosides. Such inactivation of the enzyme in living cells could conceivably result in accumulation of AdoHcy intracellularly with resultant inhibition of many methyltransferases. However, considering the very low levels of MTA [40,41] *vis-à-vis* the considerably higher concentrations of AdoHcy [46, 47] in many normal mammalian cells, it appears equivocal whether inactivation of AdoHcy by endogenous MTA could significantly perturb AdoHcy levels *in vivo*, although this might be more likely in certain malignant cells that, as previously considered, apparently lack MTA phosphorylase [59, 61] and also could be pertinent to some cytostatic effects of high concentrations of MTA added to cells in culture (see below).

Savarese *et al.* [62] raise the possibility that pentose-1-phosphate products of the action of MTA phosphorylase on MTA and other of its substrates might exert regulatory effects on certain enzymes of carbohydrate metabolism, such as phosphoglucomutase, which is believed also to catalyze the phosphoribomutase reaction.

Prior to overviewing certain cytostatic effects of exogenous MTA on cultured cells, something must be said about the cellular uptake of this nucleoside. Perfused rat liver rapidly sequesters MTA from the external medium [119]. MTA also easily penetrates into mammalian non-nucleated erythrocytes and can be metabolized therein. In order to distinguish between membrane transport and intracellular catabolism of MTA by red blood cells, Della Ragione *et al.* [120] studied the uptake and intracellular accumulation of the labeled nucleoside by ATP-depleted and also inorganic phosphate-depleted erythrocytes. They concluded that MTA uptake under physiological circumstances is the result of a tandem action of: (1) a saturable carrier-mediated membrane transport system of high capacity and relatively low affinity, and (2) a metabolic step (almost certainly catalyzed by MTA phosphorylase) of low capacity and high affinity. AdoMet apparently enters mammalian cells much less readily than MTA

[119, 121], and the passage of AdoHcy across cell plasmalemmas is sluggish if it occurs at all [121].

The inhibitory effects of MTA and MTT on lectin-induced lymphocyte proliferation [9, 56, 58] have already been mentioned. Pegg *et al.* [115] investigated the effects of MTA and related nucleosides on SV-40-virus-transformed 3T3 mouse embryo fibroblasts. MTA, SIBA and MTT (all of which directly inhibit spermine synthase to a greater extent than spermidine synthase) at 25 μM concentrations significantly retarded cell growth, and at 200 μM elicited more than 70% inhibition of proliferation. Exposure to 200 μM MTT caused a 75% decrease in spermidine but, strangely enough, produced only a considerably smaller decline in spermine. MTA and SIBA also diminished intracellular spermidine concentrations but were less effective than MTT. However, exogenous spermidine did not reverse the growth inhibitory effects of MTA, MTT or SIBA which suggests that spermidine depletion by these thioalkyl nucleosides is not the sole cause of their cytostatic effects. By contrast, depression of cell growth by α-difluoromethylornithine (a suicide inhibitor of ornithine decarboxylase) or MGBG (which inhibits AdoMet decarboxylase), which resulted in marked depletion of intracellular spermidine and spermine, was readily reversed by addition of spermidine to the medium. The inhibition of cell proliferation by MTA and SIBA (but not MTT) could conceivably be related to intracellular formation of adenine liberated by the action of MTA phosphorylase on these nucleosides. Pegg *et al.* [115] found that 100 μM adenosine, especially when combined with adenosine deaminase inhibitors, strongly depressed the growth of SV-40-3T3 cells; but adenosine apparently cannot be formed *directly* from MTA or its enzymatic cleavage product adenine in mammalian cells, and the effects of adenine on cell growth under comparable conditions were not reported.

The results just considered emphasize the difficulties in interpretation of studies on various effects of addition of unphysiologically high concentrations of MTA or other MTA phosphorylase substrates to growing mammalian cells. Furthermore, MTA can exert other effects on intact cells besides those discussed. For example, Galletti *et al.* [122] showed that 100 μM MTA added to erythrocytes evoked 50% inhibition of incorporation of radioactivity from [*methyl*-^{3}H]methionine into membrane protein methyl ester groups. SIBA and adenine but not adenosine were also highly inhibitory under the same conditions. (Other contributions by Galletti *et al.* [123–125] and Freitag and Clarke [126] provide details of the utilization of methionine, via endogenous AdoMet formation and protein methylase II action, for methylation of erythrocyte membrane proteins *in vivo* and *in vitro*.) In a similar vein, it is hard to gauge whether the inhibitory effects of MTA on α-amanitin-sensitive RNA synthesis [127] and puff induction in giant chromosomes [128] in salivary glands of *Drosophila melanogaster* are due to direct actions of MTA or to products of its metabolic degradation.

When administered to living mammalian organisms, MTA elicits a variety of pharmacologic effects including lowering of body temperature of guinea pigs [129] and depression of rabbit blood pressure [130]. MTA also elicits relaxation of intestinal strips in rabbits and contraction of isolated guinea pig uterus [130]. It seems possible that these effects could be related, at least in part, to the demonstrated interactions of MTA with specific adenosine receptors on surfaces of appropriate cells [131, 132], which also has neuropharmacologic implications [132]. The observation that MTA reverses the inhibitory effect of adenosine on ADP-induced blood platelet aggregation [133] at present defies simple explanation.

Genetic aspects of MTA metabolism

The key role of MTA phosphorylase for the metabolism and some of the biological actions of MTA is highlighted in several places in this article, particularly with respect to the concept that phosphorolytic cleavage of MTA may provide the major, if not the sole, non-dietary source of adenine in mammalian cells. Kamatani and Carson [134] have just reported some pertinent investigations on normal WI-L2 human lymphoblastic cell lines that are replete with MTA phosphorylase and on a variant of these cells that is deficient in APRT and thus cannot convert adenine to 5′-AMP. Whereas culture of the wild-type cells did not produce adenine at detectable rates, considerable excretion of adenine was observed with the APRT-deficient cell line. The rate of formation of adenine by the APRT-negative cells was about 10% of the speed of hypoxanthine production in a separate hypoxanthine-guanine phosphoribosyl transferase (EC 2.4.2.8)-deficient variant of the WI-L2 cell line, whereas adenine formation was close to that of MTA production as determined in the MTA phosphorylase-negative cells. MGBG at 10 μM almost totally inhibited synthesis of adenine by APRT-deficient cells and also of MTA by another variant that lacked MTA phosphorylase, whereas hypoxanthine production was not affected by 100 μM MGBG. Addition of 50 μM spermidine or spermine (which depress endogenous polyamine production) to APRT-negative cells decreased adenine accumulation by about 30% and diminished MTA formation by the separate MTA phosphorylase-negative line to a somewhat greater extent. It was also found that 2-fluoroadenine, a strong competitive inhibitor of MTA phosphorylase was highly toxic for the wild-type WI-L2 cells but did not significantly influence growth of the APRT-deficient line. However, 2-fluoroadenine inhibited adenine accumulation in the APRT-negative cells in a dose-dependent fashion with a reciprocal increase in MTA formation. Taken together, these findings indicate that adenine is produced predominantly via polyamine biosynthesis yielding MTA which is then cleaved by MTA phosphorylase, rather than via phosphorolysis of adenosine and/or 2-deoxyadenosine. Although the latter eventuality has been postulated [135], it seems unlikely since the concentrations of adenosine and 2′-deoxyadenosine in normal mammalian tissues are strikingly low, and also because the latter nucleosides are extremely inefficient substrates for purine nucleoside phosphorylase [15]. It is of interest that, although complete deficiency of APRT in man is apparently very rare indeed [136–138], the incidence of heterozy-

gosity for APRT deficiency is estimated to be in the range of 0.4 to 1.1% of the population [139, 140]. As discussed above, a substantial number of human malignant cells in culture lack detectable MTA phosphorylase but it is not entirely clear whether this primarily reflects any mutation of the MTA phosphorylase gene. It would be worthwhile to hunt for congenital MTA phosphorylase deficiencies in man. Since the enzyme is present in erythrocytes, screening of a large number of patients, including neonates, should be quite feasible.

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