Biochemical Pharmacology, Vol. 32, No. 12, pp. 1907–1916, 1983.
Printed in Great Britain.

0006-2952/83 $3.00 + .00

5′-DEOXY-5′-METHYLTHIOADENOSINE PHOSPHORYLASE—II

ROLE OF THE ENZYME IN THE METABOLISM AND ANTINEOPLASTIC ACTION OF ADENINE-SUBSTITUTED ANALOGS OF 5′-DEOXY-5′-METHYLTHIOADENOSINE*

TODD M. SAVARESE†‡§, DANIEL L. DEXTER‡ and ROBERT E. PARKS, JR.†

†Section of Biochemical Pharmacology, Division of Biology and Medicine, Brown University, and ‡Department of Medicine, Roger Williams General Hospital, Providence, RI 02912, U.S.A.

and

JOHN A. MONTGOMERY

Kettering-Meyer Laboratory, Southern Research Institute, Birmingham, AL 35255, U.S.A.

(*Received* 20 *September* 1982; *accepted* 22 *November* 1982)

Abstract—The biological activities of several previously synthesized [J. A. Montgomery *et al.*, *J. med. Chem.* **17**, 1197 (1974)] adenine-substituted analogs of 5′-deoxy-5′-methylthio- or 5′-deoxy-5′-ethylthioadenosine, including the 2-fluoroadenine, 2-chloroadenine, 2,6-diaminopurine, 8-azaadenine, and 4-aminopyrazolo[3,4-*d*]pyrimidine-containing derivatives, have been reexamined. It is demonstrated that many of these analogs are cleaved to their respective free base analogs by 5′-deoxy-5′-methylthioadenosine phosphorylase (MTAPase), an enzyme associated with polyamine biosynthesis, and that this reaction is necessary for the cytotoxic action of these MTA analogs to be fully expressed. Evidence to support this includes: (1) the growth of two MTAPase-containing human colon carcinoma cell lines (the HCT-15 and DLD-1 lines) was inhibited by these analogs, whereas an MTAPase-deficient cell line, the CCRF-CEM human T-cell leukemia, was relatively insensitive to their cytotoxic action; (2) extracts of the MTAPase-containing colon carcinoma cell lines were able to cleave these analogs to their respective free base analogs; in contrast, extracts of MTAPase-deficient CCRF-CEM cells were unable to cleave these analogs; (3) intact colon carcinoma cells converted these MTA analogs to their corresponding 5′-phosphorylated analog nucleotides, whereas CCRF-CEM cells did not, at least to detectable levels; and (4) the MTA analog, 5′-deoxy-5′-ethylthio-4-aminopyrazolo[3,4-*d*]pyrimidine ribonucleoside, which is not a substrate of MTAPase, did not form analog nucleotides and was essentially noncytotoxic to all cell lines tested, whereas the corresponding adenine analog, 4-aminopyrazolo[3,4-*d*]pyrimidine, readily formed analog nucleotides and was highly cytotoxic to all the lines. It is postulated that the corresponding adenine analog 5′-phosphorylated nucleotides are the primary active metabolites of these MTA analogs, having been formed by the cleavage of these nucleosides to free adenine analogs by MTAPase, followed by the conversion of these base analogs to analog nucleotides by adenine phosphoribosyltransferase and the enzymes of adenine nucleotide phosphorylation. This pathway represents a novel drug-activation system for the synthesis of analog nucleotides and has the potential to be exploited chemotherapeutically.

* This investigation was supported by PHS Grants CA 07340, CA 20892, CA 13943, and CA 09204 (Training Program in Cancer Research) awarded by the National Cancer Institute, DHHS.

§ Author to whom all correspondence should be addressed.

‖ Abbreviations used: AdoMet, *S*-adenosyl-L-methionine; 4-APP, 4-aminopyrazolo[3,4-*d*]pyrimidine; APRT, adenine phosphoribosyltransferase (EC 2.4.2.7); 2,6-DAP, 2,6-diaminopurine; 5′-ETAPPR, 4-amino-1-[5-deoxy-5-(ethylthio)-β-D-ribofuranosyl]pyrazolo[3,4-*d*]pyrimidine; 5′-ETDAPR, 2-amino-5′-deoxy-5′-(ethylthio)adenosine; 5′-ETFAR, 5′-deoxy-5′-(ethylthio)-2-fluoroadenosine; HEPES, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid; HPLC, high performance liquid chromatography; MTA, 5′-deoxy-5′-(methylthio)adenosine; MTAPase, 5′-deoxy-5′-(methylthio)adenosine phosphorylase; 5′-MTA-zaAR, 7-amino-3-[5-deoxy-5-(methylthio)-β-D-ribofuranosyl]-*v*-triazolo[4,5-*d*]pyrimidine; 5′-MTClAR, 2-chloro-5′-deoxy-5′-(methylthio)adenosine; and PNP, purine nucleoside phosphorylase (EC 2.4.2.1).

5′-Deoxy-5′-methylthioadenosine phosphorylase (MTAPase) is responsible in mammalian tissues for the metabolism of 5′-deoxy-5′-methylthioadenosine (MTA), which is generated from *S*-adenosylmethionine (AdoMet) primarily during the synthesis of the polyamines, spermidine and spermine [1, 2]; MTA can also be formed directly from AdoMet [3, 4].‖ MTAPase catalyzes the reaction:

$$\text{MTA} + P_i \rightleftharpoons$$

$$\text{Adenine} + \text{5-methylthioribose-1-phosphate}$$

Originally described in the rat ventral prostate in 1969 [5], this enzyme has been identified recently in a wide range of mammalian tissues [6–14]. Several cultured neoplastic cell lines, however, lack MTAPase activity [10, 13, 15].

An important physiological role of the enzyme is to convert MTA to salvageable intermediates. One

product, adenine, can react with 5-phosphoribosyl-1-pyrophosphate to form 5′-AMP and PP_i as catalyzed by adenine phosphoribosyltransferase (APRT), and, thereby, rejoin intracellular adenine nucleotide pools. The other product, 5-methylthioribose-1-phosphate, has been shown recently to be converted to L-methionine by a not yet fully elucidated series of reactions [16–18].

The finding that MTAPase can utilize an analog of MTA, 5′-deoxyadenosine, as a substrate, and that the metabolism of this compound results in a number of biochemical perturbations [14], encouraged us to explore the use of this enzyme and its associated salvage pathways as novel antimetabolite-activating systems. Our approach was to examine various analogs of MTA as alternative substrates of MTAPase, and to determine whether the products of this reaction were cytotoxic, or were metabolized to cytotoxic derivatives.

One class of MTA analog antimetabolites is that in which the adenine moiety of MTA is replaced by an adenine analog. In 1974, Montgomery *et al.* [19] reported the synthesis of a variety of adenine-substituted derivatives of 5′-deoxy-5′-methylthio- and 5′-deoxy-5′-ethylthioadenosine, including those containing 2-fluoroadenine, 2-chloroadenine, 2,6-diaminopurine, 8-azaadenine, or 4-aminopyrazolo-[3,4-*d*]pyrimidine (for structures, see Fig. 1). The original rationale for the formulation of these compounds was that, as analogs of MTA, they might interfere with polyamine and/or AdoMet metabolism. Some of these compounds, especially 5′-deoxy-5′-ethylthio-2-fluoroadenosine (5′-ETFAR), were found to be growth inhibitory to cultured H.Ep.-2 cells. Although the metabolism of these compounds was not studied, it was noted that an H.Ep.-2 line lacking APRT activity and resistant to 2-fluoroadenine was cross-resistant to 5′-ETFAR, indicating that cleavage of this compound to the free base was necessary for cytotoxicity [19].

This observation, and the close structural similarity of these analogs to MTA, suggested that the metabolism and action of these compounds might involve MTAPase. The present studies demonstrate that many of these analogs are, in fact, substrates for MTAPase and that their intracellular cleavage to free adenine analogs by this enzyme is a crucial step in the mechanism by which these MTA analogs exert cytotoxicity in MTAPase-containing cell lines. Preliminary reports describing portions of this work have been communicated [20, 21].

MATERIALS AND METHODS

Materials. Analogs of MTA were synthesized as reported previously [19]. 2-Chloroadenine was supplied by Dr. S. H. Chu, Brown University. 5′-Deoxy-5′-methylthioadenosine, 2,6-diaminopurine sulfate, 8-azaadenine, 4-aminopyrazolo[3,4-*d*]-pyrimidine, and buttermilk xanthine oxidase (Grade III) were purchased from the Sigma Chemical Co. (St. Louis, MO). Calf spleen purine nucleoside phosphorylase was obtained from Boehringer Mannheim (Indianapolis, IN). 5′-Deoxy-5′-ethylthioadenosine was synthesized from *S*-adenosylethionine (obtained from Sigma) as described by Zappia *et al.* [6]. 2-Fluoroadenine (NSC 27364) was obtained from the NCI.

In vitro *cytotoxicity studies.* The establishment in culture of the HCT-15 [22] and DLD-1 [23] human colon carcinoma lines has been reported. *In vitro* cytotoxicity studies were carried out using a modification of a previously described procedure [24]. On day 0, replicate 35 mm tissue culture dishes (Falcon Plastics, Oxnard, CA) were inoculated with 1×10^5 cells in RPMI 1640 medium (10% heat-inactivated fetal calf serum) supplemented with penicillin (100 units/ml), streptomycin (100 μg/ml), fungizone (25 μg/ml), tylosine (30 μg/ml) and gentamycin (20 μg/ml). The medium was buffered with 0.075% $NaHCO_3$, 10 mM HEPES, and 10 mM Tris. After

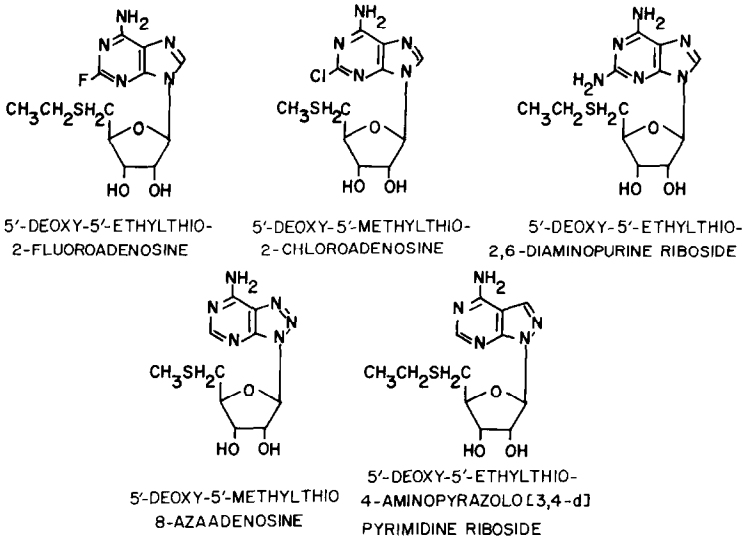

Fig. 1. Structures of several adenine-substituted analogs of MTA.

24 hr (day 1) the cells were rinsed twice with saline. Drugs were added to each plate at concentrations ranging from 10^{-7} to 2×10^{-4} M in an RPMI 1640 medium (2 ml final volume) supplemented with 15% horse serum. Horse serum, unlike fetal calf serum, contains no MTAPase activity [15] and is, therefore, appropriate for use in cell culture studies involving MTA and its analogs. Also on day 1, cells from two dishes were harvested by trypsin–EDTA treatment and counted in a hemacytometer to determine the number of cells per dish at the time of drug addition. On day 5, drug-treated and control cultures were harvested by trypsin–EDTA treatment and counted. The number of doublings for the nontreated cells was calculated from the cell number in control dishes on days 1 and 5; usually 3.4 to 4.0 cell doublings were observed during this 96-hr period. The molar drug concentration required to inhibit the number of doublings over this period by 50% (IC_{50}) was calculated from a dose–response curve, in which cell numbers were plotted against drug concentration.

The CCRF-CEM human T-cell leukemia line, whose establishment in culture has been described [25], was obtained from Dr. Michael Wiemann of the Roger Williams General Hospital, Providence, RI. CCRF-CEM cells were routinely grown as suspension cultures in an RPMI 1640 medium supplemented with 10% fetal calf serum, buffers, and antibiotics (see above) in a humidified incubator maintained at 95% air: 5% CO_2 at 37°. For cytotoxicity studies, cells were first washed several times in RPMI 1640 medium containing 15% horse serum. They were then introduced into 16 × 125 mm culture tubes (Falcon Plastics) containing 3 ml of the 15% horse serum-containing medium, to which analogs (2×10^{-4} to 10^{-7} M) had been added. The final cell concentration was 1×10^5 cells/ml. After 96 hr of incubation, the cell number was determined using a hemacytometer; during this period, untreated cells underwent between 2.5 and 3.2 doublings.

Reversed-phase high performance liquid chromatography. The liberation of adenine-like bases from adenine-substituted MTA analogs was monitored directly by reversed-phase HPLC, using a Varian 4200 high performance liquid chromatograph equipped with a Waters μBondapak C_{18} analytical column (0.39 × 30 cm). Separation of bases from nucleosides was obtained using a modification of a previously described reversed-phase HPLC procedure [14]. In these studies, the low concentrate eluent was 10 mM potassium phosphate, pH 5.5, and the high concentrate eluent was 50% methanol, 5 mM potassium phosphate, pH 5.5. The gradient profile consisted of a linear increase of high concentrate eluent from 0 to 100% at a rate of 9%/min, followed by a 6-min isocratic elution at 100% high concentrate eluent, followed by a linear decrease in the level of high concentrate eluent (−9%/min) until 0% was reached. The flow rate was 1.0 ml/min. Adenine analog bases were identified based on the retention times of authentic standards. A Schoeffel SF 770 variable wavelength detector was used to monitor the formation of adenine analogs with λ_{max} values not close to 254 nm. To facilitate identification in some cases, the u.v. spectra of unknown peaks collected from the HPLC column were determined with a Perkin Elmer 402 spectrophotometer and compared with spectra of authentic standards. Reversed-phase HPLC was also used to verify the purity of the MTA analogs; all produced single, homogeneous peaks under these chromatographic conditions.

Anion-exchange high performance liquid chromatography. Natural and analog nucleotides were measured with an anion-exchange HPLC technique that employed a Varian model 5000 liquid chromatograph equipped with a Whatman Partisil PXS 10/25 SAX column (5 × 160 mm). Separation of the various nucleotides was achieved with a programmed gradient using 1 mM potassium phosphate (pH 4.5) as the low concentrate (starting) eluent and 500 mM potassium phosphate (pH 4.5) as the high concentrate eluent. The gradient profile consisted of a linear increase of high concentrate eluent from 0 to 100% over a 40-min period, followed by a 20-min isocratic elution at 100% high concentrate eluent. The flow rate was 1.2 ml/min. In addition to u.v. monitoring at 254 nm to detect naturally occurring nucleotides, the Schoeffel SF 770 variable wavelength monitor was used to simultaneously detect analog nucleotides that absorb well at other wavelengths (295 nm for 2,6-diaminopurine-containing compounds; 278 nm for 8-azaadenine-containing compounds; 275 nm for 4-aminopyrazolo[3,4-*d*]pyrimidine-containing compounds). For nucleotide incorporation studies involving 5′-ETFAR or 5′-MTClAR, the eluting buffers were adjusted to pH 3.9 and 3.5, respectively, in order to separate the 2-haloadenine-containing nucleotides from adenine nucleotides.

Other assays. MTAPase activity was assayed using a previously described spectrophotometric method [14]. Protein was determined by the method of Bradford [26], using bovine γ-globulin (Sigma) as a standard.

RESULTS

Cytotoxicity of MTA analogs against MTAPase-containing and MTAPase-deficient cell lines. If adenine-substituted analogs of MTA, such as those in Fig. 1, are substrates of MTAPase, they would be phosphorolyzed intracellularly, liberating adenine analog bases of known cytotoxicity, such as 2-fluoroadenine [27] or 2,6-diaminopurine [28]. These base analogs could then be converted to adenine analog nucleotides by APRT [29, 30] and the enzymes of adenine nucleotide phosphorylation [31–33]. These analog nucleotides might then inhibit cell growth by becoming incorporated into nucleic acids, by interfering with nucleotide synthesis, and other effects [27, 34]. If the above hypothesis is correct, these MTA analogs should exert cytotoxic effects only on those cell lines that contain MTAPase; cell lines that lack MTAPase should be unable to activate these analogs and, therefore, should be insensitive to these agents.

To test this postulate, the cytotoxic activities of these MTA analogs against two cultured human colon carcinomas lines that contain relatively high levels of MTAPase [9], the HCT-15 and DLD-1 lines, were compared to their activities against a cell line which is deficient in MTAPase, the CCRF-CEM

Table 1. *In vitro* cytotoxicity of various adenine-substituted MTA analogs and their corresponding bases against MTAPase-containing and MTAPase-deficient human tumor cell lines

	HCT-15 colon carcinoma	DLD-1 colon carcinoma	CCRF-CEM leukemia
MTAPase activity* (nmolar units/mg protein)	1.6 ± 0.2†	2.2 ± 0.03	< 0.01
		IC_{50}‡ (μM)	
5′-Ethylthio-2-fluoroadenosine (5′-ETFAR)	0.4 ± 0.2	0.5 ± 0.2	> 15
2-Fluoroadenine	0.7 ± 0.0	0.5 ± 0.2	0.1 ± 0.0
5′-Ethylthio-2,6-diaminopurine ribonucleoside (5′-ETDAPR)	116 ± 4	186 ± 29	> 200
2,6-Diaminopurine	42 ± 3	49 ± 10	41 ± 7
5′-Ethylthio-4-aminopyrazolo[3,4-*d*]pyrimidine ribonucleoside (5′-ETAPPR)	> 200	> 200	> 200
4-Aminopyrazolo[3,4-*d*]pyrimidine	0.6 ± 0.1	0.5 ± 0.4	4 ± 3
5′-Methylthio-2-chloroadenosine (5′-MTClAR)	157 ± 40	163 ± 26	> 200
2-Chloroadenine	35 ± 1	15 ± 6	48 ± 19
5′-Methylthio-8-azaadenosine (5′-MTAzaAR)	185 ± 21	ND§	> 100
8-Azaadenine	47 ± 8	24 ± 9	68 ± 10

* MTAPase activity was determined by the spectrophotometric method described in Ref. 14.
† Data from Ref. 9.
‡ The IC_{50} values were determined as described under Materials and methods. Values represent the mean ± S.D. of two to four independent determinations.
§ Not determined.

human T-cell leukemia [15] (see Table 1). In general, MTA analogs such as 5′-ETDAPR, 5′-MTClAR, and 5′-MTAzaAR exerted a dose-dependent, growth-inhibitory effect on the MTAPase-containing colon carcinoma cell lines, although the IC_{50} values for these analogs were high (100–200 μM). The corresponding base analogs, representing the presumed product of the intracellular interaction of these MTA analogs with MTAPase, were relatively potent inhibitors of colon carcinoma cell growth, with IC_{50} values 3- to 11-fold less than those for their counterpart MTA-like nucleosides.

By far, the most cytotoxic MTA analog studied was the 2-fluoroadenine-containing derivative, 5′-ETFAR: the IC_{50} value of this analog for the HCT-15 colon carcinoma line, 0.4 μM, was some 300- to 450-fold lower than that for any other nucleoside tested. In fact, 5′-ETFAR was the only MTA analog whose IC_{50} value was similar to that of its corresponding base analog. The 4-aminopyrazolo[3,4-*d*] pyrimidine-containing MTA analog, 5′-ETAPPR, was the least growth inhibitory to the colon carcinoma lines of those tested; even at 200 μM, 5′-ETAPPR inhibited growth only 10–15% relative to controls. The poor cytotoxicity of 5′-ETAPPR was in marked contrast to the very strong growth inhibitory effect of the aglycone, 4-aminopyrazolo[3,4-*d*] pyrimidine (4-APP), suggesting that 5′-ETAPPR is cleaved at a very slow rate, if at all.

In contrast, the growth of the MTAPase-deficient CCRF-CEM leukemia line was only weakly inhibited by these adenine-substituted MTA analogs. Even at 200 μM, 5′-ETDAPR, 5′-MTClAR, and 5′-ETAPPR caused only modest growth inhibition (45% or less relative to controls). 5′-ETFAR at a concentration of 15 μM (over 35-fold greater than the IC_{50} value of this compound for HCT-15 cells) inhibited CCRF-CEM growth only by 25%. Nevertheless, the CCRF-CEM cells were sensitive to all of the corresponding base analogs, indicating that the lack of response of this line to the MTA analogs is due to its inability to cleave these nucleosides. Despite the difficulties in comparing IC_{50} values obtained from cell lines grown in monolayer versus those grown in suspension, the striking difference in the sensitivity of the MTAPase-containing and -deficient cell lines to these MTA analogs suggests that the presence of MTAPase is required for the cytotoxic action of these MTA analogs to be fully expressed.

Metabolism of MTA analogs in MTAPase-containing and MTAPase-deficient cell lines. To test the hypothesis that MTAPase is responsible for generating cytotoxic adenine-like bases from these MTA analogs, the metabolism of these MTA derivatives was compared in the MTAPase-containing and -deficient cell lines. When 105,000 *g* supernatant fluids of sonicated DLD-1 colon carcinoma cells were incubated for 2 hr with 200 μM 5′-ETDAPR in the presence of orthophosphate, a substantial portion of this nucleoside was converted to the corresponding free base, 2,6-diaminopurine (2,6-DAP), as monitored by reversed-phase HPLC (Fig. 2, A and B). The putative 2,6-DAP peak was identified based on the similarity of its retention time and u.v. spectrum with that of authentic 2,6-DAP. Extracts of the MTAPase-containing colon carcinoma lines were also able to cleave 5′-ETFAR, 5′-MTClAR, and 5′-MTAzaAR to their respective free adenine analogs. However, these extracts were unable to produce 4-APP from 200 μM 5′-ETAPPR after a 2-hr incubation period; even after 24 hr, only trace amounts of 4-APP were formed (results not shown). In contrast, 105,000 *g* supernatant fluids of sonicates of MTAPase-deficient CCRF-CEM cells, at protein concentrations equivalent to those of the colon car-

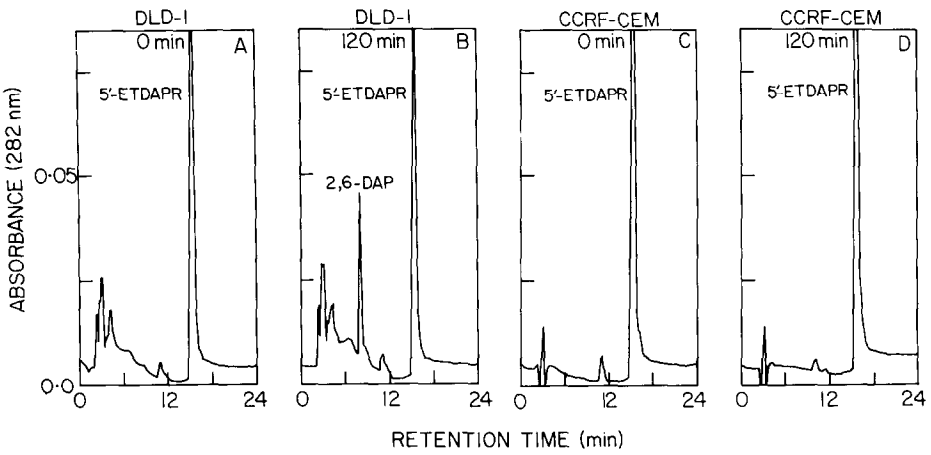

Fig. 2. (A and B): Reversed-phase HPLC profiles demonstrating the liberation of 2,6-DAP from 5'-ETDAPR in the presence of extracts of MTAPase-containing DLD-1 colon carcinoma cells. Cells (approx. 7.5×10^7) were grown in 100 mm plastic dishes in RPMI 1640 medium supplemented with 10% fetal calf serum in a humidified incubator maintained in an atmosphere of 95% air: 5% CO_2 at 37°. When the plates were confluent, cells were harvested in saline with a rubber policeman, washed in saline, and resuspended in a 50 mM potassium phosphate (pH 7.4), 1 mM dithiothreitol buffer. The suspension was then sonicated and centrifuged at 105,000 *g* for 45 min. The resulting supernatant fluid was dialyzed against several changes of phosphate buffer for 3 hr at 4°. Reaction mixtures (1 ml) containing 200 μM 5'-ETDAPR, 50 mM potassium phosphate (pH 7.4), and the dialyzed, high-speed supernatant fluid of DLD-1 cell extracts (500 μg protein) were incubated in a shaking water bath at 37°. At the indicated times, 0.5-ml aliquots of the reaction mixture were added to 0.125 ml of 20% perchloric acid at 4°, and, following centrifugation to remove precipitated material, the supernatant fluid was adjusted to pH 6.5–7.5 with 5 N KOH. The samples were then centrifuged to remove perchlorate salts, and the supernatant fluids were frozen at −20°. Separation of 2,6-DAP and 5'-ETDAPR was achieved using the reversed-phase HPLC technique described under Materials and methods. Identification of the 2,6-DAP peak is outlined in the text. (C and D): Reversed-phase HPLC profiles demonstrating the inability of extracts of MTAPase-deficient CCRF-CEM leukemia cells to liberate 2,6.-DAP from 5'-ETDAPR. Reactions and chromatography were carried out as described above for the DLD-1 cell line, except dialyzed high-speed supernatant fluids of CCRF-CEM cells (500 μg protein/ml) were used.

cinoma extracts, were unable to produce 2,6-DAP from 5'-ETDAPR (Fig. 2, C and D). In fact, none of the MTA analogs was cleaved by extracts of this cell line over a 2-hr incubation period.

Although the above finding indicated that the presence of MTAPase is required for these MTA analogs to be metabolized, it was necessary to obtain additional evidence that MTAPase, and not some other enzyme, was responsible for the cleavage of these nucleosides. To examine this, MTAPase from cultured DLD-1 colon carcinoma cells was partially purified by ammonium sulfate fractionation and Sephadex G150 gel filtration chromatography. As shown in Fig. 3, cleavage activity of two representative adenine-substituted MTA analogs, 5'-ETDAPR and 5'-MTClAR, remained associated with MTAPase activity, at least at this low level of purification (3-fold). In addition, these analogs were not cleaved in the absence of orthophosphate, ruling out any involvement of a hydrolase in the metabolism of these derivatives. The analogs were also unable to react with purine nucleoside phosphorylase (PNP), the only other known mammalian phosphorylase that is specific for purine nucleosides. The finding that these MTA analogs are not substrates of PNP was corroborated by the observation that these compounds were not phosphorolyzed by CCRF-CEM extracts, which, despite lacking MTAPase, contained PNP activity at a level (37.6 ± 10.8 nmolar units/mg protein) approximately equivalent to that found in the colon carcinoma lines [9].

In intact cells, it is expected that any adenine analog liberated from these MTA derivatives would be converted to analog adenine nucleotides by the adenine salvage pathway. To test this, an MTAPase-containing [9] subline of the DLD-1 colon carcinoma, designated DLD-1 (Clone A) [23], was incubated with 250 μM 5'-ETDAPR for 24 hr during logarithmic growth; for comparison, parallel experiments were performed using equimolar amounts of the free base analog, 2,6-DAP. The cells were then examined for analog nucleotide formation by anion-exchange HPLC (Fig. 4, A–C). The nucleotide profile of the untreated cells shows only the naturally occurring nucleotides (monitored at 254 nm); very little absorbance was noted at 295 nm, a wavelength that can be used to detect 2,6-DAP-containing nucleotides [33]. However, in the profiles treated with 5'-ETDAPR, peaks corresponding to 2,6-diaminopurine ribonucleoside diphosphate (DDP) and triphosphate (DTP) appeared. In cells treated with 2,6-DAP, the presumed cytotoxic metabolite, DTP accumulated to an even greater extent (2-fold) than in those treated with 5'-ETDAPR during the 24-hr exposure period. This finding correlated with the observation that 2,6-DAP is about 3-fold *more* cytotoxic than 5'-ETDAPR against the colon carcinoma lines (Table 1). It also suggested that the

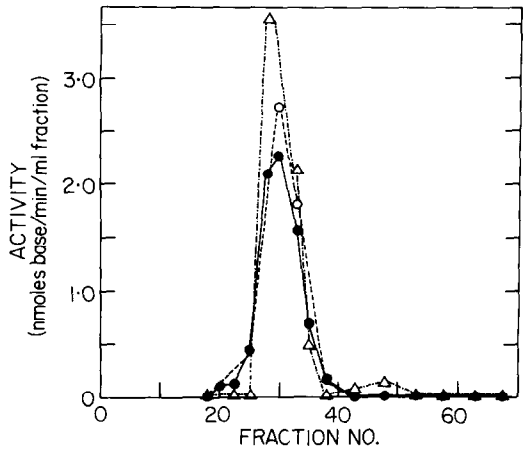

Fig. 3. Association of 5′-ETDAPR and 5′-MTClAR cleavage activities with DLD-1 colon carcinoma MTAPase after partial purification of the enzyme by ammonium sulfate fractionation and Sephadex G150 gel filtration. High speed (105,000 *g*) supernatant fluids were prepared from sonicates of DLD-1 cells (approx. 4×10^8) as described in Fig. 2. These supernatant fluids were then subjected to ammonium sulfate fractionation; the precipitate that formed between 40 and 65% ammonium sulfate saturation was resuspended in a 50 mM potassium phosphate (pH 7.4), 1 mM dithiotheitol buffer. This enzyme preparation, containing approx. 45 nmolar units of MTAPase, was then applied to a 1.75 × 80 cm Sephadex G150 column that had been equilibrated with the above-mentioned buffer. Gel filtration chromatography was carried out at a flow rate of 0.3 ml/min; 2.1-ml fractions were collected. Aliquots (0.3 ml) of the fractions were assayed for MTAPase activity in reaction mixtures (1 ml) containing 100 μM MTA, 40 mM potassium phosphate, and 0.8 μmolar units xanthine oxidase, by the spectrophotometric method as outlined previously [14]. 5′-ETDAPR and 5′-MTClAR cleavage activities were assayed by monitoring the appearance of the respective free adenine analogs by reversed-phase HPLC. Reaction mixtures (0.4 ml) containing 250 μM MTA analog, 40 mM potassium phosphate, and aliquots (0.3 ml) of the fractions were incubated in a shaking water bath at 37° for 5 min. The reaction was stopped, and the samples were processed as described in Fig. 2. The reversed-phase HPLC method employed to detect free adenine analog bases is detailed under Materials and methods. Key: MTAPase activity (●——●), 5′-ETDAPR cleavage activity (○ - - ○), and 5′-MTClAR cleavage activity (△-·-·-△).

cleavage of 5′-ETDAPR to 2,6-DAP in these cells is the rate-limiting step in the formation of 2,6-DAP-containing nucleotides from this nucleoside. Similar results were obtained with the MTAPase-containing HCT-15 colon carcinoma line.

In addition, DLD-1 (Clone A) cells were able to convert 5′-MTAzaAR to 8-azaadenine-containing nucleotides and 5′-MTClAR to 2-chloroadenine-containing nucleotides, although in both cases analog nucleotide formation from the free base exceeded that from the nucleoside by several fold. DLD-1 cells were also able to form 2-fluoroadenine nucleotides when incubated for 24 hr with 15 μM 5′-ETFAR; indeed, 2-fluoroadenine triphosphate accumulated in the 5′-ETFAR-treated cells to nearly the same extent (approx. 65%) as that which occurs in cells treated with 15 μM 2-fluoroadenine. DLD-1 (Clone A) cells, however, were not able to form detectable levels of 4-APP-containing nucleotides after 24 hr of incubation with 250 μM 5′-ETAPPR, despite the finding that 4-APP itself was readily incorporated into analog nucleotides in this cell line. These results are consistent with the previously mentioned observation that 5′-ETAPPR is not phosphorolyzed, even in extracts from MTAPase-containing cell lines.

In contrast, when MTAPase-deficient CCRF-CEM cells were incubated with 5′-ETDAPR under similar conditions (Fig. 4E), no analog nucleotide formation was detected, even when the incubation was continued for 48 hr (not shown). CCRF-CEM cells treated with 2,6-DAP, however, readily formed 2,6-DAP-containing nucleotides (Fig. 4F); in fact, by 24 hr, a large portion of the cells treated with this base analog had died, as evidenced by the depletion of natural nucleotides. None of the five MTA analogs studied here was converted to analog nucleotides in this cell line, at least to detectable levels. On the other hand, analog nucleotides were formed when these cells were incubated with each of the corresponding free base analogs.

Substrate specificity studies. The apparent kinetic parameters of MTA and some of its adenine-substituted analogs with MTAPase present in 105,000 *g* supernatant fluids of DLD-1 colon carcinoma extracts are presented in Table 2. As has been observed with MTAPase from other sources, the K_m value of the DLD-1 enzyme for MTA was in the low micromolar range [6, 12, 14]. 5′-Deoxy-5′-ethylthioadenosine had virtually the same kinetic constants as does MTA, indicating, as has been noted previously, that MTAPase is relatively nonspecific in regard to the alkyl moiety of the thioether [6, 8, 35, 36]. As for the purine ring, it is clear that substitutions can be made at the 2-position (e.g. 5′-MTClAR, 5′-ETDAPR) without loss of substrate activity. Although the values for 5′-ETFAR could not be obtained because of our limited supplies of this compound, 5′-ETFAR has been shown to behave as a good substrate of MTAPase from murine Sarcoma 180 cells ($K_m = 20$ μM, V_{max} rel. to MTA = 73%) [35]. The kinetic parameters of the 8-substituted derivative, 5′-MTAzaAR, were also not determined due to its limited availability, but preliminary studies indicated that this analog has a relatively poor turnover rate. The only analog which failed to react with MTAPase was 5′-ETAPPR; this derivative, in fact, acted as a competitive inhibitor of the enzyme (with respect to MTA), with a relatively high K_i value (35 μM). This finding is significant in that another 7-deazaadenine derivative of MTA, 5′-deoxy-5′-methylthiotubercidin, has been shown to be a noncatalyzed competitive inhibitor of the enzyme from rat ventral prostate [37], human lymphocytes [8], and Sarcoma 180 cells [38]. Taken together, these data suggest that the 7-nitrogen of the purine ring plays a key role in the catalytic mechanism of MTAPase.

DISCUSSION

These studies demonstrate that a number of adenine-substituted analogs of the natural metabolite, 5′-deoxy-5′-methylthioadenosine, are substrates of MTAPase, and that their phosphorolysis

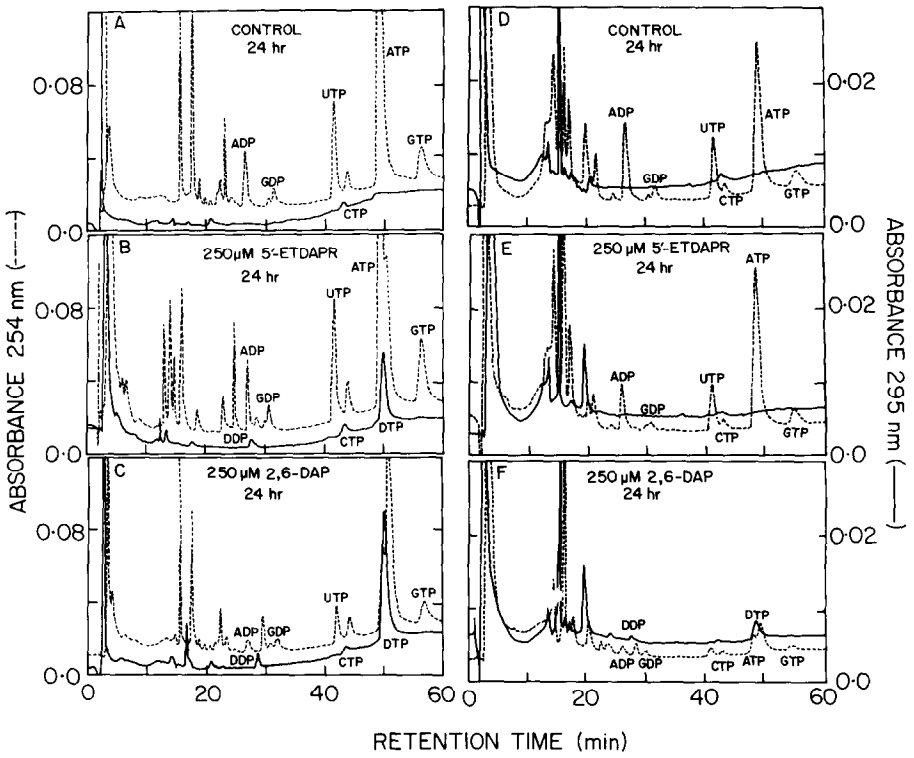

Fig. 4. (A, B and C): Anion-exchange HPLC profiles demonstrating the incorporation of 5'-ETDAPR and 2.6-DAP into 2,6-DAP-containing nucleotides in cultured DLD-1 (Clone A) colon carcinoma cells. Cells (5×10^5) were plated in 60 mm plastic dishes in RPMI-1640 medium supplemented with 15% horse serum. When the cell number reached approximately 2×10^6 per dish, the medium was replaced with that containing no additions (A), 250 μM 5'-ETDAPR (B), or 250 μM 2,6-DAP (C). After 24 hr of incubation in a humidified incubator maintained in an atmosphere of 95% air: 5% CO_2 at 37°, during which cells in the untreated dishes underwent approximately one doubling, the medium was removed and each dish was treated with 1 ml of 4% perchloric acid on ice. After the addition of 0.1 ml of 500 mM potassium phosphate (pH 7.4) to each dish, cell extracts and debris were harvested with a rubber policeman and centrifuged. The supernatant fluids (1 ml) were adjusted to pH 6.5–7.5 with 5 N KOH and frozen at −20°. The anion-exchange HPLC method employed to separate natural and analog nucleotides is described under Materials and Methods. 2,6-Diaminopurine-containing nucleotides were tentatively identified based on their ability to absorb at 295 nm [33]. The profiles shown represent the nucleotide pools of approximately 3.2×10^6 cells. (D, E and F): Anion-exchange HPLC profiles of CCRF-CEM leukemia cells treated with 5'-ETDAPR or 2,6-DAP. Cells were grown in suspension in RPMI 1640 medium supplemented with 15% horse serum under conditions identical to that described above for the DLD-1 (Clone A) colon carcinoma cells. Following harvest by centrifugation, the cells were resuspended at a concentration of 10^6 cells/ml in 10 ml media containing no additions (D), 250 μM 5'-ETDAPR (E), or 250 μM 2,6-DAP (F). After 24 hr of incubation (during which untreated cells underwent less than one-half of one doubling) the cell suspensions were centrifuged and all but 0.25 ml of the supernatant fluid was removed; to each of the cell pellets 0.75 ml of 4% perchloric acid and 0.1 ml of 500 mM potassium phosphate (pH 7.4) were added at 4°. The samples were centrifuged, and 1.0 ml of the supernatant fluids was adjusted to pH 6.5–7.5 with 5 N KOH. After centrifugation, supernatant fluids were frozen at −20°, and later analyzed for nucleotides by anion-exchange HPLC. The profiles shown represent the nucleotide pools of approximately 7.4×10^6 cells.

by this enzyme is a critical step in the conversion of these compounds to their biologically active metabolites. The evidence obtained here is consistent with the following hypothesis for the mechanism of action of these compounds. Upon transport into the cell, these analogs are phosphorolytically cleaved by cytosolic MTAPase, yielding a free base analog and, depending upon the analog, either 5-methylthioribose-1-phosphate or 5-ethylthioribose-1-phosphate. Once the free base analogs, e.g. 2-fluoroadenine, are liberated intracellularly, they can react with 5-phosphoribosyl-1-pyrophosphate as catalyzed by APRT (as has been demonstrated in isolated enzyme systems [29, 39, 40]) to yield the respective analog 5'-monophosphate nucleotides. These nucleotides, in turn, are converted to nucleotide diphosphates by adenylate kinase and are metabolized further to nucleotide triphosphates by nucleoside diphosphokinase and/or ATP-generating systems [32]. These analog adenine nucleotides then may exert growth inhibition by a variety of mechanisms; 2-fluoroadenine-containing nucleotides, for

Table 2. Apparent kinetic parameters of 5′-methylthioadenosine, 5′-ethylthioadenosine, and some of their adenine-substituted analogs with MTAPase from the DLD-1 human colon carcinoma*

	K_m (μM)	V_{max} (% rel. to MTA)	K_i (μM)
5′-Methylthioadenosine†	3 ± 0.5	100	
5′-Ethylthioadenosine†	1.6 ± 0.5	92 ± 3	
5′-Methylthio-2-chloroadenosine‡	3 ± 1	122 ± 45	
5′-Methylthio-8-azaadenosine	ND§	ND	
5′-Ethylthio-2,6-diaminopurine ribonucleoside‡	25 ± 10	103 ± 16	
5′-Ethylthio-4-aminopyrazolo[3,4-*d*]pyrimidine ribonucleoside†			35 ± 9
5′-Ethylthio-2-fluoroadenosine	ND	ND	

* Values represent the mean ± S.D. of at least two independent determinations.

† Values were determined using the spectrophotometric assay described in Ref. 14.

‡ Values were determined by monitoring the formation of base analogs by the reversed-phase HPLC method described under Materials and Methods. Reaction mixtures (1–2 ml) containing 50 mM potassium phosphate, pH 7.4, 105,000 *g* supernatant fluids of DLD-1 colon carcinoma cell sonicates (275–300 μg protein/ml reaction mix), and various concentrations of analog nucleosides were incubated in a shaking water bath at 37°; at various times up to 15 min, aliquots were removed and samples were processed for reversed-phase HPLC as outlined in Fig. 2. Peaks were identified and quantitated using authentic base analogs as standards.

§ Not determined.

example, have been shown to interfere with purine *de novo* synthesis [41], and to inhibit RNA synthesis [42].

In short, the hypothesis proposes that the cytotoxicity of these MTA analogs results primarily from their metabolic conversion to analog adenine nucleotides by the sequential action of MTAPase and APRT. The importance of MTAPase activity in the formation of analog nucleotides, and thus the cytotoxic action of these MTA derivatives, is supported by several findings. Only in MTAPase-containing cell lines (in these studies, two human colon carcinoma lines) were the MTA-like compounds converted to analog nucleotides, and, correspondingly, only in these cell lines did the MTA analogs exert significant growth inhibition. In accord with the hypothesis, the MTAPase-deficient CCRF-CEM leukemia line failed to form analog nucleotides, at least to detectable levels, from any of the MTA derivatives, and, correspondingly, was relatively insensitive to the growth inhibitory effects of these agents. Importantly, when the CCRF-CEM cells were treated with any of the various free base analogs themselves, i.e. when the MTAPase step was circumvented, analog nucleotides were readily synthesized, and cell growth was strongly inhibited. These findings indicate that CCRF-CEM cells are unresponsive to MTA analogs not because of an inability to convert base analogs to their nucleotide forms, or some insensitivity to the inhibitory effects of such analog nucleotides, but rather because of their relative inability to catalyze the initial step in the activation pathway of these MTA derivatives, i.e. phosphorolysis by MTAPase. Nevertheless, it is difficult to prove that CCRF-CEM cells are completely unable to cleave these MTA analogs. Even at a very low rate of cleavage some of these analogs could effect marked biological actions. For example, because of the potent growth-inhibitory effect of 2-fluoroadenine, the cleavage of only 0.5% of 15 μM 5′-ETFAR would be enough to account for the low but definite cytotoxicity of 5′-ETFAR on this cell line.

Similar results have been obtained using murine tumor cell lines: the MTAPase-deficient L1210 leukemia line [13] was shown to be insensitive to 5′-ETFAR (IC_{50} for growth inhibition = > 100 μM), while the MTAPase-containing L5178Y leukemia line was found to be highly responsive to this agent (IC_{50} = 0.3 μM) [21]. The finding that L1210 cells are MTAPase-deficient and, therefore, unable to activate an MTA analog such as 5′-ETFAR may explain why 5′-ETFAR had failed to produce a therapeutic response in mice bearing the L1210 leukemia [19].

The role of APRT in the activation and cytotoxic effects of MTA analogs was indicated in the original studies on these compounds, where it was noted that an APRT-deficient H.Ep.-2 subline resistant to 2-fluoroadenine was cross-resistant to the 2-fluoroadenine-containing MTA analog, 5′-ETFAR [19]. Thus, the apparent requirement for both MTAPase and APRT activities in order for the cytotoxicity of these MTA analogs to be expressed is consistent with the view that analog nucleotides are the active metabolites of these compounds.

On the other hand, additional mechanisms by which MTA analogs exert their growth inhibitory effects may exist. For example, analogs related to 5′-deoxy-5′-ethylthioadenosine, such as 5′-ETFAR or 5′-ETDAPR, would generate 5-ethylthioribose-1-phosphate upon reaction with MTAPase. Since the normal product of the MTAPase reaction, 5-methylthioribose-1-phosphate, has been shown to be converted to methionine, with carbon atoms 2-5 of the ribose forming the backbone of the amino acid [16–18], it is conceivable that its homolog, 5-ethylthioribose-1-phosphate, might be converted to the highly toxic antimetabolite, ethionine, by this same pathway. The metabolic fate of 5-ethylthioribose-1-phosphate is presently under study. Furthermore, the nucleosides themselves may have inhibitory activity. For example, 5′-MTClAR

is a weak but definite growth inhibitor of CCRF-CEM cells, despite there being no evidence for its metabolism by these cells. Since MTA interferes with a number of important biochemical reactions such as spermine synthase [43, 44], and inhibits AdoMet metabolism (for a review, see Ref. 45), it is possible that certain MTA analogs inhibit cell growth, in part, by similar mechanisms.

The MTAPase-dependent, two-step activation of adenine-substituted MTA analogs represents a unique mechanism for the delivery of adenine analogs into intracellular nucleotide pools, one that may be exploited chemotherapeutically. In order for MTA analogs to have high antineoplastic activity, this activation mechanism requires that the adenine-substituted MTA analog and its corresponding purine base serve as good substrates for MTAPase and APRT, respectively. Of the compounds studied to date, the one that best fulfills these criteria is the 2-fluoroadenine-containing derivative, 5′-ETFAR. This analog is an excellent alternate substrate of murine Sarcoma 180 MTAPase [35], while its metabolite, 2-fluoroadenine, is known to be a good substrate for APRT [29, 39, 40]. In addition, 5′-ETFAR is rapidly metabolized to 2-fluoroadenine nucleotides in MTAPase-containing cell lines such as the DLD-1 human colon carcinoma, murine Sarcoma 180 cells [35], and the L5178Y murine leukemia (T. M. Savarese, unpublished data). 5′-ETFAR, with IC_{50} values in the 10^{-7} M range, is by orders of magnitude the most potent inhibitor of cell growth of all the adenine-substituted MTA analogs in every system tested thus far, including the H.Ep.-2 line [19], the L5178Y leukemia [21], and the HCT-15 and DLD-1 human colon carcinoma lines. Nevertheless, 5′-ETFAR is not toxic when administered to mice at 100 mg/kg once every 4 days for three doses [19], suggesting that normal tissues may be relatively insensitive to the adverse effects of this antimetabolite. The high antiproliferative activity of 5′-ETFAR, at least *in vitro*, coupled with its apparent low toxicity, make this and other 2-fluoroadenine-containing MTA derivatives the most promising candidates of this class for development as antineoplastic compounds. Currently, a series of such analogs are being formulated and investigated.

As chemotherapeutic agents, adenine-substituted MTA analogs might be regarded simply as prodrugs of their corresponding free adenine base analogs. However, the additional activation step that they must undergo, i.e. phosphorolysis by MTAPase, may impart to them pharmacological properties that are significantly different from, and perhaps superior to, those of the parent free base analogs. For example, for reasons not yet understood, the free adenine analogs are highly toxic (e.g. the maximum tolerated dose of 2-chloroadenine in mice is 20 mg per kg per day [46]), whereas, on the basis of limited studies to date, the MTA analogs appear relatively nontoxic [19]. Also, since it would be expected that tissues that contain relatively high levels of MTAPase would metabolize MTA analogs to a greater extent than tissues with relatively low levels of this enzyme, the MTA-like compounds may exhibit some organ specificity. The prostate, whose function in providing the seminal fluid with millimolar levels of spermidine and spermine (as in the rat) or spermine (as in the human [47]) necessitates the presence of an active mechanism for metabolizing MTA and recycling its components, is one such potential target tissue. The use of these MTA analogs in the treatment of carcinomas of prostatic origin, or in the control of prostatic hypertrophy, should therefore be explored. These analogs might, in fact, be effective against any malignant or normal cell type that is rapidly turning over MTA, presumably as a consequence of high polyamine biosynthetic activity. The potential use of MTA analogs in the chemotherapy of cancer has been discussed elsewhere [35, 38, 48].

Acknowledgements—The authors are grateful to Drs. Ellen Spremulli, Gerald Crabtree, and S. H. Chu of Brown University for their aid and advice during these studies. The fine technical assistance of Diane Farineau and Nina Calabresi is well appreciated.

REFERENCES

1. A. E. Pegg and H. G. Williams-Ashman, *J. biol. Chem.* **244**, 682 (1969).
2. A. E. Pegg and H. G. Williams-Ashman, *Archs. Biochem. Biophys.* **137**, 156 (1970).
3. K. R. Swiatek, L. N. Simon and K. L. Chao, *Biochemistry* **12**, 4670 (1973).
4. J. Wilson, A. Corti, M. Hawkins, H. G. Williams-Ashman and A. E. Pegg, *Biochem. J.* **180**, 515 (1979).
5. A. E. Pegg and H. G. Williams-Ashman, *Biochem. J.* **115**, 241 (1969).
6. V. Zappia, A. Oliva, G. Cacciapuoti, P. Galletti, G. Mignucci and M. Carteni-Farina, *Biochem. J.* **175**, 1043 (1978).
7. G. Cacciapuoti, A. Oliva and V. Zappia, *Int. J. Biochem.* **9**, 35 (1978).
8. M. W. White, A. A. Vandenbark, C. L. Barney and A. J. Ferro, *Biochem. Pharmac.* **31**, 503 (1982).
9. G. W. Crabtree, D. L. Dexter, J. D. Stoeckler, T. M. Savarese, L. Y. Ghoda, T. L. Rogler-Brown, P. Calabresi and R. E. Parks, Jr., *Biochem. Pharmac.* **30**, 793 (1981).
10. N. Kamatani, W. A. Nelson-Rees and D. A. Carson, *Proc. natn. Acad. Sci. U.S.A.* **78**, 1219 (1981).
11. D. L. Garbers, *Biochim. biophys. Acta* **523**, 82 (1978).
12. A. J. Ferro, N. C. Wrobel and J. A. Nicolette, *Biochim. biophys. Acta* **570**, 65 (1979).
13. J. I. Toohey, *Biochem. biophys. Res. Commun.* **82**, 27 (1978).
14. T. M. Savarese, G. W. Crabtree and R. E. Parks, Jr., *Biochem. Pharmac.* **30**, 189 (1981).
15. N. Kamatani and D. A. Carson, *Cancer Res.* **40**, 4178 (1980).
16. P. Backlund, Jr. and R. A. Smith, *J. biol. Chem.* **256**, 1533 (1981).
17. P. Backlund, Jr., C. P. Chang and R. A. Smith, *J. biol. Chem.* **257**, 4196 (1982).
18. P. C. Trackman and R. H. Abeles, *Biochem. biophys. Res. Commun.* **103**, 1238 (1981).
19. J. A. Montgomery, A. T. Shortnacy and H. J. Thomas, *J. med. Chem.* **17**, 1197 (1974).
20. T. M. Savarese, D. L. Dexter, E. N. Spremulli, G. W. Crabtree, S. H. Chu, J. A. Montgomery, R. E. Parks, Jr. and P. Calabresi, *Proc. Am. Ass. Cancer Res.* **22**, 209 (1981).
21. T. M. Savarese, M. Y. Chu, S. H. Chu, G. W. Crabtree, D. L. Dexter, E. N. Spremulli, J. D. Stoeckler, P. Calabresi and R. E. Parks, Jr., in *The Biochemistry of S-Adenosylmethionine and Related Compounds* (Eds. E. Usdin, R. T. Borchardt, and C. R. Creveling), pp. 709–713. MacMillan, London (1982).

22. D. L. Dexter, J. A. Barbosa and P. Calabresi, *Cancer Res.* **39**, 1020 (1979).
23. D. L. Dexter, E. N. Spremulli, Z. Fligiel, J. A. Barbosa, R. Vogel, A. VanVoorhees and P. Calabresi, *Am. J. Med.* **71**, 949 (1981).
24. D. L. Dexter, G. H. Heppner and P. Calabresi, *Biochem. Pharmac.* **28**, 1933 (1979).
25. G. E. Foley, H. Lazarus, S. Farber, B. G. Uzman, B. A. Boone and R. E. McCarthy, *Cancer, N.Y.* **18**, 522 (1965).
26. M. M. Bradford, *Analyt. Biochem.* **72**, 248 (1976).
27. L. L. Bennett, Jr. and D. Smithers, *Biochem. Pharmac.* **13**, 1331 (1964).
28. J. H. Burchenal, A. Bendich, G. B. Brown, G. B. Elion, G. H. Hitchings, C. P. Rhoads and C. C. Stock, *Cancer, N.Y.* **2**, 119 (1949).
29. J. K. Roy, C. A. Haavik and R. E. Parks, Jr., *Proc. Am. Ass. Cancer Res.* **3**, 146 (1960).
30. I. Lieberman and P. Ove, *J. biol. Chem.* **235**, 1765 (1960).
31. A. Kornberg and W. E. Pricer, Jr., *J. biol. Chem.* **183**, 481 (1951).
32. R. E. Parks, Jr. and P. R. Brown, *Biochemistry* **12**, 3295 (1973).
33. R. P. Agarwal, S. Cha, G. W. Crabtree and R. E. Parks, Jr., in *Symposium of Chemistry and Biology of Nucleosides and Nucleotides* (Eds. R. K. Robins and R. E. Harmon), p. 159. Academic Press, New York (1978).
34. R. W. Brockman, *Adv. Cancer Res.* **7**, 129 (1963).
35. R. E. Parks, Jr., J. D. Stoeckler, C. Cambor, T. M. Savarese, G. W. Crabtree and S. H. Chu, in *Molecular Actions and Targets for Cancer Chemotherapeutic Agents* (Eds. A. C. Sartorelli, J. S. Lazo and J. R. Bertino), p. 229. Academic Press, New York (1981).
36. R. W. Wolford, M. R. MacDonald, B. Zehfus, T. J. Rogers, and A. J. Ferro, *Cancer Res.* **41**, 3035 (1981).
37. J. K. Coward, N. C. Motola and J. D. Moyer, *J. med. Chem.* **20**, 500 (1977).
38. R. E. Parks, Jr., T. M. Savarese and S. H. Chu, in *New Approaches to the Design of Antineoplastic Agents* (Eds. T. I. Kalman and T. Bardos), pp. 141–154. Elsevier, New York (1982).
39. R. E. A. Gadd and J. F. Henderson, *Can. J. Biochem.* **48**, 295 (1970).
40. T. A. Krenitsky, S. A. Neil, G. B. Elion and G. H. Hitchings, *J. biol. Chem.* **244**, 4779 (1969).
41. H. T. Shigeura, G. E. Boxer, M. L. Meloni and S. D. Sampson, *Biochemistry* **5**, 994 (1966).
42. H. T. Shigeura, G. E. Boxer, S. D. Sampson and M. L. Meloni, *Archs. Biochem. Biophys.* **111**, 713 (1965).
43. H. Hibasami and A. E. Pegg, *Biochem. biophys. Res. Commun.* **81**, 1398 (1978).
44. R-L. Pajula, A. Raina and T. Eloranta, *Eur. J. Biochem.* **101**, 619 (1979).
45. H. G. Williams-Ashman, J. Seidenfeld and P. Galletti, *Biochem. Pharmac.* **31**, 277 (1982).
46. H. E. Skipper, J. A. Montgomery, J. R. Thomson and F. M. Schabel, Jr., *Cancer Res.* **19**, 425 (1959).
47. H. Tabor and C. W. Tabor, *Pharmac. Rev.* **16**, 245 (1964).
48. T. M. Savarese, G. W. Crabtree and R. E. Parks, Jr., *Biochem. Pharmac.* **28**, 2227 (1979).