STUDIES ON THE INHIBITION BY NUCLEOSIDE-TRIPHOSPHATES OF SHEEP BRAIN 5-NUCLEOTIDASE*

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5-nucleotidase, first identified as a distinct enzyme in heart muscle (Reis, 1934) has since been found in a variety of tissues (Reis, 1951), and purified from bull seminal plasma (Heppel and Hilmoe, 1951), snake venom (Hurst and Butler, 1951), potatoes (Kornberg and Pricer, 1950) and liver (Song and Bodansky, 1966). An enzyme activity in central nervous system, catalyzing the dephosphorylation of AMP, with a pH optimum around the neutrality, was originally found in crude homogenates (Reis, 1951) and appears to be distinct from alkaline and acid phosphatase. The properties of the enzyme, however, have never been reported in purified systems. The evidence from the present study indicates that sheep brain 5-nucleotidase is allosterically inhibited by very low concentrations of ATP, UTP, and CTP. GTP is without effect.

METHODS

The activity was measured spectrophotometrically by coupling the 5-nucleotidase reaction to the deamination of adenosine formed, in the presence of an excess of adenosine deaminase. The standard

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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 the enzyme preparation. 0.3 µg of commercial adenosine deaminase (obtained from Boehringer & Soehne GmbH, Mannheim, Germany), were 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 cuvette. The velocity of the reaction was strictly proportional to the amount of 5-nucleotidase up to rates higher than 0.200 absorbance units per minute. All rates studies reported here were conducted at rates of less than 0.080 absorbance units per minute.

The partially purified 5-nucleotidase was prepared as follows. Each step was carried out in the cold, unless otherwise stated. Sheep brains from freshly killed animals were homogenized with two volumes of 0.9% NaCl in a Waring Blendor. The extract was centrifuged at 27,000 x g and the supernatant fluid was heated 10' at 56°C. The precipitate was centrifuged at 27,000 x g, and the supernatant fluid was brought to pH 5 with 1 N acetic acid, and centrifuged at 10,000 x g. The supernatant fluid, adjusted to pH 7.4 with 0.5 N NaOH, was precipitated with ammonium sulphate between 0.33 and 0.52% saturation, solubilized in the minimal volume of Tris-HCl buffer 0.05 M pH 7.4 and dialyzed overnight against distilled water. All insoluble material, which formed during the dialysis, was removed by centrifugation. 3 ml portions of the extract were adsorbed on a Sephadex G-100 column (2.5 x 57 cm) equilibrated with 0.050 M Tris-HCl buffer, and eluted with the same buffer at a flow rate of approximately 18 ml per hour. The 5-nucleotidase activity was constantly recovered between the 85th and the 115th ml of the eluate, as a smooth symmetrical peak. The final preparation did not catalyze the splitting of inorganic phosphate from phenylphosphoric acid, 2'-AMP, 3'-AMP and nucleoside di- and triphosphates, and was free of any detectable adenosine and AMP deaminase activity. IMP was hydrolyzed at approximately the same rate as AMP.

RESULTS AND DISCUSSION

5-nucleotidase from sheep brain displays uncomplicated reaction kinetics: the substrate saturation curve is in accord with Michaelis Menten kinetics (fig. 1). The reaction is proportional to enzyme concentration up to 110 μg of protein, and the time course is linear for about 15 minutes. In Tris-HCl buffer the pH optimum is 7.3.

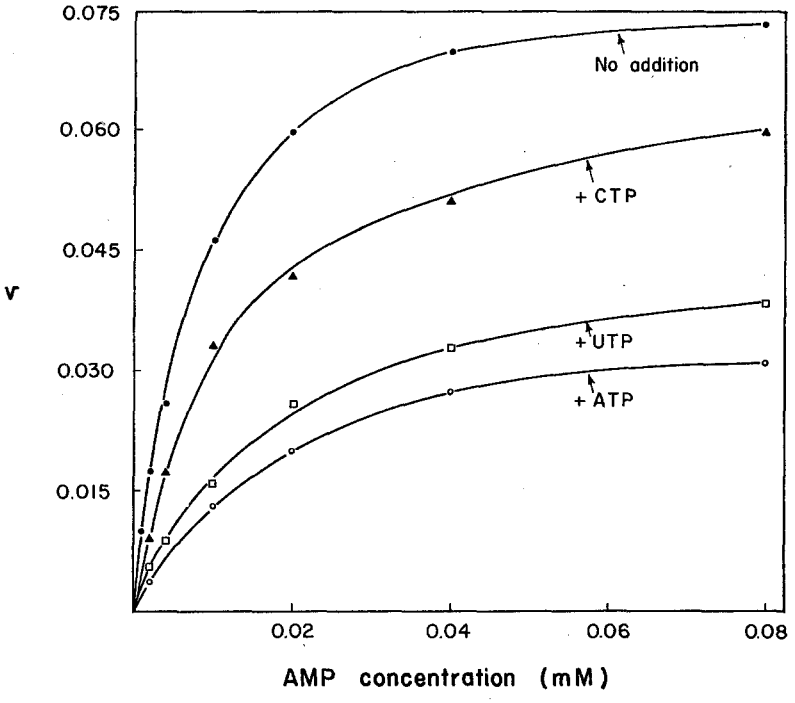

Fig. 1. Substrate saturation curve in the absence and in the presence of inhibitors. The initial reaction velocity is expressed as optical density change per minute at 265 mμ. ATP and UTP concentrations were 5 μM, CTP concentration was 20 μM.

The enzyme is strongly inhibited by ATP, UTP, and CTP. GTP is without effect (fig. 2). The enzyme is not inhibited by inorganic pyrophosphate, ribose-5-phosphate, purine and pyrimidine ribonucleosides and bases, and by nucleoside 2'- or 3'-phosphates. The inhibition is asymptotic to a finite value, and 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 inhibitory effect on adenosine deaminase.

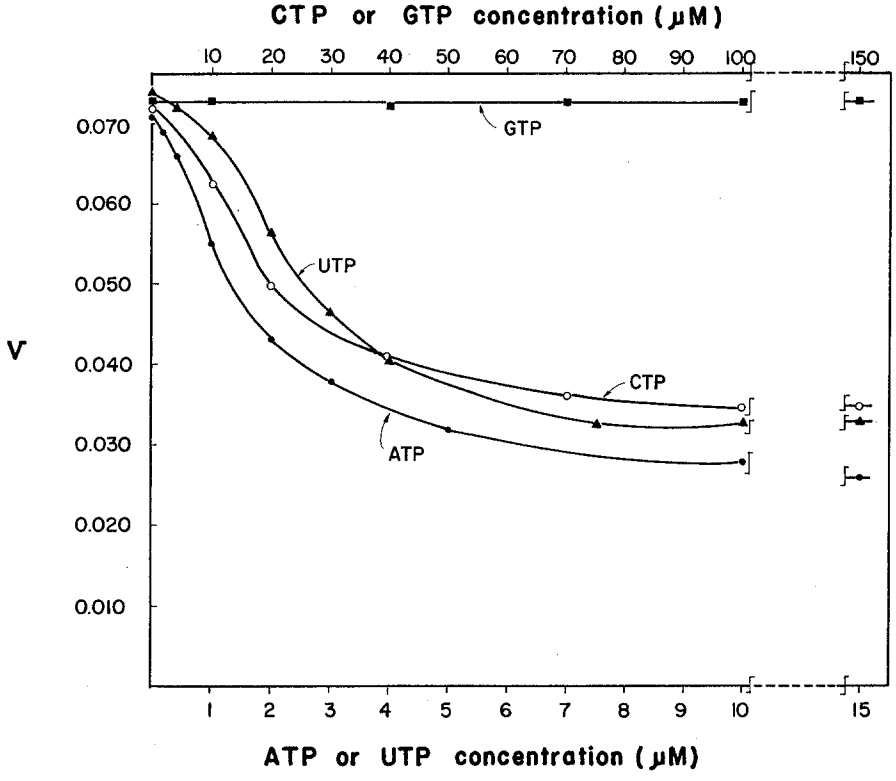

Fig. 2. Effect of varying concentration of nucleoside-triphosphates on the initial velocity of 5-nucleotidase. The final AMP concentration was 0.08 mM. The reaction was started by addition of the enzyme. The initial velocity is expressed as in fig. 1.

Even in the presence of inhibitors, there is no indication of sigmoidal kinetics indicative of cooperative binding of substrate molecules on the enzyme molecule (fig. 1). Lineweaver-Burk plots drawn from the curves of fig. 1 showed that the inhibition is of the mixed competitive and non-competitive type with respect to AMP. However, a cooperative effect between inhibitor molecules is apparent in the sigmoidal form of the inhibition curves obtained

when 5-nucleotidase is assayed in the presence of increasing concentration of ATP, UTP or CTP (fig. 2). According to Monod et al. (1965) such sigmoidal curves can be ascribed to cooperative interactio between different inhibitor sites on the enzyme molecule.

If the Hill plot of coordinates is applied to kinetic measurements of the 5-nucleotidase by plotting $\log \frac{v}{Vmax - v}$ against log of AMP concentration, a straight line of positive slope n=1 is obtained (see fig. 3, inset). Since the addition of inhibitors does not alter the shape of the substrate saturation curve, this

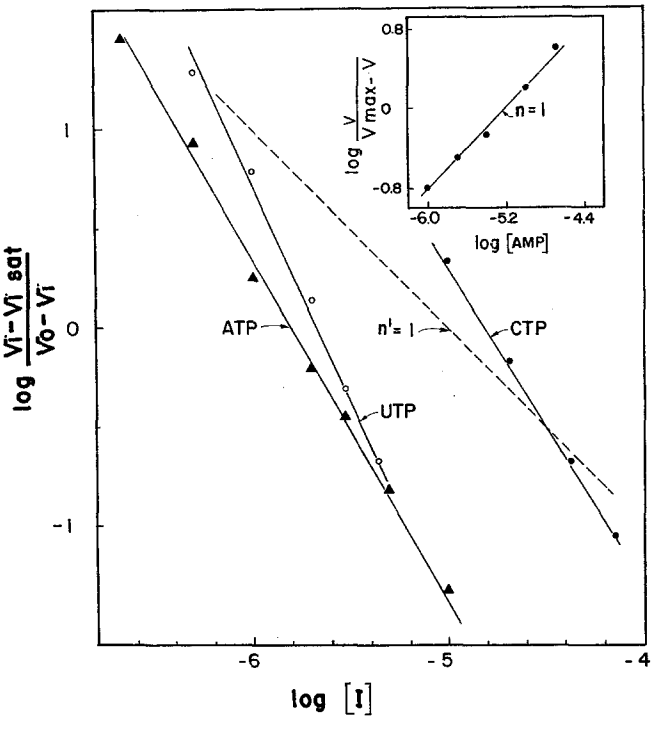

Fig. 3. Determination of interaction coefficient, n', between inhibitor binding sites. The data are plotted from those of fig. 2. The values used for vi(sat) were 0.027, 0.034 and 0.038 O.D. units per minute in the presence of ATP, UTP, and CTP respectively. The calculated values for n' were 1.72, 2.2 and 1.62 for ATP,UTP, and CTP respectively. Inset. Determination of the interaction coefficient, n, between substrate binding sites. The data are plotted from those of fig. 1 (upper curve). The same interaction coefficient, n=1, was obtained from the other curves of fig. 1.

is consistent with the existence of independent binding sites on the enzyme molecules. When the Hill system of coordinates is applied to kinetic measurements of 5-nucleotidase, by plotting log $\frac{vi - vi(sat)}{vo - vi}$ (where vi is the reaction velocity in the presence of the inhibitors, vi(sat) is the reaction velocity at saturating concentration of inhibitors and vo is the reaction velocity in the absence of inhibitors) against log of inhibitors concentration (Jensen and Nester, 1966), a straight line of negative slope n', higher than 1 is obtained (fig. 3), suggesting that one enzyme molecule interacts with more than one inhibitor molecule.

Inorganic phosphate, even at 10 mM does not inhibit the activity of sheep brain 5-nucleotidase. However, when added at 0.2 mM, it causes almost complete reversal of the inhibition exerted by ATP, but not by CTP and UTP.

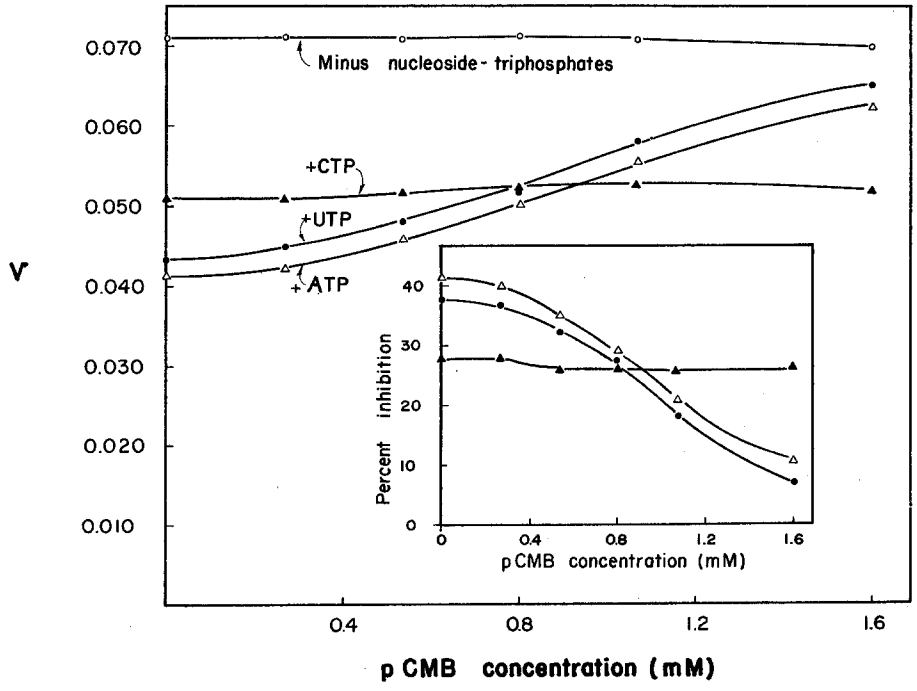

Fig. 4. Effect of pCMB on activity of 5-nucleotidase and on its inhibition by 2 μM ATP, 4 μM UTP and 20 μM CTP. The inset shows that there is a decline in sensitivity to ATP and UTP, but not to CTP inhibition. The initial velocity is expressed as in fig. 1.

Almost complete desensitization to inhibition by ATP and UTP was observed by assaying for enzyme activity in the presence of increasing concentrations of p-chloromercuribenzoate (pCMB). This treatment did not lead to desensitization to CTP inhibition (fig. 4), suggesting that the binding sites for CTP might be different from those for ATP and UTP.

The existence of distinct binding sites for the different nucleoside-triphosphates is further strengthened by the above mentioned observation that inorganic phosphate overcomes the inhibition by ATP, but not that by CTP and UTP, and by preliminary experiments which have shown that the effect of mixing two nucleoside-triphosphates results in inhibitions significantly exceeding the sum of their fractional inhibitions.

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