Razin, S., Morowitz, H. J., and Terry, T. T. (1965), *Proc. Natl. Acad. Sci. U. S. 54*, 219.
Salton, M. R. J., and Netschey, A. (1965), *Biochim. Biophys. Acta 107*, 539.
Schatz, G., Penefsky, H. S., and Racker, E. (1967), *J. Biol. Chem. 242*, 2552.
Vorbeck, M. L., and Marinetti, G. V. (1965), *Biochemistry 4*, 296.

Sheep Brain 5′-Nucleotidase. Some Enzymic Properties and Allosteric Inhibition by Nucleoside Triphosphates*

Pier Luigi Ipata

ABSTRACT: Enzymological studies of the action of inhibitors, adenosine triphosphate (ATP), uridine triphosphate (UTP), and cytidine triphosphate (CTP), upon partially purified preparations of sheep brain 5′-nucleotidase are described. The inhibition is of the mixed competitive and noncompetitive type with respect to adenosine monophosphate and is potentiated at high temperatures. Plots of kinetic data take the form of sigmoidal inhibition curves. Cooperative inhibitions are observed between inhibitor binding sites; however, first-order kinetics are observed in substrate saturation curves, suggesting the absence of interaction between substrate binding sites. Inorganic phosphate does not inhibit the activity of 5′-nucleotidase; at 0.2 mM, however, it reverses the inhibition by ATP, but not that by UTP and CTP. 5′-Nucleotidase is desensitized to ATP and UTP inhibition, but not to CTP inhibition, by treatment with *p*-mercuribenzoate.

The 5′-nucleotidases (5′-ribonucleoside phosphohydrolase, EC 3.1.3.5) comprise an apparently large and diverse group of enzymes, widely distributed in animal and plant tissues (Reis, 1934, 1951; Heppel and Hilmoe, 1951; Hurst and Butler, 1951; Kornberg and Pricer, 1960; Song and Bodansky, 1967).

An enzyme activity in the central nervous system, catalyzing the dephosphorylation of AMP[1] with a pH optimum around neutrality, was originally found by Reis (1951) in crude homogenates, and appears to be distinct from alkaline and acid phosphatase.

Our interest in brain 5′-nucleotidase was kindled by the observation of appreciable 5′-nucleotidase activity in crude sheep brain homogenates and by the inhibition exerted by ATP and other nucleoside triphosphates on the enzyme activity (Ipata, 1967).

The kinetic studies reported herein on the inhibition of 5′-nucleotidase by ATP, UTP, and CTP show that the inhibition is of an allosteric nature. The kinetics of 5′-nucleotidase *vs.* AMP is in accord with Michaelis–Menten theory.

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[1] Abbreviations used: PMB, *p*-mercuribenzoate; ATP, UTP, CTP, GTP, adenosine, uridine, cytidine, and guanosine triphosphates; AMP, adenosine monophosphate; IMP, inosine monophosphate.

Experimental Procedure

Materials. Nucleosides and nucleotides were obtained either from Sigma Chemical Co. or from Boehringer und Soehne. Whale skeletal myoglobin was obtained from Serevac Laboratories. Bovine serum albumin (containing serum albumin dimer), catalase, and pancreatic ribonuclease were obtained from Sigma Chemical Co. Adenosine deaminase was obtained from Boehringer und Soehne. Tris (Sigma) was used as a buffer in most experiments. Other chemicals were of reagent grade or of the highest quality available.

5′-Nucleotidase Assay Procedures. The enzyme activity was measured spectrophotometrically at 265 mμ by coupling the 5′-nucleotidase reaction to the deamination of adenosine formed, in the presence of an excess of adenosine deaminase. The standard reaction mixture contained, in a final volume of 1 ml, 0.033 M Tris-HCl buffer (pH 7.4), varying concentrations of substrate adjusted to pH 7.4, and about 100 μg of protein. Commercial adenosine deaminase (0.3 μg) was added to the reaction mixture, and the decrease in optical density at 265 mμ was followed with a recording spectrophotometer at room temperature. AMP was omitted in the reference cuvet. The velocity of the reaction was strictly proportional to the amount of 5′-nucleotidase up to rates higher than 0.200 absorbance unit/min. All rates studies reported here were conducted at rates of less than 0.080 absorbance unit/min.

Occasionally, the activity was measured from the

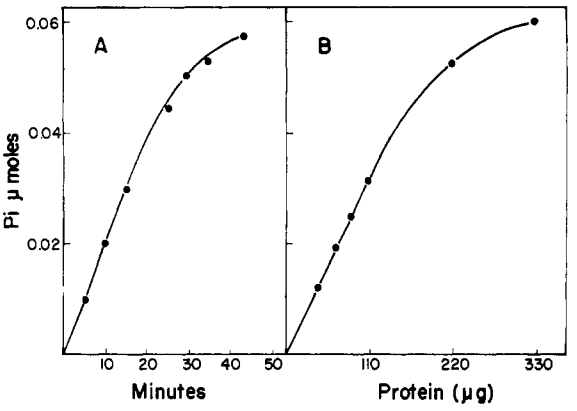

FIGURE 1: Effect of incubation time (A) and enzyme concentration (B) on the reaction rate. The final AMP concentration was 2.16 mM. Activities were measured from the amount of inorganic phosphate released from AMP as described under Experimental Procedure. For A, 110 μg of protein was used. For B, the reaction was stopped after 15 min had elapsed. The inorganic phosphate was determined on 0.2-ml portions of the supernatant fluid.

amount of inorganic phosphate released from AMP. The reaction mixture contained 0.6 ml of 0.05 M Tris-HCl buffer (pH 7.4) and varying amounts of AMP and 5′-nucleotidase. The final volume was 1 ml. Reaction temperature was 37°. The reaction was stopped with 1.8 ml of 10% trichloroacetic acid; tubes were centrifuged, and inorganic phosphate was determined on 0.2-ml portions of the supernatant fluid according to Fiske and Subbarow (1925). Modifications of the standard assay conditions are reported in the presentation of the experimental data.

Gel Filtration for Molecular Weight Determination. Sephadex G-100 gel columns were prepared and calibrated with myoglobin, ribonuclease, serum albumin (containing serum albumin dimer), and adenosine deaminase as internal standards according to the procedure of Andrews (1964). The dimensions of the columns were 2.4 × 50 cm and the flow rate was adjusted at approximately 30 ml/hr. Protein solutions were applied to the top of the column in a volume of 2 ml by increasing the density with sucrose (5 mg) and layering the sample under the buffer above the column bed.

Sucrose Density Gradients. Linear gradients of sucrose from 5 to 20% (w/v) containing 0.05 M Tris-HCl buffer (pH 7.4) were prepared in a volume of 4.7 ml. The internal standard and 5′-nucleotidase preparation were layered on each sucrose density gradient in a volume of 0.1 ml. These were centrifuged for 18 hr at 3° and at 38,000 rpm in the SW 39 rotor of the Model L preparative ultracentrifuge (Spinco). Tubes were pierced with a 21-gauge syringe needle with the aid of a fractionator similar to that described by Martin and Ames (1961). Fractions (44) (7 drops each) were collected and assayed for enzyme activities.

Preparation of Partially Purified 5′-Nucleotidase. The procedure described previously (Ipata, 1967) was essentially followed. In the fractionation procedure reported below, the temperature was maintained between 0 and 4° unless otherwise noted. The purification scheme is summarized in Table I.

PREPARATION OF THE CRUDE EXTRACT. Sheep brains from freshly killed animals were cut into small pieces. These were washed several times with 0.9% NaCl and homogenized in a Waring Blendor. When the temperature of the extract approached 4°, the homogenization was halted and started again after cooling of the extract below 0°. The total homogenization time was 15 min. The extract was then centrifuged at 27,000*g* for 30 min. The supernatant fluid was retained and considered as crude extract (fraction I of Table I).

TABLE I: Purification of 5′-Nucleotidase from Sheep Brain.

Fraction	Vol (ml)	Protein (mg)	5′-Nucleotidase (units)[a]	Sp Act. (units/mg of protein)
I	680	14,300	400,400	28
II	542	6,950	305,800	44
III	540	4,600	253,000	55
IV	15	850	102,000	120
V	180	37	70,300	1,900

[a] One unit equals an activity equivalent to a decrease of 0.001 ODU/min per mg of protein.

HEAT TREATMENT. Fraction I was transferred into a large beaker and heated with continuous gentle stirring at 55° for 10 min. At the end of the heating period the beaker and the contents were cooled quickly by swirling in a water and ice slurry. The precipitate was removed by centrifugation at 27,000*g* and the supernatant fluid was retained (fraction II).

PRECIPITATION AT pH 5. Acetic acid (1 N) was slowly added to fraction II under continuous stirring. When the pH reached a value of 5, the suspension was centrifuged at 10,000*g*, and the supernatant fluid was adjusted to pH 7.4 with 0.5 N NaOH (fraction III).

AMMONIUM SULFATE FRACTIONATION. Fractionation of fraction III by $(NH_4)_2SO_4$ was accomplished by adding sufficient solid ammonium sulfate to give a final saturation of 33%. The precipitate was removed by centrifugation and more solid ammonium sulfate was added to bring the level of saturation to 52%. The precipitate from this fraction, redissolved in 0.05 M Tris-HCl buffer (pH 7.4) (fraction IV), contained most of the 5′-nucleotidase activity.

SEPHADEX G-100 FRACTIONATION. A column (2.4 × 57) was prepared according to the procedure of Andrews (1964) and equilibrated with 0.05 M Tris-HCl buffer

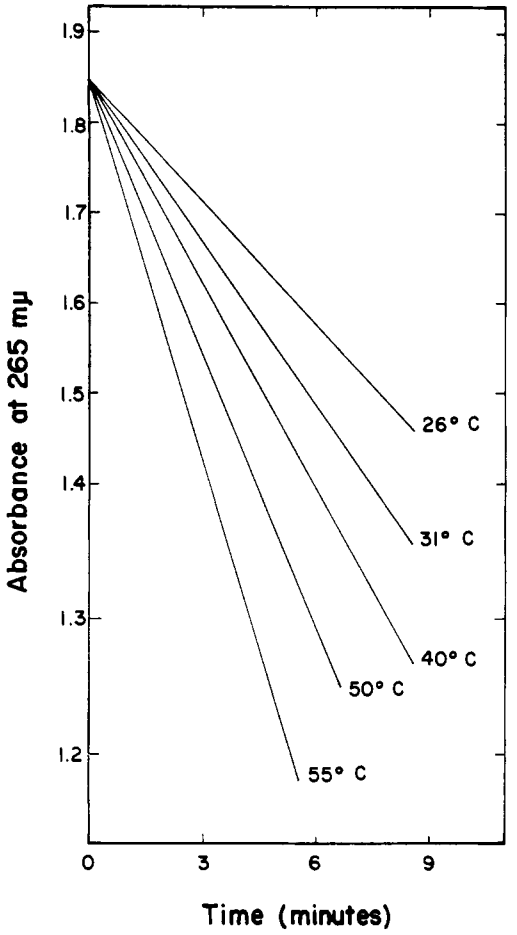

FIGURE 2: Plots of velocities (expressed as absorbance at 265 mμ as a function of time) at different temperatures. The protein concentration was 110 μg/reaction mixture. The final AMP concentration was 0.14 mM.

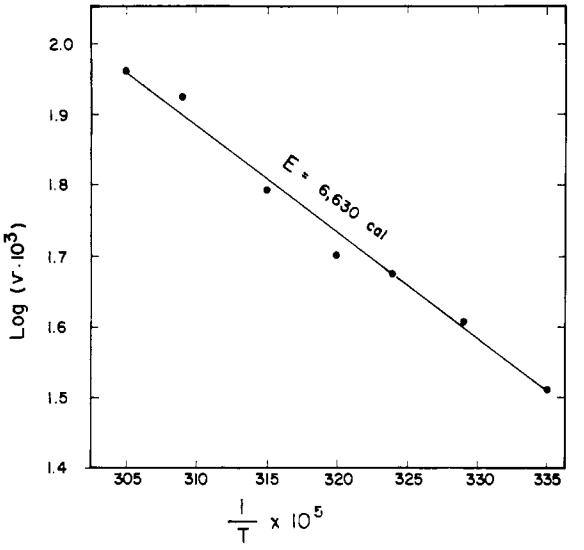

FIGURE 3: Arrhenius plot. The data include initial velocity determinations at temperatures between 26 and 55°, expressed as ΔE at 265 mμ/min, plotted against reciprocal temperatures (Kelvin scale). 5′-Nucleotidase was not inactivated during the 10-min incubation period up to the maximal temperature tested. E equals activation energy.

(pH 7.4). Then 3 ml of fraction IV was applied to the top of the column and eluted at 4° in 3-ml fractions with the same buffer at a flow rate of about 18 ml/hr. This procedure resulted in complete separation of 5′-nucleotidase from adenosine deaminase. The procedure was repeated several times to ensure fractionation of the entire fraction IV. The enzyme was eluted as a sharp symmetrical peak between the 85th and 115th ml. The fractions of peak activity were pooled and stored at −20°. When these were thawed some of the protein was insoluble. The supernatant fluid remaining after centrifugation at 27,000*g* for 20 min was retained (fraction V).

The final preparation did not catalyze the splitting of inorganic phosphate from phenylphosphoric acid, adenosine 2′-monophosphate, adenosine 3′-monophosphate, and nucleoside di- and triphosphates, and was free of any detectable adenosine and AMP deaminase activity. Inosinic acid was hydrolyzed at approximately the same rate as AMP. The enzyme preparation was stored at −20°. The frozen sample was rapidly thawed just prior to use and was used as such or dialyzed 5 hr against 1000 volumes of distilled water when rates studies were conducted as a function of hydrogen ion concentration. Proteins were determined spectrophotometrically (Warburg and Christian, 1942).

Results

Reaction Kinetics. Sheep brain 5′-nucleotidase displays uncomplicated reaction kinetics. Figure 1 shows that the reaction rate is proportional to enzyme concentration up to 110 μg of protein/ml of reaction mixture, and that the time course of the reaction is linear for about 15 min. Figure 2 shows that the linearity of the reaction rate is mantained through a fairly wide range of temperature. Figure 3 is a standard Arrhenius plot, the logarithm of enzyme velocity plotted against the reciprocal of absolute temperature. The activation energy calculated from the negative slope, $-E/R$, has a value of 6630 cal/mole. The evaluation of the K_m for AMP by the use of double-reciprocal plots is shown in Figure 4. A value of 7.5 μM was obtained.

Effect of pH. The data relevant to the influence of hydrogen ion concentration upon both enzyme stability and enzyme activity appear in Figure 5. The enzyme is quite stable at 37° in the pH range 5–9 for durations of at least 15 min. In Tris-acetate buffer the enzyme has a peak of activity at about pH 7.3.

Stability to Heating. 5′-Nucleotidase preparations in Tris-HCl buffer (0.05 M, pH 7.4) (1 mg of protein/ml) were held at various temperatures for 10 min. Tubes

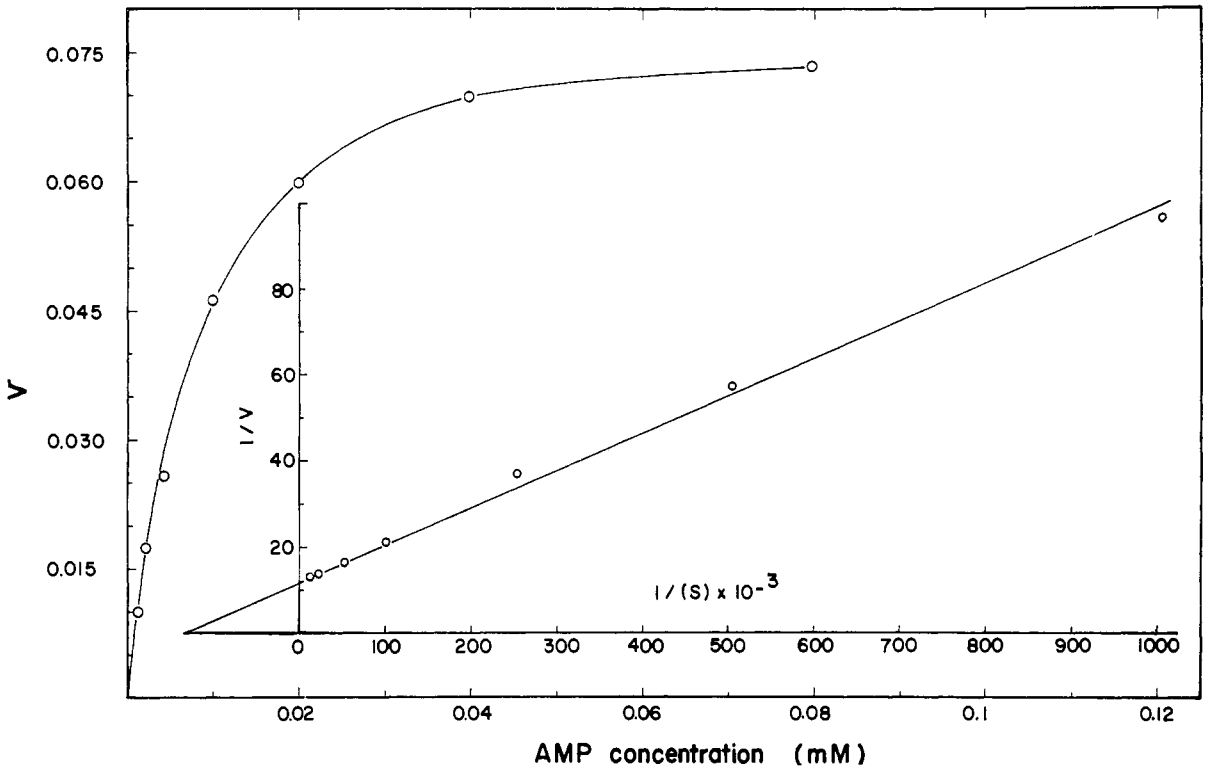

FIGURE 4: Effect of AMP concentration on initial reaction velocities. The velocity is expressed as Δabsorbance per minute at 265 mμ. The Michaelis constant was calculated from the plot $1/v$ *vs.* $1/S$ according to the method of Lineweaver and Burk (1934).

were rapidly chilled, and the enzyme activity was tested. No loss in activity took place up to 57°; the enzyme is inactivated 25% at 62° and 82% at 70°.

Effect of Divalent Cations on Enzyme Activity. The effect of divalent cations at a final concentration of 5 mM is reported in Table II. No requirement of added Mg^{2+} or Mn^{2+} ions for maximal activity could be demonstrated, as concentrations of these ions of 10^{-6}–10^{-2} M had no effect on enzyme activity.

TABLE II: Effect of Divalent Cations on 5′-Nucleotidase Activity.[a]

Additions	% Inhibn
None	0
Mn^{2+}	0
Mg^{2+}	0
Ca^{2+}	16.8
Co^{2+}	19.5
Cu^{2+}	55.2
Ni^{2+}	58.4
Zn^{2+}	96.3

[a] Enzyme activity was assayed spectrophotometrically as described under Experimental Procedure. Cations at a final concentration of 5 mM were added simultaneously with substrates at initiation of reactions. The final AMP concentration was 0.08 mM; 110 μg of protein was used per reaction mixture.

Molecular Weight Determination. The molecular weight of 5′-nucleotidase was determined by gel filtration according to the method of Andrews (1964) and by sucrose density gradient according to Martin and Ames (1961). The G-100 gel elution profile takes the form of a single sharp symmetrical peak, and 5′-nucleotidase and serum albumin dimer are eluted as coincident peaks. Enzyme samples taken from eluates corresponding to the active peak did not reveal any differences in properties, such as sensitivity to inhibitor, stability to heating, or pH optimum. The elution volume corresponding to 5′-nucleotidase led to a calculation of 134,000 as its molecular weight. This result compares favorably with a molecular weight of 142,000 determined by the sucrose gradient technique (Figure 6).

Inhibitors. 5′-Nucleotidase from sheep brain is strongly inhibited by ATP, UTP, and CTP. (Figure 7 shows that for each nucleoside triphosphate the inhibition is of the mixed competitive and noncompetitive type with respect to AMP. The following compounds at 0.5 mM final concentration had no inhibitory effect on enzyme activity: guanosine triphosphate, guanosine diphosphate, adenosine 2′-monophosphate,

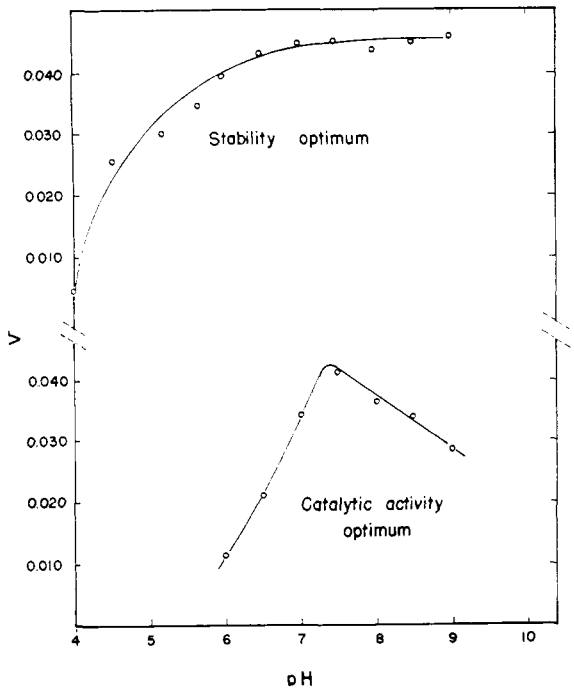

FIGURE 5: Effect of hydrogen ion concentration on reaction velocity. The optimal pH for enzyme stability was determined by storage of the dialyzed enzyme preparation at 37° for 20 min at the indicated pH, followed by the spectrophotometric assay for enzyme activity in Tris-acetate buffer (0.033 M, pH 7.4). The optimal pH for catalytic activity was determined in 0.033 M Tris-acetate buffer. The velocity is expressed as in Figure 4.

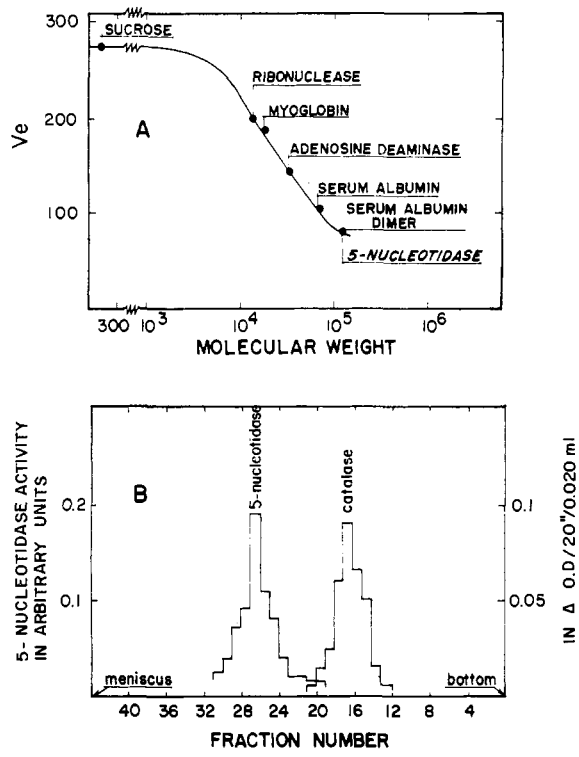

FIGURE 6: (A) Plot of elution volumes (V_e) against log (molecular weight) for 5′-nucleotidase and other marker proteins on Sephadex G-100. Adenosine deaminase, bovine serum albumin, serum albumin dimer, myoglobin, and ribonuclease (approximately 3 mg of total protein) were dissolved in 2 ml of the 5′-nucleotidase preparation. The density of the protein solution was increased by addition of sucrose (5 mg). The solution was applied to the top of a Sephadex G-100 column (2.4 × 50 cm) by layering under the buffer already present. The column was equilibrated and eluted with 0.05 M Tris-HCl buffer (pH 7.4) containing KCl (0.1 M). Fractions (3 ml) were collected at a flow rate of approximately 30 ml/hr. Void volumes were estimated by the absorption at 280 mμ for serum albumin and serum albumin dimer, and at 407 mμ for myoglobin. Void volumes for adenosine deaminase and ribonuclease were estimated by assaying the effluent by the methods of Kalckar (1947) and of Klee and Richards (1957), respectively. 5′-Nucleotidase was located by assaying the effluent spectrophotometrically as described under Experimental Procedure. (B) Determination of 5′-nucleotidase molecular weight by sucrose gradient centrifugation technique. The 5′-nucleotidase preparation (0.1 ml) containing 0.04 mg of catalase was layered on a linear gradient of sucrose, prepared according to Martin and Ames (1961) in 0.05 M Tris-HCl buffer (pH 7.4). After 18 hr of centrifugation at 38,000 rpm, 3°, the gradient was fractionated and analyzed. Catalase was assayed on 0.020 ml of each fraction as described by Martin and Ames (1961). 5′-Nucleotidase was assayed from the amount of inorganic phosphate liberated from an excess of AMP after incubation of each fraction at 37° for 1 hr.

adenosine 3′-monophosphate, uridine 3′-monophosphate, uridylic, cytidylic, and guanylic acids, inorganic pyrophosphate, adenine, cytidine, hypoxanthine, ribose 5′-phosphate, guanine, and uridine. Adenosine, uridine, and cytidine diphosphates were also inhibitory, but to a minor extent, as compared with equal concentrations of the corresponding nucleoside triphosphates. The inhibition by nucleoside triphosphates is freely reversible, as shown by the fact that treatment of the enzyme with 10 μM ATP or UTP, a concentration more than sufficient to cause maximal inhibition, followed by dilution, resulted in restoration of enzyme activity. Similar results were obtained by treatment of the enzyme with nucleoside triphosphates, followed by extensive dialysis. None of the substances tested had any inhibitory effect on adenosine deaminase. The effect of varying concentration of nucleoside triphosphates on the 5′-nucleotidase activity did not obey a simple mass theory; sigmoidal forms of inhibition curves, asymptotic to a finite value, were obtained when 5′-nucleotidase was assayed in the presence of increasing concentration of nucleoside triphosphates, and the values of the interaction coefficient (n') for ATP, CTP, and UTP, calculated according to Jensen and Nester (1966), were 1.7, 2.2, and 1.6, respectively

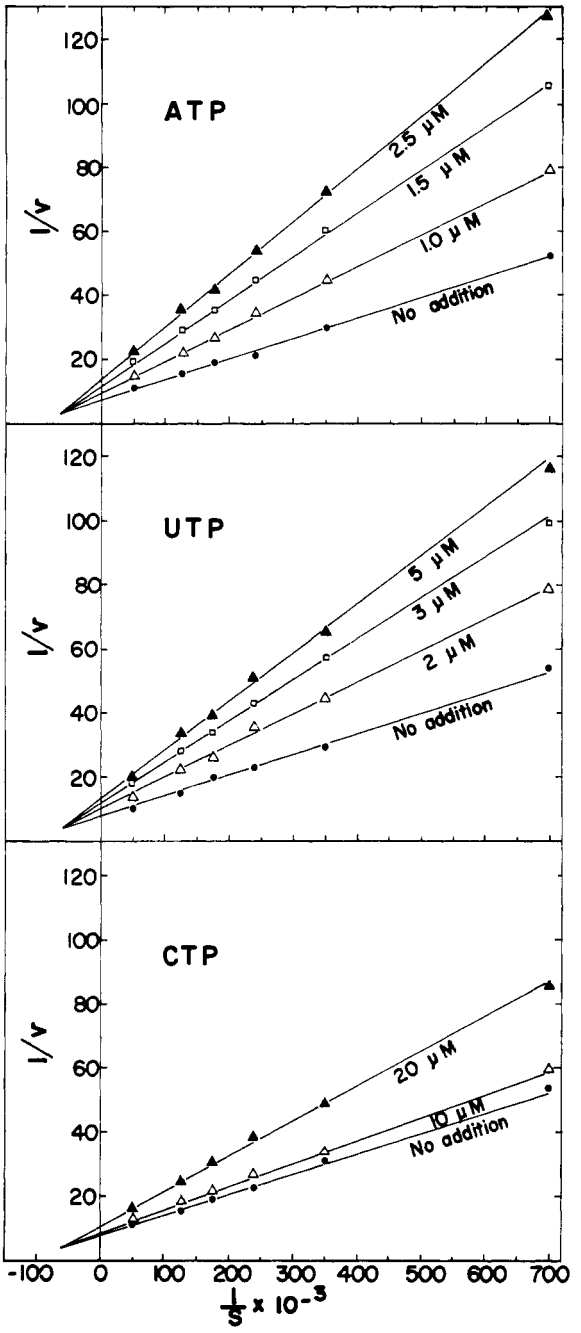

FIGURE 7: Double-reciprocal plots of AMP concentration and initial reaction velocities in the absence and in the presence of nucleoside triphosphates. The initial velocity is expressed as Δabsorbance per minute at 265 mμ under the standard assay conditions.

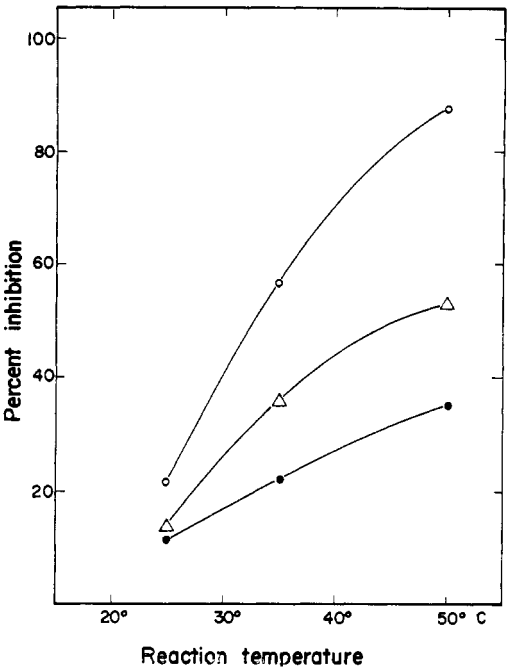

FIGURE 8: Effect of incubation temperature upon inhibition of 5′-nucleotidase activity by 1 μM ATP (○—○—○), 2 μM UTP (△—△—△), and 15 μM CTP (●—●—●). Percentage inhibition equals $(v_0 - v_i)/v_0$. The activity was measured spectrophotometrically in the presence of 110 μg of enzyme preparation, as described under Experimental Procedure. The final AMP concentration was 0.08 mM.

(Ipata, 1967). This suggests the existence of at least two interacting inhibitor sites per enzyme molecule. If the empirical equation proposed by Hill (1910) is applied to kinetic measurements of 5′-nucleotidase by plotting log $v/(V_{max} - v)$ against log S, a line of positive slope, $n = 1$, is obtained (Ipata, 1967). Since the addition of inhibitors never led to enzyme kinetics indicative of cooperative binding of substrate molecules (see Figure 7), this is consistent with the existence of independent binding sites for AMP in the enzyme molecule.

Influence of Temperature on Nucleoside Triphosphate Inhibition. The inhibition of 5′-nucleotidase by nucleoside triphosphates is remarkably temperature dependent. Well within the limits of thermal stability of the enzyme, an increase in the inhibitory action of ATP, UTP, and CTP accompanies the increase of reaction temperature. Thus, ATP concentrations which cause 22% inhibition at 25° inhibit the enzyme activity 88% at 50°. The effect of temperature is less pronounced with UTP and CTP (Figure 8). Figure 9 shows the sigmoidal form of inhibition curves obtained at different temperatures as a function of ATP concentration.

Effect of Inorganic Phosphate on Enzyme Activity. Sheep brain 5′-nucleotidase is not inhibited by inorganic phosphate up to concentrations of 10 mM, the maximum tested; however at 0.2 mM, inorganic phosphate completely reversed the inhibition by ATP, but not that by CTP and UTP (Table III).

Cooperative Inhibition. The studies described above suggested that the enzyme might possess more than one inhibitor binding site. To get further insight on this point, the effect of mixing two nucleoside

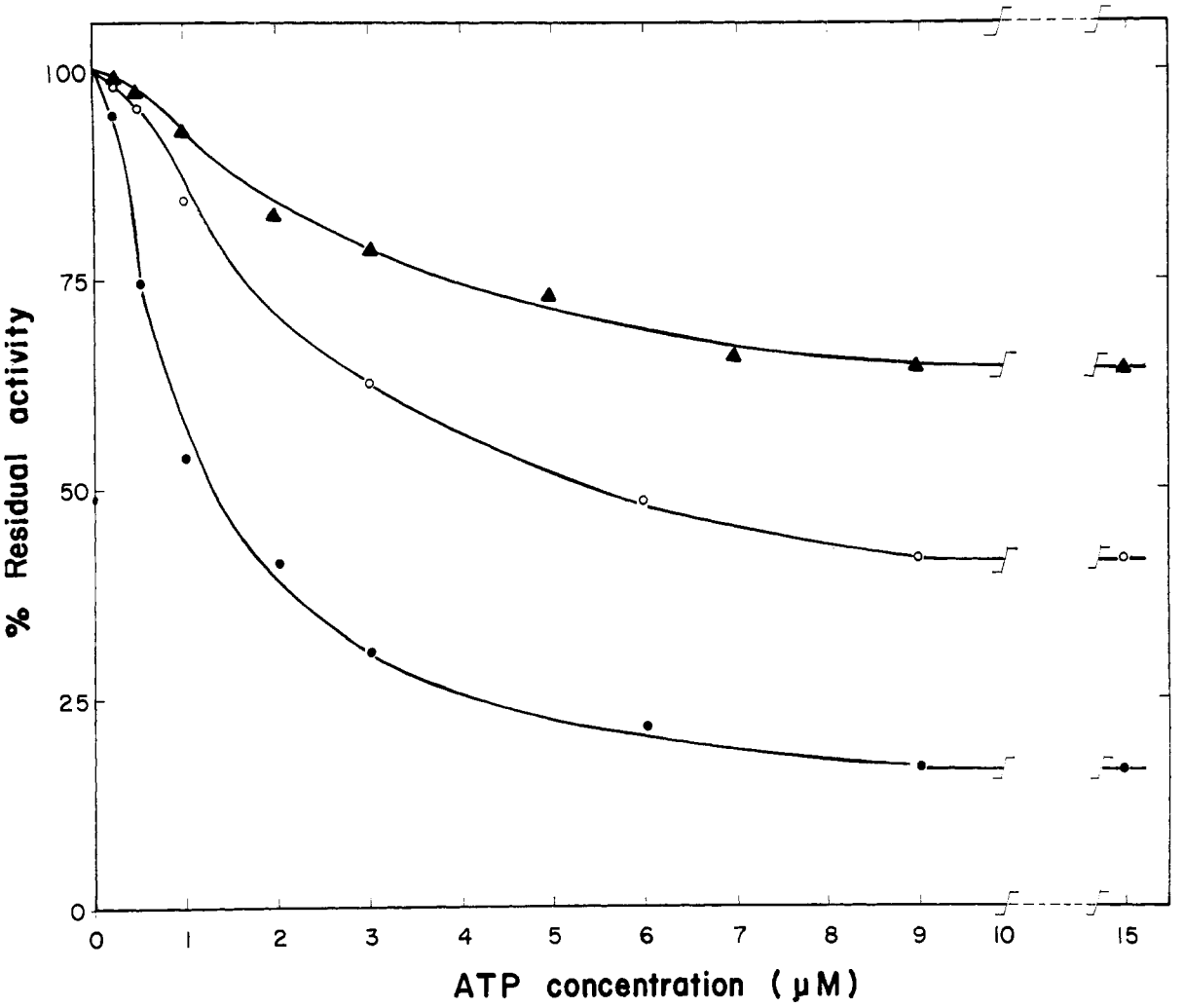

FIGURE 9: Per cent residual activity as a function of ATP concentration at 19 (▲—▲—▲), 25 (○—○—○), and 50° (●—●—●). The activity was measured spectrophotometrically as described under Experimental Procedure. The final AMP concentration was 0.08 mM.

triphosphates on enzyme activity was tested. It was reasoned that if two inhibitors were bound at a single site they should compete with each other, and the residual activity observed by mixing two nucleoside triphosphates should be equal to the product of the separate residual activities. The data reported in Table IV show that the effect of two nucleoside triphosphates acting together led to a residual activity significantly lower than the product of the separate residual activities.

Studies were also conducted with noninhibitory analogs. Inosine, adenine, and ribose 5′-phosphate (each 1 mM) had no effect upon the inhibition of 5′-nucleotidase by 5 μM ATP or UTP and by 20 μM CTP.

Densensitization of 5′-nucleotidase to ATP and UTP but not to CTP inhibition by treatment with PMB has been reported previously (Ipata, 1967). Preincubation of enzyme preparation at 37° for 5, 10, and 15 min in the presence of PMB never led to any major reduction in sensitivity of the enzyme to nucleoside triphosphates. Heating of the enzyme at 45, 50, and 56° in the presence or absence of substrate or inhibitors did not lead to densensitization to nucleoside triphosphate inhibition.

Discussion

The data presented above indicate the presence in sheep brain of a 5′-nucleotidase with a molecular weight of about 140,000 characterized by strong inhibition by ATP, UTP, and CTP. The inhibition by nucleoside triphosphates is noteworthy enough to deserve brief discussion. It is becoming increasingly clear that, in some regulatory enzymes belonging to both synthetic and degradative pathways, modifiers occupy sites on the enzyme molecule distinct from those for the substrates (Monod *et al.*, 1965). It is postulated that the binding of modifiers may lead to a reversible con-

TABLE III: Effect of Inorganic Phosphate on 5′-Nucleotidase Activity and on Its Inhibition by Nucleoside Triphosphates.[a]

Additions	% Init Act.
None	100
ATP (5 μM)	52
P_i (2.0 mM)	100
ATP (5 μM) + P_i 0.20 (mM)	98
UTP (8 μM)	54
UTP (8 μM) + P_i (0.20 mM)	55
CTP (30 μM)	61
CTP (30 μM) + P_i (0.20 mM)	62
CTP (30 μM) + P_i (1.00 mM)	61

[a] Enzyme activity was assayed spectrophotometrically as described under Experimental Procedure. The final AMP concentration was 0.08 mM. The reaction was started by addition of enzyme; 110 μg of protein was used per reaction mixture.

TABLE IV: Cooperative Inhibition of 5′-Nucleotidase by Pairs of Nucleoside Triphosphates.[a]

Nucleoside Triphosphate 1	Nucleoside Triphosphate 2	Concn (μM)	% Residual Act.	% Residual Act. by 1 + 2	Predicted Residual Act.
ATP		1	77		
	UTP	1	93	51	72
ATP		2	59		
	UTP	2	77	23	46
ATP		10	38		
	UTP	10	47	10	18
ATP		1	77		
	CTP	10	86	52	66
ATP		2	59		
	CTP	20	69	25	41
ATP		10	38		
	CTP	100	53	15	21
CTP		10	86		
	UTP	1	93	71	80
CTP		20	69		
	UTP	2	77	43	53
CTP		100	53		
	UTP	10	47	18	25

[a] 5′-Nucleotidase activity was measured spectrophotometrically as described under Experimental Procedure in the presence of either each nucleoside triphosphate alone or of a mixture of two nucleoside triphosphates in terms of total nucleoside triphosphate concentration. The predicted residual activity is obtained from the product of the residual activities in the presence of each inhibitor alone. For example, there was 59% residual activity in the presence of 2 μM ATP and 77% residual activity in the presence of 2 μM UTP. The predicted activity in the presence of 2 μM ATP and 2 μM UTP is (0.59 × 0.77) × 100 or 46%.

formational alteration in the protein, resulting either in activation or inhibition of enzyme activity. In 5′-nucleotidase from sheep brain homotropic interaction between inhibitor molecules occur as predicted by Monod *et al.* (1965). This cooperative effect is apparent in the sigmoidal form of inhibition curves, and by graphical evaluation of the interaction coefficient $n' > 1$. The cooperative inhibition of 5′-nucleotidase by pairs of nucleoside triphosphates cannot be explained by interaction of these nucleotides at a single binding site and indicates that two ribonucleotides can be bound simultaneously by the enzyme and that the binding of one inhibitor increases the effect of binding of the second. The existence of distinct sites is further strengthened by the observations (a) that inorganic phosphate reverses the inhibition by ATP, but not that by CTP and UTP, and (b) that PMB desensitizes the enzyme to ATP and UTP inhibitions, but not to CTP inhibition.

Cooperative interaction between substrate molecules that do not follow Michaelis–Menten kinetics has been often reported in the literature (Atkinson *et al.*, 1965; Changeux, 1963; Gerhart and Pardee, 1963; Scarano *et al.*, 1967). However, for several regulatory enzymes which do not belong to the *K* system, interaction between substrate molecules is absent (Casky *et al.*, 1964; Jensen and Nester, 1965; Martin, 1963; Patte *et al.*, 1963). This seems to be the case for 5′-nucleotidase from sheep brain, since the substrate saturation curve can be fitted to a rectangular hyperbola, rather than the sigmoidal curve characteristic of cooperative interaction between substrate molecules, and the Hill plot yields an interaction coefficient $n = 1$.

The effect of temperature on nucleoside triphosphate inhibition shows that the degree of inhibition of the enzyme by ATP, UTP, and CTP varies over a large range of temperature. Presumably, the temperature effect could be a consequence of (a) a conformational change of enzyme protein, rendering the binding sites more accessible to the inhibitors, or (b), less likely, a temperature-dependent alteration of inhibitor molecules. The inhibition curves, obtained at fixed substrate concentration as a function of ATP concentration at various temperatures (Figure 9) strikingly resemble those based on Rubin and Changeux's (1966) computations for the case of "exclusive substrate binding and variable preferential affinity of inhibitors."

Desensitization of various enzymes to allosteric inhibitors has been achieved by many physical and chemical treatments (Stadtman, 1966). Desensitization to allosteric effectors indicates the nonidentity of catalytic and effector sites. The effect of PMB on enzyme

inhibition by nucleoside triphosphate shows that the binding sites for ATP and UTP may be selectively destroyed.

No attempt has been made to use the data presented in this paper to relate the sheep brain 5′-nucleotidase to any of the models that have been proposed for allosteric enzymes. The purification to homogeneity should be accomplished before any model can be proposed.

The results described in this paper indicate that the inhibition of sheep brain 5′-nucleotidase by nucleoside triphosphates may have a potential role "*in vivo*." It has been shown by Fritzson (1967) that during liver regeneration the activity of 5′-nucleotidase shows cyclic variations which are inversely related to the growth rate of the liver. In this case the regulation of 5′-nucleotidase activity is apparently due to a modulation of enzyme synthesis and breakdown. Fritzson has proposed that the level of 5′-nucleotidase would regulate the intracellular concentration of ribomononucleotides for RNA synthesis. The inhibition of 5′-nucleotidase by ATP and other nucleoside triphosphates could have a similar role, and would prevent the breakdown of AMP to adenosine and inorganic phosphate. It must be emphasized that AMP is a key intermediate for purine nucleotide interconversion in brain. It is acted upon by a specific ATP-activated adenylate deaminase (Mendicino and Muntz, 1955; Setlow and Lowenstein, 1967) giving IMP for guanyl compounds synthesis. The metabolic significance of the regulation of this enzyme has been discussed by Setlow *et al.* (1966).

ATP has therefore a double effect on AMP metabolism in brain, being an activator of its deamination and an inhibitor of its dephosphorylation. The result would be to convey AMP to inosinic acid for the synthesis of guanyl compounds, or to ADP, for ATP synthesis, avoiding its breakdown to degradation products.

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References

Andrews, P. (1964), *Biochem. J. 91*, 222.

Atkinson, D. E., Hataway, J. A., and Smith, E. C. (1965), *Biochem. Biophys. Res. Commun. 18*, 1.

Casky, C. T., Ashton, D. M., and Wyngaarden, J. B. (1964), *J. Biol. Chem. 239*, 2570.

Changeux, J.-P. (1963), *Cold Spring Harbor Symp. Quant. Biol. 28*, 497.

Fiske, C. H., and Subbarow, Y. (1925), *J. Biol. Chem. 66*, 357.

Fritzson, P. (1967), *Eur. J. Biochem. 1*, 12.

Gerhart, J. C., and Pardee, A. B. (1963), *Cold Spring Harbor Symp. Quant. Biol. 28*, 491.

Heppel, L. A., and Hilmoe, R. J. (1951), *J. Biol. Chem. 188*, 665.

Hill, A. V. (1910), *J. Physiol. 40*, 4 p.

Hurst, R. O., and Butler, G. C. (1951), *J. Biol. Chem. 193*, 91.

Ipata, P. L. (1967), *Biochem. Biophys. Res. Commun. 27*, 337.

Jensen, R. A., and Nester, E. W. (1965), *J. Mol. Biol. 12*, 468.

Jensen, R. A., and Nester, E. W. (1966), *J. Biol. Chem. 241*, 3373.

Kalckar, H. M. (1947), *J. Biol. Chem. 167*, 445.

Klee, W., and Richards, F. M. (1957), *J. Biol. Chem. 229*, 489.

Kornberg, A., and Pricer, W. E. (1960), *J. Biol. Chem. 186*, 557.

Lineweaver, H., and Burk, D. (1934), *J. Am. Chem. Soc. 56*, 658.

Martin, R. G. (1963), *J. Biol. Chem. 238*, 257.

Martin, R. G., and Ames, B. (1961), *J. Biol. Chem. 236*, 1372.

Mendicino, J., and Muntz, J. A. (1955), *J. Biol. Chem. 233*, 178.

Monod, J., Wyman, J., and Changeux, J.-P. (1965), *J. Mol. Biol. 12*, 88.

Patte, J. C., Le Bras, G., Loviny, T., and Cohen, G. N. (1963), *Biochim. Biophys. Acta 67*, 16.

Reis, J. L. (1934), *Bull. Soc. Chim. Biol. 16*, 385.

Reis, J. L. (1951), *Biochem. J. 48*, 548.

Rubin, M. M., and Changeux, J.-P. (1966), *J. Mol. Biol. 21*, 265.

Scarano, E., Geraci, G., and Rossi, M. (1967), *Biochemistry 6*, 192.

Setlow, B., Burger, R., and Lowenstein, J. M. (1966), *J. Biol. Chem. 241*, 1244.

Setlow, B., and Lowenstein, J. M. (1967), *J. Biol. Chem. 242*, 607.

Song, C. S., and Bodansky, O. (1967), *J. Biol. Chem. 242*, 694.

Stadtman, E. R. (1966), *Advan. Enzymol. 28*, 41.

Warburg, O., and Christian, W. (1942), *Biochem. Z. 310*, 35.