# 5′-Nucleotidase from Smooth Muscle of Small Intestine and from Brain. Inhibition by Nucleotides†

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ABSTRACT: 5′-Nucleotidase prepared from muscle of small intestine of pig is strongly inhibited by nucleoside di- and triphosphates and their phosphonate analogs. Substrate kinetics approximate the Michaelis–Menten form for AMP, which shows a $K_m$ of 3–6 $\mu M$ at pH 5.3–7.2. Inhibition is characterized as partial competitive, except at pH 5.3, where inhibition by ATP is noncompetitive. The $K_i$ values for several inhibitors have been determined, and their departure from completeness of competitive inhibition has been studied. Inhibitor cooperativity of the type reported for the enzyme from sheep brain (P. L. Ipata (1968), *Biochemistry 7*, 507) was not observed for the enzyme from gut. In addition we failed to confirm sigmoid inhibition kinetics with 5′-nucleotidase from sheep brain.

The purification and some general properties of 5′-nucleotidase from smooth muscle of small intestine were described previously. The enzyme is inhibited by nucleoside di- and triphosphates. The ADP analog $\alpha,\beta$-methylene adenosine diphosphonate (Myers et al., 1965)[1] is the most effective inhibitor found so far (Burger and Lowenstein, 1970). The enzyme is located primarily although not exclusively in or on the plasma membrane of the cell (Baer and Drummond, 1968; Bajusz and Jasmin, 1964; Emmelot and Bos, 1965; Hardonk, 1968; Song and Bodansky, 1967; Song et al., 1967), and the purification procedure adopted by us is consistent with this distribution (Burger and Lowenstein, 1970). 5′-Nucleotidase is probably the enzyme responsible for the production of adenosine, a powerful vasodilator. The diameter of the blood vessels which are mainly responsible for controlling the rate of blood flow is determined by smooth muscle (Berne, 1963; Jacob and Berne, 1960; Thulesius, 1967; Rubio and Berne, 1969; Berne and Rubio, 1973). The present paper describes some kinetic properties of the enzyme from smooth muscle, in particular its inhibition by ADP and AOPCP. Some experiments on the inhibition of the enzyme from brain are also reported.

## Experimental Section

*Materials.* Materials and methods were described previously (Burger, 1968; Burger and Lowenstein, 1970). In most cases a continuous assay was used. Briefly, in this assay the adenosine formed from AMP in the 5′-nucleotidase reaction was converted to inosine by an excess of adenosine deaminase. The reaction was followed and recorded spectrophotometrically at 262.5 nm, and was allowed to proceed to completion. The AMP concentration was determined at suitable intervals from the absorbance shown on the chart recording, and the corresponding reaction velocity was determined from the slope of the curve at the same point. The absorbance of the completed reaction served as an internal reference. The reaction rates obtained in this manner were found to be equivalent to initial rates. Details of this assay were given previously (Burger and Lowenstein, 1970), when it was described as assay 1a. The kinetic parameters determined in this manner were checked by a computer method which required as primary data only the AMP concentration as a function of time.

In assay 1a, adenosine is removed by deamination to inosine. The possibility of product inhibition by adenosine, inosine, or orthophosphate was tested by observing the effects of these substances at concentrations of 5, 10, and 20 $\mu M$ on the initial reaction rate in the presence of 2.5 and 9.5 $\mu M$ AMP, equivalent to 0.5 and 2 times the $K_m$ for AMP, respectively. No inhibition was detected under these conditions.

The experiments with 5′-nucleotidase prepared from smooth muscle which are reported below were carried out with preparations with specific activities in the range of 5–10 $\mu$mol per mg of protein per min.

5′-Nucleotidase from sheep brain was prepared according to the method of Ipata (1968). The highest specific activity obtained by Ipata (1968) was 0.21 $\mu$mol per mg of protein per min (fraction V); however, most of the kinetic work reported in his paper was carried out with enzyme of a specific activity of about 0.013 $\mu$mol per mg of protein per min (fraction IV). The experiments reported below which deal with 5′-nucleotidase prepared from sheep brain were carried out with a preparation of fraction IV. It had a specific activity of 0.009 $\mu$mol per mg of protein per min at 24°.

## Results

*Substrate Kinetics.* The relation of AMP concentration to reaction velocity approximates Michaelis–Menten kinetics. Double reciprocal plots are linear, within the range 1–10 $\mu M$ AMP, in the presence of either 50 m$M$ Tris-HCl (pH 7.2) or of 50 m$M$ MES-Tris, pH 6.5, 6.2, 5.7, or 5.3. The $K_m$ values for AMP fall between 3 and 6 $\mu M$ in every case except in 50 m$M$ MES-Tris buffer (pH 5.7), where the $K_m$ is 15 $\mu M$. We reported previously that a second pH optimum occurs at pH 5.7 with this buffer (Burger and Low-

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[1] The following nonstandard abbrevations are used: AOPCP, $\alpha,\beta$-methylene adenosine diphosphonate; isoAMP, 3-$\beta$-D-ribofuranosyladenine 5′-monophosphate (Leonard and Laursen, 1965); $v$, uninhibited reaction velocity; $v_i$, inhibited reaction velocity.

enstein, 1970).

*Effect of Inhibitors.* The similarity in structure between the 5′-nucleotidase substrates and its nucleoside di- and triphosphate inhibitors (Burger and Lowenstein, 1970) suggests that the inhibition results from the competition of the inhibitors for the catalytic site of the enzyme. In apparent harmony with this, Baer et al. (1966) reported competitive inhibition kinetics for the inhibition of rat heart 5′-nucleotidase by ATP. On the other hand, Edwards and Maguire (1970) reported nucleoside triphosphates, including ATP, to exhibit mixed competitive–noncompetitive kinetics for the same enzyme. We have attempted to distinguish kinetically between partial and complete competitive inhibition, since only the latter is consistent with a direct competition for the active site. The two types of competitive inhibition are distinguished from other kinds of inhibition by the alteration of the apparent Michaelis constant $K_m$ without a change in $V_{max}$. They are not readily distinguishable by plotting $1/v$ against $1/S$. However, they may be distinguished by plotting $1/v$ against $I$ (Dixon, 1953). In this plot complete competitive inhibition (as well as noncompetitive inhibition) yields a straight line, whereas partial competitive inhibition yields a curve which is concave downwards (Webb, 1963a). Complete and partial competitive inhibition also differ from each other in the functions describing the change in $K_m'$. In the case of complete competitive inhibition, $K_m'$ rises without bound; in the case of partial competitive inhibition, $K_m'$ approaches some upper limit. In simple examples of complete competitive inhibition $K_m'$ rises linearly with inhibitor concentration; in simple examples of partial competitive inhibition $K_m'$ rises according to a Langmuir adsorption isotherm of inhibitor concentration. Partial inhibition may result from the binding of inhibitor to a special site on the enzyme, whence it indirectly lessens the affinity of the enzyme for substrate (Gerhart and Pardee, 1964).

*Inhibition by ATP and isoAMP.* Inhibition of 5′-nucleotidase by ATP, using AMP as substrate and 50 m$M$ Tris-HCl (pH 7.2) as buffer, is competitive. The $K_i$ is about 0.15 $\mu M$. Plots of $K'_{m_{AMP}}$ against inhibitor concentration, and of $1/v$ against inhibitor concentration (Dixon, 1953) show that the inhibition is of the partial competitive type (Figure 1). Similar experiments showed that the inhibition of the enzyme by ATP at pH 6.5, in 50 m$M$ MES-Tris buffer, is also of the partial competitive type, with a $K_i$ of 0.08 $\mu M$.

In contrast to its behavior at pH 7.2 and 6.5, inhibition of 5′-nucleotidase by ATP in 50 m$M$ MES-Tris buffer (pH 5.3) is noncompetitive and the $K_i$ is 0.6 $\mu M$. It has been reported that the inhibition of 5′-nucleotidase from ascites cells by ATP is competitive ($K_i$ at pH 7.5 = 0.4 $\mu M$), whereas UTP, GTP, and CTP show mixed competitive and noncompetitive inhibition (Murray and Friedrichs, 1969).

Inhibition of the enzyme by isoAMP is of the complete competitive type, with a $K_i$ of 5 $\mu M$.

*Inhibition by ADP.* We reported previously that ADP is a strong competitive inhibitor of 5′-nucleotidase (Burger and Lowenstein, 1970). The method of Dixon yields a $K_i$ for ADP of 0.022 $\mu M$ (Figure 2). In the low range of ADP concentrations used in the experiment, namely below 0.8 $\mu M$, the inhibition approximates the kinetics of complete competitive inhibition, that is to say the data replotted according to Dixon (Figure 2B) yield straight lines (Webb, 1963a). However, when the range of ADP concentrations examined is extended to 3 $\mu M$ (Figure 3A) partial competitive kinetics is evident in the curvature of the Dixon plot

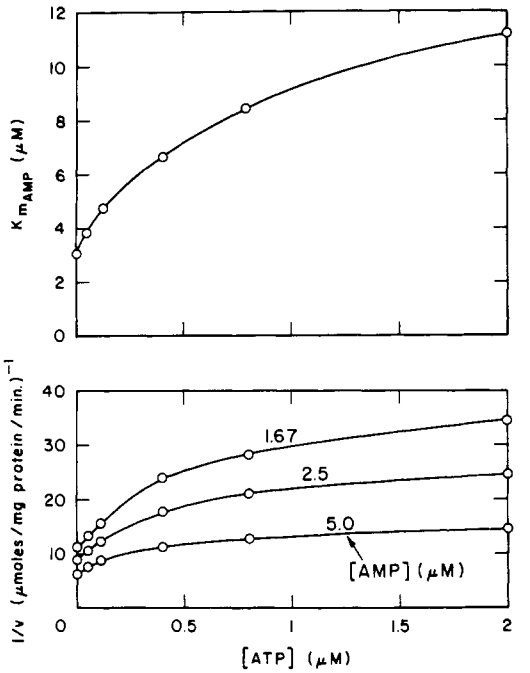


FIGURE 1: Effect of ATP on 5′-nucleotidase from smooth muscle at pH 7.2. Reaction mixtures contained 50 m$M$ Tris-HCl buffer (pH 7.2), 26.4 $\mu$g/ml of 5′-nucleotidase (specific activity 0.25), 1 $\mu$g/ml of adenosine deaminase, and AMP and ATP as indicated. Reactions were started by addition of AMP and run at 24°. Top: the $K_m$ values obtained from a double reciprocal plot of the primary data are plotted as a function of ATP concentration. Bottom: the data are plotted according to the method of Dixon (1953).

(not shown). At higher concentrations still, the Dixon plot becomes linear, suggesting complete competitive inhibition at high inhibitor concentrations. The situation is even clearer when the E-type plot of Webb (1963b) is employed (Figure 3). Here $v/(v - v_i)$ ($1/i$ in the nomenclature of Webb) is plotted against 1/[inhibitor]. An intercept of 1.0 indicates complete inhibition, while a higher value indicates a partial inhibition. When the data are replotted in this way the points for [ADP] < 3 $\mu M$ define a straight line with a $v/(v - v_i)$ intercept of 1.4. However, at sufficiently high concentrations of ADP a weaker but completely competitive inhibition is manifest by an abrupt curvature toward an intercept of 1.

*Tests for Multiple Enzyme Species.* It might be argued that the same result would be obtained if our preparation contained two different phosphatases subject to different types of inhibition. This possibility was largely ruled out by substrate specificity tests conducted previously (Burger and Lowenstein, 1970). To investigate this point further, the experiment shown in Figure 3 was repeated in the presence of 3.3 m$M$ glucose 1-phosphate, with the same results as those shown in Figure 3 (a $v/(v - v_i)$ intercept of 1.4). This shows that glucose 1-phosphate does not act as a competitive substrate, and that the results shown in Figure 3 were not due to a combination of 5′-nucleotidase and a nonspecific phosphatase. It does not rule out the presence of two distinct 5′-nucleotidases with different inhibition characteristics. We consider this possibility unlikely on the basis of heat inactivation studies (Burger and Lowenstein, 1970). The heat treatment resulted in parallel inactivation of 5′-nucleotidase activity measured at pH 6.5, 5.8, and 5.2, in the absence and presence of 17 $\mu M$ ADP or 33 $\mu M$ ATP.

*Inhibition by AOPCP and IDP.* The ADP analog AOPCP is an extremely powerful competitive inhibitor of

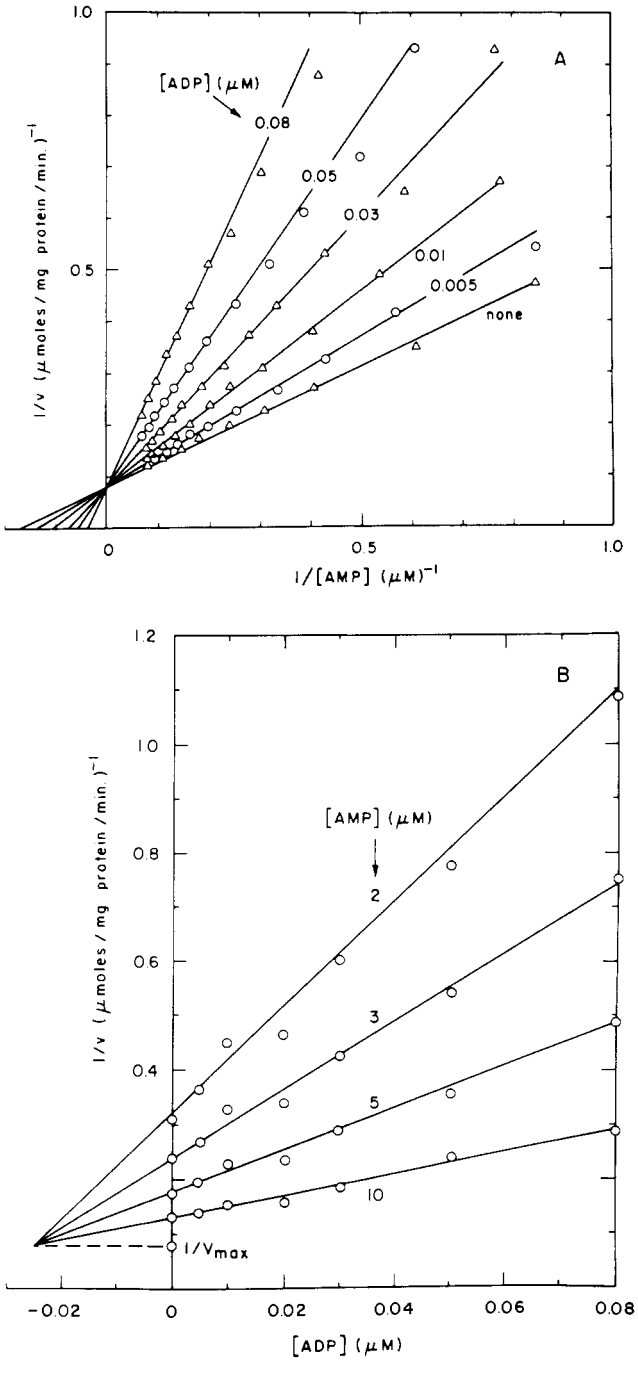


FIGURE 2: Effect of ADP on 5′-nucleotidase from smooth muscle at pH 6.5. Reaction mixtures contained 50 m*M* MES-Tris buffer (pH 6.5), 20 $\mu M$ AMP, 1.11 $\mu$g/ml of 5′-nucleotidase (specific activity 7.0), 1 $\mu$g/ml of adenosine deaminase, and ADP as indicated. (A) Double reciprocal plot; (B) plot of $1/v$ against [I].

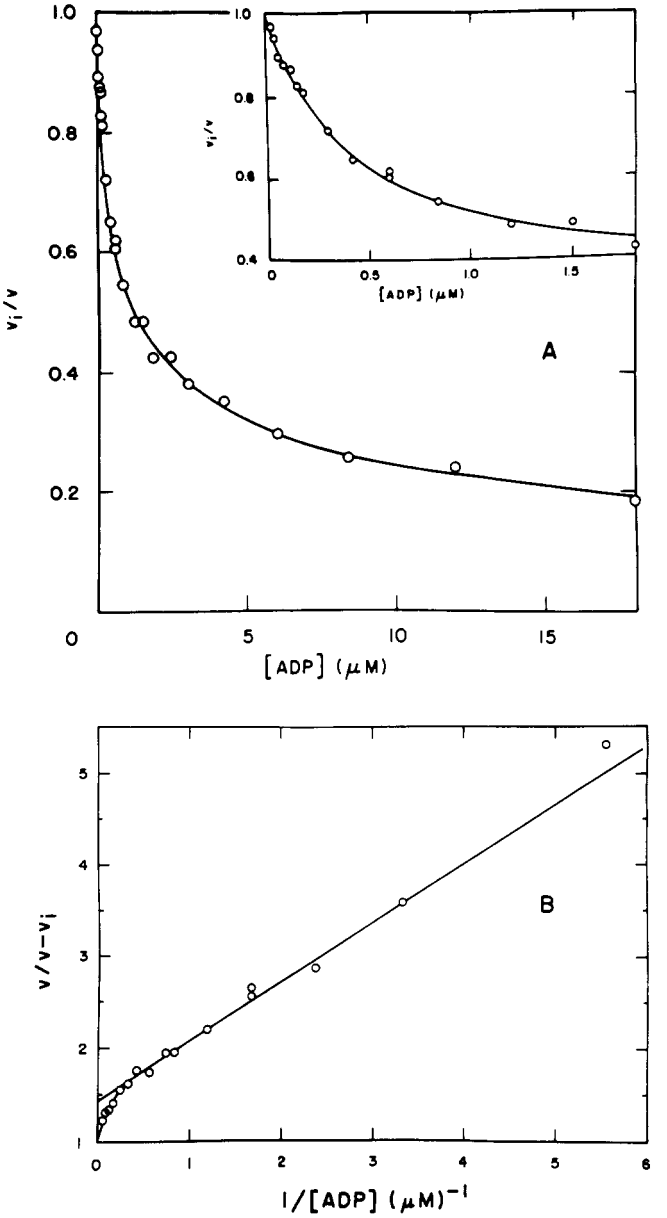


FIGURE 3: Effect of ADP on 5′-nucleotidase from smooth muscle at pH 6.5 over a wider range of inhibitor concentrations. Conditions were as described for Figure 2, except that the 5′-nucleotidase concentration was 1.23 $\mu$g/ml (specific activity 7.0) and the initial AMP concentration was 100 $\mu M$. The velocity of each reaction was measured spectrophotometrically before the addition of ADP. When the AMP concentration had fallen to 80 $\mu M$, ADP was added in the amounts shown and the assay was continued. (A) Plot of fractional activity as a function of [I]; $v$ is the uninhibited activity, and $v_i$ is inhibited activity, both at the same substrate concentration. (B) Plot of reciprocal fractional inhibition as a function of $1/[I]$ (Webb, 1963b).

5′-nucleotidase. Graphical determination of the inhibition constant by the method of Dixon (1953) yields a $K_i$ of about 0.002 $\mu M$. This determination was done just as that depicted in Figure 2, except for the use of 0.0005–0.005 $\mu M$ AOPCP instead of ADP. In the low range of inhibitor concentration used in this experiment, the inhibition approximates kinetics of complete competitive inhibition (Webb, 1963b). However, when a more extended range of AOPCP concentrations is examined, namely the region of 0.010–0.20 $\mu M$, partial competitive inhibition becomes evident (Figure 4). Partial competitive inhibition is also shown by IDP ($K_i$ = 0.37 $\mu M$), which is a very much weaker inhibitor than AOPCP (Figure 4). These results confirm the extremely low $K_i$ for AOPCP reported previously for the enzyme from smooth muscle (Burger and Lowenstein, 1970). The enzyme from mouse liver has also been reported to be inhibited by AOPCP, but to a much weaker extent. In this case the $K_i$ was 63 $\mu M$ and the inhibition was noncompetitive (Evans and Gurd, 1973).

*Tests for Inhibitor Cooperativity.* According to Ipata (1967, 1968) the inhibition of 5′-nucleotidase from sheep brain by ATP is a sigmoidal function of inhibitor concentration. This is not seen in the case of the inhibition of 5′-nucleotidase from smooth muscle by ADP (Figure 3) or

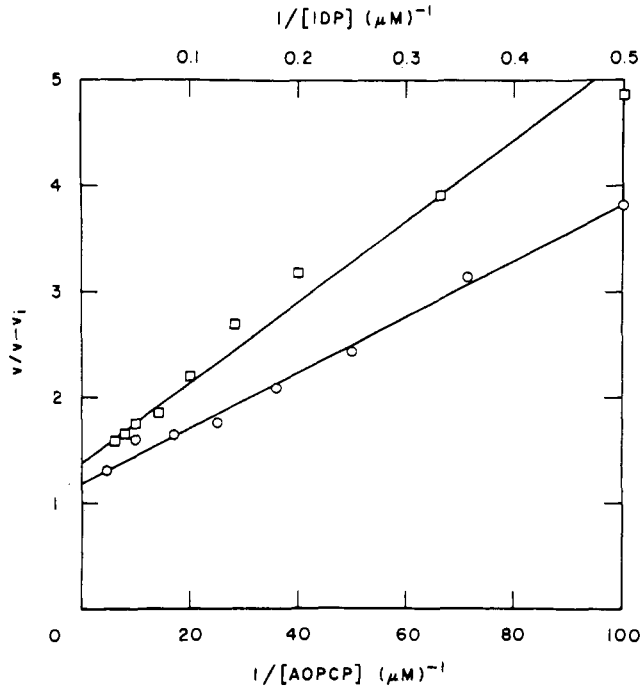


FIGURE 4: Effect of AOPCP and IDP on 5′-nucleotidase from smooth muscle at pH 6.5. Conditions were as described for Figure 2 except that the 5′-nucleotidase concentration was 0.62 μg of protein/ml (specific activity 9.3). (○) AOPCP; (□) IDP.

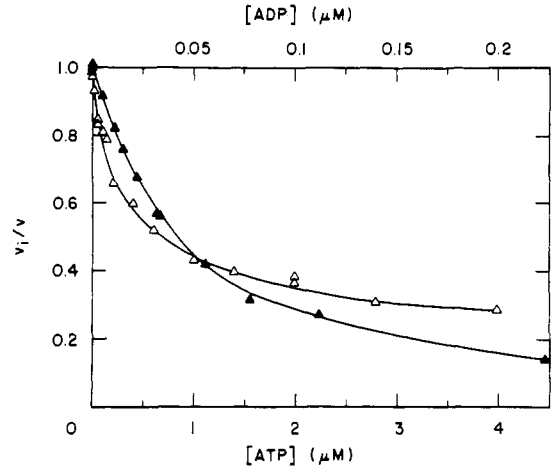


FIGURE 5: Effects of ADP and ATP on 5′-nucleotidase from sheep brain. Reaction mixtures involving tests with ADP (△) contained 67 μM ADP, 50 mM MES-Tris buffer (pH 6.5), .49 mg/ml of 5′-nucleotidase (specific activity 0.0145), and 1 μg/ml of adenosine deaminase. The incubations were run at 37°. Reaction mixtures involving tests with ATP (▲) were similar except that the buffer used was Tris-HCl (pH 7.2) and that the temperature was 24° (specific activity 0.009).

ATP (not shown). Our observations taken in conjunction with those of Ipata suggested a fundamental difference between the enzymes from smooth muscle and brain. This led us to test the effects of ADP and ATP on the enzyme prepared from sheep brain according to the method of Ipata (1968). Neither ADP nor ATP was found to yield sigmoidal inhibition curves when tested over concentration ranges of 0.002–0.20 μM ADP or 0.1–4.5 μM ATP, which cover the inhibition range of 5–70% (Figure 5).

Inhibitor cooperativity can also be evaluated by comparing the effects of various inhibitors separately and in combination. Such a comparison was carried out by Ipata (1968), who found that the inhibitory effect of pairs of different nucleoside triphosphates used together was greater than that predicted from their separate effects using models which did not involve cooperative interaction. He concluded that two different nucleoside triphosphates can be bound to the same enzyme molecule simultaneously, and that the inhibitors show cooperative interaction.

Table I shows that an explanation of the inhibition of 5′-nucleotidase from smooth muscle by pairs of different nucleoside diphosphates does not require a model entailing their cooperative interaction. Three models which did not involve cooperative interaction were considered by us and their prediction tested. The strongest noncooperative inhibition will result when each inhibitor affects a special site which is specific for each inhibitor. The predicted fractional activity is

$$v_{i_1+2}/v = (v_{i_1}/v)(v_{i_2}/v) \quad (1)$$

where $v$ is the uninhibited activity, $v_{i_1}$ and $v_{i_2}$ are the inhibited activities in the presence of inhibitors 1 and 2, and $v_{i_1+2}$ is the inhibited activity in the presence of both inhibitors. This is referred to as model 1 in Table I. By contrast, in the case of two complete competitive inhibitors acting at a single site of an enzyme which obeys the Michaelis–Menten relation

$$\phi = \sigma/(1 + \sigma + \iota) \quad (2)$$

where $\phi = v/V_{max}$; $\sigma = S/K_m$; and $\iota = I/K_i$ (Dixon and Webb, 1964), one obtains

$$v/v_{i_1+2} = (v/v_{i_1}) + (v/v_{i_2}) - 1 \quad (3)$$

where the symbols have the same meaning as above. This is referred to as model 2 in Table I. Another simple model assumes that the inhibition obtained with any nucleoside diphosphate can be related to a concentration of ADP which yields the same degree of inhibition, and that the effect of

Table I: Noninteraction of Nucleoside Diphosphate Inhibitors.

| Inhibitor 1 | (μM) | Inhibitor 2 | (μM) | Percent of Uninhibited Activity[a] Found | Predicted by Model 1 | 2 | 3 |
|---|---|---|---|---|---|---|---|
| ADP | 1.44 | | | 47 | | | |
| AOPCP | 0.05 | | | 55 | | | |
| CDP | 3.3 | | | 58 | | | |
| GDP | 3.3 | | | 71 | | | |
| IDP | 6.7 | | | 72 | | | |
| TDP | 1.0 | | | 41 | | | |
| UDP | 1.0 | | | 61 | | | |
| ADP | 1.44 | CDP | 3.3 | 40 | 27 | 35 | 41 |
| ADP | 1.44 | TDP | 1.0 | 33 | 20 | 28 | 35 |
| ADP | 1.44 | UDP | 1.0 | 43 | 28 | 37 | 42 |
| AOPCP | 0.05 | IDP | 6.7 | 45 | 39 | 45 | 50 |
| AOPCP | 0.05 | TDP | 1.0 | 35 | 23 | 31 | 37 |
| CDP | 3.3 | GDP | 3.3 | 54 | 41 | 47 | 52 |
| CDP | 3.3 | UDP | 1.0 | 49 | 36 | 42 | 50 |
| GDP | 3.3 | IDP | 6.7 | 59 | 51 | 55 | 58 |
| IDP | 6.7 | UDP | 1.0 | 54 | 44 | 49 | 54 |

[a] Enzyme activity was measured as described for Figure 3. "Uninhibited activity" refers to the activity of the enzyme in the presence of 80 μM AMP without addition of the inhibitor(s). The activity observed in the presence of pairs of inhibitors is compared with the activities predicted by three noncooperative models described in detail in the text. The models are: 1, maximum noncooperative inhibition additivity; 2, inhibitor additivity following kinetics of complete competitive inhibition; and 3, inhibitor additivity obeying the partial competitive kinetics of ADP.

Table II: Summary of $K_i$ and $K_m$ Values.[a]

| Substance Tested | $K_i$ or $K_m$ ($\mu M$) at pH 5.3 | 6.5 | 7.2 |
|---|---|---|---|
| $K_i$ | | | |
| AOPCP | | 0.002 | |
| ADP | | 0.022 | 0.040* |
| ATP | 0.60 | 0.080 | 0.15 |
| IDP | | 0.37 | |
| isoAMP | | | 5.0 |
| IMP | | | 14.0* |
| $K_m$ | | | |
| AMP | 5.3 | 6.0 | 3.0 |

[a] $K_i$ and $K_m$ values were determined as described in the text, using data shown in Figures 1, 2, and 4, and results mentioned in the text. Values marked with an asterisk (*) are not mentioned elsewhere. The continuous assay was used with 50 m$M$ Tris-HCl buffer (pH 7.2) or 50 m$M$ MES-Tris buffer, pH 6.5 or 5.3, at 25°. Adenosine and orthophosphate did not inhibit at concentrations as high as 20 $\mu M$.

two different nucleoside diphosphates is equal to that resulting from the sum of two equivalent ADP concentrations. This is referred to as model 3 in Table I. In applying this model we used the data in Figure 3 to determine the equivalent ADP concentrations.

The results shown in Table I agree most closely with the predictions made by model 3, which of the models considered, predicts the lowest inhibition by combinations of inhibitors. Our results do not support the hypothesis of Ipata (1968) that the inhibition of 5′-nucleotidase by nucleoside diphosphates involves positive cooperativity between the inhibitors, and that different nucleoside diphosphates act at different sites.

Slight deviations from straight lines were observed in some plots of $1/v$ against $1/S$, both in the presence and absence of inhibitors. These deviations were all in the same direction, namely from the straight line toward the abscissa at low values of $S$ (Figure 2). The deviations were quite small under all conditions employed by us, but they were observed with both the smooth muscle and sheep brain enzyme. This type of deviation is diagnostic of negative cooperativity with respect to the substrate (Conway and Koshland, 1968).

## Discussion

A summary of $K_i$ values of various inhibitors at three different pH values is given in Table II. The particular pH values were chosen for the following reasons. At pH 5.3 the enzyme activity is near its low pH optimum, and its inhibition by ATP is near maximum. At pH 6.5 the enzyme shows maximum inhibition by ADP. At pH 7.2 the activity of the enzyme in the absence of $Mg^{2+}$ ions is at its second optimum (Burger and Lowenstein, 1970). The table indicates the remarkably low $K_i$ values for AOPCP and ADP. The $K_i$ values of two substrates, isoAMP and IMP, and the $K_m$ value for AMP are shown for comparison. They are two to three orders of magnitude greater than the $K_i$ values for AOPCP and ADP.

The results shown in Figures 2 and 3 and in Table I indicate that the preparation of 5′-nucleotidase from smooth muscle studied by us does not show cooperativity with respect to nucleoside di- and triphosphate inhibitors. This is in contrast to the results obtained by Ipata (1967, 1968) and Ipata and Cercignani (1970) with the enzyme from sheep brain. We were also unable to observe inhibitor cooperativity with enzyme from sheep brain (Figure 5). The enzyme from rat heart also does not show cooperativity with respect to nucleoside diphosphate (Sullivan and Alpers, 1971) and nucleoside triphosphate (Edwards and Maguire, 1970; Sullivan and Alpers, 1971) inhibitors.

The possible physiological significance of 5′-nucleotidase in smooth muscle and its regulation were discussed previously (Burger and Lowenstein, 1970).

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