Biochem. J. (1973) **135**, 43–57
Printed in Great Britain

Regulatory Properties of Adenosine Triphosphate–L-Methionine *S*-Adenosyltransferase of Rat Liver

By J. B. LOMBARDINI,* TING-CHAO CHOU† and PAUL TALALAY
Department of Pharmacology and Experimental Therapeutics, The Johns Hopkins University School of Medicine, Baltimore, Md. 21205, *U.S.A.*

(*Received* 29 *January* 1973)

1. Double-reciprocal plots of the reaction velocity of yeast, rat liver and *Escherichia coli* ATP–L-methionine *S*-adenosyltransferases (EC 2.5.1.6) as a function of the L-methionine concentrations (under saturating ATP conditions) demonstrate downward deflexions from linearity for the yeast and *E. coli* adenosyltransferases and an upward deflexion for the rat liver enzyme. 2. The activities of partially purified preparations of rat liver ATP–L-methionine *S*-adenosyltransferase are enhanced by low concentrations of non-substrate analogues of L-methionine [e.g. 1-aminocyclopentanecarboxylic acid (cycloleucine) and L-2-amino-4-hexynoic acid], or by inorganic tripolyphosphate, an ATP analogue. When the concentrations of these analogues were raised further, the activity decreased. Double-reciprocal plots became linear in the presence of these modifier analogues. The inhibitions are common to all the L-methionine adenosyltransferases examined, but the activation(s) were only found with rat and mouse liver enzymes and not with enzymes obtained from several other tissues of these or other species. 3. The rate of formation of *S*-adenosyl-L-methionine bears a sigmoidal relation to the L-methionine concentrations when ATP is saturating. The activating effects of the L-methionine analogues and of tripolyphosphate are observed at low L-methionine concentrations, and become obliterated as the L-methionine concentration is raised. These findings are analysed in terms of various regulatory enzyme models.

The enzymic formation of (−)-*S*-adenosyl-L-methionine by nucleophilic transfer of the adenosyl moiety of ATP to the sulphur atom of L-methionine was discovered by Cantoni (1952, 1953) and has since been studied extensively (Cantoni & Durell, 1957; Mudd & Cantoni, 1958; Mudd, 1963; Mudd & Mann, 1963; Greene, 1969; Chou & Talalay, 1972). With partially purified preparations of rabbit liver (Cantoni & Durell, 1957) and yeast (Mudd & Cantoni, 1958) adenosyltransferase (ATP–L-methionine adenosyltransferase, EC 2.5.1.6), the following stoicheiometry has been established:

$$\text{L-Methionine} + \text{ATP} \xrightarrow{K^+,\ Mg^{2+}} \text{(−)-}S\text{-adenosyl-L-methionine} + PP_i + P_i$$

The mechanism involves a complete dephosphorylation of ATP, with the formation of enzyme-bound tripolyphosphate, which is then asymmetrically hydrolysed to pyrophosphate and P_i. Mudd (1962) has further shown that the yeast enzyme possesses tripolyphosphatase activity that is specifically and profoundly enhanced by low concentrations of *S*-adenosyl-L-methionine.

The metabolic importance of *S*-adenosyl-L-methionine, a high-energy sulphonium compound, is threefold: first, as a methyl donor involved in more than 40 enzymic transmethylation reactions (Mudd & Cantoni, 1964; Lombardini & Talalay, 1971*a*); secondly, as a propylamine donor for the formation of spermidine and spermine (Tabor *et al.*, 1958; Jänne *et al.*, 1971; Cohen, 1971); thirdly, as a regulator or effector of certain enzymic reactions (Lombardini & Talalay, 1971*a*), most notably its own synthesis (Chou & Talalay, 1972).

The kinetics of the yeast adenosyltransferase reaction have been reinvestigated by Chou & Talalay (1972) with a highly purified enzyme preparation. A rapid and extremely sensitive assay technique, which separates *S*-adenosyl-L-[*Me*-^{14}C]methionine from L-[*Me*-^{14}C]methionine by differential adsorption on cellulose phosphate discs rather than ion-exchange columns, has been developed (Chou & Lombardini, 1972). For the yeast enzyme, double-reciprocal plots of the initial reaction velocity as a function of L-methionine concentration display considerable deviations from Michaelis–Menten kinetics and are characterized by downward inflexions (Chou & Talalay,

* Present address: Department of Pharmacology and Therapeutics, Texas Tech University School of Medicine, Lubbock, Tex. 79409, U.S.A.

† Present address: Division of Pharmacology, Sloan-Kettering Institute for Cancer Research, New York, N.Y. 10021, U.S.A.

1972; Lombardini *et al.*, 1970), which have been attributed to activation of the rate-limiting step of the enzymic reaction by low concentrations of *S*-adenosyl-L-methionine (Chou & Talalay, 1972). Preliminary kinetic studies on partially purified adenosyltransferases from rat liver and *Escherichia coli* also demonstrated deviations from Michaelis–Menten kinetics (Lombardini *et al.*, 1970), but the character of these deviations is not the same in all systems.

Our efforts have been directed to the rational design of inhibitors of *S*-adenosyltransferases as potential chemotherapeutic agents. To this end, we investigated numerous steric, electronic and conformational analogues of L-methionine (Lombardini *et al.*, 1970) and ATP (T.-C. Chou & P. Talalay, unpublished work). In the course of these studies, we observed that low concentrations of such inhibitors as 1-aminocyclopentanecarboxylic acid (cycloleucine) or L-2-amino-4-hexynoic acid activated (10–20%), rather than inhibited, the rat liver enzyme. This phenomenon was a unique property of rat liver adenosyltransferase and was not observed with enzymes from two microbial sources (Lombardini & Talalay, 1971*a*).

Efforts to magnify and to analyse this unusual biphasic (activation and inhibition) action of L-methionine analogues on the rat liver adenosyltransferase, led to the observation that low concentrations of tripolyphosphate exerted similar biphasic stimulatory and inhibitory effects.

A detailed study of the enzymes from other rat tissues (kidney, testis, spleen, brain, heart, intestine, skeletal muscle, blood and Walker 256 tumour) and from chicken tissues (liver, kidney, brain and spleen) has shown that the activation phenomenon is a peculiar characteristic of the rat and mouse liver enzymes rather than a general property of rodent and avian tissue adenosyltransferases.

Experimental

Materials

All solutions were prepared from reagent-grade chemicals in deionized glass-distilled water. L-Methionine was supplied by Schwarz/Mann, Orangeburg, N.Y., U.S.A. Sodium tripolyphosphate was purchased from Alfa Products, Beverly, Mass., U.S.A. D-Norleucine, *O*-acetyl-L-serine and 1-aminocyclopentanecarboxylic acid (cycloleucine) were purchased from Cyclo Chemical, Los Angeles, Calif., U.S.A. L-Norvaline was purchased from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. *S*-Carbamoyl-L-cysteine was supplied by Aldrich Chemical Co., Milwaukee, Wis., U.S.A. *p*-Hydroxymercuribenzoate was obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. L-[*Me*-^{14}C]Methionine (53.6μCi/μmol) was purchased from Amersham/Searle Corp., Arlington Heights, Ill., U.S.A., and was purified by passage through Dowex AG 50W (X2; NH_4^+ form) columns (0.6cm×12cm) at neutral pH. The ion-exchange resin was obtained from Bio-Rad Laboratories, Richmond, Calif., U.S.A. Cellulose phosphate ion-exchange paper discs (23mm diameter; catalogue no. P81) were purchased from Reeve Angel, Clifton, N.J., U.S.A. L-2-Amino-4-hexynoic acid (Coulter & Talalay, 1968; A. W. Coulter & J. Salt, unpublished work) and the *cis* and *trans* isomers of DL-2-amino-4-hexenoic acid (Skinner *et al.*, 1961) were synthesized by Dr. A. W. Coulter of this Department. Female rats (140–160g) were purchased from Sprague–Dawley, Madison, Wis., U.S.A. *E. coli* strain B (mid-exponential phase) cells were obtained frozen from Grain Processing, Muscatine, Iowa, U.S.A. Fleischmann baker's yeast and chicken livers were purchased from local food markets. Female White Leghorn chickens were obtained from Truslow Farms, Chestertown, Md., U.S.A.

Preparation of ATP–L-methionine S-adenosyltransferases

The yeast adenosyltransferase was purified from baker's yeast by the procedure of Stekol (1963) as modified by Chou & Talalay (1972). The *E. coli* adenosyltransferase was purified by the method of Tabor & Tabor (1972) and the rat liver adenosyltransferase was purified from female Sprague–Dawley rats by the procedures previously described (Cantoni & Durell, 1957; Lombardini *et al.*, 1970; Pan & Tarver, 1967). The specific activities of the preparations of yeast, *E coli* and rat liver adenosyltransferases were 4.7–30, 2.5–4.2 and 2.4–6.7μmol of *S*-adenosyl-L-methionine synthesized/30min per mg of protein at 37°C respectively, under the assay conditions of reaction system A, with 10mM-L-methionine (see Table 1). All enzyme specific activities and protein concentrations were determined under the conditions of Lombardini *et al.* (1970). The purified rat liver enzyme contained significant pyrophosphatase activity but no detectable adenosine triphosphatase activity.

The following procedures were carried out near 4°C. Rat tissues other than liver (for the experiments described in Fig. 4 and Table 2) were homogenized in 2.5vol. of 50mM-potassium phosphate buffer, pH7.0, containing 5mM-2-mercaptoethanol and 20% (v/v) glycerol. The homogenates were centrifuged twice for 20min at 15000*g*. The supernatant fluid was fractionated with solid $(NH_4)_2SO_4$ without adjustment of pH. The fraction precipitating at 33–56% saturation was dissolved in a small volume of the homogenization buffer and dialysed overnight against 2 litres of the buffer. This procedure resulted in approximately a threefold increase in specific activity.

Table 1. *Composition of reaction mixtures used for assay of ATP–L-methionine S-adenosyltransferase*

The reaction mixtures had a final volume of 100 μl and the total L-methionine radioactivity per vessel was approx. 410000 d.p.m. The times of incubation at 37°C are given with individual experiments.

Reaction mixture A		Reaction mixture B	
Component	Concn. (mM)	Component	Concn. (mM)
Tris–HCl (pH 7.6)	160	Tris–histidine (pH 9.0)	90
KCl	200	KCl	100
$MgCl_2$	300	$MgCl_2$	15
Glutathione	8	2-Mercaptoethanol	5
L-Methionine	Varied*	L-Methionine	Varied*
ATP	20	ATP	10

* Specific enzyme activities were determined at 10.0 mM-L-methionine and are expressed as μmol of *S*-adenosyl-L-methionine formed/30 min per mg of protein at 37°C in reaction mixture A. The L-methionine concentrations for all other experiments are given in the appropriate protocols.

Table 2. *Concentrations of cycloleucine and L-2-amino-4-hexynoic acid required for 50% inhibition of adenosyltransferases from various sources*

The rat liver, yeast and *E. coli* enzymes were partially purified preparations assayed under the conditions described in the Experimental section (and in Fig. 3). The enzyme preparations for measurements on rat spleen, kidney and Walker 256 tumour were carried out as specified in the Experimental section and Fig. 4. The L-methionine concentration in all experiments was 37.5 μM. The amounts of enzyme protein used to initiate the reaction and other details are given in Figs. 3 and 4. I_{50} is the concentration (mM) of inhibitors required to obtain 50% inhibition.

Source of enzyme	I_{50} (mM)			
	Cycloleucine		L-2-Amino-4-hexynoic acid	
Reaction mixture ...	A	B	A	B
Liver	2.6	10.0	1.3	3.7
Spleen	2.2	1.0	0.8	0.4
Kidney	1.4	0.8	0.5	0.4
Walker 256 tumour	1.7	0.8	0.8	0.3
Yeast	5.4	1.5	2.3	1.0
E. coli	4.0	1.4	3.2	1.1

For the preparation of the adenosyltransferases from rat tissues described in Table 5, the tissues were homogenized in 2.5 vol. of 50 mM-Tris–HCl buffer, pH 7.6, containing 5 mM-2-mercaptoethanol, and centrifuged at 15000*g* for 15 min. The supernatant fluid was used as the source of the enzyme.

Chicken tissues were homogenized in 2.5 vol. of 50 mM-potassium phosphate buffer, pH 7.0, containing 5 mM-2-mercaptoethanol and 20% (v/v) glycerol. The homogenates were centrifuged twice for 20 min at 15000*g*. The clear supernatant fluid was used as the source of the enzyme.

The purified preparations of rat liver, yeast and *E. coli* adenosyltransferases were stored in small portions at −15°C. These preparations were thawed and diluted with solutions of bovine plasma albumin (2 mg/ml; pH 7.0) immediately before their addition to the incubation systems.

Enzymic assays

Measurement of adenosyltransferase activity was based on the conversion of L-[*Me*-^{14}C]methionine into *S*-adenosyl-L-[*Me*-^{14}C]methionine, which carries a positive charge and is quantitatively retained on discs of cellulose phosphate ion-exchanger (H^+ form) after extensive washing with water to remove the unchanged L-[*Me*-^{14}C]methionine (Chou & Lombardini, 1972). The incubations were conducted at 37°C with agitation in glass-stoppered conical tubes

(13ml capacity) in a final volume of 0.1ml in either reaction mixture A or reaction mixture B, which differed in both their final pH and ionic strength (Table 1). The incubation times are specified in the individual protocols, and were selected so that product formation was linear with time. The reaction was terminated by removal of a sample (50μl), which was spotted on to paper discs, processed and counted as described by Chou & Lombardini (1972).

Calculation of the inhibitory potency of the L-methionine analogues was based on the method of Dixon (1953), since the regulatory properties of the enzyme precluded the accurate determination of the K_m for L-methionine and, consequently, K_i values could not be calculated. The initial reaction velocities were measured at a relatively low fixed concentration of L-methionine and a series of inhibitor concentrations. The I_{50} values (the concentration of inhibitor giving 50% of control activity) were determined graphically by plotting the reciprocal velocity against inhibitor concentration (Lombardini *et al.*, 1970).

Tripolyphosphatase activity was measured by determining the production of P_i from tripolyphosphate. The incubations were conducted for 15min at 37°C in a final volume of 100μl containing the following: sodium tripolyphosphate (2mM), Tris–histidine (pH9.0, 90mM), KCl (100mM), $MgCl_2$ (15mM), 2-mercaptoethanol (5mM) and appropriate quantities of enzyme. The reaction was terminated and phosphate determined by a modification of the procedure of Baginski *et al.* (1967). The following reagents were added in rapid succession directly to the reaction vessels: (a) 10% (w/v) trichloroacetic acid (300μl) containing 2% (w/v) ascorbic acid and 0.1% EDTA; (b) water (200μl); (c) 2% (w/v) ammonium molybdate (150μl); (d) 2% (w/v) sodium arsenite and 4% (w/v) sodium citrate contained in 10% trichloroacetic acid (300μl). The reaction vessels were thoroughly agitated on a Vortex Junior mixer after the addition of each reagent. After 20min, which allows maximum development of the blue phosphomolybdate complex, the absorbance of the solution was determined at 860nm and compared with a standard curve.

Tumour maintenance

The Walker 256 tumour was maintained in male Sprague–Dawley rats by intramuscular transplant as described by Talalay *et al.* (1952).

Results

General kinetic properties of adenosyltransferases of yeast, E. coli and rat liver

The adenosyltransferases prepared from yeast, *E. coli* and from rat liver demonstrate contrasting kinetic properties when the reaction velocity is graphically represented (double-reciprocal plots) as a function of the L-methionine concentrations (Fig. 1). The results for the yeast and *E. coli* adenosyltransferases exhibit downward deflexions from linearity, in contrast with the rat liver adenosyltransferase, which displays an upward deflexion. The kinetics for the yeast enzyme have been analysed in detail by Chou & Talalay (1972) and it is assumed that their interpretations may also be relevant for the *E. coli* enzyme.

In view of the peculiarity of the rat liver enzyme, efforts were made to study whether its mechanism was in fact similar to that of the rabbit liver, *E. coli* and yeast enzymes. Mudd (1962) has reported that highly purified preparations of yeast adenosyl-

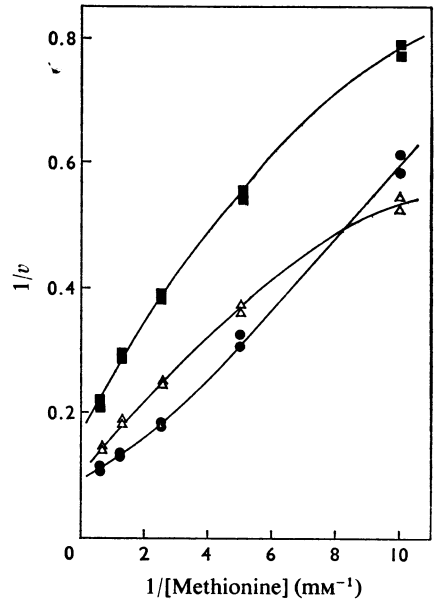

Fig. 1. *Velocity of S-adenosyl-L-methionine formation as a function of L-methionine concentration for rat liver, yeast and E. coli enzymes*

Incubations (reaction mixture B) were conducted as described in the Experimental section for 30min at 37°C. The following amounts of partially purified enzyme protein were used to initiate the reaction (specific activities expressed as μmol of *S*-adenosyl-L-methionine/30min per mg of protein): ●, rat liver adenosyltransferase, 3.2μg, specific activity 5.7; ■, yeast adenosyltransferase, 2.4μg, specific activity 5.5; △, *E. coli* adenosyltransferase, 2.4μg, specific activity 2.5. v is expressed as total nmol of *S*-adenosyl-L-methionine formed under these conditions in the reaction system.

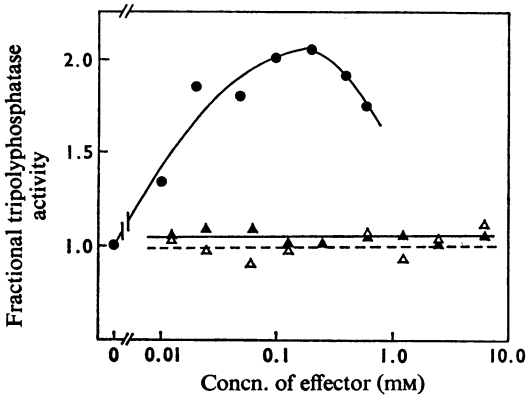

Fig. 2. *Fractional activity of the hydrolytic cleavage of tripolyphosphate by partially purified rat liver adenosyltransferase as a function of the logarithm of the concentrations of three effectors, S-adenosyl-L-methionine* (●), *cycloleucine* (▲) *and* L-*2-amino-4-hexynoic acid* (△)

The components of the reaction mixture are described in the Experimental section. The reaction was initiated with 4.7μg of rat liver adenosyltransferase (tripolyphosphatase) and the incubations were conducted at 37°C for 15min. The enzymic reaction was terminated by trichloroacetic acid precipitation of the protein and then the entire incubation mixture was analysed for phosphate by the procedure of Baginski *et al.* (1967). Since the enzyme preparation is contaminated with pyrophosphatase activity, the stoicheiometry of the tripolyphosphatase reaction could not be unequivocally determined.

transferase also possess tripolyphosphatase activity, which is stimulated by *S*-adenosyl-L-methionine. This has been confirmed and carefully examined by Greene (1969) and by Chou & Talalay (1972). The regulatory importance of *S*-adenosyl-L-methionine, which stimulates both its own synthesis and the tripolyphosphatase activities of the enzyme, has been studied in detail for the yeast adenosyltransferase (Chou & Talalay, 1972). Purified preparations of rat liver adenosyltransferase also possess tripolyphosphatase activity, which is markedly stimulated by *S*-adenosyl-L-methionine (Fig. 2).

Two analogues of L-methionine (cycloleucine and L-2-amino-4-hexynoic acid) inhibit the synthesis of *S*-adenosyl-L-methionine by the yeast and *E. coli* enzymes. These analogues display biphasic concentration-dependent activation–inhibition effects on the synthesis of *S*-adenosyl-L-methionine by the rat liver enzyme (Lombardini *et al.*, 1970). However, a wide range of concentrations of these compounds was without effect on the tripolyphosphatase activity of the rat liver enzyme (Fig. 2).

Effects of L-*methionine analogues on isofunctional adenosyltransferases*

Effects of a wide range of concentrations of the two analogues of L-methionine, cycloleucine and L-2-amino-4-hexynoic acid, on partially purified preparations of adenosyltransferases obtained from yeast, *E. coli* and rat liver were examined in reaction mixtures A and B at a L-methionine concentration of 37.5μM (Fig. 3). Low concentrations of the analogues activated the liver enzyme slightly but invariably (up to 20%) in reaction mixture A and by 50–70% in reaction mixture B. In confirmation of earlier findings from this laboratory (Lombardini *et al.*, 1970), higher concentrations of the analogues inhibited the same enzyme in both systems.

In contrast, no activations of the microbial enzymes were observed at any concentration of the analogues in either reaction mixture. The activational effects did not depend on enzyme purity, since they are demonstrable in centrifuged crude rat liver homogenates. Similar activations were observed with centrifuged homogenates of the livers of CF-1 mice (T.-C. Chou, unpublished work).

With the use of reaction mixture A or B, adenosyltransferase preparations from other normal or malignant rat tissues, including spleen, kidney and the Walker 256 tumour, failed to display any activation over a wide range of concentrations of cycloleucine or L-2-amino-4-hexynoic acid (Fig. 4), and inhibition was invariably observed at sufficiently high concentrations.

The concentrations of analogues required to achieve 50% inhibition of the reaction (Table 2) were significantly lower (two- to three-fold) in reaction mixture B (pH9.0 and low ionic strength) than in reaction mixture A (pH7.6 and high ionic strength) (Figs. 3 and 4 and Table 2). This observation applies to the microbial enzymes and all mammalian tissue preparations except the liver, where larger activations occurring in reaction mixture B probably obscure this phenomenon.

The remarkable specificity of the activational effects of the L-methionine analogues on the adenosyltransferases of rat and mouse liver, and the absence of this property in other mammalian and microbial enzymes, raised questions with respect to the evolutionary importance of this phenomenon. We consequently examined the effects of a wide range of concentrations (0.05–20.0mM) of cycloleucine or L-2-amino-4-hexynoic acid on centrifuged crude homogenates of a variety of chicken tissues in reaction mixtures A and B, at a L-methionine concentration of 37.5μM. At no concentration was any activation observed and the enzymes of all chicken tissues were inhibited if sufficiently high concentrations of analogues were attained (Table 3). It may be noted that the chicken tissues (other than liver) appear to be slightly less

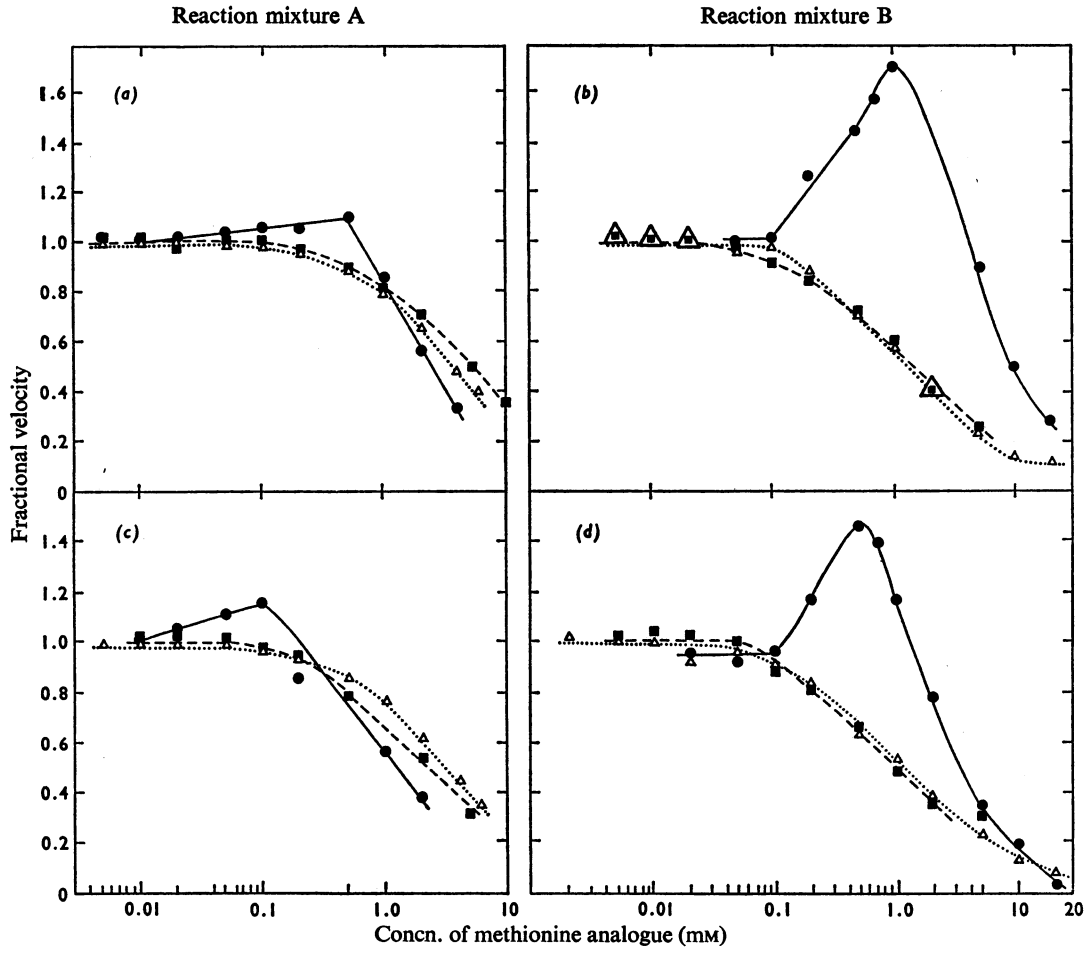

Fig. 3. *Effect of* L-*methionine analogues, cycloleucine* (*a, b*) *and* L-2-*amino*-4-*hexynoic acid* (*c, d*), *in rat liver* (●), *yeast* (■) *and E. coli* (△) *adenosyltransferases*

The fractional velocity is plotted as a function of the logarithm of the inhibitor concentration. The amounts of enzyme protein used to initiate the reaction and the incubation times at 37°C were as follows (specific activities expressed as μmol of *S*-adenosyl-L-methionine/30min per mg of protein): rat liver (specific activity 6.7), 1.7μg of protein, 6min incubation; yeast (specific activity 4.7), 1.2μg of protein, 10min incubation; *E. coli* (specific activity 4.2), 1.5μg of protein, 30min incubation. The compositions of reaction mixtures A (*a, c*) and B (*b, d*) are described in Table 1. The L-methionine concentration was 37.5μM.

sensitive to the inhibitors than are the corresponding rat tissues (Table 2). The I_{50} values for cycloleucine and L-2-amino-4-hexynoic acid for chicken liver are lower than for rat liver, presumably because of the activational effects observed in the latter tissue.

The availability of a large number of L-methionine analogues of varying inhibitory potency for adenosyltransferases permitted an examination of the amino acid specificity of the activation of the rat liver enzyme. Six additional compounds were selected for this comparison: the *cis* and *trans* isomers of DL-2-amino-4-hexenoic acid, as well as *S*-carbamoyl-L-cysteine, *O*-acetyl-L-serine, D-norleucine and L-norvaline. The two last-named compounds are such weak inhibitors that the I_{50} values could not be measured, whereas the *cis* isomer of the ethylenic amino acid is a far weaker inhibitor than the *trans* isomer (Lombardini *et al.*, 1970). Table 4 shows an interesting relation between the concentrations of the analogue required for maximum activation ($A_{max.}$) of the liver enzyme and that necessary for 50% inhibition (I_{50}). The $I_{50}/A_{max.}$ ratios vary between 3 and 10, and it is consequently not unexpected that the I_{50} values for D-norleucine and L-norvaline are too high

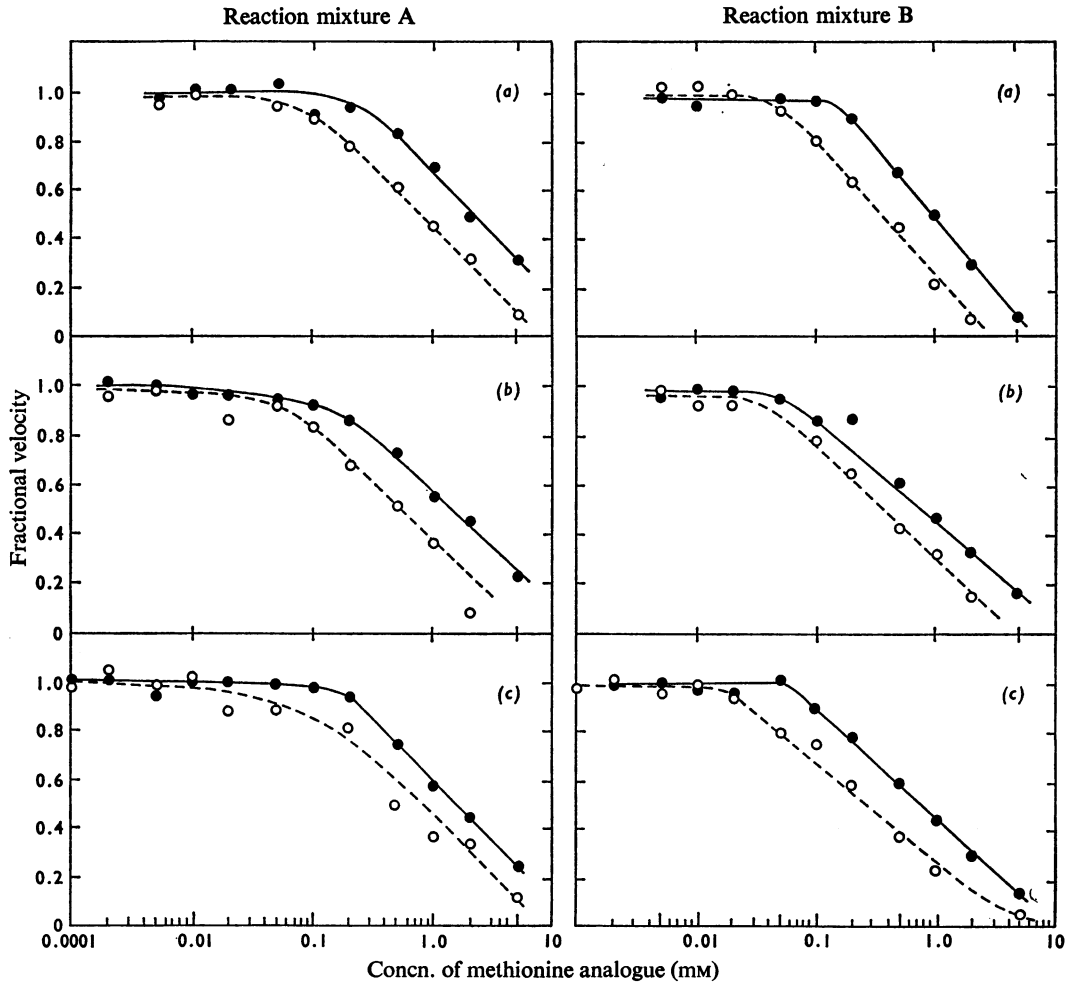

Fig. 4. *Effects of the* L-*methionine analogues, cycloleucine* (●) *and* L-2-*amino-4-hexynoic acid* (○) *on rat spleen* (*a*), *kidney* (*b*) *and Walker* 256 *tumour* (*c*) *adenosyltransferases*

The fractional velocity as a function of the logarithm of the inhibitor concentration. The enzymes were prepared as described in the Experimental section. The enzymic reaction was initiated by addition of appropriate amounts of protein: spleen, 120μg; kidney, 70μg; Walker 256 tumour, 90μg. Reaction vessels were incubated at 37°C for 10min with shaking. Compositions of reaction mixtures A (left-hand panel) and B (right-hand panel) are described in Table 1. The L-methionine concentration was 37.5μM.

to be measured directly because of limitations of solubility. The ability of these compounds to activate the liver enzyme was thus directly related to their inhibitory potency.

Influence of L-*methionine concentration on activating and inhibiting effects of* L-*methionine analogues*

A more detailed examination of the stimulatory effects of cycloleucine and L-2-amino-4-hexynoic acid on the rat liver enzyme revealed that at saturating ATP concentrations this phenomenon was highly sensitive to L-methionine concentrations. When the L-methionine concentration was varied over a wide range, the initial velocity of the reaction was found to depend in a sigmoidal fashion on the L-methionine concentration, and the double-reciprocal plots of $1/v$ against $1/s$ curved upwards (Fig. 5), as has been described for many regulatory enzymes (Stadtman, 1966). Linearity of product formation with time and enzyme concentration is observed under these experimental conditions. The addition of methionine

Table 3. *Concentrations of cycloleucine and* L-2-*amino-4-hexynoic acid required for* 50% *inhibition of chicken adenosyltransferases*

The chicken liver, spleen, kidney and brain enzymes were 15000*g* supernatants of crude homogenates. The conditions of preparation and components of the incubation systems are described in the Experimental section. The amounts of enzyme protein used to initiate the reaction and the incubation times at 37°C were: liver, 210μg of protein, 10min incubation; spleen, 370μg of protein, 20min incubation; kidney, 360μg of protein, 5min incubation; brain, 120μg of protein, 20min incubation. The L-methionine concentration in all experiments was 37.5μM.

Source of enzyme	I_{50} (mM)			
	Cycloleucine		L-2-Amino-4-hexynoic acid	
Reaction mixture ...	A	B	A	B
Liver	1.1	3.2	0.8	2.3
Spleen	5.6	2.1	1.9	
Kidney	2.0	1.5	0.9	
Brain	5.0	1.3	2.2	

Table 4. *Concentrations of various amino acids required for maximum activation* ($A_{max.}$) *and* 50% *inhibition* (I_{50}) *of purified rat liver adenosyltransferase in reaction mixture B*

The rat liver enzyme, a partially purified preparation with a specific activity of 6.2μmol of *S*-adenosyl-L-methionine formed/30min per mg of protein, was assayed under the conditions described in the Experimental section. Incubations were conducted for 6min at 37°C with a L-methionine concentration of 37.5μM. The reaction was initiated by addition of 1.7μg of enzyme protein.

Compound	$A_{max.}$ (mM)	I_{50} (mM)	$I_{50}/A_{max.}$
L-2-Amino-4-hexynoic acid	0.7	3.5	5.0
1-Aminocyclopentanecarboxylic acid	1.0	10.0	10.0
DL-2-Amino-4-*trans*-hexenoic acid	2.0	16.5	8.25
DL-2-Amino-4-*cis*-hexenoic acid	10.0	86.0*	8.6
S-Carbamoyl-L-cysteine	10.0	62.0	6.2
O-Acetyl-L-serine	14.0	42.0	3.0
D-Norleucine	27.0	—†	—
L-Norvaline	30.0	—†	—

* Obtained from Dixon plots by extrapolation.
† Too high to be measured.

analogues tends to obliterate this sigmoidal relationship (Fig. 6). Thus low concentrations of the analogues (results for L-2-amino-4-hexynoic acid are entirely similar but are not shown) activate whereas higher concentrations inhibit the reaction. The double-reciprocal plots (Fig. 6) show that the system displays typical Michaelis–Menten behaviour in the presence of the modifier. Thus for a given concentration of the modifier, if the concentration of the methionine analogue is varied, either activation or inhibition of the overall reaction may be observed, depending on the prevailing concentration of L-methionine in the reaction system. The Hill plots (Hill, 1913; Atkinson *et al.*, 1965) of the same experimental results gave values for the Hill coefficient (*n*) of 1.2 in the absence of the cycloleucine and 1.0 in its presence (Fig. 9). It has been pointed out that the magnitudes of the Hill coefficients are a function of the number of binding sites and the strength of the co-operative interactions between them, so that no simple interpretation of these values is possible (Atkinson, 1966).

Effects of tripolyphosphate on isofunctional adenosyltransferases

It is well known that tripolyphosphate is a powerful inhibitor of the synthesis of *S*-adenosyl-L-methionine by the yeast adenosyltransferase and that it is com-

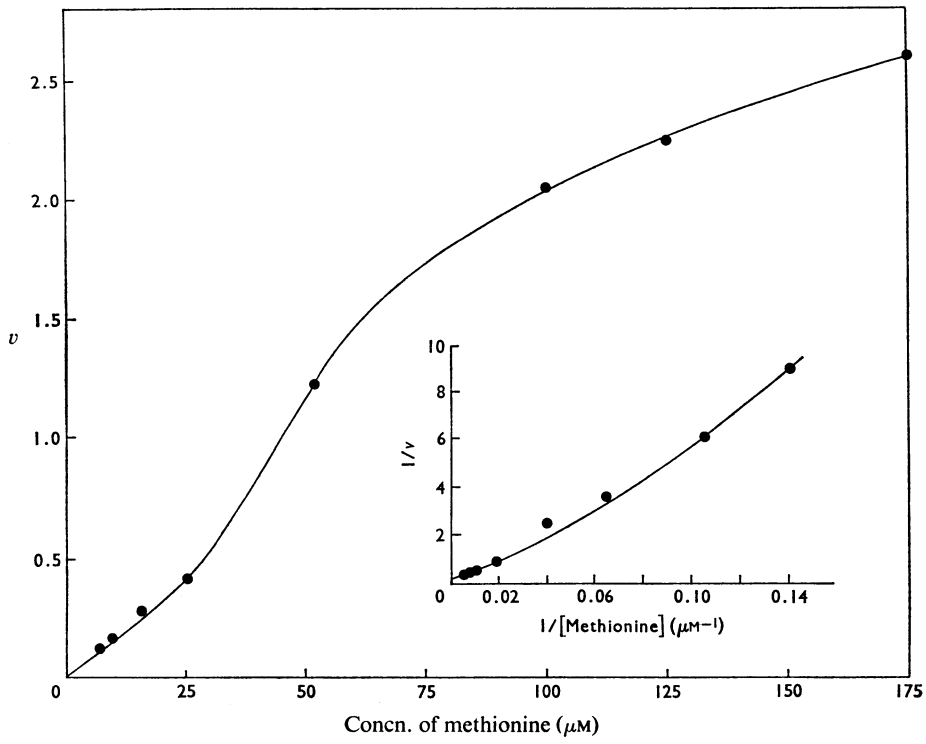

Fig. 5. *Dependence of reaction velocity of rat liver ATP–*L*-methionine S-adenosyltransferase on* L*-methionine concentrations*

The assay system, reaction mixture B, is described in the Experimental section and contained 7.8 μg of enzyme protein with a specific activity of 3.8 μmol of *S*-adenosyl-L-methionine/30 min per mg of protein. The ATP concentration was saturating (10 mM). Reaction vessels were incubated at 37°C for 5 min with shaking. Double-reciprocal plots of the same results are shown in the inset. Only the range of 7–175 μM-L-methionine is shown, to emphasize the sigmoidal portion of the relationship. Concentrations up to 2 mM-L-methionine gave typical saturation kinetics. The velocities are expressed as total nmol of *S*-adenosyl-L-methionine formed in the reaction systems.

petitive with ATP (Greene, 1969; Chou & Talalay, 1972). It was of interest that the inhibitory effect of quite low concentrations of tripolyphosphate could be demonstrated in a variety of crude centrifuged homogenates of mammalian tissues (Table 5). However, low concentrations of tripolyphosphate markedly stimulated the activity of partially purified and crude rat liver adenosyltransferase ($A_{max.} = 0.20$ mM). In this case, also, the $I_{50}/A_{max.}$ ratio is near 5.0. The contrasting response of the rat liver and yeast enzymes to tripolyphosphate is clearly displayed in Fig. 7.

The stimulatory effect of tripolyphosphate on adenosyltransferase activity in crude centrifuged homogenates of various rat tissues appears to be confined to the liver enzyme. Table 5 also shows that rat liver has the highest adenosyltransferase activity, with the following tissues in order of decreasing activity: kidney, testis, spleen, brain, heart, small intestine, skeletal muscle, whole blood and serum. Chicken liver is also not stimulated by tripolyphosphate.

The activating effect of tripolyphosphate is, like that of the methionine analogues, strongly dependent on L-methionine concentration (Fig. 8). The sigmoidal curve observed at low L-methionine concentrations was obliterated by low concentrations of tripolyphosphate. In contrast, at higher L-methionine concentrations, sufficient concentrations of tripolyphosphate were invariably inhibitory. The Hill (Hill, 1913; Atkinson *et al.*, 1965) plots (Fig. 9) of the same experimental values gave $n = 1.3$ for the Hill coefficient in the absence of tripolyphosphate and $n = 0.8$–0.9 in the presence of this modifier.

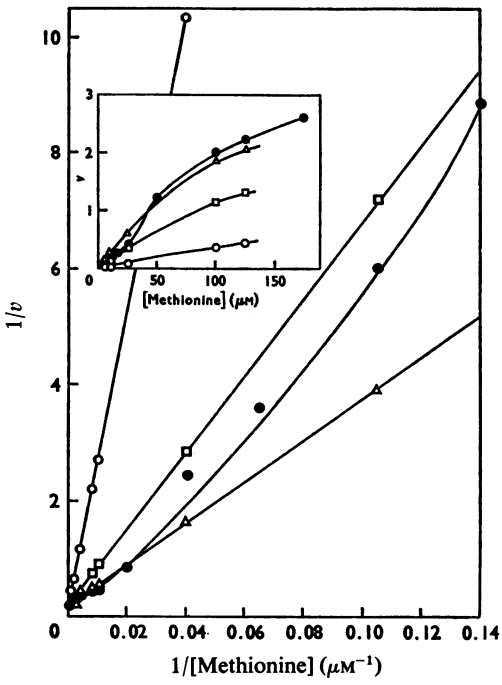

Fig. 6. *Double-reciprocal plots of the reaction velocity as a function of the* L-*methionine concentrations for rat liver ATP–*L-*methionine S-adenosyltransferase in the presence of various concentrations of cycloleucine*

The control results (no inhibitor present, ●) are the same as presented in Fig. 5. Cycloleucine was added to the reaction system at concentrations of 0.5 (△), 2.0 (□) and 10.0 (○) mM. The inset contains the data plotted as the dependence of reaction velocity (nmol of *S*-adenosyl-L-methionine formed) on the L-methionine concentrations.

Combined effects of methionine analogues and of tripolyphosphate

The effects of the simultaneous presence of tripolyphosphate and of cycloleucine are shown in Table 6. These experiments were carried out at lower concentrations of L-methionine (25 μM) than those used above (Figs. 6 and 8), to enhance the activational effects. The magnitude of the activational effects may be quantitated by the use of the fractional activity (or velocity) concept, designated v_f, which is defined as the ratio of the velocity in the presence of a modifier to that in the absence of that modifier. The maximal activational effect exerted by tripolyphosphate under these conditions is larger ($v_f = 3.5$) and occurs at lower concentrations ($A_{max.} = 0.2$ mM) than that of cycloleucine ($v_f = 2.5$ at $A_{max.} = 1.0$ mM).

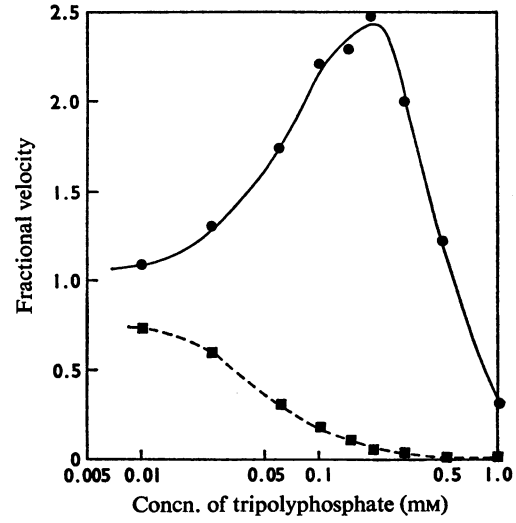

Fig. 7. *Effects of various concentrations of tripolyphosphate on the initial velocity of yeast* (■) *and rat liver* (●) *adenosyltransferases*

Incubations (reaction mixture B) were conducted as described in the Experimental section for 10 min at 37°C with yeast adenosyltransferase (0.2 μg, specific activity 28 μmol of *S*-adenosyl-L-methionine/30 min per mg of protein) or rat liver adenosyltransferase (9.4 μg of protein, specific activity 2.4 μmol of *S*-adenosyl-L-methionine/30 min per mg of protein). The control velocity in the absence of inhibitor was 1.0.

The findings in Table 6 may be more clearly understood by comparing the observed fractional velocities with those expected if both compounds exerted their effects independently of one another. If we assume that tripolyphosphate does not interfere with the modifying effect of cycloleucine (and vice versa), then the product of the fractional activities observed in the presence of fixed concentrations of tripolyphosphate alone, and cycloleucine alone, should give the expected fractional velocity that pertains in the combined presence of the two modifiers at the same concentrations.

When the ratio of observed to calculated fractional velocity is 1.0, the assumption of non-interference of action is confirmed. This situation (ratio = 0.9–1.0) is observed in the upper left corner of Table 6, where the concentrations of both tripolyphosphate and cycloleucine are low, suggesting a summation (or non-interference) of the activational effects of the two compounds. As the concentrations of both tripolyphosphate and cycloleucine are gradually increased, this ratio decreases to a value of about

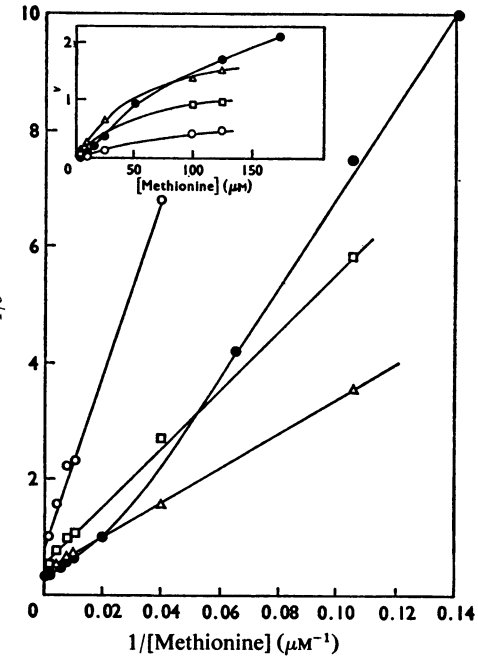

Fig. 8. *Double-reciprocal plots of the reaction velocity of rat liver ATP–L-methionine S-adenosyltransferase as a function of the L-methionine concentrations in the presence of different concentrations of tripolyphosphate*

Incubations (reaction mixture B) were conducted as described in the Experimental section. The tripolyphosphate concentrations were (△) 0.06, (□) 0.2 and (○) 0.7mM; ●, no tripolyphosphate. The reactions were initiated with 4.7μg of enzyme protein and incubated for 5min at 37°C. The inset contains the same results plotted as dependence of the reaction velocity on the L-methionine concentrations.

0.45 under the selected experimental conditions (bottom right of Table 6). In addition, it may be observed that the ratio of observed to expected fractional velocities approaches a minimum (<0.3) when the concentrations of both tripolyphosphate (0.2mM) and cycloleucine (1–2mM) exert their maximal activational effects if present alone. Further increases in either the tripolyphosphate or the cycloleucine concentration, or both, invert this ratio to the higher values (e.g. >0.4). The tendency for the ratio to go through a minimum and then to rise again toward the lower right corner of Table 6 suggests that the inhibitions exerted by high concentrations of the compounds may be additive. These findings argue for interference (or antagonism) between the activational effects of tripolyphosphate and those of cycloleucine. This interference is maximal at concentrations where each compound alone exerts its maximal activational effect.

Combined effects of tripolyphosphate and p-hydroxymercuribenzoate

The results reported in Table 7 suggest that tripolyphosphate at either activating or inhibitory concentrations potentiates the inhibition by *p*-hydroxymercuribenzoate. An equally plausible explanation of the results is that the activational effect of tripolyphosphate is inverted to inhibition in the presence of *p*-hydroxymercuribenzoate. Caution should be exercised in these interpretations since the fractional velocity does not in any way permit conclusions relative to mechanisms, sites of effects, or prove causal relationships. However, the findings do indicate mutual interference between the effects of the two modifiers. Similar observations on the destruction of co-operative effects have been reported for a number of regulatory enzymes (Stadtman, 1966; Atkinson, 1966).

Discussion

The purified adenosine triphosphate–L-methionine *S*-adenosyltransferase of rat liver displays tripolyphosphatase activity, which is profoundly stimulated by low concentrations of *S*-adenosyl-L-methionine (Fig. 2) and thus resembles the yeast enzyme (Mudd, 1962). The interpretation advanced for these observations is that tripolyphosphate is an obligatory intermediate that is far more tightly bound than its products, and their release permits the enzyme to recycle (Mudd, 1965).

Earlier work from this laboratory (Chou & Talalay, 1972) disclosed that the reaction velocity of yeast adenosyltransferase was time-dependent in the very early stages and that this lag could be markedly lengthened by adding tripolyphosphate or shortened by *S*-adenosyl-L-methionine. Chou & Talalay (1972) demonstrated a direct stimulation by *S*-adenosyl-L-methionine of its own synthesis. The deviations of the kinetics of yeast adenosyltransferase from first-order Michaelis–Menten theory were ascribed to time-dependent formation of *S*-adenosyl-L-methionine and time-dependent activation of the inherent tripolyphosphatase activity by *S*-adenosyl-L-methionine. We repeated the reaction-velocity measurements for the yeast adenosyltransferase and extended them to lower L-methionine concentrations (5–50μM) than those used previously (Chou & Talalay, 1972). A downward deflexion of double-reciprocal plots was once again noted but no suggestion of any sigmoidal relationship could be detected.

The discovery that the rat liver adenosyltransferase is activated by low concentration of non-substrate L-methionine analogues (Lombardini & Talalay,

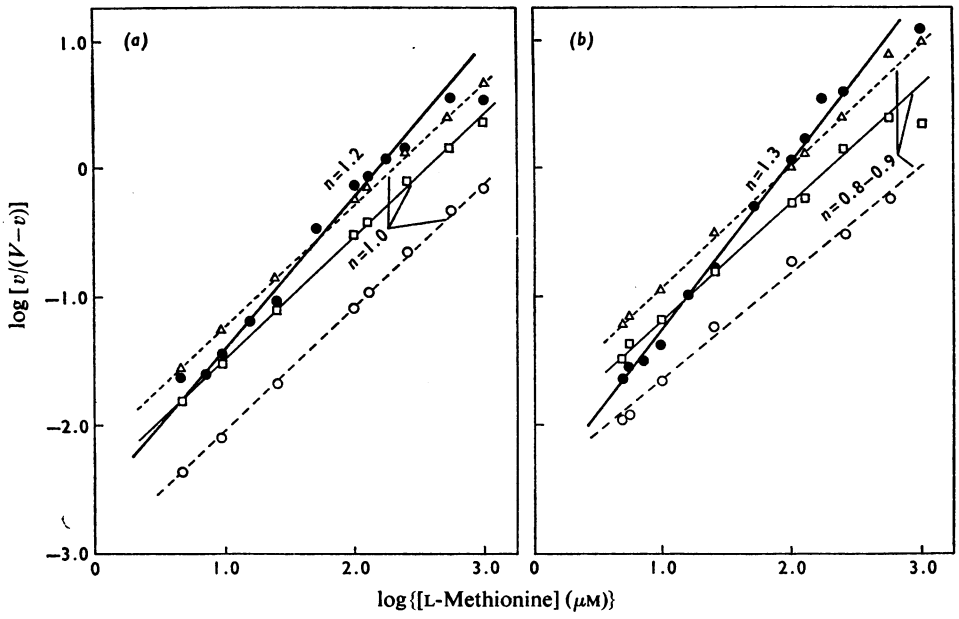

Fig. 9. *Hill plots showing the effects of cycloleucine (a) and tripolyphosphate (b) on rat liver ATP–L-methionine S-adenosyltransferase*

The results are replotted from Figs. 6 and 8. Three concentrations of cycloleucine (*a*: △, 0.5, □, 2.0 and ○, 10.0 mM) and three concentrations of tripolyphosphate (*b*: △, 0.06, □, 0.2, ○, 0.7 mM) are shown; ●, control.

Table 5. *Activities of adenosyltransferases from various rat tissues in the presence and absence of* 0.1 *mM-tripolyphosphate*

Each tissue was pooled from three adult male Sprague–Dawley rats, homogenized with 2 vol. of 5 mM-Tris–HCl buffer, pH 7.6, containing 5 mM-2-mercaptoethanol and then centrifuged at 15000*g* for 15 min. A sample (20 μl) of the supernatant (equivalent to 6.7 mg or 6.7 μl of the original tissue) was used for the assay. Incubations were carried out in reaction mixture B at 37°C for 5 min under conditions described in the Experimental section. The experimental values have been recalculated per g wet wt. of tissue and for 30 min incubations. Each value is the mean ± S.D. of three determinations.

	Adenosyltransferase activity (nmol of *S*-adenosyl-L-methionine/30 min per g of tissue)	
Tissue	Control	+Tripolyphosphate (0.1 mM)
Liver	645.0 ± 7.0	1225 ± 34
Kidney	160.3 ± 1.7	126.0 ± 2.0
Testis	130.5 ± 0.5	88.0 ± 1.0
Spleen	129.5 ± 4.5	111.0 ± 1.5
Brain	70.8 ± 2.2	61.8 ± 3.2
Heart	49.5 ± 3.5	40.8 ± 2.2
Small intestine	35.3 ± 1.1	27.0 ± 0.2
Skeletal muscle	15.1 ± 2.7	7.2 ± 0.2
Blood	4.6 ± 0.2	3.5 ± 0.1
Serum	1.1 ± 0.2	1.0 ± 0.2

Table 6. *Combined effects of various concentrations of cycloleucine and tripolyphosphate on adenosyltransferase activity of purified rat liver adenosyltransferase*

Incubations were carried out in the standard 100μl assay system (reaction mixture B), and measurements were performed with cellulose phosphate paper discs under the conditions described in the Experimental section. The rat liver enzyme was a partially purified preparation with a specific activity at saturating L-methionine concentration of 5.9μmol of *S*-adenosyl-L-methionine synthesized/30min per mg of protein. In this experiment the amount of enzyme protein used to initiate the reaction was 1.2μg and the L-methionine concentration was 25μM. The time of incubation was 5min, in which 0.083nmol of *S*-adenosyl-L-methionine was synthesized in the control vessels and designated as 1.0. All other values are expressed in terms of fractional velocities (v_f). The values in parentheses give the ratio of the observed value of the fractional velocity to the expected value, on the assumption that there is no interference between the effects of the two modifiers. For instance, at 0.2mM-tripolyphosphate and 1.0mM-cycloleucine, the expected $v_f = 2.23 \times 3.54 = 7.89$ (for non-interference) and the observed value for $v_f = 2.28$, giving a ratio of observed to expected value of 0.289. Values shown in **bold** type indicate the concentration regions where the effects of the modifiers show maximum additivity (top left) and maximum interference (bottom right).

Concn. of cycloleucine (mM)	Concn. of tripolyphosphate (mM) ...	0	0.01	0.05	0.2	1.0
0		1.00	1.31	2.50	3.54	1.07
0.1		1.31	**1.63**	**2.96**	2.95*	0.920
			(0.948)	**(0.902)**	(0.636)	(0.657)
0.2		1.36	**1.80**	3.02	3.16	0.900
			(1.01)	(0.888)	(0.657)	(0.616)
0.5		1.73*	**2.24**	3.02	2.59	0.940
			(0.987)	(0.698)	(0.423)	(0.508)
1.0		2.23	2.30*	2.74	**2.28**	0.810
			(0.788)	(0.491)	**(0.289)**	(0.339)
2.0		2.03	1.84	1.71	**1.67**	0.650
			(0.692)	(0.337)	**(0.232)**	(0.300)
5.0		0.99	1.00	1.00	**0.91***	0.460
			(0.769)	(0.403)	**(0.259)**	(0.434)
10.0		0.60	0.470	0.500	**0.50**	0.300
			(0.595)	(0.333)	**(0.236)**	(0.469)

* Duplicate values differed by more than ±5%.

1971*a*; Lombardini *et al.*, 1970) and of tripolyphosphate (present paper) led to a more careful examination of the kinetics of the rat liver enzyme. The results of these studies show clearly that the initial reaction velocity bears a sigmoidal relationship to the concentrations of L-methionine (Figs. 5, 6 and 8). Such sigmoidal saturation curves are the hallmark of regulatory enzymes; the precise molecular explanation must await complete purification of the adenosyltransferase enzyme and studies of its molecular structure. Rat liver adenosyltransferase displays kinetic characteristics that in some ways resemble those of a number of previously described regulatory enzymes. The behaviour of the NAD^+-linked isocitrate dehydrogenase of *Neurospora crassa* (Sanwal *et al.*, 1965), which oxidizes (2*R*,3*S*)-isocitrate, is probably not entirely parallel to the present case since the non-substrate analogues, citrate and (2*S*,3*S*)-isocitrate, are strong activators of the enzymic reaction at low substrate concentration but neither of these effectors becomes inhibitory at high concentrations. These results suggest that the effectors are not bound at the catalytic site. Yet these effectors [citrate and (2*S*,3*S*)-isocitrate] shift the velocity of the isocitrate dehydrogenase reaction from sigmoidal to hyperbolic saturation kinetics, thus simulating the effect of AMP.

In contrast with this, cycloleucine and L-2-amino-4-hexynoic acid, which are both activators (at low concentrations) and potent competitive inhibitors (at high concentrations) of the rat liver adenosyltransferase, are probably bound to the catalytic site, although two classes of sites, such as regulatory (activator) and catalytic (inhibitor), cannot be excluded.

The peculiar activating and inhibitory effects of cycloleucine and L-2-amino-4-hexynoic acid are dependent on L-methionine concentration, and in almost every aspect resemble the effects of succinate and malate on aspartate transcarbamoylase of *E. coli*

Table 7. *Combined effects of various concentrations of tripolyphosphate and p-hydroxymercuribenzoate on purified rat liver adenosyltransferase*

Incubations were carried out in the standard 100 μl assay system with reaction mixture B (except that 2-mercaptoethanol was omitted), and measurements were carried out as described in the Experimental section. The rat liver enzyme was a partially purified preparation with a specific activity at saturating L-methionine concentrations of 2.4 μmol of *S*-adenosyl-L-methionine synthesized/30 min per mg of protein. In this experiment the amount of enzyme protein used to initiate the reaction was 5 μg per vessel and the L-methionine concentration was 39 μM. The time of incubation was 5 min at 37°C, which resulted in the synthesis of 0.049 nmol of *S*-adenosyl-L-methionine in the control vessel (designated as 1.0). The results are expressed as fractional velocities. The values in parentheses give the ratio of the observed fractional velocity to the value expected if there were no interference between the two modifiers. See Table 6 for further explanation.

Concn. of *p*-hydroxymercuribenzoate (mM)	Concn. of tripolyphosphate (mM) ... 0	0.05	0.10	0.2	0.5
0	1.00	1.80	2.43	1.76	0.89
0.05	0.41	0.21	0.19	0.12	0.06
		(0.29)	(0.19)	(0.17)	(0.16)
0.1	0.22	0.07	0.05	0.03	0*
		(0.18)	(0.008)	(0.01)	(0)
0.2	0.17	0.05	0.02	0*	0*
		(0.15)	(0.005)	(0)	(0)
0.5	0.15	0.03	0*	0*	0*
		(0.13)	(0)	(0)	(0)

* Activity too low to be measured accurately.

(Gerhart & Pardee, 1963). Aspartate transcarbamoylase is activated by low concentrations of succinate and malate, neither of which is capable of replacing aspartate as a substrate. Moreover, the activating effect is only observed at low aspartate concentrations, and increasing the concentrations of analogues to higher values causes competitive inhibition of the enzyme. The explanation offered for these observations is based on the assumption of co-operative effects between successively bound substrate molecules. At low aspartate concentrations, when most of the substrate-binding sites are unoccupied, the analogues bind to unoccupied substrate sites, cause co-operative effects mimicking the substrate, and activate the enzyme by increasing affinity for aspartate at other unoccupied substrate sites. As the analogue concentrations are increased, they enter into competition with the substrate, aspartate, and the activity of the enzyme is diminished. The rat liver adenosyltransferase behaves quite similarly, and the same binding site may be receptive to both substrates and analogues, and thus activation at low analogue concentrations and inhibition at high analogue concentrations with hyperbolic kinetics is observed. Cycloleucine and L-2-amino-4-hexynoic acid shift the substrate-saturation curve, which is sigmoidal in the absence of these effectors, to a hyperbolic curve in their presence.

The activating and inhibitory effects of tripolyphosphate which also depend on the concentration of L-methionine could be rationalized on a similar single-binding-site model for tripolyphosphate since this compound is an obligatory enzyme-bound intermediate for the yeast enzyme and, presumably, also for the liver enzyme. As the L-methionine concentration is raised (at saturating concentrations of ATP), the tripolyphosphate-binding site becomes progressively more fully occupied by endogenous tripolyphosphate and the external tripolyphosphate becomes inhibitory, whereas at low L-methionine and tripolyphosphate concentrations the co-operative effects between the externally added and endogenously formed tripolyphosphate stimulate the enzymic reaction.

The simplest view is that the modifier and catalytic site(s) of the liver adenosyltransferase may in fact be identical. This minimal model receives some support from three observations: (*a*) the substrate and modifiers are structural analogues; (*b*) the activational and the inhibitory effects of cycloleucine and of L-2-amino-4-hexynoic acid are competitively overcome by saturating concentrations of L-methionine; (*c*) only those amino acid analogues that inhibit the adenosyltransferase reaction display activational effects at low concentrations and there is a direct correlation between the potency order for activation and inhibition.

The finding of a seemingly unique regulatory property of rat and mouse liver enzymes, and the absence of this type of phenomenon in enzymes from other

animal and microbial sources, poses interesting questions with respect to its functional significance. The unpublished work of the authors showing that cultured rat liver tumour (HTC) cells and ox liver do not display this regulatory property, as well as the finding that at least one avian (chicken) liver does not, suggests that it may be a characteristic of only normal rat and mouse liver enzymes.

We express our appreciation to Dr. A. W. Coulter for generous gifts of samples of L-2-amino-4-hexynoic acid and the *cis* and *trans* isomers of DL-2-amino-4-hexenoic acid. Dr. Donald S. Coffey and Dr. Allan H. Fenselau kindly provided much valuable advice. We are grateful to Mrs. Mary Karen Burch for skilful technical assistance. These investigations were supported by Pharmacology–Toxicology Program–Project Grant GM 16492 and Training Grant GM 1183 from the National Institutes of Health, Bethesda, Md., U.S.A., and by grants in aid from the Gustavus and Louise Pfeiffer Foundation of New York, N.Y., U.S.A.

References

Atkinson, D. E. (1966) *Annu. Rev. Biochem.* **35**, 85–124
Atkinson, D. E., Hathaway, J. A. & Smith, E. C. (1965) *J. Biol. Chem.* **240**, 2682–2690
Baginski, E. S., Foa, P. P. & Zak, B. (1967) *Clin. Chim. Acta* **15**, 155–158
Cantoni, G. L. (1952) *J. Amer. Chem. Soc.* **74**, 2942–2943
Cantoni, G. L. (1953) *J. Biol. Chem.* **204**, 403–416
Cantoni, G. L. & Durell, J. (1957) *J. Biol. Chem.* **225**, 1033–1048
Chou, T.-C. & Lombardini, J. B. (1972) *Biochim. Biophys. Acta* **276**, 399–406
Chou, T.-C. & Talalay, P. (1972) *Biochemistry* **11**, 1065–1073
Cohen, S. S. (1971) *Introduction to the Polyamines*, Prentice-Hall, Englewood Cliffs,
Coulter, A. W. & Talalay, P. (1968) *J. Biol. Chem.* **243**, 3238–3247
Dixon, M. (1953) *Biochem. J.* **55**, 170–171
Gerhart, J. C. & Pardee, A. B. (1963) *Cold Spring Harbor Symp. Exp. Biol.* **28**, 491–496
Greene, R. C. (1969) *Biochemistry* **8**, 2255–2265
Hill, A. V. (1913) *Biochem. J.* **7**, 471–480
Jänne, J., Schenone, A. & Williams-Ashman, H. G. (1971) *Biochem. Biophys. Res. Commun.* **42**, 758–764
Lombardini, J. B. & Talalay, P. (1971*a*) *Advan. Enzyme Regul.* **9**, 349–384
Lombardini, J. B. & Talalay, P. (1971*b*) *Fed. Proc. Fed. Amer. Soc. Exp. Biol.* **30**, 628
Lombardini, J. B. & Talalay, P. (1972) *Fed. Proc. Fed. Amer. Soc. Exp. Biol.* **31**, 553
Lombardini, J. B., Coulter, A. W. & Talalay, P. (1970) *Mol. Pharmacol.* **6**, 481–499
Mudd, S. H. (1962) *J. Biol. Chem.* **237**, PC1372–PC1375
Mudd, S. H. (1963) *J. Biol. Chem.* **238**, 2156–2163
Mudd, S. H. (1965) in *Transmethylation and Methionine Biosynthesis* (Shapiro, S. K. & Schlenk, F., eds.), pp. 33–47, University of Chicago Press, Chicago and London
Mudd, S. H. & Cantoni, G. L. (1958) *J. Biol. Chem.* **231**, 481–492
Mudd, S. H. & Cantoni, G. L. (1964) *Compr. Biochem.* **15**, 1–47
Mudd, S. H. & Mann, J. D. (1963) *J. Biol. Chem.* **238**, 2164–2170
Pan, F. & Tarver, H. (1967) *Arch. Biochem. Biophys.* **119**, 429–434
Sanwal, B. D., Stachow, C. S. & Cook, R. A. (1965) *Biochemistry* **4**, 410–421
Skinner, C. G., Edelson, J. & Shive, W. (1961) *J. Amer. Chem. Soc.* **83**, 2281–2286
Stadtman, E. R. (1966) *Advan. Enzymol. Relat. Subj. Biochem.* **28**, 41–154
Stekol, J. A. (1963) *Methods Enzymol.* **6**, 566–577
Tabor, H. & Tabor, C. W. (1972) *Methods Enzymol.* **17**, part B, 393–397
Tabor, H., Rosenthal, S. M. & Tabor, C. W. (1958) *J. Biol. Chem.* **233**, 907–914
Talalay, P., Takano, G. M. V. & Huggins, C. (1952) *Cancer Res.* **12**, 834–837