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Effects of Inhibitors of Adenosine Triphosphate:L-Methionine S-Adenosyltransferase on Levels of S-Adenosyl-L-methionine and L-Methionine in Normal and Malignant Mammalian Tissues

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SUMMARY

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Low levels of ATP:L-methionine *S*-adenosyltransferase (adenosyltransferase, EC 2.5.1.6) have been detected by a sensitive radioactive assay procedure in transplantable neoplasms, including the Walker 256 tumor of the rat, and the Lewis lung tumor and B-16 melanoma of C_{57} black mice. These enzymatic activities and those of a number of normal rodent tissues are all significantly inhibited by 1-aminocyclopentanecarboxylic acid (cycloleucine) and by L-2-amino-4-hexynoic acid, which are structural and conformational analogues of L-methionine.

Following single intraperitoneal injections to rats, the plasma cycloleucine levels remain almost constant for at least 96 hr, whereas the L-2-amino-4-hexynoic acid plasma levels fall with a half-life of about 60 hr. Both cycloleucine and L-2-amino-4-hexynoic acid are highly concentrated in the Walker 256 tumor, while the acetylenic amino acid is concentrated in the Lewis lung tumor.

Administration of pharmacological doses of cycloleucine (5.19 mmoles/kg) or L-2-amino-4-hexynoic acid (2.64 mmoles/kg) elevates plasma and tissue levels of these amino acids in a dose- and time-dependent manner to concentrations which are probably sufficiently high to achieve significant inhibition of the tissue adenosyltransferases. Mice and rats treated with these adenosyltransferase inhibitors display dramatic elevations of free L-methionine levels in all tissues examined (liver, spleen, kidney, brain, plasma, Walker 256 tumor, and Lewis lung tumor). These effects are dose-dependent. The levels of five other amino acids (isoleucine, leucine, tyrosine, phenylalanine, and valine) in liver or spleen show only minor

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changes in response to this treatment, except for a doubling of the phenylalanine level in the spleen. Treatment of animals with high doses of other amino acid analogues that do not inhibit the adenosyltransferases (L-norvaline, L-valine, DL-homoserine) had no effect on the L-methionine levels.

Treatment with pharmacological doses of cycloleucine and L-2-amino-4-hexynoic acid depresses the levels of (−)-*S*-adenosyl-L-methionine (Ado-Met) by 20–40% in most normal and malignant tissues (including spleen, kidney, pancreas, brain, adrenal, Walker 256 tumor of rats, and Lewis lung and L1210 tumors of mice). These effects are time- and dose-dependent. Amino acids (L-norvaline, L-serine, and L-valine) that are not adenosyltransferase inhibitors are without effect on tissue Ado-Met concentrations. In contrast to the findings in other tissues, the Ado-Met levels of liver are invariably increased following administration of adenosyltransferase inhibitors.

INTRODUCTION

S-Adenosyl-L-methionine plays a strategic role in providing activated methyl groups for a wide variety of biological methylation reactions, and the propylamine fragments for spermidine and spermine biosynthesis (1–6). The mechanisms that regulate tissue levels and turnover of Ado-Met[2] and L-methionine are not well understood, and there are few clues as to the factors controlling the metabolic flux of Ado-Met and its utilization for methylation reactions and polyamine biosynthesis.

It is believed that the ATP:L-methionine *S*-adenosyltransferase (EC 2.5.1.6) reaction is the principal, if not the sole, mechanism for the synthesis of Ado-Met in animal tissues, in accordance with the following stoichiometry (7):

$$\text{L-Methionine} + \text{ATP} \xrightarrow{Mg^{++},\ K^{+}} (-)\text{-}S\text{-adenosyl-L-methionine} + PP_i + P_i$$

S-Adenosyl-L-homocysteine, which is one of the products of transmethylation reactions, does not appear to undergo direct remethylation to Ado-Met in animal tissues (8), and is cleaved to adenosine and L-homocysteine, in which the sulfur atom then undergoes further oxidation and is eventually largely eliminated as inorganic sulfate.

A close relationship between the cellular levels of L-methionine and those of Ado-Met was first pointed out by Schlenk and colleagues (8, 9), who demonstrated the accumulation of large amounts of Ado-Met, easily detectable by ultraviolet absorption, in yeast cells incubated in media rich in methionine. A similar but somewhat less dramatic phenomenon is observed in mammalian systems (10). *S*-Adenosyl-L-methionine levels rise in all tissues (4-fold in liver and 30–100% in other tissues) upon the administration of L-methionine (100 mg/kg). Moreover, Baldessarini and others have reported that Ado-Met levels are depressed by the administration of certain (but not all) compounds which are capable of acting as methyl group acceptors (11, 12). This finding and the observation that monoamine oxidase inhibitors, such as pargyline (11, 12), also cause the depression of Ado-Met levels in brain and liver, have led Baldessarini to suggest that the availability of methylation acceptors and presumably the rate of Ado-Met utilization may also exercise control over Ado-Met levels. More recent reports (13–15), which establish that the administration to rodents of the anti-parkinsonian agent 3,4-dihydroxy-L-phenylalanine (L-dopa) causes a rapid decline in brain, kidney, and adrenal (but not muscle) Ado-Met levels, are also in line with this view, since L-dopa and its decarboxylation product (dopamine) both undergo rapid enzymatic *O*-methylation at the expense of Ado-Met.

Recent efforts in this laboratory to design structural and conformational analogues of L-methionine as inhibitors of the adenosyl-

[2] The abbreviations used are: Ado-Met, (−)-*S*-adenosyl-L-methionine; adenosyltransferase, ATP: L-methionine *S*-adenosyltransferase (EC 2.5.1.6); cycloleucine, 1-aminocyclopentanecarboxylic acid; hexynoic acid, L-2-amino-4-hexynoic acid.

transferases derived from rat liver and microbial sources have led to the discovery that 1-aminocyclopentanecarboxylic acid (cycloleucine), L-2-amino-4-hexynoic acid, and related compounds are inhibitors of this enzyme in competition with L-methionine (16). Since cycloleucine suppresses the growth of a number of tumors and microorganisms, it was suggested that this compound may owe its growth-inhibitory properties at least in part to inhibition of the ATP:L-methionine *S*-adenosyltransferase reaction (5, 16). Validation of this suggestion requires re-examination of reports that a variety of mouse and rat neoplasms, with the exception of certain Morris hepatomas, do not contain detectable amounts of adenosyltransferase activity (17, 18). However, adenosyltransferase activity is similar in the leukocytes of patients with chronic myelocytic leukemia and their normal counterparts, whereas the concentrations of Ado-Met in the malignant cells are markedly elevated above the relatively low levels of normal white blood cells (19, 20).

This paper establishes that several solid malignant tumors contain low but clearly detectable levels of adenosyltransferase activity. Our experiments deal with the effects *in vivo* of the administration of adenosyltransferase inhibitors on the levels of Ado-Met and L-methionine in normal and malignant rodent tissues. If any pharmacological effects of these inhibitors are to be ascribed to inhibition of the enzymatic synthesis of Ado-Met, it would be expected (a) that tissue levels of L-methionine would rise and those of Ado-Met would decline, (b) that these effects would occur at appropriate tissue concentrations of the inhibitors, and (c) that the time course of inhibition would follow the tissue levels of the inhibitors. The object of this study was to examine these propositions.

The development of highly specific and sensitive double-isotope, enzymatic derivative procedures for Ado-Met (10, 21) and for L-methionine (22) has made these experiments feasible. These methods have established that liver and many other rodent tissues contain 40–140 nmoles of Ado-Met and 50–180 nmoles of L-methionine per gram of wet tissue weight.

EXPERIMENTAL PROCEDURE

Materials

All solutions were prepared in deionized, glass-distilled water from reagent grade chemicals. The sources of the chemicals were as follows: L-methionine, L-valine, L-serine, L-norleucine, and ammonium sulfate (special enzyme grade) were supplied by Schwarz/Mann. *S*-Adenosyl-L-methionine hydrogen sulfate was purchased from Boehringer-Mannheim. 1-Aminocyclopentanecarboxylic acid (cycloleucine) was supplied by Cyclo Chemical Corporation. Glutathione was obtained from P-L Biochemicals. The amino acid mixture used for calibration of the amino acid analyzers was purchased from the Beckman Instrument Company. 2-Mercaptoethanol was supplied by Eastman Organic Chemicals and distilled under reduced pressure (b.p. 58–60° at 23 mm Hg). L-Norvaline, tris(hydroxymethyl)-aminomethane base, 5-hydroxytryptamine (serotonin)–creatinine sulfate complex, and *N*-acetyl-5-hydroxytryptamine (*N*-acetyl-serotonin) were obtained from Sigma Chemical Company. The primary and secondary scintillators PPO (2,5-diphenyloxazole) and POPOP {*p*-bis[2-(5-phenyloxazolyl)]benzene} used in making Bray's scintillation fluid (23) were purchased from New England Nuclear. Spectroscopic quality *p*-dioxane and glycerol were purchased from Matheson, Coleman, and Bell. Tetralithium [8-^{14}C]-adenosine 5′-triphosphate (53.4 μCi/μmole in 50% ethanol), *S*-adenosyl-L-[*methyl*-^{14}C]-methionine (44.5 μCi/μmole), L-[*methyl*-^{14}C]-methionine (53.6–60.0 μCi/μmole), L-[*methyl*-^{3}H]methionine (2.6 mCi/μmole), and [^{3}H]acetic anhydride (100 μCi/μmole) were purchased from Schwarz/Mann, New England Nuclear, or Amersham/Searle Corporation. The cation and anion exchange Dowex resins AG 50W-X2 (100–200 mesh) and AG 1-X2 (100–200 mesh) were supplied by Bio-Rad Laboratories. L-2-Amino-4-hexynoic acid was synthesized and characterized by infrared, mass, and nuclear magnetic resonance spectroscopy in this laboratory by Dr. A. W. Coulter (24).[3] Animals were obtained from the following suppliers: Sprague-Daw-

[3] A. W. Coulter and J. Salt, unpublished work.

ley male rats, 85–170 g, from Sprague-Dawley, Madison, Wis.; C_{57} black male mice from Huntington Farms, West Conshohocken, Pa.; and DBA/2 female and C_{57} black male mice from Jackson Memorial Laboratories, Bar Harbor, Maine. *Escherichia coli* strain B (midlogarithmic) cells were obtained from Grain Processing, Muscatine, Iowa. The Walker 256 tumor, the Lewis lung tumor, and the B-16 melanoma were gifts from Mr. Isidore Wodinsky, Arthur D. Little, Inc., Cambridge, Mass. The L1210 leukemia was a gift from Dr. Albert H. Owens of this University.

Methods

Preparation of ATP:L-methionine S-adenosyltransferases. Comparison of the specific activities of the adenosyltransferases of rat and mouse livers and tumors was carried out on tissues prepared in a similar manner. The animals were killed by cervical dislocation and the tissues were immediately excised and washed with cold 50 mM potassium phosphate buffer (pH 7.0) containing 5 mM 2-mercaptoethanol. The tissues were then weighed and homogenized in a Potter-Elvehjem apparatus with 2.5 volumes of the above buffer and centrifuged two times at 20,000 × *g* for 20 min. The clear supernatant fluid was used for the assays of enzyme activities (Table 1).

For the inhibitor studies the adenosyltransferases from rat and mouse tissues were prepared as follows. Rats bearing the Walker 256 tumor, mice bearing the Lewis lung tumor, or normal rats and mice were killed and the appropriate tissues were pooled. The tissues were homogenized with 2.5 volumes of 40 mM potassium phosphate buffer (pH 6.9) containing 5 mM 2-mercaptoethanol and 20% glycerol and centrifuged twice at 20,000 × *g* for 20 min. The supernatant fluid was fractionated with saturated ammonium sulfate (pH 7.0) containing 5 mM 2-mercaptoethanol. The fraction precipitating between 33 and 56% saturation was dissolved in the initial buffer and dialyzed overnight against 2 liters of the same buffer. The adenosyltransferase preparation obtained from rat liver was further purified by DEAE-cellulose chromatography (16, 25).

Assay of ATP:L-methionine S-adenosyltransferase. The column chromatographic procedure of Mudd *et al.* (26) as modified by Lombardini *et al.* (16) was employed to determine the specific activities of the isofunctional enzymes prepared from different tissues (Table 1). Incubation mixtures of 250 μl contained (in micromoles): glutathione, 2; KCl, 50; Tris-HCl, pH 7.6, 40; ATP, 5; $MgCl_2$, 75, L-methionine, 0.5, containing 500,000 cpm of L-[*methyl*-^{14}C]methionine; and the enzyme preparation. For inhibition studies the quantity of L-methionine in the reaction system was 9.38 nmoles. Incubations were carried out at 37° with agitation. The reaction was terminated by diluting with 10 ml of cold H_2O and applying the mixtures to Dowex AG 50W-X2 (100–200 mesh) columns (6 × 30 mm) in the ammonium cycle. A minimum of 100 ml of H_2O was used to wash unreacted L-[*methyl*-^{14}C] methionine through the columns. The Ado-Met was eluted from the columns with two 5-ml aliquots of concentrated ammonium hydroxide, and each aliquot was counted in a liquid scintillation spectrometer with 15 ml of Bray's scintillation fluid (23) with an efficiency of 70%. The specific activity is expressed as micromoles of Ado-Met formed per milligram of protein in a 30-min incubation at 37° (16).

Tissue preparation for analysis of S-adenosyl-L-methionine. Animals were killed by cervical fracture and the tissues were immediately excised and washed with cold 0.9% NaCl. The tissues were then frozen in liquid nitrogen, weighed, and homogenized with 0.33 M perchloric acid (usually 2 volumes) in a conical Potter-Elvehjem apparatus. The homogenate was centrifuged for 15 min at 10,000 × *g*, and the supernatant fluid was adjusted to pH 6.5 with potassium bicarbonate and centrifuged (10 min at 10,000 × *g*). Aliquots of the supernatant fluid were then analyzed as described below.

Assay of S-adenosyl-L-methionine. The double-isotope method of Baldessarini and Kopin (10, 21) was employed to determine tissue content of Ado-Met. This technique utilized Ado-Met as a methyl donor for the enzymatic synthesis of melatonin from *N*-acetylserotonin by hydroxyindole *O*-meth-

yltransferase. This enzyme was purified according to published procedures (10, 27). The assay mixture contained the following components in a final volume of 1.0 ml: 100 μmoles of potassium phosphate buffer (pH 6.9), 10 nmoles of *N*-[^{3}H]acetylserotonin (240,000 cpm) prepared according to Baldessarini and Kopin (10, 21), 1 nmole of exogenous carrier *S*-adenosyl-L-[*methyl*-^{14}C]-methionine (25,000 cpm), 50–100 μl of partially purified hydroxyindole *O*-methyltransferase containing approximately 2 mg of protein (specific activity, 14.5 nmoles of melatonin formed per milligram of protein per hour), and an aliquot of centrifuged neutralized tissue extract (100–250 μl). Water was added to a final volume of 1.0 ml, and the reaction mixture was incubated for 1 hr at 37°. When a large number of tissues were to be processed at the same time it was advantageous to prepare the tissue extracts and to add an aliquot of the extract to the reaction mixtures (omitting the methyltransferase) as soon as possible. These samples could then be frozen overnight, and the addition of the transferase, the incubation, and subsequent extraction of product could be performed on the following day. The reaction was stopped by addition of 2 ml of 1 N sodium hydroxide and the [*N-acetyl*-^{3}H; *methoxy*-^{14}C]melatonin was extracted into 6 ml of chloroform which was washed twice with 2 ml of 1 N sodium hydroxide. Aliquots of the chloroform phase were evaporated to dryness in scintillation vials in a desiccator under vacuum, and counted for ^{3}H and ^{14}C with 10 ml of Bray's scintillation fluid. (Efficiency for ^{14}C was 90% and for ^{3}H was 36%; correction was needed only for the spill of ^{14}C into the ^{3}H channel.) The ratio of ^{3}H to ^{14}C changed linearly with dilution by endogenous Ado-Met in the tissues, and the quantity of Ado-Met was graphically determined from a calibration curve constructed with unlabeled *S*-adenosyl-L-methionine as a standard.

Tissue preparation and analysis of amino acids. Tissue extracts in 0.33 M perchloric acid were prepared according to the above procedure for determining Ado-Met levels. For experiments in which the double-isotope enzyme derivative method for L-methionine (22) was used, the following additional steps were included. After neutralization of the tissues with $KHCO_3$, an aliquot (usually 0.3 ml) of the centrifugate was passed over an anion exchange resin (AG 1-X2, 100–200 mesh, chloride form, 2 × 20 mm) to remove endogenous ATP, which interferes with the assay by diluting the specific activity of the [8-^{14}C]ATP. Controls with either L-[*methyl*-^{14}C]methionine or [8-^{14}C]ATP indicated that there was both quantitative removal of endogenous ATP and quantitative recovery of L-methionine (22).

Assay of L-methionine. A rapid, highly sensitive, enzymatic double-labeled isotope dilution method for the quantitative determination of L-methionine has been devised in this laboratory (22). The assay mixture contains the following components (in micromoles) in a final volume of 350 μl: glutathione, 2; KCl, 50; Tris-HCl, pH 7.6, 80; $MgCl_2$, 75; L-[*methyl*-^{3}H]methionine containing 80,000 cpm; [8-^{14}C]ATP, 0.015, containing 500,000 cpm; approximately 1.0 mg of *E. coli* adenosyltransferase [specific activity, 2.5 μmoles of Ado-Met formed per milligram of protein in 30 min under specified conditions (16)]; and a processed aliquot of centrifuged tissue supernatant fluid. A standard curve is constructed by adding exogenous unlabeled L-methionine and calculating the isotope ratio of ^{14}C:^{3}H, taking into consideration both the ^{14}C spill (approximately 30%) into the tritium channel and the tritium spill (approximately 1–2%) into the ^{14}C channel. The simultaneous equations were solved on a calculator.

Other amino acid analyses. These were performed on either the Beckman 120C amino acid analyzer, using the long column (0.9 × 60 cm) and the standard buffer system(28), or the single column system (0.7 × 125 cm) of the Technicon amino acid analyzer (29). Both instruments were calibrated with a standard amino acid mixture and L-norleucine as an internal standard.

Determination of tissue inhibitor concentrations. Cycloleucine was quantitatively determined on the Beckman 120C amino acid analyzer. The cyclic amino acid was eluted in almost the same relative position (with respect to pH and ionic strength) as norleucine, and thus there was no overlap with the natural amino acids and other

ninhydrin-positive components present in tissues. Standards of cycloleucine gave only about 25% of the color intensity of the standard α-amino acids. The direct quantitative analysis of L-2-amino-4-hexynoic acid on the Beckman amino acid analyzer was hindered by the complete overlap of its elution position with that of L-alanine in the normal buffer systems. However, the ratios of the absorbances of the ninhydrin reaction products of these amino acids as detected by the 570 nm and 440 nm colorimeters were very different, and could be used to determine both amino acids reliably. Thus the ratio of chart areas measured at 570 nm to those measured at 440 nm was 7.10 for alanine and 1.61 for L-2-amino-4-hexynoic acid. The appropriate constants were obtained for standard quantities of each amino acid and for mixtures. The quantity of each amino acid in mixtures could be calculated with an accuracy of ±5–10% by solution of two simultaneous equations.

Protein concentrations. These were determined by the method of Lowry *et al.* (30).

Tumor transfer. The Walker 256 tumor was carried intramuscularly in male Sprague-Dawley rats (31). Approximately 1 g of tumor cells was homogenized by hand in a ground-glass homogenizer with 1 ml of sterile 0.9% NaCl. An aliquot, usually 0.1–0.2 ml, of this suspension was injected intramuscularly into the right thigh of the rat. Within 4–5 days the tumor was palpable and the tumors were usually harvested on the seventh day.

The L1210 leukemia was carried in DBA/2 female mice. The spleen from a leukemic animal was homogenized by hand in a loosely fitting glass homogenizer with 12–15 ml of sterile 0.9% NaCl. An aliquot (0.1 ml) of this solution was injected subcutaneously into the inner left thigh. After 5–6 days the tumor weighed 300–500 mg and the animals were killed. The animals normally die by the eight or ninth day as a result of systemic infiltration of the leukemia.

The Lewis lung tumor and melanoma B-16 were carried in C_{57} black male mice and transplanted every 2 weeks by implantation of an approximately 2-mm cube of tumor subcutaneously by trochar into the right axillary area.

Injections. Compounds were administered usually by intraperitoneal injections in 0.9% NaCl (1–1.5 ml for the rats and 0.3 ml for the mice).

RESULTS

Activities of adenosyltransferase in normal and malignant tissues. Using less sensitive assay techniques, several investigators could not detect adenosyltransferase activity in certain tumors, i.e., rat Novikoff solid and ascites hepatomas (17) and a variety of mouse neoplasms, including hepatic adenocarcinoma BW7756 (18). However, various Morris hepatomas (5123D, 7793, and 7795) contain levels of adenosyltransferase comparable to rat liver (17). The extremely sensitive radioactive enzyme assay of Mudd *et al.* (26), and its modifications (16), have clearly established that centrifuged crude homogenates of the Walker 256 tumor, the Lewis lung tumor, and the B-16 melanoma of C_{57} black mice all contain low but easily measurable levels of adenosyltransferase that amount to some 3–8% of the specific activity of liver (Table 1). The tumor activity levels are comparable to those of many normal tissues (26). Liver is invariably the most active tissue, and pancreas and kidney contain high activities. There is therefore good evidence for the virtually ubiquitous tissue distribution of the adenosyltransferases.

The injection of cycloleucine or of L-2-amino-4-hexynoic acid had little or no effect on the enzyme activities observed in centrifuged crude homogenates of normal and malignant tissues, when the assays were carried out under conditions which dilute the tissue levels of these compounds to non-inhibitory levels. Although several instances are known in which enzyme inhibitors affect the balance between rates of enzyme synthesis and degradation, there is no evidence for this phenomenon in the present case (Table 1).

Inhibition of isofunctional rodent adenosyltransferases by cycloleucine and L-2-amino-4-hexynoic acid. Although cycloleucine and L-2-amino-4-hexynoic acid have been shown to inhibit the adenosyltransferases of rat liver, yeast, and *E. coli* (16), it became necessary to establish that these inhibitory

effects pertained also to the isofunctional enzymes obtained from other tissues. Table 2 gives the concentrations of these substances required for 50% inhibition (I_{50} values) of adenosyltransferase activity of centrifuged crude homogenates of liver, spleen, and the Walker 256 tumor of Sprague-Dawley rats, as well as the Lewis lung tumor of C_{57} black mice. These values vary between 0.8 and 2.6 mM with 37.5 μM L-methionine, under the reaction conditions stated. The adenosyltransferases of various chicken tissues are likewise inhibited by cycloleucine and L-2-amino-4-hexynoic acid (33).

TABLE 1

Activities of ATP:L-methionine adenosyltransferase in centrifuged homogenates of normal and malignant mouse and rat tissues

The tissues were prepared and activities measured as described under EXPERIMENTAL PROCEDURE. The formation of Ado-Met was shown to be linear with respect to time and protein concentration. The final L-methionine concentration in the assay system was 2.0 mM. The treated animals (male C_{57} black mice or male Sprague-Dawley rats) received intraperitoneal injections of cycloleucine (7.75 mmoles/kg for mice and 5.19 mmoles/kg for rats) or L-2-amino-4-hexynoic acid (3.94 mmoles/kg for mice and 2.64 mmoles/kg for rats) at 18 and 2.5 hr prior to removal of tissues for analysis. The activities are expressed as means ± standard deviations. The number of animals involved in each set of determinations is given in parentheses.

Treatment of animals	Male C_{57} black mice		
	Normal liver	Lewis lung tumor	B-16 melanoma
	nmoles Ado-Met formed/30 min/mg protein		
Untreated	126 ± 7 (5)	5.0 ± 0.7 (5)	4.0; 4.9 (2)
Cycloleucine	125 ± 16 (3)	5.7 ± 0.3 (3)	
L-2-Amino-4-hexynoic acid	152 ± 10 (3)		

Treatment of animals	Male Sprague-Dawley rats	
	Liver	Walker 256 tumor
	nmoles Ado-Met formed/30 min/mg protein	
Untreated	73.0 ± 12.9 (7)	5.5 ± 1.4 (5)
Cycloleucine	80.3; 93.1 (2)	6.8 (1)
L-2-Amino-4-hexynoic acid	85.7; 90.0 (2)	

TABLE 2

Inhibition of adenosyltransferases of various normal and malignant tissues by cycloleucine and L-2-amino-4-hexynoic acid

The determinations were carried out on crude centrifuged homogenates of appropriate tissues obtained from Sprague-Dawley rats and C_{57} black mice under conditions described by Lombardini *et al.* (16), at a final L-methionine concentration of 37.5 μM. The concentrations of inhibitor (I_{50}) required to reduce the activity to one-half were determined graphically by the method of Dixon (32). The number of animals involved in each set of determinations is given in parentheses. The I_{50} values are expressed as means ± standard deviations when measurements were made on more than one animal.

Tissue	Concentration of analogue required for 50% inhibition	
	Cycloleucine	L-2-Amino-4-hexynoic acid
	mM	*mM*
Rat liver	2.62 ± 0.27 (5)	1.87 ± 0.43 (3)
Rat spleen	2.15 ± 0.05 (2)	1.0 (1)
Walker 256 tumor	2.41 ± 0.13 (3)	0.83 ± 0.05 (3)
Lewis lung tumor	2.13 ± 0.09 (3)	0.90 ± 0.08 (3)

Blood and plasma levels of cycloleucine and L-2-amino-4-hexynoic acid. Following the intraperitoneal injection of 10–100-mg doses of cycloleucine to male Sprague-Dawley rats weighing 133–137 g (i.e., 0.58–5.7 mmoles/kg), the blood levels attained plateaus of 0.4–4.4 mM in 2–4 hr and remained essentially unchanged for as long as 96 hr (Fig. 1). At the highest dose levels the rats do not survive beyond about 7 days. These findings confirm earlier reports (34) that cycloleucine is cleared extremely slowly from the rat, and is not appreciably metabolized in this species. Cycloleucine does not accumulate significantly within the cellular elements of the blood, since the concentrations of this amino acid in perchloric acid extracts of either heparinized plasma and/or whole blood were almost the same (ratio = 1.13 ± SD of 0.05; N = 5) at time intervals from 15 min to 24 hr after administration of the cycloleucine. In a more limited study, following the intraperitoneal administration of L-2-amino-4-hexynoic acid (2.64 mmoles/kg), the plasma levels rose somewhat more slowly to a maximum of 2.5 mM and fell very slowly with a half-life of approximately 60 hr (not shown). No information is available on the possible metabolism of L-2-amino-4-hexynoic acid.

Tissue levels of cycloleucine and L-2-amino-4-hexynoic acid. Table 3 shows the levels of cycloleucine and L-2-amino-4-hexynoic acid in various Sprague-Dawley rat and C_{57} black mouse tissues following the intraperitoneal injection of these compounds. The administration of two doses of 5.19 mmoles of cycloleucine per kilogram or two doses of 2.64 mmoles of L-2-amino-4-hexynoic acid per kilogram resulted in liver and spleen levels that were of the order of 3.5–23.3 μmoles/g. It is of considerable interest that these amino acids were significantly concentrated (2.9–4.6-fold) in the Walker 256 tumor in relation to the other tissues studied. The hexynoic acid was concentrated about 2-fold in the Lewis lung tumor over the levels in the liver. If uniformly distributed in tissue water, these levels of the L-methionine analogues would be sufficiently high to achieve substantial inhibitions of the respective tissue adenosyltransferases.

Levels of S-adenosyl-L-methionine in normal and malignant tissues. In contrast to the relatively low adenosyltransferase activities of tumors, the Ado-Met levels of tumors and those of other tissues with similarly low enzyme levels are comparable to the Ado-Met levels of liver, which is the richest animal source of this enzyme. Tables 4, 5, and

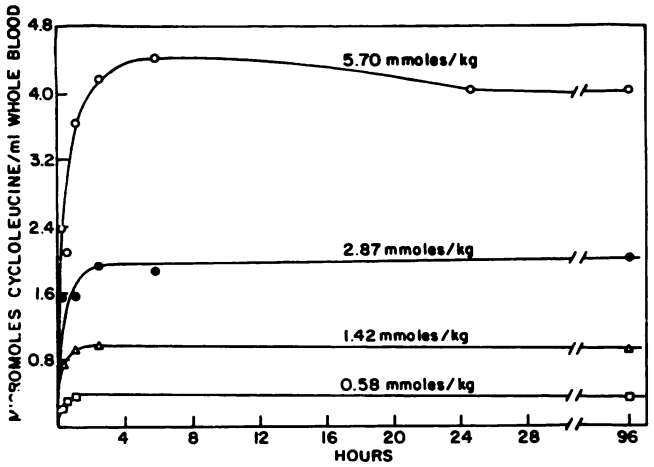

FIG. 1. *Blood levels of cycloleucine in rats as a function of time and dose*

Male Sprague-Dawley rats (133–137 g, body weight) received the indicated doses of cycloleucine (0.58–5.7 mmoles/kg) in 1.5 ml of 0.9% NaCl by intraperitoneal injection. Aliquots (75 μl) of whole blood were collected at intervals from the tails, deproteinized with perchloric acid, and analyzed for cycloleucine content on the amino acid analyzer as described under EXPERIMENTAL PROCEDURE.

TABLE 3

Tissue concentrations of cycloleucine or L-2-amino-4-hexynoic acid following their administration to rats and mice

The rats received intraperitoneal injections of cycloleucine (5.19 mmoles/kg) or L-2-amino-4-hexynoic acid (2.64 mmoles/kg) at 18 and 2.5 hr prior to removal of tissues for analysis. The mice were treated on the same schedule. Each dose of cycloleucine was 7.75 mmoles/kg, and that of L-2-amino-4-hexynoic acid was 3.94 mmoles/kg. The number of animals involved in each set of determinations is given in parentheses.

Tissue	Concentration of	
	Cycloleucine	L-2-Amino-4-hexynoic acid
	μmoles/g wet tissue (mean ± SD)	
Sprague-Dawley rat		
Liver	10.0 ± 1.8 (6)	3.5 ± 0.6 (3)
Spleen	7.8 ± 1.7 (4)	
Walker 256 tumor	29.0 ± 3.3 (4)	16.2 ± 2.4 (4)
Mouse (C_{57} black)		
Liver	23.3 ± 0.4 (3)	8.8 ± 0.3 (3)
Lewis lung tumor	23.2 ± 4.1 (3)	15.8 ± 1.0 (3)

6 give the Ado-Met levels of various tissues of Sprague-Dawley rats and C_{57} black and DBA/2 mice. The Ado-Met levels fall into the range between 40 and 140 nmoles/g of wet tissue weight. The only consistently discernible pattern is the somewhat higher concentration of Ado-Met in the liver than in other normal tissues. It should be noted that the Walker 256 and Lewis lung tumors are also relatively rich sources of this substance.

The administration of cycloleucine or of L-2-amino-4-hexynoic acid produces characteristic tissue changes in Ado-Met levels. In the spleen, kidney, pancreas, brain, and adrenal of Sprague-Dawley rats the Ado-Met levels drop by 25–40%, and in the Walker tumor by 35–40%. In every instance L-2-amino-4-hexynoic acid produces a slightly larger effect than cycloleucine. In contrast, the liver levels of Ado-Met in both normal and tumor-bearing rats increased to about double control values (Table 4). Entirely parallel observations were made in normal C_{57} black and DBA/2 mice and in those bearing the Lewis lung and L1210 leukemia tumors, respectively. The administration of the L-methionine analogues depresses Ado-Met levels in the spleen and tumors and elevates those in the liver (Tables 5 and 6). The findings in the DBA/2 mice bearing L1210 leukemia are complicated by the infiltration of both spleen and liver with leukemic elements, but the basic observations show entirely similar trends in all of the situations examined. The injection into rats of L-norvaline (Table 4; as well as L-serine or L-valine, not shown) at comparable doses (670 mg/kg) was without influence on Ado-Met levels of liver and spleen. The latter three amino acids have virtually no inhibitory effects on the adenosyltransferases, thus suggesting that the effects of cycloleucine and L-2-amino-4-hexynoic acid on the Ado-Met levels are related to the inhibition of its synthesis.

The effects of the L-methionine analogues on Ado-Met levels are dose-dependent (Table 7) and occur relatively soon after the administration of the inhibitors. Although the effects described in Tables 4–7 were all examined 18 hr after the initiation of treatment with adenosyltransferase inhibitors, the effects are manifest within 30–120 min (Table 8). Thus, following the intraperitoneal administration of 4.57 mmoles of cycloleucine per kilogram to male Sprague-Dawley rats, the spleen Ado-Met levels showed a near maximum decline of 38% in 30 min and remained depressed for at least 4 hr, whereas the liver Ado-Met levels rose more slowly, reaching a plateau of 1.5 times control values within 2 hr and remaining elevated for at least 2 hr more.

Similar results were obtained following the

TABLE 4

Tissue levels of S-adenosyl-L-methionine in male Sprague-Dawley rats treated with adenosyltransferase inhibitors

The treated rats received intraperitoneal injections of cycloleucine (5.19 mmoles/kg), L-2-amino-4-hexynoic acid (2.64 mmoles/kg), or L-norvaline (5.73 mmoles/kg) at 18 and 2.5 hr prior to removal of tissues for analysis. The number of animals involved in each set of determinations is given in parentheses.

Treatment of animals	*S*-Adenosyl-L-methionine						
	Liver	Spleen	Kidney	Pancreas	Brain	Adrenals[a]	Walker 256 tumor
	nmoles/g wet tissue (mean ± SD)						
Normal							
Untreated	123 ± 12 (7)	73.6 ± 4.6 (8)	76.8 ± 3.7 (4)	47.9 (2)	42.7 ± 3.4 (4)	87.1 (4)	
Cycloleucine	193 ± 14 (7)	55.8 ± 5.3 (7)	57.5 (2)	35.6 (2)	33.1 (2)	65.0 (4)	
L-2-Amino-4-hexynoic acid	227 ± 18 (7)	50.3 ± 5.1 (7)	54.4 ± 2.3 (3)		26.0 ± 3.5 (3)		
L-Norvaline (control)	132 ± 17 (5)	85.5 ± 4.0 (5)	76.6 ± 1.5 (5)		42.2 ± 5.2 (5)		
Walker 256 tumor-bearing							
Untreated	125 ± 14 (8)	113 ± 9 (7)	71.5 ± 6.7 (4)		39.6 ± 2.2 (4)	79.0 (4)	146 ± 7.0 (7)
Cycloleucine	239 ± 20 (7)	72.1 ± 14.0 (7)	55.8 ± 2.3 (4)		29.6 ± 1.3 (4)	61.5 (4)	95.9 ± 9.0 (7)
L-2-Amino-4-hexynoic acid	264 ± 32 (7)	60.8 ± 9.3 (7)					86.6 ± 11.3 (7)

[a] Pooled samples.

TABLE 5

Tissue levels of S-adenosyl-L-methionine in C_{57} black mice treated with adenosyltransferase inhibitors

The treated mice received intraperitoneal injections of cycloleucine (7.75 mmoles/kg) or L-2-amino-4-hexynoic acid (3.94 mmoles/kg) at 18 and 2.5 hr prior to removal of tissues for analysis. The number of animals involved in each set of determinations is given in parentheses.

Treatment	*S*-Adenosyl-L-methionine		
	Liver	Spleen	Lewis lung tumor
	nmoles/g wet tissue (mean ± SD)		
Normal mice			
Untreated	138 ± 16 (10)	65.7 ± 17.0 (10)	
Cycloleucine	237 ± 18 (6)	42.8[a] (6)	
L-2-Amino-4-hexynoic acid	264 ± 13 (7)	46.4 ± 6.0 (7)	
Lewis lung tumor-bearing mice			
Untreated	121 ± 10 (8)	80.8 ± 9.8 (4)	102 ± 10 (9)
Cycloleucine	175 ± 19 (9)	48.6 ± 5.7 (9)	58.8 ± 18.9 (9)
L-2-Amino-4-hexynoic acid	185 ± 8 (7)	41.9 ± 9.0 (7)	48.0 ± 12.5 (7)

[a] Pooled samples.

TABLE 6

Tissue levels of S-adenosyl-L-methionine in DBA/2 mice treated with adenosyltransferase inhibitors

The mice received intraperitoneal injections of cycloleucine (7.75 mmoles/kg) or DL-2-amino-4-hexynoic acid (7.88 mmoles/kg) at 18 and 2.5 hr prior to removal of tissues for analysis. The number of animals involved in each set of determinations is given in parentheses.

Treatment of animals	*S*-Adenosyl-L-methionine		
	Liver	Spleen	L1210 leukemia
	nmoles/g wet tissue (mean ± SD)		
Normal mice			
Untreated	93.2 ± 17.8 (11)	71.7 ± 4.9 (9)	
Cycloleucine	163 ± 9 (11)	49.8 ± 3.0 (11)	
DL-2-Amino-4-hexynoic acid	216 ± 41 (9)	59.0 ± 10.6 (9)	
L1210 tumor-bearing mice			
Untreated	134 ± 11 (13)	140 ± 21 (13)	91.2 ± 9.8 (13)
Cycloleucine	177 ± 20 (12)	102 ± 28 (12)	54.9 ± 7.8 (12)
DL-2-Amino-4-hexynoic acid	204 ± 30 (6)	63.5 ± 8.1 (6)	46.8 ± 6.2 (6)

injections of L-2-amino-4-hexynoic acid (2.64 mmoles/kg). Spleen levels again fell nearly to a minimum within 30 min and remained depressed for at least 4 hr. The liver Ado-Met levels first declined somewhat and reached their maximum in 4 hr (1.5 times control levels). It may be concluded from these results that the tissue Ado-Met levels respond very rapidly to the administration of the adenosyltransferase inhibitors and that the time course of these responses is entirely consistent with the tissue levels of the inhibitors (Fig. 1 and Table 3).

Tissue levels of L-methionine and other amino acids following administration of adenosyltransferase inhibitors. Since the administration of L-methionine elevates the tissue levels of Ado-Met (10), it became of interest to determine the effects of inhibitors of the adenosyltransferase reaction on tissue L-methionine concentrations. The levels of six amino acids (methionine, isoleucine, leucine, tyrosine, phenylalanine, and valine) in the livers (Fig. 2) and spleens (Fig. 3) of Sprague-Dawley rats were determined on the amino acid analyzer. The L-methionine levels were also estimated by the double-isotope enzymatic derivative method (22),

TABLE 7

Effect of varying doses of cycloleucine and L-2-amino-4-hexynoic acid on hepatic levels of S-adenosyl-L-methionine and L-methionine in male Sprague-Dawley rats

Male Sprague-Dawley rats weighing 115–135 g received intraperitoneal injections at 18 or at 18 and 2.5 hr prior to removal of tissue for analysis, for those receiving one and two doses, respectively. The number of animals involved in each set of determinations is given in parentheses.

Treatment	Dose	No. of doses	Ado-Met levels	Methionine levels
	mmoles/kg		*nmoles/g wet tissue (mean ± SD)*	
Untreated			114.9 ± 8.3 (8)	56.6 ± 7.2 (7)
Cycloleucine	5.19	2	164.2 ± 13.3 (6)	160.0 ± 4.6 (6)
	5.19	1	151.7 ± 21.8 (6)	81.0 ± 7.9 (6)
	2.60	1	128.4 ± 15.4 (6)	45.3 ± 6.7 (6)
L-2-Amino-4-hexynoic acid	2.64	2	211.3 ± 2.9 (3)	202.4 ± 31.6 (6)
	2.64	1	154.7 ± 3.8 (3)	100.8 ± 35.0 (6)
	1.32	1	114.0 ± 3.5 (3)	63.6 ± 15.7 (6)

TABLE 8

Tissue levels of S-adenosyl-L-methionine at various time intervals after injection of adenosyltransferase inhibitors to male Sprague-Dawley rats

There were three animals in each group. Cycloleucine (5.19 mmoles/kg) or L-2-amino-4-hexynoic acid (2.64 mmoles/kg) was injected intraperitoneally at zero time.

Time after treatment	Liver		Spleen	
	Cycloleucine	L-2-Amino-4-hexynoic acid	Cycloleucine	L-2-Amino-4-hexynoic acid
hr	*nmoles Ado-Met/g tissue (mean ± SD)*			
0	115 ± 14	97.7 ± 18.7	133 ± 1	81.2 ± 1.2
0.5	149 ± 19	77.4 ± 12	82.8 ± 3.5	42.6 ± 1.7
1	167 ± 7	83.7 ± 7.2	95.8 ± 25	37.2 ± 2.4
2	177 ± 12	104 ± 13	78.7 ± 25	36.0 ± 1.5
4	177 ± 10	156 ± 29	76 ± 16.5	45.5 ± 8.6

and there was good agreement between the two methods. Following treatment with cycloleucine or L-2-amino-4-hexynoic acid, the L-methionine levels become significantly elevated (2–4-fold) in both tissues. In the liver (Fig. 2) the other five amino acids (isoleucine, leucine, tyrosine, phenylalanine, and valine) are lowered slightly by cycloleucine administration. In the spleen (Fig. 3) isoleucine, leucine, and tyrosine levels are not significantly affected by either the cyclic or acetylenic compounds; however, phenylalanine is almost doubled by both inhibitors. L-Methionine levels of brain and kidney have also been measured after administration of L-2-amino-4-hexynoic acid and are elevated 2–3-fold.

Table 9 shows that the levels of L-methionine are dramatically elevated (2–7-fold) in both the Walker 256 tumor of the Sprague-Dawley rat and the Lewis lung tumor of the C_{57} black mouse following administration of the adenosyltransferase inhibitors. The effects of the hexynoic acid are greater than those of the cycloleucine, which is consistent with the fact that the acetylenic compound is a more powerful adenosyltransferase inhibitor.

The increases in tissue L-methionine levels in the liver and spleen after treatment with adenosyltransferase inhibitors occur quite rapidly (Table 10) and parallel the changes in Ado-Met levels (cf. Table 8). The magnitudes of the elevations in L-methionine

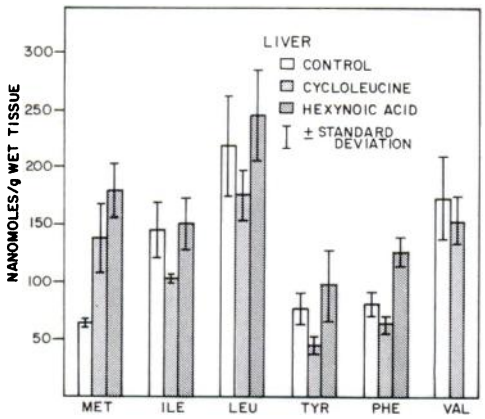

FIG. 2. *Effect of ATP:L-methionine adenosyltransferase inhibitors on levels of six amino acids in rat liver*

Three groups of male Sprague-Dawley rats (108–124 g; four animals per group) received intraperitoneal injections of 0.9% NaCl solutions alone or containing either cycloleucine (5.19 mmoles/kg) or L-2-amino-4-hexynoic acid (2.64 mmoles/kg) at 18 and 2.5 hr prior to removal of tissues for analysis. The tissues were processed and analyzed for methionine, isoleucine, leucine, tyrosine, phenylalanine, and valine by the amino acid analyzer technique as described under EXPERIMENTAL PROCEDURE. Similar values for L-methionine were determined by the double-isotope enzymatic derivative method (22). The results shown were obtained by amino acid analyzer and are expressed as mean values ± standard deviations.

concentrations are, like those of Ado-Met, dependent upon the doses of the adenosyltransferase inhibitors (Table 7). The general increases of L-methionine tissue levels that follow treatment with cycloleucine or L-2-amino-4-hexynoic acid are also reflected in elevations of the plasma concentrations of this amino acid. Figure 4 shows that cycloleucine administration elevates plasma L-methionine levels to nearly twice control values in 2 hr, and this plateau is maintained for at least 24 hr. The acetylenic amino acid produces a more gradual rise in plasma L-methionine levels, which reach a plateau in 4 hr and also remain elevated for at least 24 hr (Fig. 4).

If the effects of cycloleucine and L-2-amino-4-hexynoic acid on tissue L-methionine and Ado-Met levels are exerted by a common mechanism, it seems reasonable to assume that doses of these compounds producing near maximal effects, when given alone, should not produce additive effects when given in combination. Experimental support for the correctness of this supposition is given in the experiment shown in Fig. 5.

Since one of the effects of treatment of the rats with high doses of cycloleucine is anorexia, there existed the possibility that starvation might be responsible for the changes in the L-methionine levels. Rats deprived of food for 18 hr exhibited the same L-methionine levels as control animals which were fed ad libitum. Prior treatment of the rats with L-norvaline, or with L-valine, or with DL-homoserine (at 670 mg/kg), had no detectable effect on the L-methionine levels

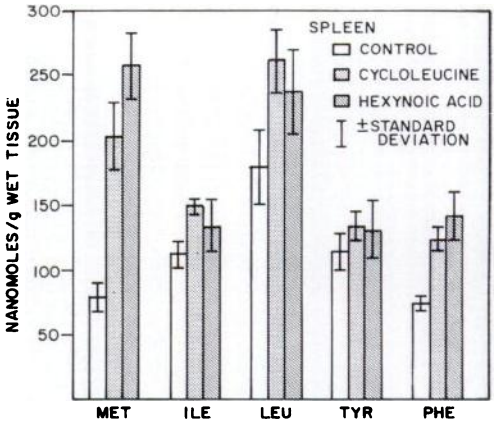

FIG. 3. *Effect of ATP: L-methionine adenosyltransferase inhibitors on levels of five amino acids in rat spleen*

Three groups of male Sprague-Dawley rats (108–124 g; four animals per group) received intraperitoneal injections of 0.9% NaCl solutions alone or containing either cycloleucine (5.19 mmoles/kg) or L-2-amino-4-hexynoic acid (2.64 mmoles/kg) at 18 and 2.5 hr prior to removal of tissues for analysis. The tissues were processed and analyzed for methionine, isoleucine, leucine, tyrosine, and phenylalanine by the amino acid analyzer technique as described under EXPERIMENTAL PROCEDURE. L-Methionine was also determined by the double-isotope enzymatic derivative method (22), and close agreement between the values obtained by the two methods was observed. The results shown are those obtained from the amino acid analyzer and are expressed as mean values ± standard deviations.

TABLE 9

Effect of treatment with adenosyltransferase inhibitors on L-methionine levels of Walker 256 and Lewis lung tumors

The treated animals (male Sprague-Dawley rats or C_{57} black mice) received intraperitoneal injections of cycloleucine (5.19 mmoles/kg for rats and 7.75 mmoles/kg for mice) or L-2-amino-4-hexynoic acid (2.64 mmoles/kg for rats and 3.94 mmoles/kg for mice) at 18 and 2.5 hr prior to removal of tissues for analysis. The number of animals involved in each set of determinations is given in parentheses.

Treatment of tumor-bearing animals	L-Methionine	
	Walker 256 tumor, Sprague-Dawley rats	Lewis lung tumor, C_{57} black mice
	nmoles/g wet tissue (mean ± SD)	
Untreated	73 ± 13 (7)	109 ± 13 (5)
Cycloleucine	274 ± 33 (6)	210 ± 67 (6)
L-2-Amino-4-hexynoic acid	482 ± 101 (7)	315 ± 123 (6)

TABLE 10

Tissue levels of L-methionine at various time intervals after injection of adenosyltransferase inhibitors to male Sprague-Dawley rats

There were three animals in each group. Cycloleucine (5.19 mmoles/kg) or L-2-amino-4-hexynoic acid (2.64 mmoles/kg) was injected intraperitoneally at zero time.

Time after treatment	Liver		Spleen	
	Cycloleucine	L-2-Amino-4-hexynoic acid	Cycloleucine	L-2-Amino-4-hexynoic acid
hr	*nmoles L-methionine/g wet tissue (mean ± SD)*			
0	65.3 ± 1.7	68.7 ± 1.9	58.8 ± 2.2	66.3 ± 1.6
0.5	87.8 ± 5.1	88.7 ± 12.3	105 ± 12	92.9 ± 5.9
1	93.8 ± 4.3	112 ± 5	93.6 ± 4.2	109 ± 10
2	86.5 ± 3.3	115 ± 15	97.1 ± 6.0	124 ± 9
4	83.6 ± 7.3	116 ± 17	105 ± 7	130 ± 14

of the liver, spleen, brain, or kidney. These amino acids are all extremely weak inhibitors of the adenosyltransferase reaction.

Effects of L-methionine administration. The effects of L-methionine administration on the liver and spleen levels of L-methionine and Ado-Met *in vivo* are shown in Fig. 6. Both the liver and spleen L-methionine levels increased 5–6-fold within 5 min after intraperitoneal injection of 0.67 mmole of L-methionine per kilogram of body weight. The Ado-Met levels in these tissues also became elevated. However, the Ado-Met levels of the liver showed a much greater response to L-methionine elevations than those of the spleen. Thirty minutes after treatment of the rodents with L-methionine the liver Ado-Met concentration increased 4-fold while the spleen level was elevated by only 25% (Fig. 6). Baldessarini (11) had earlier shown that after intraperitoneal injection of L-methionine (0.67 mmole/kg) the liver Ado-Met levels increased dramatically while those of other tissues (heart, spleen, kidney, lung, brain, and adrenal) increased to a much lesser extent.

DISCUSSION AND INTERPRETATIONS

In this paper we have established that ATP:L-methionine S-adenosyltransferase activity can be detected in all tissues examined, provided that sufficiently sensitive methods of assay are employed. Earlier reports that certain tumors are devoid of adenosyltransferase activity may therefore be in error, since such activity has been clearly demonstrated by us in other tumors, such as the transplantable Walker 256 tumor of the

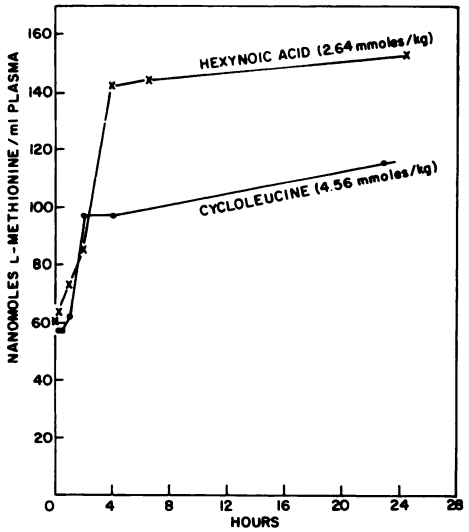

FIG. 4. *Response of plasma* L-*methionine levels of the rat to administration of cycloleucine or* L-*2-amino-4-hexynoic acid*

Two male Sprague-Dawley rats (155 g and 170 g) received intraperitoneal injections of 0.9% NaCl solutions containing either L-2-amino-4-hexynoic acid (hexynoic acid, 2.64 mmoles/kg) or cycloleucine (4.56 mmoles/kg). Whole blood (100 μl) was collected from the tail, heparinized, and centrifuged in a Beckman 152 microfuge for 3 min to remove the cells. An aliquot of the plasma (40 μl) was deproteinized with an equal volume of 0.33M perchloric acid and centrifuged for 3 min. An aliquot of the supernatant fluid was analyzed for L-methionine concentration by the double-isotope enzymatic derivative method (22) as described under EXPERIMENTAL PROCEDURE.

Sprague-Dawley rat and the B-16 melanoma and the Lewis lung tumor of C_{57} black mice.

During the course of a systematic search for structural, electronic, and conformational analogues of L-methionine, we observed that both cycloleucine (1-aminocyclopentanecarboxylic acid) and L-2-amino-4-hexynoic acid inhibited the isofunctional adenosyltransferases of bakers' yeast, *E. coli*, and rat liver, in competition with L-methionine (5, 16). These observations have now been extended to show that crude and partially purified preparations of all adenosyltransferases examined are inhibited by these L-methionine analogues. Cycloleucine has been shown by radioautography to be localized in the Walker 256 tumor of the rat, although the results presented were not quantitative (35). Since cycloleucine displays a number of pharmacological properties, such as tumor inhibition (36, 37), inhibition of the growth of certain microorganisms (38), and suppression of certain aspects of the immune response (39), we posed the question whether these effects might be related to the inhibition of adenosyltransferase activity *in vivo*. The experiments which have been performed show quite conclusively that the intraperitoneal administration of pharmacological doses of cycloleucine or L-2-amino-4-hexynoic acid elevates the tissue (and plasma) levels of these analogues to 3.5–30 μmoles/g (or ml). The plasma levels remain elevated almost at a plateau for at least 24 hr, and probably rather longer, so that both amino acids are cleared from the rodent body extremely slowly. The tissue and plasma levels of the inhibitory analogues that are attained under these circumstances are most probably high enough to produce significant inhibition of the tissue adenosyltransferases, if one assumes that tissue levels of L-methionine are in the range of 50–180 nmoles/g. It was noted that the analogues tended to concentrate in some tissues, notably the Walker 256 tumor.

In the presence of pharmacological levels of the cyclic or acetylenic amino acid we have observed prompt and dramatic elevations of the L-methionine levels (2–7-fold) in all rodent tissues examined (including plasma, liver, spleen, Walker 256, and Lewis lung tumors). These effects are dose-dependent, and were not observed when high doses of amino acids (e.g., L-norvaline, L-valine, or DL-homoserine) that are not adenosyltransferase inhibitors were administered to the animals. The elevations of the L-methionine levels persisted for many hours, and thus paralleled the elevated levels of the analogues in the body. The increases in L-methionine concentrations were particularly striking in the Walker tumor (almost 7-fold) and the Lewis lung tumor (almost 3-fold). The magnitudes of these elevations are entirely consistent with the low levels of adenosyltransferase activity in the tumors, where virtually complete blockade of this enzyme might be expected to lead to marked

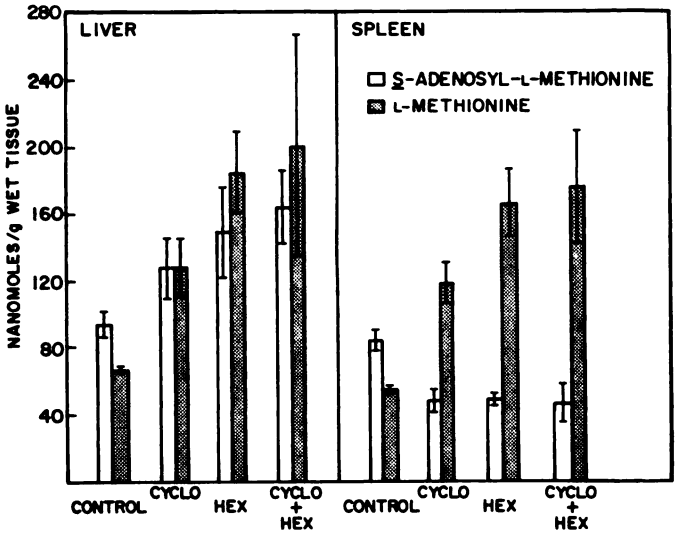

FIG. 5. *Effect of inhibitors of ATP:L-methionine S-adenosyltransferase, either singly or in combination, on rat liver and spleen levels of S-adenosyl-L-methionine and L-methionine*

Four groups of male Sprague-Dawley rats (88–99 g; three animals per group) received by intraperitoneal injection 0.9% NaCl solution alone or containing either cycloleucine (cyclo, 5.19 mmoles/kg) or L-2-amino-4-hexynoic acid (hex, 2.64 mmoles/kg), or a combination of both inhibitors (cycloleucine, 5.19 mmoles/kg, plus hexynoic acid, 2.64 mmoles/kg), at 18 and 2.5 hr prior to removal of tissues for analysis. The tissues were processed and analyzed for both S-adenosyl-L-methionine and L-methionine by the double-isotope enzymatic methods (10, 22) as described under EXPERIMENTAL PROCEDURE. The data are expressed as the mean values ± standard deviations.

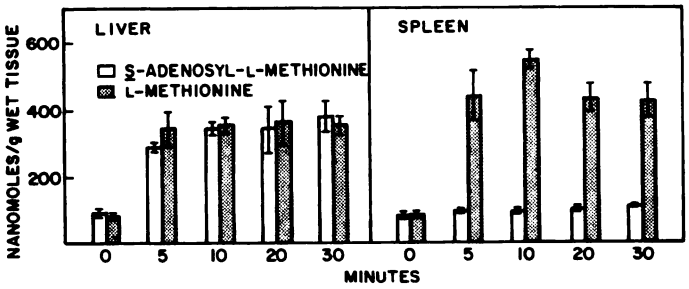

FIG. 6. *L-Methionine and S-adenosyl-L-methionine levels of rat liver and spleen after injection of L-methionine*

Five groups of male Sprague-Dawley rats (110–121 g; three animals per group) received by intraperitoneal injection 0.67 mmole/kg of L-methionine. The animals were killed at the indicated time intervals, and the tissues were processed and then subjected to analysis for S-adenosyl-L-methionine and L-methionine content by the double-isotope enzymatic methods (10, 22) as described under EXPERIMENTAL PROCEDURE. The results are expressed as mean values ± standard deviations.

accumulations of L-methionine. Another major pathway for the utilization of L-methionine is its incorporation into proteins. The L-methionine-tRNA aminoacyl synthetases are fully saturated at quite low levels (less than 10 μM) of L-methionine (40), and are not inhibited by the acetylenic or cyclic amino acid analogues.[4] No information is available on the effects of these analogues on other metabolic reactions of L-methionine, and consequently caution must be exercised in attributing the observed phenomena ex-

[4] F. R. Mangan and P. Talalay, unpublished observations.

clusively or even principally to inhibition of the adenosyltransferase reaction. Although exhaustive studies of the effects of these analogues on the tissue levels of all other amino acids have not been carried out, the limited results shown in Figs. 2 and 3 suggest that at least in liver and spleen the L-methionine levels are most responsive to treatment with adenosyltransferase inhibitors.

The accumulation of L-methionine in response to administration of the adenosyltransferase inhibitors is accompanied by dose-dependent changes in the tissue levels of *S*-adenosyl-L-methionine. In all tissues examined, including certain transplantable tumors, but with the notable exception of the liver, the *S*-adenosyl-L-methionine levels are depressed by 20–40%. Other amino acids, such as L-norvaline, L-valine, or L-serine, that do not inhibit the adenosyltransferases do not affect the levels of Ado-Met or of L-methionine. These findings can be rationalized on the assumption that under normal conditions the levels of Ado-Met are controlled by the rate of synthesis rather than that of utilization, and that consequently inhibition of the synthetic enzyme causes accumulation of the substrate (L-methionine) and depression of the level of the product (Ado-Met). It should be noted that in the presence of the inhibitors the Ado-Met levels are depressed, even in the face of marked elevations of L-methionine in these tissues. Thus these depressions assume added significance, suggesting relatively complete blockade of the synthesis of Ado-Met, since artificial elevations of L-methionine normally result in increases in tissue Ado-Met levels (see Fig. 6 and ref. 10).

The pattern of Ado-Met levels in the liver is quite different from that in all the other tissues. Administration of adenosyltransferase inhibitors *in vivo* causes elevation (1.5–2-fold) in hepatic Ado-Met levels in both mice and rats. Although no unique explanation can be offered for this finding, certain factors may contribute to this apparent paradox. It will be recalled that the adenosyltransferase activity of liver is far higher (10–20-fold) than that of any other mammalian tissue. Consequently, even if a high degree of inhibition of this enzyme were achieved in the liver, there may be sufficient residual adenosyltransferase activity to maintain appreciable rates of synthesis of Ado-Met. As already pointed out by others (10) and confirmed by us (Fig. 6), tissue levels of Ado-Met become elevated when L-methionine is administered. Whereas these elevations are modest (25–50%) in most tissues, the response of the liver is most dramatic (5–6-fold). Since L-methionine accumulates in all the tissues (including plasma and liver) when adenosyltransferase inhibitors are administered, this would tend to raise Ado-Met levels. If the high adenosyltransferase activities of the liver are only partially inhibited, an increased synthesis of Ado-Met in this tissue could be the result of the redistribution of L-methionine from other tissues to the liver. Evidence bearing on this proposal might be obtained by direct perfusion of isolated livers with inhibitory analogues in the absence or presence of various levels of L-methionine, and measurement of Ado-Met levels in the tissue.

One unusual feature of the rat and mouse liver adenosyltransferases may conceivably have a bearing on the elevations of hepatic Ado-Met levels which have been discussed. The rodent liver enzyme is endowed with unique regulatory properties. The velocity of the reaction bears a sigmoidal relation to L-methionine levels. Low concentrations of cycloleucine and L-2-amino-4-hexynoic acid stimulate the adenosyltransferase activity of rodent liver by obliterating the sigmoidal kinetics observed at low L-methionine concentrations and raising the affinity of the enzyme for L-methionine (34). It is not clear whether this specific cooperative property of the rodent liver enzyme is in any way causally related to the elevations of the liver Ado-Met levels which are observed when the L-methionine analogues are administered *in vivo*.

As in the case of the changes in tissue L-methionine levels, the response of Ado-Met levels is dependent on the dose of the adenosyltransferase inhibitor, and the time course parallels the changes in tissue levels of cycloleucine and L-2-amino-4-hexynoic acid as well as those of L-methionine.

The facts enumerated above strongly sug-

gest that pharmacological doses of cycloleucine and L-2-amino-4-hexynoic acid inhibit ATP : L-methionine *S*-adenosyltransferase activity *in vivo*. There are reasons supporting the view that inhibition of this enzyme may be at least in part responsible for the tumor-inhibitory properties of cycloleucine, and possibly for some of its other pharmacological activities. L-2-Amino-4-hexynoic acid has so far undergone only preliminary screening against L1210 leukemia in the DBA/2 mouse, and prolongs the survival time of these animals by a modest degree (25–30%) without much evidence of toxicity (Cancer Chemotherapy National Service Center, NSC 134451). In view of the fact that these analogues appear to accumulate in tumors, the evaluation of their chemotherapeutic potential in other systems appears warranted.

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