5'-Nucleotidase from rat heart membranes

Inhibition by adenine nucleotides and related compounds

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ADP and ATP and their analogues were evaluated as inhibitors of 5'-nucleotidase purified from heart plasma membrane. ADP analogues are more powerful inhibitors than the corresponding ATP analogues. The most powerful inhibitor found is adenosine 5'-[$\alpha\beta$ -methylene]diphosphate (AOPCP) for which the enzyme shows a K_i of 5 nM at pH 7.2. Measurements of pK_i values for ADP and AOPCP as a function of pH indicate that the major inhibitory species of both nucleotides is the dianion. In the physiological range of pH values, AOPCP is a more powerful inhibitor than ADP principally because a higher percentage of AOPCP exists in the dianion form. The methylenephosphonate analogue of AMP (ACP), though not a substrate, is a moderately effective inhibitor. The corresponding analogues of ADP (ACPOP) and ATP (ACPOPOP) are as good inhibitors as ADP and ATP respectively. The thiophosphate analogues of ADP all inhibit 5'-nucleotidase, although not as powerfully as ADP, the most effective of these analogues being adenosine 5'-O-(1-thiodiphosphate) diastereoisomer B {ADP[α S](B)}. Other nucleotides inhibit the enzyme, but none is as effective as AOPCP. Inorganic tripolyphosphate and methylenediphosphonate are better inhibitors of the enzyme than is inorganic pyrophosphate. Inorganic thiophosphate is a better inhibitor than is orthophosphate. Hill plots of the ADP and AOPCP inhibition yield slopes close to 1; Hill plots of the ATP inhibition yield slopes of about 0.6. $MgADP^-$ is not an inhibitor, and $MgATP^{2-}$ is at best a very weak inhibitor of the enzyme.

Abbreviations used: ϵ AMP, 1, N^6 -ethenoadenosine 5'-monophosphate; ϵ ADP, 1, N^6 -ethenoadenosine 5'-diphosphate; AMP[S], adenosine 5'-O-(thiomonophosphate); ADP[β S], adenosine 5'-O-(2-thiodiphosphate); ADP[α S](A), adenosine 5'-O-(1-thiodiphosphate) diastereoisomer A; ADP[α S](B), adenosine 5'-O-(1-thiodiphosphate) diastereoisomer B; AOPCP, adenosine 5'-[$\alpha\beta$ -methylene]diphosphate; AOPCPOP, adenosine 5'-[$\alpha\beta$ -methylene]triphosphate; APOPOP, adenosine 5'-[$\beta\gamma$ -methylene]triphosphate; ACP, 5'-deoxyadenosine 5'-methylenephosphonate (alternative designation: 6'-deoxyhomoadenosine-6'-phosphonate); ACPPOP, the ADP analogue corresponding to ACP; ACPOP, the ATP analogue corresponding to ACP; P_i , orthophosphate; PP_i , pyrophosphate; PPP_i , linear tripolyphosphate; PCP, methylenediphosphonate; thio P_i , thiophosphate; v_0 , uninhibited reaction velocity; v_i , inhibited reaction velocity.

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5'-Nucleotidase activity is inhibited by a variety of nucleoside di- and tri-phosphates. ADP is a much more powerful inhibitor than ATP (Burger & Lowenstein, 1970; Sullivan & Alpers, 1971). An analogue of ADP, in which the pyrophosphate bridge oxygen is replaced by a methylene group (AOPCP), is an even better inhibitor (Burger & Lowenstein, 1975). We now throw light on why this is so.

The type of inhibition to which 5'-nucleotidase is subject is a matter of controversy. Inhibition by ATP has been reported to be a competitive (Burger & Lowenstein, 1975; Murray & Friedrichs, 1969), non-competitive (Tanaka *et al.*, 1973; Edwards & McGuire, 1970) and mixed (Stefanovic *et al.*, 1976). In most instances previous workers do not appear to have checked the purity of their ATP. Commercially available ATP is frequently contaminated by several percent of ADP. Since ADP is at least 150 times more powerful an inhibitor

than ATP, results concerning the nature of the ATP inhibition obtained from such studies may be equivocal. Moreover, earlier studies involved the use of partially purified enzyme from various sources, and this left open the possibility of the complicating presence of more than one type of 5'-nucleotidase, or of non-specific phosphatases, or both.

ADP and AOPCP were previously reported to be mixed inhibitors of the enzyme from smooth muscle of small intestine (Burger & Lowenstein, 1975). [We previously used the terms 'partial competitive inhibition' and 'complete competitive inhibition', but now prefer the terms 'mixed inhibition' and 'competitive inhibition' respectively, as suggested by Cornish-Bowden (1979).] AOPCP was reported to be a non-competitive inhibitor of the enzyme from mouse liver plasma membrane (Evans & Gurd, 1973). It seems likely that these differing results were obtained either because the inhibitors were tested over an insufficient range of inhibitor and H^+ concentrations, or because 5'-nucleotidases from different sources show different kinetic behaviour, or both.

Mg^{2+} ions reverse the inhibition of 5'-nucleotidase by ATP and ADP. Previous work left unclear whether the reversal is partial or complete (Burger & Lowenstein, 1970; Sullivan & Alpers, 1971; Tanaka *et al.*, 1973). We have carried out new studies that clarify this point.

Materials and methods

Adenosine, AMP, ADP, AOPCP, ϵ AMP, ϵ ADP and PCP were purchased from P-L Biochemicals. AMP[S], ATP and adenosine deaminase came from Boehringer-Mannheim. Sodium pyrophosphate and sodium triphosphate were obtained from Sigma Chemical Co. AOPCPOP and AOPOPCP came from Miles Laboratories, and sodium thiophosphate from Alfa Products. ACP, ACPOP and ACPOPOP were gifts from Dr. John G. Moffatt (Syntex Research, Palo Alto, CA, U.S.A.) (Jones & Moffatt, 1968). ADP[β S], ADP[α S](A) and ADP[α S](B) were gifts from Dr. Perry A. Frey (University of Wisconsin, Madison, WI, U.S.A.). [^3P]AMP was obtained from Amersham. The purity of all nucleotides was checked by t.l.c. on Silica gel 60F₂₅₄ (Merck) with the mixture isobutyric acid/18M-NH₃/water (63:2:35, by vol.) (Krebs & Hems, 1953). If necessary the nucleotides were purified by paper chromatography on Whatman no. 3 paper with the same mixture. ATP was purified by chromatography on Dowex-1 (Cl-form) to remove a small amount of ADP.

5'-Nucleotidase was purified from rat hearts and assayed by using assay 1 or assay 3 (Naito & Lowenstein, 1981). Unless otherwise indicated, the

continuous spectrophotometric assay (assay 1) mixture contained 4 μ g of adenosine deaminase and 50 mM-triethanolamine/HCl buffer, pH 7.5.

Results and discussion

AMP analogues

The enzyme is relatively unspecific with respect to the purine or pyrimidine base of the nucleoside monophosphate substrate, although AMP yields the highest V_{max}/K_m value (Naito & Lowenstein, 1981, and references cited therein). The enzyme hydrolyses ϵ AMP with a K_m similar to and a V_{max} about one-half that for AMP (Table 1). AMP[S] is also a substrate, with a K_m actually lower than that for AMP, but a V_{max} only one-sixteenth that for AMP. Murray & Atkinson (1968) first showed that AMP[S] is a substrate for 5'-nucleotidase. Edwards & McGuire (1970) reported that a preparation of the rat heart enzyme, with a specific activity of about 0.1 unit (μ mol/min)/mg, hydrolysed AMP[S]

Table 1. K_m and K_i values for 5'-nucleotidase from rat heart
Reaction rates were measured in 50 mM-triethanolamine/HCl buffer, pH 7.5, at 30°C. Assay 1 was used, except in experiments with ACP, ATP and adenosine, for which assay 3 was used. The specific activity of the enzyme was 247 units/mg and each reaction mixture contained 2–10 ng of enzyme. K_i values were calculated from Dixon plots for three AMP concentrations. Inhibitor concentrations were used in the following ranges: ADP, 0.025–0.4 μ M; AOPCP, 2.5–30 nM; AOPCPOP, 0.04–0.24 μ M; AOPOP, 0.2–2.0 μ M; ACP, 20–100 μ M; ACPOP, 0.02–0.2 μ M; ACPOPOP, 0.4–10 μ M; ϵ ADP, 0.2–1.0 μ M; ADP[β S], 0.5–6.0 μ M; ADP[α S](A), 1–8 μ M; ADP[α S](B), 0.05–0.7 μ M; P_i , 1–10 mM; PCP, 40–400 μ M; PP_i , 50–300 μ M; PPP_i , 30–150 μ M; thio P_i , 100–1000 μ M; adenosine, 200–1000 μ M. The K_i values for ATP were obtained from double-reciprocal plots at 2 μ M- and 80 μ M-ATP respectively.

Substrate	K_m (μ M)	V_{max} (%)	Relative V_{max}/K_m
AMP	4.7	100	21
ϵ AMP	6.5	45	6.9
AMP[S]	2.3	6	2.6
Inhibitor	K_i (μ M)	Inhibitor	K_i (μ M)
ADP	0.082	ADP[β S]	1.4
ATP	1.5 and 4.4	ADP[α S](A)	2.0
AOPCP	0.006	ADP[α S](B)	0.31
AOPCPOP	0.066	P_i	3000
AOPOP	0.54	PCP	90
AOPOP	45	PP_i	200
ACPOP	0.067	PPP_i	110
ACPOPOP	1.3	Thio P_i	420
ϵ ADP	0.47	Adenosine	650

with a $V_{\max}$ of 36% and a K_m of 69% of the values observed with AMP. The purified plasma-membrane enzyme used by us had a specific activity of 247 units/mg. AMP[S] is not hydrolysed by alkaline phosphatase from calf intestine (Eckstein, 1970), but it is converted into AMP by phosphodiesterase from snake venom (Eckstein & Baer, 1969).

ACP, the phosphonate analogue of AMP, is not a substrate, as is to be expected; instead it inhibits the enzyme with a K_i of 45 μM , which is about 10 times greater than the K_m for AMP.

Inhibition by ADP and ATP

ADP and AOPCP are both powerful inhibitors of 5'-nucleotidase. Plots of $1/v$ against $1/[S]$ showed that ADP is a competitive inhibitor of the pure enzyme from rat heart. Plots of the data are not shown, but their quality was similar to that reported by Burger & Lowenstein (1975). The E-type plot of Webb (1963) $\{v/(v-v_i)\}$ plotted against $1/[ADP]$ yielded a straight line that intercepted 1.0 on the ordinate. Plots of $1/v$ against $[ADP]$ (Dixon, 1953) yielded straight lines and a K_i for ADP of 0.082 μM , one-fourth the value reported by Sullivan & Alpers (1971) for the partially purified enzyme from rat heart. The partially purified enzyme from smooth muscle of small intestine was previously reported to show mixed inhibition with ADP (Burger & Lowenstein, 1975).

ATP is a mixed inhibitor at concentrations less than 50 μM and a competitive inhibitor at concentrations greater than 50 μM . The E-type plot of Webb (1963) yielded a curved line that intercepted 1.0 on the ordinate. At the highest concentrations of the purified ATP used (100 μM), contamination contributed 0.024 μM -ADP, which is 30% of the K_i of ADP. Reciprocal plots yielded K_i values of 1.5 and 4.4 μM at 2 μM - and 80 μM -ATP respectively. The results of these experiments were replotted in terms of $\log[(v_0-v_i)/v_i]$ versus $\log[ADP]$ or $\log[ATP]$ (Hill, 1913). The slope h for ADP is 0.91, whereas that for ATP is 0.64 (Fig. 1), suggesting negative co-operativity with ATP but not ADP.

Inhibition by ADP analogues

ADP and all ADP analogues tested are competitive inhibitors of 5'-nucleotidase, AOPCP being the most powerful, with a K_i of 6 nM at pH 7.5 (Table 1). The extremely low K_i for AOPCP is not observed with ACPOP. ADP[β S], ADP[α S](A), and ADP[α S](B) show K_i values of 1.4, 2.0 and 0.3 μM respectively. Thus the enzyme shows some discrimination between the A and B stereoisomers of ADP[α S] (Eckstein, 1979; Cusack *et al.*, 1983). None of these thiophosphate analogues inhibits as strongly as ADP. Substitution of sulphur on the β -phosphate of ADP would be expected to lower its

Fig. 1. Hill plots of inhibition of 5'-nucleotidase by ADP (●) and ATP (○)

Reaction mixtures contained 50 mM-triethanolamine/HCl buffer, pH 7.5, 1.0 μg of adenosine deaminase, 1.67 mM-sodium cholate, ADP as indicated, and 12.2 ng of 5'-nucleotidase. The final volume was 3.0 ml. The continuous spectrophotometric assay was used (assay 1). Each reaction was started by adding AMP and was run at 30°C. The lines show the least-squares fit of the data. When the experiment with ATP was repeated with [^{32}P]AMP and AMP concentrations of 10, 20 and 50 μM , the three Hill plots each had a slope of 0.66.

pK_3 (Yount, 1975); substitution on the α -phosphate may have a similar effect. If the active species of the inhibitor is the dianion, the lower pK_3 of these analogues may partly explain their weaker inhibitory effects. ADP[β S] inhibits alkaline phosphatase from *Escherichia coli* (Goody & Eckstein, 1971). ϵ ADP is about as strong an inhibitor as ADP[α S](B).

Inhibition by ATP analogues

AOPCPOP, AOPOPCP and ACPOPOP show K_i values of 0.066, 0.54 and 1.3 μM , respectively. Note that the compound most closely related to AOPCP is the most powerful inhibitor among the phosphonate analogues of ATP. The K_i for AOPCPOP is 11 times greater than that for AOPCP, whereas the K_i for ATP is about 20 times greater than the K_i for ADP. The K_i for ACPOPOP is almost identical with the K_i for ATP, a situation that parallels the K_i values for ACPOP and ADP.

Other inhibitors

PP_i and PCP are competitive inhibitors in the concentration ranges tested, with K_i values of 200 and 90 μM respectively. The greater inhibitory power of AOPCP as compared with ADP is

reflected in an attenuated manner by PCP as compared with PP_i . The longer bond lengths of the P—C bond as compared with the P—O—P bond angle (Yount, 1975) may play a role in the tighter binding of the methylenediphosphonate analogues. PPP_i shows competitive inhibition within the concentration range tested (30–150 μM), with a K_i of 110 μM . Whereas nucleoside diphosphates are better inhibitors than nucleoside triphosphates, this tendency is reversed with the corresponding inorganic polyphosphates.

The K_i for P_i of 3.0 mM is in the physiological range of P_i concentrations. The K_i for adenosine of 0.65 mM is much greater than the physiological range of adenosine concentrations. The K_i for thio P_i is 0.42 mM, which is considerably lower than K_i for P_i of 3.0 mM, a relationship that is echoed by the K_m values for AMP[S] and AMP of 2.3 and 4.7 μM respectively (Table 1). 5'-Nucleotidase from rat heart is not inhibited by 10 mM-NaF.

Effect of Mg^{2+} on ADP and ATP inhibition

Inhibition of 5'-nucleotidase by ADP and ATP in the presence and in the absence of Mg^{2+} ions is shown in Fig. 2. The abscissas show unchelated nucleotide concentrations. For example, in Fig. 2(a) a $\bullet$ symbol at 0.15 μM free ADP shows the same activity as a $\circ$ symbol at 0.16 μM free ADP in which the reaction mixture also contained 0.04 μM -MgADP and a $\triangle$ symbol at 0.14 μM free ADP in which the reaction mixture also contained 1.86 μM -MgADP. Similarly, in Fig. 2(b), a $\bullet$ symbol at 6 μM free ATP shows the same activity as a $\circ$ symbol at 5.6 μM free ATP in which the reaction mixture also contained 4.4 μM -MgATP and a $\triangle$ symbol at 6.1 μM free ATP in which the reaction mixture also contained 93.9 μM -MgATP. In other words, the observed inhibitions corresponded closely to the concentrations of free nucleotides and not to the concentrations of the Mg^{2+} chelates of the nucleotides. This is evidence that the free nucleotides are inhibitory, whereas their Mg^{2+} chelates are not. The highest concentrations of MgADP and MgATP tested in the results shown in Fig. 2 were almost 2 and 100 μM respectively. Higher concentrations of these chelates were tested at constant free [ADP] and [ATP] (Fig. 3). No significant additional inhibition is observed until 15 μM -MgADP is reached, but 4.5% additional inhibition occurs by the time 30 μM -MgADP is reached. Similarly, no significant additional inhibition is observed until 1 mM-MgATP is reached, but 8% additional inhibition occurs by the time 2 mM-MgATP is reached.

The small inhibition observed as $[MgCl_2]$ is raised in the absence of ADP and ATP (Fig. 3) is consistent with the hypothesis that the substrate is free AMP, that MgAMP is not a substrate, and

Fig. 2. Inhibition of 5'-nucleotidase as a function of the concentrations of free ADP and of free ATP

Reaction mixtures contained 10 μM -AMP, 50 mM-triethanolamine/HCl buffer, pH 7.5, 1.67 mM-cholesterol, and $MgCl_2$ and ADP or ATP as indicated. The reactions were started by adding 4.1 and 2.1 ng of 5'-nucleotidase/ml in the experiments with ADP and ATP respectively. (a) $\bullet$, ADP without $MgCl_2$; $\circ$ and $\triangle$, 0.2 μM - and 2 μM -ADP respectively, and $MgCl_2$ to yield the concentrations of free ADP shown in the abscissa. The latter were calculated by using the stability constant of 3330M^{-1} for MgADP (Phillips *et al.*, 1966). The continuous spectrophotometric assay (assay 1) was used with 4 μg of adenosine deaminase. (b) $\bullet$, ATP without $MgCl_2$; $\circ$ and $\triangle$, 10 μM - and 100 μM -ATP respectively, and $MgCl_2$ to yield the concentrations of free ATP shown in the abscissa. The latter were calculated by using the stability constant of 50000M^{-1} (Phillips *et al.*, 1966). The ATP preparation contained 0.024% ADP. The radioactivity assay was used (assay 3), with $[^{32}\text{P}]\text{AMP}$ (2.5 Ci/mol).

that the stability constant for MgAMP is 100M^{-1} (Taqui Khan & Martell, 1967). The excess $MgCl_2$ added to maintain 2 μM free ADP accounts for all of the extra inhibition observed as $[MgADP]$ is raised (Fig. 3, upper panel). This is not the case

Fig. 3. Effects of $MgCl_2$, $MgADP$ and $MgATP$ on 5'-nucleotidase

Assay 3 was used with $100\mu M$ - $[^{32}P]AMP$ (0.65 Ci/mol). All assay mixtures for $MgADP$ contained $2\mu M$ free ADP. For example, $3mM$ - $MgCl_2$ was used with $22\mu M$ total ADP. All assay mixtures for $MgATP$ contained $100\mu M$ free ATP. For example, $480\mu M$ - $MgCl_2$ was used with $500\mu M$ total ATP. The ATP preparation contained 0.024% ADP. The controls (100%) lacked ADP, ATP and $MgCl_2$. The enzyme had a specific activity of 247 units/mg.

with the extra inhibition observed as $[MgATP]$ is raised, because here free $[Mg^{2+}]$ is small compared with $[MgATP]$ (Fig. 3, lower panel). These results imply that $MgATP$ is an inhibitor of 5'-nucleotidase, albeit a very weak one. The difference between $MgADP$ and $MgATP$ is probably due to the difference in charges, the former being chiefly $MgADP^-$ and the latter chiefly $MgATP^{2-}$ at pH 7.5. If nucleoside polyphosphates inhibit by binding to a metal in the enzyme, the two negative charges of $MgATP^{2-}$ would contribute to a tighter binding than would the single negative charge of $MgADP^-$. Edwards *et al.* (1982) reported that plasma-membrane 5'-nucleotidase of B-lymphocytes is activated 2-fold by $10mM$ - $MgCl_2$.

Effect of pH on K_i , K_m and V_{max} .

The effect of pH on K_i values for ADP and AOPCP was studied by using the method of Dixon & Webb (1964). Maximum inhibition by ADP is reached at about pH 6, where the K_i is 21 nM (Fig. 4a). The pK_i decreases at higher pH values and the

Fig. 4. Effect of pH on pK_m values for AMP and pK_i values for ADP and AOPCP of 5'-nucleotidase

The continuous spectrophotometric assay was used. Reactions were run in the presence of $1.67mM$ -cholate and the following $50mM$ buffers: ○ and ●, Tris/HCl; △ and ▲, triethanolamine/HCl; and □ and ■, Mes/Tris. At pH 6.03, Mes/Tris buffer contained 0.001% Triton X-100 instead of sodium cholate. The pH values were measured at the end of the reactions. In panel (a), closed symbols show pK_i and open symbols pK_m . Values for K_m , V_{max} , and K_i were determined from double reciprocal plots. The curves and the pK values were calculated and drawn according to the equation $y = c/(1 + [H^+]/K_a + K_b/[H^+])$, using the computer program of Cleland (1979). The calculated pK values are shown by the symbol × with horizontal error bars showing the standard error.

plot of pK_i versus pH shows one change in slope, corresponding to a pK of 6.51 ± 0.13 at $30^\circ C$, and attains a constant slope, of -1 at $pH > 7.5$. The pK_3 of ADP is 6.4 – 6.6 at $25^\circ C$ (Weitzel & Spehr,

1958; Taqui Khan & Martell, 1967). Our results indicate that ADP^{2-} binds to 5'-nucleotidase more strongly than does ADP^{3-} .

Maximum inhibition of the enzyme by AOPCP is reached at about pH 7.2 ($[\text{H}^+] = 63 \text{ nM}$), where the K_i is 4.9 nM (Fig. 4a). The plot of $\text{p}K_i$ versus pH attains a constant slope of -1 at pH above 8.3 and shows two changes in slope corresponding to a $\text{p}K_a$ of 6.75 ± 0.15 and a $\text{p}K_b$ of 7.71 ± 0.08 . Since AOPCP does not have a $\text{p}K$ between 6.0 and 7.5, it seems likely that the $\text{p}K_a$ of 6.75 reflects a functional group of the enzyme, possibly an imidazole of histidine. The $\text{p}K_3$ of AOPCP is 8.3 at 25°C (Schliselfeld *et al.*, 1982; Vogel & Bridger, 1982). [Myers *et al.* (1965) reported that the secondary ionization of AOPCP has a $\text{p}K$ of 8.15.] Our results suggest that AOPCP^{2-} also binds to 5'-nucleotidase more strongly than does AOPCP^{3-} . In the region where the slope of $\text{p}K_i$ versus pH is -1 for both inhibitors, the difference between the two lines is $1.9 \text{ p}K_i$ units; in other words, the K_i for AOPCP is 79.4 times smaller than the K_i for ADP. The difference in $\text{p}K_3$ for AOPCP and ADP is $8.3 - (6.4 \text{ to } 6.6) = 1.9 \text{ to } 1.7$. Thus the observed difference in $\text{p}K_i$ can be accounted for by the difference in $\text{p}K_3$ for ADP and AOPCP and the most powerful inhibitory species are the dianions of ADP and AOPCP.

The plot of $\text{p}K_m$ versus pH (Fig. 4a) shows a constant K_m value between pH 7.4 and 8.1, a positive slope below pH 7.4, and a negative slope above pH 8.1. Computer analyses of the data in accordance with Cleland (1979) yield a $\text{p}K_a$ of 6.58 ± 0.03 and $\text{p}K_b$ of 8.93 ± 0.03 . The secondary ionization of AMP has a $\text{p}K$ of 6.2–6.4 at 25°C (Tucci *et al.*, 1961; Taqui Khan & Martell, 1967). The dependence of V_{max}/K_m upon pH is shown in Fig. 4(b). The $\text{p}K_a$ value derived from this curve is 6.78 ± 0.03 , which is close to the $\text{p}K_2$ of AMP and the $\text{p}K$ of a 'normal' imidazole group of histidine. The $\text{p}K_b$ value derived from this curve is 8.74 ± 0.03 , which is reasonably close to the $\text{p}K_b$ value of 8.93 obtained from the plot of $\text{p}K_m$ versus pH; it presumably corresponds to the ionization of an active group on the free enzyme. Dornand *et al.* (1978) have reported that thiol-complexing reagents have no effect on 5'-nucleotidase from plasma membrane of pig lymphocytes, suggesting that a thiol-group is not responsible for $\text{p}K_b$. Other possible ionizing groups in this pH region are α -amino groups, the ϵ -amino group of lysine and water co-ordinated to a metal (Krishnaswamy & Kenkare, 1970).

$\log V_{\text{max}}$ is relatively constant over the pH range employed (Fig. 4c). A $\text{p}K_a$ value of 5.71 ± 0.04 and a $\text{p}K_b$ value of 9.97 ± 0.04 are calculated for what is presumably the enzyme-substrate complex; the lower of these values could be due to the ionization

of an imidazole group of histidine, the upper value to water co-ordinated to a metal or an ϵ -amino group of lysine. These $\text{p}K$ values are extrapolated and probably not very accurate.

ADP and AOPCP are greatly superior as inhibitors to PP_i and PCP. If 5'-nucleotidase contains a bivalent metal at its active site, as do alkaline phosphatases (Plocke *et al.*, 1962; Fernley, 1971; Cathala *et al.*, 1975), then ADP and AOPCP may inhibit by virtue of being active-site-directed chelating agents.

In their original publication, Myers *et al.* (1963) stated that they had synthesized AOPOPCP because of an anticipated difference in its $\text{p}K_4$ compared with that of ATP, and that they expected this property to be of interest in kinetic studies conducted over a pH range, in the event that AOPOPCP is a substitute for or an inhibitor of ATP in a given enzyme system. This expectation has been met in striking fashion for AOPCP in the present investigation.

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