Metabolism of *S*-Adenosylhomocysteine and *S*-Tubercidinylhomocysteine in Neuroblastoma Cells†

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ABSTRACT: The metabolism of the methylase product inhibitor *S*-adenosylhomocysteine and its 7-deaza analogue *S*-tubercidinylhomocysteine has been studied in cultured N-18 neuroblastoma cells. The latter compound, designed to resist metabolic degradation, has been shown to be inert under the same conditions where *S*-adenosylhomocysteine is rapidly and extensively degraded. The product analyses elucidated by high-performance liquid chromatography indicate that the primary route of *S*-[8-^{14}C]adenosylhomocysteine metabolism in these cells leads to adenosine. This product does not accumulate but is rapidly converted to nucleotides or oxypurines by the action of adenosine kinase and adenosine deaminase, respectively. The presence of the potent adenosine deaminase inhibitor coformycin leads to a pronounced inhibition of oxypurine formation, an increase in nucleotide formation, and a slight accumulation of the primary metabolic products adenosine and adenine.

S-Adenosylmethionine is the methyl donor in a number of important transmethylation reactions, transfer of the methyl group yielding *S*-adenosyl-L-homocysteine (SAH)[1] as one of the products. SAH has been shown to inhibit competitively most of the methyltransferases which utilize *S*-adenosylmethionine as the methyl donor. For example, catechol *O*-methyltransferase (COMT) (EC 2.1.1.6) (Coward et al., 1972), histamine *N*-methyltransferase (EC 2.1.1.8) (Zappia et al., 1969; Baudry et al., 1973), indoleethylamine *N*-methyltransferase (EC 2.1.1.49) (Lin & Narasimhachari, 1975), phenylethanolamine *N*-methyltransferase (EC 2.1.1.28) (Deguchi & Barchas, 1971), and tRNA methyltransferase (EC 2.1.1.29–36) (Pegg, 1971; Glick et al., 1975) are all subject to product inhibition by SAH; however, the K_i values for these reactions vary over a wide range. Thus the role of SAH as a regulator of the transmethylation process is of initial interest. Because of the different sensitivities of the various methylating enzymes toward SAH, the control of tissue SAH levels can be of physiological importance in regulating cellular transmethylation. In this respect, the metabolic reactions that affect the level of SAH in the cell are of considerable interest. We have shown (Coward et al., 1974) that *S*-tubercidinylhomocysteine (STH), the 7-deaza analogue of SAH, is a potent inhibitor of COMT in vitro with K_i = 30 μM compared with K_i = 20 μM for SAH inhibition of the same enzyme. However, the 7-deaza analogue, designed to resist metabolic breakdown, is a much more effective inhibitor of dopamine methylation in neuroblastoma cells than the natural product inhibitor SAH (Michelot et al., 1977). A similar pattern is observed in RNA methylation inhibition by STH and SAH in phytohemagglutinin-stimulated rat lymphocytes (Chang & Coward, 1975) and in Novikoff hepatoma cells (Kaehler et al., 1977, 1979). Since SAH and STH have similar K_i values in a number of cell-free enzyme systems (Coward et al., 1974), it is evident that the greater potency of the 7-deaza analogue in whole cells is due to differences in transport and/or metabolism in these systems.

Two major pathways for the enzymatic cleavage of SAH have been demonstrated (De La Haba & Cantoni, 1959; Duerre, 1962; Walker & Duerre, 1975). SAH hydrolase (EC 3.3.1.1), which has been shown to be present in yeasts, plants, birds, and mammals, catalyzes the reversible hydrolysis of SAH to adenosine and L-homocysteine. The enzyme system favors synthesis rather than hydrolysis (De La Haba & Cantoni, 1959); however, a mechanism has recently been proposed (Palmer & Abeles, 1976) for the hydrolysis reaction. Secondly, SAH nucleosidase (EC 3.2.2.9), which has been demonstrated to date only in procaryotes, catalyzes the cleavage of the glycosyl linkage of SAH to yield adenine and *S*-ribosylhomocysteine (Duerre, 1962). SAH has also been shown to be deaminated to *S*-inosinylhomocysteine (SIH) by adenosine deaminase (EC 3.5.4.4) from *Aspergillus oryzae* (Schlenk & Zydek, 1968; Schlenk et al., 1971) but not by adenosine deaminase from intestine (Kalckar, 1947). The major urinary metabolite of SAH after intravenous injection into the rat is *S*-adenosyl-γ-thio-α-ketobutyrate (SATKB), a product presumably formed by L-amino acid oxidase (EC 1.4.3.2) (Duerre et al., 1969). Recently, the metabolism of SAH in rat and mouse brain, kidney, and liver homogenates has been described (Cortese et al., 1974; Schatz et al., 1977), the major metabolic pathway involving cleavage of the homocysteine–ribose bond with subsequent metabolism of adenosine in a tissue-dependent manner to oxypurinic products. Another possible route of SAH metabolism, demonstrated in yeast, may involve remethylation to *S*-adenosylmethionine (Shapiro & Ehninger, 1969) followed by enzymatic cleavage of the S–C bond of the methionine moiety to give methylthioadenosine (MTA) (Gefter et al., 1966). The above pathways are summarized in Figure 1.

It is of importance to note, in this respect, that tubercidin derivatives do not act as substrates for an enzyme which catalyzes the phosphorolytic cleavage of the purine–ribose bond of the corresponding adenosine derivatives (Coward et al., 1977) or the deamination of the 6-amino function (Suhadolnik, 1970), and recent studies indicate that STH is not a substrate for purified SAH hydrolase (EC 3.3.1.1) isolated from rat liver

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[1] Abbreviations used: COMT, catechol *O*-methyltransferase; SAH, *S*-adenosyl-L-homocysteine; STH, *S*-tubercidinylhomocysteine; SIH, *S*-inosinylhomocysteine; SATKB, *S*-adenosyl-γ-thio-α-ketobutyrate; MTA, 5′-methylthioadenosine; AMP, ADP, and ATP, 5′-mono-, 5′-di-, and 5′-triphosphate derivatives of adenosine; IMP, IDP, and ITP, 5′-mono-, 5′-di-, and 5′-triphosphate derivatives of inosine; TbMP, tubercidin 5′-monophosphate; 7DzIno, 7-deazainosine; 7DzHyp, 7-deazahypoxanthine; Tb, tubercidin; 7DzAde, 7-deazaadenine; Xan, xanthine; Hyp, hypoxanthine; Ino, inosine; Ado, adenosine; Ade, adenine; 5′-ClAdo, 5′-chloroadenosine; 5′-ClTb, 5′-chlorotubercidin; HMPTA, hexamethylphosphoric triamide; LC, high-pressure liquid chromatography.

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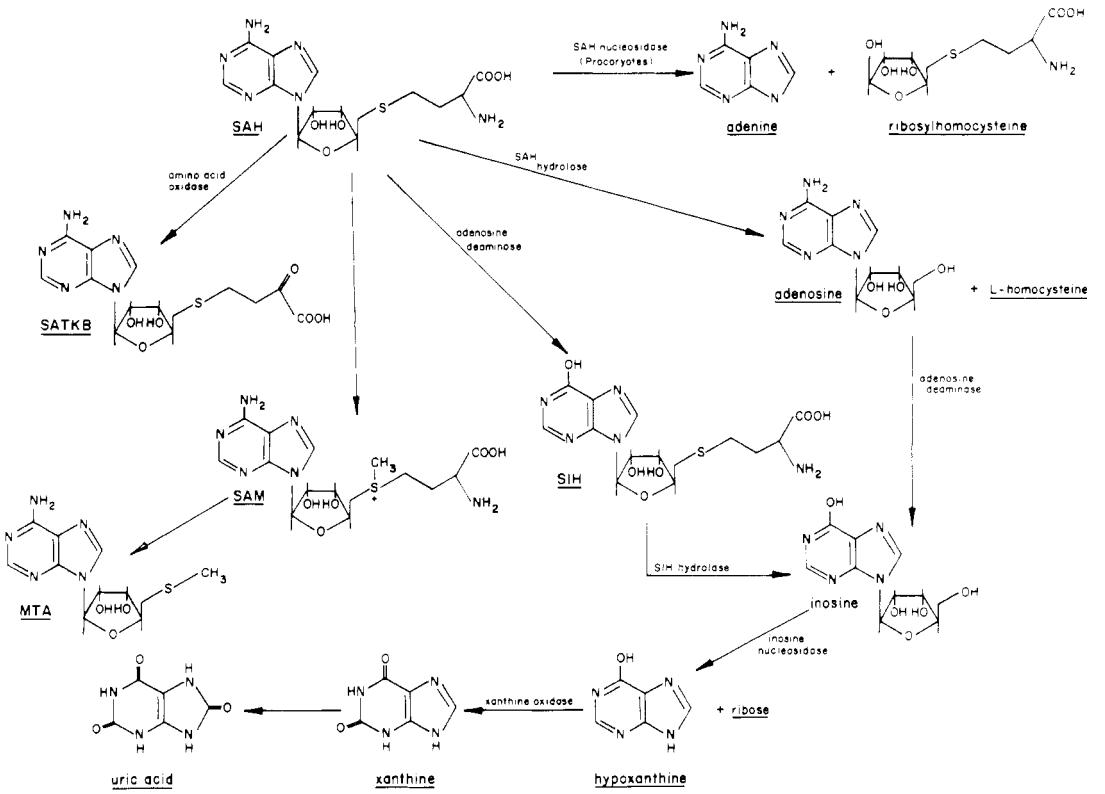

FIGURE 1: Possible pathways of metabolism of SAH.

(Chiang et al., 1977). Taken in total, these data suggest that the greater efficacy of STH relative to SAH as a transmethylase inhibitor in whole cells is due to its greater stability toward the metabolic degradations outlined above. Of course, it is possible that differences in the transport of SAH and STH may be also of relevance. No detailed studies of SAH uptake in mammalian cells have been reported, although results from in vivo experiments in the dog (Walker & Duerre, 1975) suggest that the cells of various organs are impermeable to exogenously administered SAH. It has been stated (Trewyn & Kerr, 1976) that SAH does not penetrate cells in culture, whereas other structural analogues do. The metabolism of *S*-[8-^{14}C]adenosyl-L-homocysteine and *S*-[*carboxyl*-^{14}C]-tubercidinylhomocysteine in neuroblastoma cell culture is the subject of this report.

Materials and Methods

Inosine diphosphate and inosine triphosphate were purchased from Fluka Ag. *S*-Adenosyl-γ-thio-α-ketobutyrate was a gift from Dr. J. A. Duerre, University of North Dakota, and coformycin was a gift from Dr. S. Kondo, Microbial Chemistry Research Foundation, Tokyo. *S*-Inosinylhomocysteine, *S*-tubercidinylhomocysteine, and methylthioadenosine were prepared in our own laboratories. Tubercidin was kindly donated by Dr. C. B. Whitfield, Upjohn, Kalamazoo, MI, and the 7-deaza analogues of inosine, hypoxanthine, adenine, and adenosine monophosphate were gifts from Dr. L. B. Townsend, University of Utah. L-Homocystine, L-methionine, and all other purinic standards were puchased from Sigma. *S*-Adenosyl-L-homocysteine was obtained from Boehringer Mannheim Biochemicals. Reagent grade phosphoric acid, acetic acid, sodium acetate, trichloroacetic acid, and hydriodic acid were purchased from Fisher. Thionyl chloride was purchased from Fisher and freshly distilled before use. Hexamethylphosphoric triamide (HMPTA) was purchased from Aldrich and stored over molecular sieves before use. Liquid ammonia was purchased from New England Welding and was used undistilled. Sodium dihydrogen phosphate was purchased from Baker and the optical density of a 1.0 M solution was measured at 254 nm to select a batch which gave the lowest absorption value in order to minimize base-line drift in gradient elutions. Methanol and acetonitrile (both distilled in glass) were purchased from Burdick and Jackson. [8-^{14}C]Adenosine (no. NEC-524) (specific activity 54.7 mCi/mmol) and Formula 963 scintillation fluid were obtained from New England Nuclear. [1-^{14}C]-L-Methionine (no. CFA-443) (specific activity 60 mCi/mmol) was obtained from Amersham. Dulbecco's modified Eagle's medium (no. H.21), phosphate-buffered saline (no. 408), penicillin, streptomycin solution (no. 514), and 2.5% trypsin solution (no. 509) were purchased from Flow Laboratories. Trypan blue (Direct Blue, C.I. no. 23850) was obtained from Matheson, Coleman and Bell. Plastic flasks and Petri dishes were from Falcon Co. (no. 3013 and 3001, respectively). All buffers used were prepared in deionized, distilled water and were sterile filtered with a 47-mm Gelman (GA-8 Metricel) 0.2-μm membrane filter (no. 74588) to remove particulate matter and degassed in vacuo before use. Centrifugations were carried out on a Sorvall superspeed RC2B centrifuge at 4 °C. Cell populations were measured on cell suspensions by using either a Coulter counter, Model ZB_I, or a hemocytometer.

High-Pressure Liquid Chromatography. Gradient elutions and analyses carried out at column inlet pressures of greater than 500 psi were performed on a Du Pont Model 830 liquid chromatograph. Nongradient elutions at column inlet pressures of less than 500 psi were carried out on a modular LC unit, details of which will be forwarded on request. All column eluents were monitored with a UV detector operating at 254 nm. Radioactive effluents were monitored by collecting fractions of known volume on a LKB Model 7000 fraction collector. To each fraction was added sufficient scintillation fluid to form either a homogeneous solution or a clear gel. A

Packard Tri-Carb Model 3320 scintillation spectrometer was used to measure the radioactivity of the samples. Radiochromatograms were constructed by plotting the radioactivity of the fractions against their retention time. Partisil-10 SCX and Partisil-10 SAX microparticle columns 25 × 0.46 cm (Whatman) were used for cation exchange and anion exchange chromatography, respectively. Reverse-phase chromatography was carried out on a Partisil-10 ODS or ODS 2 microparticle column, 25 × 0.46 cm, loaded with either 4.5% or 15% octadecyl silica (Whatman). Chromatographic standards were prepared as 1 mM solutions in distilled water where solubility permitted. Adenine was prepared as a 1 mM solution in 10 mM HCl, and xanthine and uric acid were prepared as 1 mM solutions in 0.1 M phosphate buffer, pH 7.5. 5′-Chlorotubercidin, 5′-chloroadenosine, tubercidin, and 7-deazaadenine were prepared as 1 mM solutions in aqueous methanol. Samples were introduced with a Hamilton syringe Model HP-1805. The revelant chromatographic operating conditions appear in the legends of the figures.

Recovery Studies. The sum of the radioactivity in the collected fractions was compared with the total amount of radioactivity initially applied to the column, which was determined by adding an appropriate volume of radioactive sample directly to a blank tube of column effluent generated by the fraction collector.

Preparation of [8-^{14}C]-5′-Chloroadenosine. A modification of the procedure described by Kikugawa & Ichino (1971) was carried out. [8-^{14}C]Adenosine (0.125 mg, 54.7 mCi/mmol) was diluted with nonradioisotopic adenosine (2.375 mg) to give a final specific activity of 2.7 mCi/mmol. HMPTA (25 μL) and thionyl chloride (2.5 μL) were added to [8-^{14}C]adenosine (2.5 mg) contained in a 0.5-mL glass culture tube, the mixture was capped and vortexed, and the resulting yellow solution was left to stand at room temperature for 12 h. Distilled water (100 μL) was added to quench the reaction; the mixture was vortexed and applied to a 1.0-mL Bio-Rad AG 50W-X8 resin column (100–200 mesh, H^+ form) which had been prewashed with distilled water (40 mL). The column was washed with distilled water (30 mL) and then 50% NH_4OH solution, and the ammoniacal liquors were collected until no significant radioactivity (<500 cpm/mL) could be detected in the eluates. Prior to lyophilization, a small aliquot of the ammoniacal fraction was subjected to LC analysis on a Partisil-10 ODS column loaded with 4.5% octadecyl silica by using methanol:water (20:80 v/v) as the eluting solvent. This chromatographic system effects a satisfactory separation of adenosine and 5′-chloroadenosine and is of preparative value. Examination of the radiochromatogram of the ammoniacal eluate showed that no significant amount of [8-^{14}C]adenosine was present and that [8-^{14}C]-5′-chloroadenosine was formed in almost quantitative yield (24.4 μCi, 97%).

Preparation of S-[8-^{14}C]Adenosyl-L-homocysteine. A synthetic procedure described by Borchardt et al. (1976a) was adopted. L-Homocystine (6.0 mg) was placed in a two-necked, 10-mL flask fitted with a sodium hydroxide drying tube and a glass coated stirrer bar was added. The whole apparatus was then continuously flushed with dry nitrogen gas and the flask immersed in an acetone–dry ice cooling bath. Liquid ammonia (5 mL) was quickly added and the mixture stirred for 10 min. Sodium metal, previously chipped clean and kept under dry ether, was added to the reaction until the resulting deep blue coloration persisted for more than 20 min. The color was then discharged by addition of a few crystals of ammonium chloride, affording a clear, pale straw-colored solution. This solution was quickly transferred to a 10-mL flask containing the lyophilized sample of [8-^{14}C]-5′-chloroadenosine (2.58 mg, 24.4 μCi) prepared as described previously and the mixture allowed to stir for 10 h at cooling bath temperature. The bath was removed and the mixture allowed to warm to ambient temperature overnight. Distilled water (2.5 mL) was added to the pale yellow residue and the resulting solution adjusted to pH 6.0 with 1 N HCl. A small aliquot of this solution was subjected to LC analysis on a Partisil-10 ODS column loaded with 4.5% octadecyl silica by using methanol:water (20:80 v/v) as the eluting solvent. Examination of the radiochromatogram obtained showed that [8-^{14}C]-5′-chloroadenosine (63.5%) and *S*-[8-^{14}C]adenosyl-L-homocysteine (32%) were the only major radioisotopic components. Separation of the desired product was achieved by lyophilizing the reaction mixture, taking up the residue in 0.5 mL of methanol–water (20:80 v/v) and injecting 0.1-mL aliquots of this solution on the chromatographic system described above. At this concentration, the radioisotopic components of the mixture are detectable by UV absorption; thus the UV absorbing fractions corresponding to SAH were collected and combined, the solvent removed by lyophilization and the residue dissolved in sufficient doubly distilled water to give ca. 10^{-2} μCi/μL. To check the radioisotopic purity of the products, a small aliquot was analyzed by reverse-phase chromatography and was shown to be homogenous. In a similar manner, unreacted [8-^{14}C]-5′-chloroadenosine was quantitatively recovered for reutilization.

Preparation of 5′-Chlorotubercidin. A modification of the existing procedure for the preparation of 5′-chlorotubercidin from tubercidin via chlorination with thionyl chloride in HMPTA (Borchardt et al., 1976a; Coward et al., 1977) afforded the title compound in high yield and purity. HMPTA (4.5 mL), previously dried over molecular sieves, and thionyl chloride (0.5 mL) were mixed together in a two-necked, round-bottomed flask containing a magnetic stirrer and immersed in a water bath at ambient temperature. The mixture was stirred for 10 min, tubercidin (0.5 g) added in one portion, and the reaction stirred for 18 h at ambient temperature. The orange-colored solution was poured into a distilled water–ice mixture (35 mL), the reaction flask was washed with cold water, and the washings were added to the water–ice mixture. The resulting yellow-colored solution was stirred for 5 min and the pH adjusted to 9–10 with 0.88 M ammonia solution. A yellowish-brown oil was deposited which was dissolved by heating the mixture on a steam bath, with stirring for 15 min. The deep yellow solution was filtered while still hot and the filtrate cooled to 0 °C in an ice–salt bath. Chloroform (15–20 mL) was added to the cooled solution; the biphasic mixture was stirred at 0 °C for 1.5 h and then stored at 4 °C overnight. The resulting flocculent precipitate was isolated by filtration and washed with a little cold water. The moist, white solid was recrystallized from hot water to afford 5′-chlorotubercidin as white silky crystals (0.487-g dry weight, 91%), melting at 162–184 °C with gradual sublimation. This material was homogenous by TLC on silica gel (R_f 0.75) by using methanol–chloroform (15:85 v/v) as eluting solvent and by LC on Partisil-10 ODS (t_R = 25.5 min) by using methanol:water (20:80 v/v) as eluent, at a flow rate of 1.0 mL/min. The NMR (Me_2SO-d_6) spectrum was fully consistent with the structure, but contrary to a previous report (Borchardt et al., 1976b) the C_7 and C_8 protons were each observed as doublets ($J_{7,8}$ = 3.8 Hz) centered at δ 6.64 and 7.33, respectively.

Preparation of S-[carboxyl-^{14}C]Tubercidinylhomocysteine. Initial conversion of methionine to homocysteine thiolactone hydriodide was carried out by using a modification of a

procedure originally described by Baernstein (1934). L-Methionine (0.88 mg) was placed in a 1.0-mL single necked, round-bottomed flask and L-[1-^{14}C]methionine (0.042 mg in 0.167 mL of deaerated water, 16.67 μCi) added to give a final specific activity of 2.7 mCi/mmol. The solution was lyophilized, HI (0.2 mL, 47%) quickly added to the residue, and a slow stream of dry nitrogen gas immediately bubbled through the solution. The solution was then heated in an oil bath at 140–145 °C, under nitrogen, for 6 h. The reaction flask was then cooled, distilled water (1.0 mL) was added, and the solution plus flask and condenser washings was lyophilized. The semicrystalline residue obtained was dissolved in distilled water (1.0 mL) and again lyophilized. The residue was dissolved in distilled water (0.1 mL) and a small aliquot taken out and subjected to LC analysis on a Partisil-10 SCX column by using 0.25 M sodium acetate, pH 4.5, as eluting solvent. Examination of the radiochromatogram from this analysis showed a 91% conversion of L-methionine into homocysteine thiolactone hydriodide. A solution of NaOH (0.1 mL, 4 N) was added to the above aqueous solution of [1-^{14}C]homocysteine thiolactone hydriodide and the mixture heated at 50 °C, under nitrogen for 45 min. 5′-Chlorotubercidin (2 mg) was then added to the solution in one portion and the mixture heated at 50 °C under nitrogen for 18 h. On cooling, the pH of the reaction mixture was adjusted to between 6–7 with 4 N HCl and a small aliquot of the reaction mixture analyzed by LC on a Partisil-10 SCX cation-exchange column. *S*-[*carboxyl*-^{14}C]Tubercidinylhomocysteine was obtained from this reaction in yields ranging from 35 to 56%. Separation and purification of the title compound from the above reaction mixture was carried out by injecting 50-μL aliquots of the neutralized reaction mixture onto a Partisil-10 reverse-phase ODS column (CH_3OH:H_2O, 20:80 v/v) and collecting an appropriate cut of the UV-detectable band corresponding to STH. Lyophilization of the appropriate pooled fractions afforded a residue which was dissolved in sufficient distilled water to give a solution containing ca. 10^{-2} μCi/μL. The radioisotopic purity of this product (>98%) was evaluated by analysis of a small aliquot on a Partisil-10 SCX cation exchange column.

Cell Culture. As previously described (Michelot et al., 1977), stock cultures of N-18 neuroblastoma cells were maintained in 25-cm^3 plastic flasks in growth medium supplemented with 10% fetal calf serum, penicillin (100 units/mL), streptomycin (100 μg/mL), and 2.2 g of sodium bicarbonate per L in an atmosphere of 5% CO_2 and 95% air saturated by H_2O at 37 °C. Cells were subcultured by mechanically dislodging them from the plastic surface without the aid of trypsin. For each experiment, the cells were plated onto 35-mm diameter plastic Petri dishes at a concentration of 10^4 to 10^5 cells per mL in 2 mL of medium supplemented with 10% fetal calf serum. The dishes were incubated at 37 °C for 4 days, after which time the monolayers were 70–80% confluent. The cells were fed with fresh medium 24 h prior to experimentation. After this period, the monolayers were 90–100% confluent and the majority of cells had formed extensive neurites.

Metabolism of S-[8-^{14}C]Adenosyl-L-homocysteine and S-[carboxyl-^{14}C]Tubercidinylhomocysteine. To minimize possible extracellular metabolism of SAH or STH, the medium was separated from the monolayer on removal from the incubator and the cells were rinsed gently three times with 0.5 mL of fresh, prewarmed isotonic phosphate-buffered saline consisting of the following: 129 mM NaCl, 2.5 mM KCl, 7.4 mM Na_2HPO_4, 1.3 mM KH_2PO_4, 0.63 mM $CaCl_2$, 0.7 mM $MgSO_4$, 5.3 mM glucose, and 46 mM sucrose. Monlayers were immediately incubated in this buffer (1.0 mL per 35-mm Petri dish) at 37 °C in an atmosphere of 5% CO_2 and 95% air for periods of 1, 3, or 6 h in the presence of [8-^{14}C]SAH (0.13 μCi, 50 μM) or [*carboxyl*-^{14}C]STH (0.13 μCi, 50 μM). In some experiments, cells were incubated for 10 min with coformycin at a concentration of 10 μM or 1×10^{-3} μM before incubation with SAH. After incubation the dishes were stored frozen (−4 °C) and then allowed to warm to room temperature over ca. 10 min. Cell monolayers were scraped off the surface of the dish by using a thin Perspex square, and the resulting cell suspension was pipetted into 5-mL plastic centrifuge tubes. The suspensions were frozen and thawed three times over ca. 30 min with an acetone/dry ice bath; after this treatment, microscopic examination showed that all the cells were lysed. To each homogenate was added 30 μL of a freshly prepared 10% w/v solution of trichloroacetic acid to give a mixture of between pH 2 and 3. These mixtures were incubated for 10 min on a water bath at 40 °C to precipitate the protein and the acidified homogenates centrifuged at 27000*g* for 90 min. After this centrifugation, the radioactivity remaining in the supernatant was 80–97% of the initial radioactivity in the incubation medium. The supernatants were separated, adjusted to pH 5.0 with 1.0 N NaOH, and stored frozen prior to chromatographic analysis.

Viability Studies. Cell viability was determined by decanting off the incubation buffer and incubating the monolayer for 5 min at 37 °C in a solution of 0.25% trypsin in phosphate-buffered saline. The viability of the cells in suspension was then determined after staining the cells with a solution of 0.4% Trypan blue (Phillips, 1973); control cells were at least 80% viable by this assay. Viability of monolayers in the presence of 50 μM SAH or 50 μM STH and with combinations of 50 μM SAH and 10 μM coformycin was in general 90–95% of drug-free controls under the conditions described above. Cells incubated for longer times (12 h) were shown to be only 45–50% viable.

Results

Since cellular metabolism of SAH might be expected to give rise to a mixture of polar biological substances having both acidic and basic functional groups, we chose initially to examine the separation of a contrived mixture of authentic SAH metabolites on reverse phase chromatography. Preliminary experiments on a 4.5% loaded ODS column separated all 6-aminopurines, except SAH and adenosine, with no resolution of oxypurines. We then examined the 6-aminopurinic standards on a 15% loaded octadecyl silica column in the expectation that this might effect a better separation of the SAH–adenosine solute pair. The retention times of all components are markedly extended and a clean separation of SAH and adenosine is achieved (Figure 2). MTA is not eluted during the time run used in the above analysis. The radiochromatogram of a cell extract after 6-h incubation with [8-^{14}C]SAH, containing authentic markers for the substances of interest, is shown in Figure 2. As can be seen, almost all the radioactivity not associated with the SAH band migrates with the mixed oxypurinic coeluting peak; thus it was necessary to develop a chromatographic system which would enable the distribution of radioisotopic labeling in the markers associated with the oxypurinic band to be determined.

We chose to develop a reverse-phase chromatographic separation of the components of the coeluting oxypurinic band by using an acidic solvent system, since it is known (Berg, 1977) that continual use of solvents above pH 7.5 rapidly degrades the octadecyl silica bonding of the column. The most

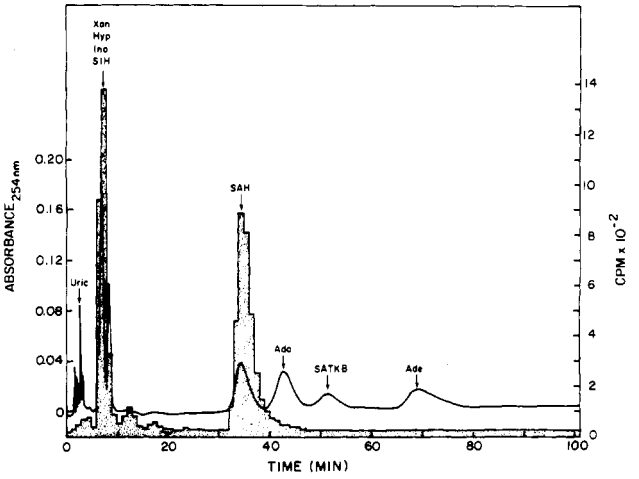

FIGURE 2: Chromatogram of a 50-μL sample of cell extract containing authentic markers obtained on a Partisil-10 ODS reverse-phase column with 15% loading of octadecyl silica. The column was eluted isocratically with 1.0 M sodium acetate buffer, pH 4.6, at a flow rate of 1.8 mL/min and a column inlet pressure of 1000 psig at ambient temperature. Shaded areas are radiolabeled metabolites.

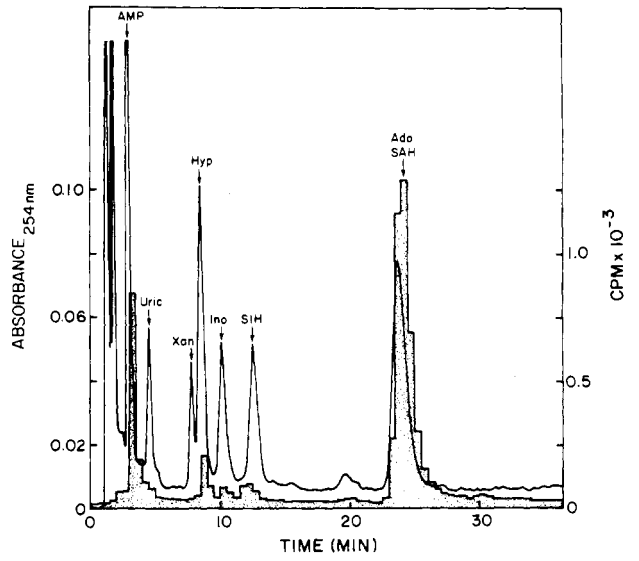

FIGURE 3: Chromatogram of a 50-μL sample of cell extract containing authentic markers obtained on a Partisil-10 ODS column with 15% loading of octadecyl silica. The column was eluted with distilled water in the primary reservoir and acetonitrile–acetic acid–water (10:1:89) in the secondary reservoir. An increasing linear gradient of the acetonitrile–acetic acid–water mixture from 1 to 100% at 1% per min and at a flow rate of 2.2 mL/min was used at a column inlet pressure of 1000 psig at ambient temperature. Shaded areas are radiolabeled metabolites.

efficient separation was achieved by using an increasing linear gradient of an acetonitrile–water–acetic acid mixture (10:89:1, repsectively) (see Figure 3). Under these conditions, all the oxypurines could be separated efficiently in less than 15 min, the order of elution being uric acid, xanthine, hypoxanthine, inosine, and SIH. The first four components are eluted in the order of their N^1 pK_a values (i.e., 5.75, 7.7, 8.8, and 8.9, respectively) which agrees with a previous report (Schatz et al., 1977); the pK_a value for N^1 of SIH has not been determined. Some 6-aminopurine derivatives are not efficiently separated on this system; e.g., adenosine coelutes with SAH, whereas MTA is not eluted during the time run used in the above analysis. The radiochromatogram of a cellular extract after 6-h incubation with [8-^{14}C]SAH and containing a mixture of authentic markers is shown in Figure 3. An early

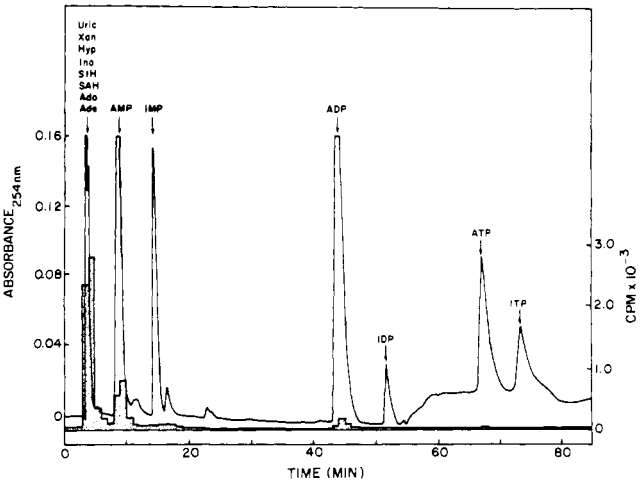

FIGURE 4: Chromatogram of a 50-μL sample of cell extract containing authentic markers obtained on a Partisil-10 SAX column. A concave exponential gradient (Du Pont setting no. 5) of 0.01 M NaH_2PO_4, pH 3.3 (primary), against 0.5 M NaH_2PO_4, pH 3.3 (secondary), was used from 1 to 100% secondary at 2%/min and held at 100% secondary for an additional 35 min. An elution flow rate of 1.0 mL/min was used at a column inlet pressure of 400 psig at ambient temperature. Shaded areas are radiolabeled metabolites.

band of radioactivity well separated from the oxypurinic region is present and may be attributable to metabolites of an extremely polar nature. A number of authentic metabolic standards of a polar nature were examined as possible candidates for SAH metabolites on the above chromatographic system; of those investigated, only nucleotides eluted with the early radioactive band.

The separation of nucleotides has received much attention over the last few years (Anderson & Murphy, 1976; Schmukler, 1970; Virkola, 1970; Kennedy & Lee, 1970; Brown, 1970; Brown et al., 1973; Brown & Parks, 1974; Kirkland, 1970), separations being based on ion-exchange and reverse-phase techniques by using both isocratic and gradient elution. However, no really efficient method has been reported for the separation of adenosine and inosine nucleotides. We have found that AMP, ADP, ATP, IMP, IDP, and ITP can be efficiently separated by using a concave exponential sodium dihydrogen phosphate concentration gradient at pH 3.3. On this system all the nonnucleotide standards elute early and do not interfere with the nucleotide separation. A radiochromatogram of a cell extract incubated with [8-^{14}C]SAH for 3 h is shown in Figure 4.

None of the above chromatographic systems is amenable to the separation of MTA, which elutes very slowly on reverse-phase chromatography in all the solvent systems investigated. A relatively quick and efficient separation of MTA from other 6-aminopurinic standards was developed to monitor the presence of this potential metabolite in [8-^{14}C]SAH-treated neuroblastoma cells. This method utilized a Partisil-10 SCX column with 0.05 M phosphate buffer, pH 4.5, as eluent at a column inlet pressure of 360 psi to give a flow rate of 0.65 mL/min at ambient temperature. On this system, MTA (t_R = 21.5 min) elutes before adenine (t_R = 34.0 min), while the adenosine–SAH solute pair is not resolved (t_R = 16.5 and 17.5 min, respectively). Some partial separation of the oxypurine standards is observed, uric acid eluting early (t_R = 8.5 min), while hypoxanthine (t_R = 12.0 min) is separated from the xanthine, inosine, SIH coeluting band (t_R = 9.75 min). By using this system, analysis of the cell extract obtained after incubation with [8-^{14}C]SAH showed the absence of MTA (Table I).

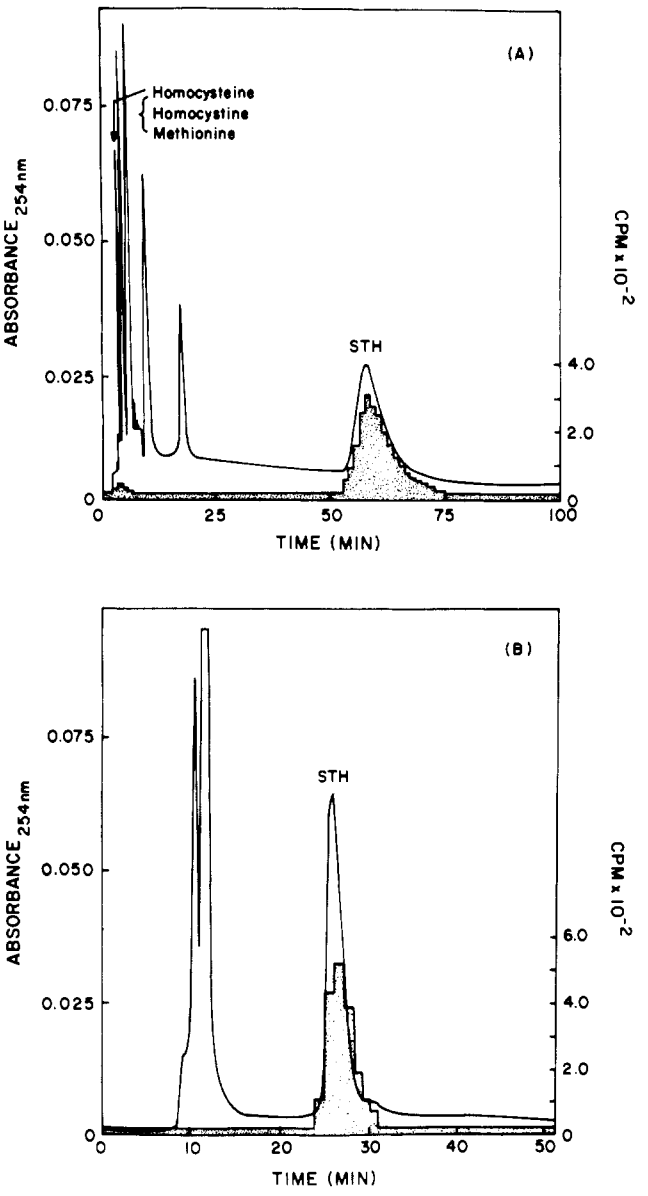

FIGURE 5: Chromatogram of a sample of cell extract after incubation with [*carboxyl*-^{14}C]STH for 6 h and containing an authentic marker of STH obtained on (A) a Partisil-10 ODS column with 4.5% loading of octadecyl silica eluted with 0.1 M sodium acetate buffer, pH 5.5, at a flow rate of 1.0 mL/min and a column inlet pressure of 500 psig at ambient temperature and (B) a Partisil-10 SCX column eluted with 0.1 M sodium phosphate buffer, pH 2.2, at a flow rate of 0.25 mL/min and a column inlet pressure of 110 psig at ambient temperature. Shaded areas are radiolabeled.

Based on the results obtained with SAH as described above, the separation of STH and its potential heterocyclic metabolites, tubercidin monophosphate (TbMP), 7-deazainosine (7-DzIno), 7-deazahypoxanthine (7-DzHyp), tubercidin (Tb), and 7-deazaadenine (7-DzAde), was initially performed on reverse-phase chromatography (see Figure 5A for chromatographic conditions). The order of elution was the same as that observed with the corresponding SAH analogues, although greater retention was obtained in the 7-deaza series. This system provided an efficient separation of TbMP (t_R = 9.9 min), 7-DzIno (t_R = 21.0 min), 7-DzHyp (t_R = 30.2 min), and 7-DzAde (t_R = 96.0 min); however, the STH–Tb solute pair (t_R = 56.1 and 51.0 min, respectively) was not base-line resolved. The resolution of this latter solute pair could not be improved by using the 15% octadecyl loaded Partisil-10 ODS 2 system. An alternative separation was achieved by

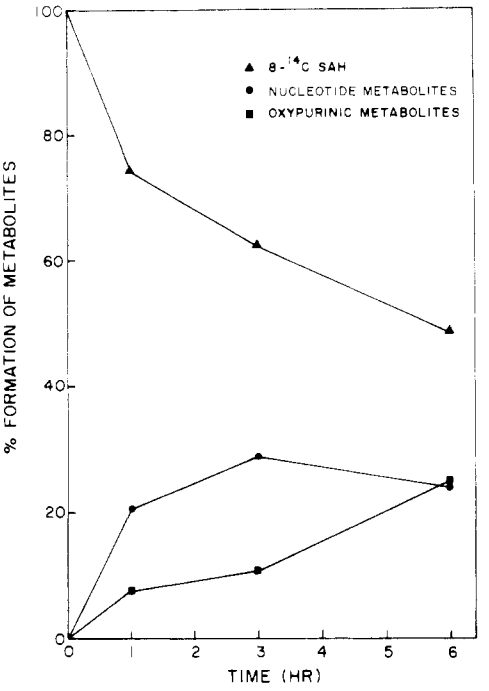

FIGURE 6: Time course of metabolism of [8-^{14}C]SAH in cultured neuroblastoma cells.

Partisil-10 SCX cation-exchange chromatography, which afforded base-line resolution of Tb (t_R = 19.4 min), SIH (t_R = 25.6 min), and 7-DzAde (t_R = 35.5 min), although, as expected, TbMP and 7-DzIno (t_R = 12.0 min) and 7-DzHyp (t_R = 14.9 min) were eluted with little retention (see Figure 5B for chromatographic conditions). These two chromatographic systems used in conjunction allow the individual determination of ring-labeled STH and its five potential metabolites to be achieved.

Although the preparation of [^{3}H]tubercidin has been described in the literature (Acs et al., 1964), the facile exchange of tritium with solvent H_2O, as has been established with analogously labeled adenosine derivatives (Baker & Haskell, 1975), argues against using this isotope for metabolic studies. Since ^{14}C-labeled tubercidin is not available nor is an efficient synthesis of tubercidin available, we chose to use [*carboxyl*-^{14}C]STH for our studies. Initial results showed that [*carboxyl*-^{14}C]STH is nearly completely (ca. 90%) recovered unchanged from the cell extracts, and that no label is observed in the void volume of the LC column, where sulfur-containing amino acids such as methionine, homocysteine, and homocysteine would be expected to elute (ODS-1; see Figure 5A for column conditions).

Table I summarizes the results obtained from the analysis of cellular extracts after incubation with [8-^{14}C]SAH on the four chromatographic systems described above (see Figures 2–4). On all chromatographic systems examined, the recovery of radioactivity from the analytical column was always within 10% of quantitative. The metabolism of [8-^{14}C]SAH in the cell system studied appears to proceed in a relatively linear fashion over the time period studied (see Figure 6), approximately 50% metabolism occurring after 6 h of incubation.

Control Experiments. To determine the stability of [8-^{14}C]SAH and [*carboxyl*-^{14}C]STH in the incubation medium, 0.13 μCi of each radiolabeled compound was added separately to prewarmed isotonic phosphate-buffered saline to give a final concentration of 50 μM. These samples were then incubated at 37 °C for 6 h as described previously. The two dishes were frozen (−4 °C) and thawed and the contents treated in an

Table I: Analysis of [8-^{14}C]SAH Metabolites Isolated from Cultured N-18 Neuroblastoma Cells[a]

expt'l conditions	uric acid[b]	XAN[b]	HYP[b]	INO[b]	SIH[b]	SAH[c]	ADO[c]	SATKB[c]	ADE[c]	MTA[d]	AMP[e]	IMP[e]	ADP[e]	IDP[e]	ATP[e]	ITP[e]
1-h incubation at 37 °C	0	0	3.1	2.5	2.3	74.6	0	0	0	0	13.3	4.2	1.8	0	0.9	0
3-h incubation at 37 °C	0	0	5.2	2.0	3.5	62.5	0	0	0	0	19.7	4.0	4.0	0	0.7	0
6-h incubation at 37 °C	0	0	19.2	2.5	2.6	48.5	0	0	0	0	20.0	2.2	1.2	0	<0.5	0
6-h incubation with 10^{-5} M coformycin at 37 °C	0	0	3.9	0	3.7	46.0	4.6	0	5.2	0	27.4	4.8	2.2	0	<0.5	0

[a] Data presented as % total radioactivity in each metabolite. [b] Determined by Partisil-10 ODS 2 chromatography (for conditions, see Figure 3). [c] Determined by Partisil-10 ODS 2 chromatography (for conditions, see Figure 2). [d] Determined on a Partisil-10 SCX column eluted with 0.05 M sodium phosphate buffer, pH 4.5, at a flow rate of 0.65 mL/min and a column inlet pressure of 360 psig at ambient temperature. [e] Determined by Partisil-10 SAX chromatography (for conditions, see Figure 4).

analogous manner to that described previously for the cell metabolism experiments. LC analysis of the resulting solutions showed that no significant chemical breakdown of either [8-^{14}C]SAH or [*carboxyl*-^{14}C]STH occurred in the incubation buffer under experimental conditions (data not shown). Incubation of [8-^{14}C]SAH (0.13 μCi, 50 μM) in growth medium supplemented with 10% fetal calf serum, penicillin (100 units/mL), and streptomycin (100 μg/mL) for 6 h at 37 °C, and then treatment of the mixture as described above, afforded a solution in which appreciable breakdown of the radiolabeled compound had occurred (LC analysis on a Partisil-10 ODS 2 column; see Table II). This breakdown appears to be enzymatic in nature since on repeating the experiment with supplemented growth medium that had previously been heated at 80 °C for 3.5 h, negligible breakdown of [8-^{14}C]SAH was observed. Incubation of [*carboxyl*-^{14}C]STH (0.13 μCi, 50 μM) in supplemented growth medium under the conditions described above resulted in no significant breakdown of the radiolabeled drug.

The following control experiment was carried out to determine the extent of extracellular metabolism of SAH in the incubation buffer during the course of the metabolism experiments. Previously washed cells were incubated in phosphate-buffered saline (1.0 mL) for 1 h at 37 °C. The buffer was then carefully removed from the monolayer and centrifuged at 27000*g* for 90 min. Microscopic examination of the supernatant showed the absence of cells. The supernatant was then quickly transferred to a 35-mm Petri dish, 0.13 μCi of [8-^{14}C]SAH added, and the dish incubated for 6 h at 37 °C as previously described. Aliquots (0.15 mL) of the solution were aseptically removed from the dish at 1- and 3-h intervals and immediately frozen at −4 °C. After 6 h, the remaining solution and the 1- and 3-h samples were treated in an analogous manner to that described previously for the cell metabolism experiments. Appreciable metabolic breakdown of [8-^{14}C]SAH was observed in all three samples when analyzed by LC on a Partisil-10 ODS 2 column (Table II). Decreasing the contact time of the buffer with the cell monolayer to 10 min and repeating the above procedure afforded similar results. No metabolism of [*carboxyl*-^{14}C]STH was seen under any of these experimental conditions.

Discussion

From the data in Table I it is evident that two major pathways of SAH metabolism operate in neuroblastoma cells: first, hydrolysis of the homocysteine–ribose bond by *S*-adenosylhomocysteine hydrolase to give adenosine and homocysteine; and, second, either deamination of the 6-aminopurine function of adenosine by adenosine deaminase or phosphorylation by adenosine kinase. The fact that mainly mononucleotides are isolated is probably due to the extraction procedure, in that most triphosphates formed would be hydrolyzed to monophosphates during the low pH protein precipitation at 40 °C. Interestingly, no significant adenosine or adenine levels could be detected in the cell extract at any of the incubation times examined, although nucleotide formation appears to occur earlier on in the time course and then levels off (see Figure 6). It has been shown that intracellular levels of nucleosides and their bases are relatively low compared with corresponding nucleotide levels (Plagemann, 1969, 1970) and the presence of the latter species as SAH metabolites suggests that the adenosine formed as a result of SAH hydrolase activity is rapidly channeled into phosphorylating pathways or converted into inosine by adenosine deaminase (Snyder et al., 1976). Adenosine may also be a potential precursor of adenine (Snyder & Henderson, 1973; Snyder et

Table II: Determination of Extracellular Metabolism of [8-^{14}C] SAH in Cultured N-18 Neuroblastoma Cells

exptl conditions	incubation time (h)	% metabolites[a]					
		hypoxanthine	inosine	SIH	adenine	adenosine	SAH
incubation of [8-^{14}C]SAH in supplemented growth medium at 37 °C	1	0.7	4.5	0	0	24.2	65.3
	3	18.3	34.6	0	0.9	1.7	43.2
	6	39.8	14.0	0	0.4	1.1	44.5
incubation of [8-^{14}C] SAH in heat-deactivated supplemented growth medium at 37 °C	6	0	0	1.64	1.20	0	96.1
incubation of [8-^{14}C] SAH in "1-h cell contact" buffer at 37 °C	1	2.4	33.6	0	0.8	9.9	51.2
	3	8.4	35.4	1.7	1.0	2.5	50.2
	6	16.5	29.4	1.9	1.0	0	50.1

[a]Data presented as % total radioactivity in each metabolite. Determined by Partisil-10 ODS 2 chromatography. Although not shown in Figure 3, adenine (t_R = 20 min), adenosine (t_R = 22.5 min), and SAH (t_R = 24.5 min) are sufficiently well resolved for rapid analysis in these control experiments.

al., 1976). Although, in general, levels of SIH and inosine were relatively low at all incubation times studied, hypoxanthine formation gradually increased to a maximum of 19.2% at 6-h incubation. Low levels of IMP were also observed, although IDP and ITP could not be detected. The origin of IMP could be from hypoxanthine or AMP via the enzymes hypoxanthine phosphoribosyltransferase (EC 2.4.2.2) or adenylate deaminase (EC 2.7.4.3); both enzymes are known to be involved in adenosine metabolism (Snyder et al., 1976). It should be noted that the material identified as IMP might contain some GMP (t_R = 18 min), since all of the radioactivity is not exactly coincident with the IMP marker (t_R = 15 min). No MTA or SATKB could be detected in any of the cell extracts analyzed and the absence of xanthine and uric acid as metabolites of SAH confirms the recent observation that murine neuroblastoma cells are devoid of xanthine oxidase (EC 1.2.3.2) (Seegmiller, 1977).

In the presence of 10^{-5} M coformycin, a potent adenosine deaminase inhibitor, inosine could not be detected as a metabolite of [8-^{14}C]SAH after 6-h incubation and the hypoxanthine level dropped from 19.2% in the noninhibitor-treated cell homogenate to 3.9%. In contrast, the AMP level increased from 20.0% to 27.4%. The overall extent of SAH metabolism in the presence of coformycin was of the same order as that observed in the noninhibitor-treated cell culture. Of some importance is the observation that significant amounts of adenosine and adenine can be detected after incubation with 10^{-5} M coformycin, both presumably formed as a result of deaminase inhibition. In this respect it is interesting to note that increased amounts of adenosine and adenine have also been detected in erythrocytes and plasma of adenosine deaminase deficient patients along with elevated adenine nucleotide levels (Mills et al., 1976). In the presence of 10^{-9} M coformycin, no inhibition of adenosine deaminase was observed. This is consistent with the finding that a minimum concentration of 1 μg/mL (3.5 μM) of coformycin is required to completely inhibit adenosine deaminase activity in intact mammalian cells (Lomax & Henderson, 1972).

Of particular concern was the level of extracellular metabolism of SAH observed in the control experiments (see Table II). This metabolism may be a result of the diffusion of enzymes out of the cell into the surrounding buffer during incubation and appears to occur fairly rapidly after addition of buffer to the monolayer. Alternatively, this SAH metabolism may be a result of inadequate washing of growth medium from the cell monolayer since incubation of [8-^{14}C]SAH in supplemented growth medium alone leads to appreciable enzyme degradation of SAH (see Table II). Of additional interest is the observation that in these control

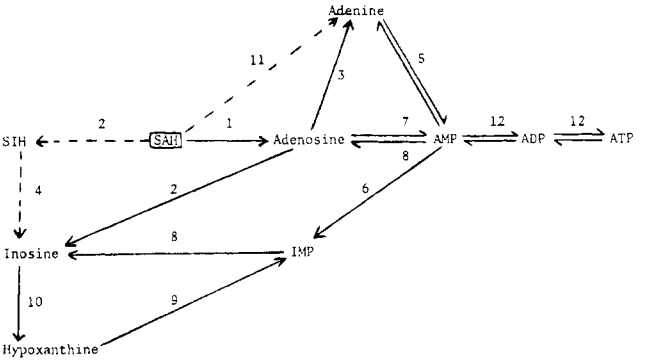

FIGURE 7: Pathways of SAH metabolism in neuroblastoma cells. (1) SAH hydrolase (EC 3.3.1.1); (2) adenosine deaminase (EC 3.5.4.4); (3) adenosine nucleosidase (EC 3.2.2.7); (4) SIH hydrolase; (5) adenine phosphoribosyltransferase (EC 2.4.2.7); (6) adenylate deaminase (EC 3.5.4.6); (7) adenosine kinase (EC 2.7.1.20); (8) 5′-nucleosidase (EC 3.1.3.5); (9) hypoxanthine phosphoribosyltransferase (EC 2.4.2.8); (10) inosine nucleosidase (EC 3.2.2.2); (11) SAH nucleosidase (EC 3.2.2.9); (12) adenylate kinase (EC 2.7.4.3).

experiments no phosphorylated metabolites were detected. It is possible that the extracellular metabolism observed above may be due to some cell lysis occurring in the incubation buffer, resulting in the dead cells releasing their contents into the surrounding medium. This phenomenon has been observed previously with N-18 neuroblastoma cells when incubated in serum-deficient medium, the activity of soluble acetylcholinesterase in the medium increasing with cell death (Lanks, 1977). However, in our experiments, cell viability measured by dye exclusion was always at least 80%.

LC analysis of cellular extracts after incubation with [*carboxyl*-^{14}C]STH showed that little or no metabolism of STH occurred even after a 6-h incubation period with ca. 90% of the radioactivity originally applied to the cell culture being eluted with authentic STH in two LC systems (see Figures 5A and 5B). In addition, STH is not enzymatically degraded when incubated in growth medium alone. These data are consistent with the observation that 7-deaza nucleosides do not act as a substrate for adenosine deaminase (EC 3.5.4.4) (Suhadolnik, 1970), SAH hydrolase (EC 3.3.1.1) (Chiang et al., 1977), or the enzyme(s) that catalyze(s) cleavage of the heterocyclic base–ribose bond of adenosine (Coward et al., 1977).

In conclusion, based on the data presented in this study, a tentative scheme for the metabolism of SAH in neuroblastoma cells can be constructed (see Figure 7). Our findings are consistent with the suggestion that differences in the efficacy of SAH and STH as transmethylation inhibitors in whole cell systems are due to the stability of the 7-deaza analogue toward

metabolic degradation and provide interesting possibilities for the control of intracellular levels of SAH which may be important in regulating cellular transmethylation. It is important to note that studies involving the determination of cellular uptake of SAH are complicated by the rapid and extensive degradation of this molecule demonstrated in this work. In contrast, the inert nature of STH should make it more amenable to investigations into the uptake and transport of this important class of methylase inhibitors.

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