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Kinetics and the Mechanism of Action of Adenosine Aminohydrolase†

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ABSTRACT: The kinetics of calf duodenum adenosine aminohydrolase were investigated. Inosine was found to be a competitive inhibitor and ammonia a noncompetitive inhibitor. These results are consistent with an ordered release of products with ammonia being the first. Comparison of certain kinetic constants to K_{eq} suggest that the minimal mechanism is ordered uni-bi in which the isomerization of an enzyme–product complex plays a significant role. Methanol gave complex inhibition kinetics, which could be interpreted in terms of mixed dead-end and alternate product inhibition and was consistent with a crypto Ping-Pong mechanism in which the hydrolytic step occurs after the rate-limiting release of ammonia. A single cationic group with a pK_a of 4.8 was found to control the rate-limiting step. Two groups with pK_a of 5.7 and 8.7 were shown to be essential for binding of the substrate, with the latter being equated with a thiol residue on the basis of inactivation by *p*-chloromercuribenzoate. These data are consistent with a previously proposed mechanism in which a tetrahedral thiopurinyl-enzyme compound is a compulsory intermediate.

Adenosine aminohydrolase has a widespread distribution in animal tissues (Conway and Cooke, 1939; Makarewicz and Zydowo, 1962) and in all species the duodenum and spleen are the richest sources (Brady and O'Donovan, 1965). A number of these enzymes have been purified, including those from calf duodenum (Brady and O'Connell, 1960; Chilson and Fisher 1963) and chicken duodenum (Hoagland and Fisher, 1967).

Although much work has been done on the specificity of the calf duodenum enzyme, especially by Schaeffer and his coworkers (Schaeffer *et al.*, 1964; Schaeffer and Bhargava, 1965; Schaeffer and Vogel, 1965), little is known about the mechanism of action. In common with most other aminohydrolases this enzyme appears to require a thiol residue for full activity (Ronca *et al.*, 1967; Pfrogner, 1967) and although fairly specific for the purine ribonucleoside moiety (Frederiksen, 1966) it is relatively nonspecific in regard to the substituent on the C_6 of the purine ring (Chassy and Suhadolnik, 1967; Baer and Drummond, 1966). In 1969 Bolen and Fisher proposed that the chicken duodenal enzyme operated by an ordered sequential mechanism. They based this conclusion on the irreversibility of the reaction and the observation that log K_m/V_m was a linear function of the surface tension in a wide variety of solvents. In contrast to this Wolfenden (1966), using the nonspecific adenosine aminohydrolase from *Aspergillus oryzae*, observed a constant maximum velocity for a series of C_6-substituted purine ribonucleosides and proposed a Ping-Pong or substitution mechanism in which a purinyl ribonucleoside–enzyme compound is formed as a compulsory intermediate. It was considered that a kinetic approach through product and alternate product inhibition (Cleland, 1963a) and evaluation of the kinetic constants (Hsu *et al.*, 1966) might resolve certain aspects concerning the mechanism of adenosine aminohydrolase.

Materials

Adenosine, 2′-deoxyadenosine, inosine, 6-methylaminopurine ribonucleoside, 6-methoxypurine ribonucleoside, 6-hydrazinopurine ribonucleoside, 6-chloropurine ribonucleoside, and *p*-chloromercuribenzoate were obtained from Sigma Chemical Co. (St. Louis, Mo.). Diisopropyl fluorophosphate

† From the Department of Biochemistry, Trinity College, Dublin 2, Ireland. *Received March 23, 1972.* Supported by the Irish Cancer Society.

was a product of Calbiochem (Los Angeles, Calif.). Deuterium oxide (99.8 atom %) was purchased from ICN (Irvine, Calif.). Alcohols, dioxane, and other reagents were purchased from B. D. H. Chemicals Ltd. (Poole, Dorset, England), and were of Analar grade wherever possible.

Adenosine aminohydrolase was obtained commercially from Sigma Chemical Co. (types I and II) and also prepared by the method of Brady and O'Connell (1962). All preparations were devoid of ribonucleosidase activity and all showed essentially the same kinetic properties.

Methods

Kinetic Measurements. Two methods were used to measure the initial rates of deamination. The first was based on the spectral change on conversion of adenosine to inosine (v_{in}) and involved measuring the decrease in optical density at 265 nm with a Unicam SP-800 recording spectrophotometer, fitted with a thermostatted cell holder and coupled to an accessory recorder *via* the SP-850 scale-expansion unit. The incubation mixture (3 ml), containing 150 μmoles of buffer at the appropriate pH, substrate, and any inhibitors were preincubated in the cell holder for 5 min prior to the addition of the enzyme. The nature and pH of the buffer and concentration of substrate and inhibitors varied considerably and these are indicated in the appropriate places. However, for standard assays a potassium phosphate buffer (pH 6.8) and an adenosine concentration of 0.1 mM was used at 37°. For the determination of the kinetic constants of substrates other than adenosine or 2′-deoxyadenosine the wavelengths used were those reported by Baer and Drummond (1966). For the ammonia inhibition experiments high concentrations of NH_4Cl were used and the reactions were run in covered cells. Parallel experiments in NaCl were also run and correction applied for ionic strength effects.

The second method of assay was based on the determination of ammonia by the colorimetric method of Fawcett and Scott (1960). The adaptation of this procedure for the discontinuous method of initial rate measurement of ammonia production (v_{NH_3}) has been described by Wisdom and Orsi (1969).

Inactivation by p-Chloromercuribenzoate. The rate of inactivation of the enzyme by *p*-chloromercuribenzoate was measured by a method similar to that used by Holloway *et al.* (1964). One milliliter of reaction mixture containing 150 μg of type II enzyme, 0.1 μmole of *p*-chloromercuribenzoate, and 50 μmoles of glycine–HCl buffer at the appropriate hydrogen ion concentration (Figure 5) was incubated at 37°. At zero time and various time intervals samples (100 μl) were removed and assayed immediately for remaining activity by the standard procedure. Control experiments without *p*-chloromercuribenzoate showed that nonspecific pH inactivation did not contribute significantly to the loss of activity.

Data Processing. Using the method of least squares and assuming equal variance for the velocities (Wilkinson, 1961), the kinetic data were fitted to eq 1, where V is the maximum velocity, K_a the Michaelis constant, and (A) the substrate concentration. All calculations were performed on an IBM

$$v = \frac{V(A)}{K_a + (A)} \quad (1)$$

360/44 digital computer with program HYPER of Cleland (1967). This program provides values for K_a, V, K_a/V, and $1/V$, the standard errors of their estimates and weighting factors (reciprocals of the squares of the standard errors) for further analysis. In the inhibition experiments the slopes (K_a'/V') and intercepts ($1/V'$) were plotted graphically against the inhibitor concentration to determine the type of inhibition. In two instances the slopes were parabolic functions of the inhibitor concentration and were fitted to eq 2.

$$\text{slope} = \frac{K_a}{V}(1 + (I)/K_{i_1} + (I)^2/K_{i_2}) \quad (2)$$

In one experiment the intercepts were a hyperbolic function of the inhibitor concentration and were fitted to eq 3.

$$\text{intercept} = \frac{1}{V}\frac{(1 + (I)/K_{inum})}{(1 + (I)/K_{iden})} \quad (3)$$

In all other cases the slopes and intercepts were linear functions of the inhibitor concentration and they were fitted to eq 4a and 4b.

$$\text{slope} = \frac{K_a}{V}(1 + (I)/K_{is}) \quad (4a)$$

$$\text{intercept} = \frac{1}{V}(1 + (I)/K_{ii}) \quad (4b)$$

TABLE I: Substrates of Adenosine Aminohydrolase.[a]

Substrate	K_m (μM)	V_m
Adenosine	35	100
2′-Deoxyadenosine	50	94
6-Hydrazinopurine ribonucleoside	94	24
6-Chloropurine ribonucleoside	130	11
6-Methoxypurine ribonucleoside	210	0.6
6-Methylaminopurine ribonucleoside	95	0.3

[a] Values for K_m and V_m were determined in phosphate buffer (pH 6.8) at 37°. The V_m values are all recorded as a percentage of that for adenosine.

Results

Substrate Specificity. The wide specificity, for the substituent on the C_6 position of the purine ring, was confirmed (Table I). The kinetic constants agree well with those previously obtained (Chassy and Suhadolnik, 1967; Baer and Drummond, 1966) except for the K_m for chloropurine ribonucleoside which was smaller by a factor of five. The most noticeable feature of these results is the 330-fold range in V_m but only 6-fold range in K_m. Chassy and Suhadolnik (1967), who also observed this large range in V_m, reported that the 6-hydroxylamino-, 6-bromo-, and 6-iodopurine ribonucleosides were also substrates. Although the specificity of the calf duodenal enzyme is similar to that of the enzyme from *A. oryzae*, the two enzymes differ in that the latter shows a relatively constant V_m for a similar range of C_6-substituted substrates (Wolfenden, 1966).

Product Inhibition. Inosine was found to be an effective inhibitor only in the neutral form producing linear competitive inhibition (Figure 1). The K_{is} value of 2.2 mM was very similar to the value obtained with 2′-deoxyadenosine as the variable

TABLE II: Inhibition Constants for Adenosine Aminohydrolase.[a]

Substrate	Inhibitor	Rate	Type of Inhibn	Inhibition Constants (M)						
				K_{is}	Slope K_{il}	K_{i2}	Intercept K_{ii}	K_{inum}	K_{iden}	Other Constants
Adenosine	NH_3	v_{in}	Linear NC	$K_aK_{ip}/K_{ia} = 1.8^b$			$K_{ip} = 2.3$			$K_{ia} = 1.47 \times 10^{-4c}$
Adenosine	Inosine	v_{NH_3}	Linear C	$K_{iq} = 2.14 \times 10^{-3}$						
2′-dAdo	Inosine	v_{NH_3}	Linear C	$K_{iq} = 2.2 \times 10^{-3}$						
Adenosine	Methanol	v_{in}	*S*-parabolic *I*-linear NC		$\frac{K_eK_{ir}}{K_{ie}} = 2.1$	$\frac{K_eK_{ir}K_{IR}}{K_{ie}} = 0.94$	$K_{irr} = 1.67$			$K_{IR} = 0.45^d$
Adenosine	Methanol	v_{NH_3}	*S*-linear *I*-hyperbolic NC	$K_{IR} = 0.35^d$				$K_{irr} = 1.48$	$\frac{K_eK_{ir}}{K_{ie}} = 2.5$	
Adenosine	Ethanol	v_{in}	*S*-parabolic *I*-linear NC		$\frac{K_bK_{it}}{K_{ib}} = 1.23$	$\frac{K_bK_{it}K_{IT}}{K_{ib}} = 0.65$	$K_{itt} = 2.84$			$K_{IT} = 0.53^d$
Adenosine	*i*-PrOH	v_{in}	Linear C	$K_I = 0.31^d$						
Adenosine	1-Propanol	v_{in}	Linear C	$K_I = 0.44^d$						

[a] Summary of results of product and alcohol inhibition experiments. Data taken from Figures 1 to 3. [b] Michaelis constants and inhibition constants of reactants: A, adenosine; P, ammonia; Q, inosine; R, methanol; C, 6-methoxypurine ribonucleoside; T, ethanol; B, 6-ethoxypurine ribonucleoside. [c] Calculated using a value of 1.15×10^{-4} M for K_a at pH 9.2. [d] These represent inhibitions for dead-end inhibitors.

TABLE III: Effect of the Solvent on Ionization of the Group Determining the Rate-Limiting Step.[a]

Solvent	pK_a	
	Cationic	Neutral Acid
Water	4.8	5.0
Sucrose (M)	4.8	5.0
KCl (1.5 M)	4.9	5.6
Dioxane (3.75 M)	4.8	4.5

[a] The variation of V_m with pH was determined as described in Figure 4. The reactions were done at 37° at an ionic strength of 0.033. The buffers used were: (a) neutral acid–sodium citrate (pH 4.0–6.4), and (b) cationic–hydroxylamine (pH 4.5–6.5), Data were fitted to eq 5.

substrate (Table II). This result agrees with those obtained by Wolfenden (1969) for both the fungal and calf duodenum enzymes, except for the K_{is} value for the latter enzyme which was larger by a factor of ten.

Inhibition by ammonia was also pH dependent in that only the base form was effective. Although it was a very poor inhibitor, it produced linear noncompetitive inhibition (Figure 2) with inhibition constants of 1.8 and 2.3 M for slope and intercept effects, respectively. There were indications of substrate inhibition at high concentrations of ammonia. Although Wolfenden (1969) found the fungal enzyme to be inhibited noncompetitively by ammonia he was unable to show any inhibition of the calf enzyme even by high concentrations at pH 9.2.

Inhibition by Alcohols. Both 2-propanol and 1-propanol were rather ineffective linear competitive inhibitors. In contrast methanol, which was also a poor inhibitor, gave complex inhibition kinetics. When the reaction rate was measured by inosine formation the slope replot was a parabola whereas the intercept replot was linear (Figure 3). This inhibition may be described as *S*-parabolic *I*-linear noncompetitive (Cleland, 1963a). However, when ammonia production was used as a measure of the reaction rate the slope replot was linear and the intercept replot a hyperbola (Figure 3) (*S*-linear *I*-hyperbolic noncompetitive inhibition). Ethanol as an inhibitor behaved like methanol rather than either of the two propanols.

The kinetic constants obtained from these inhibition experiments are summarized in Table II. The meaning that may be attributed to these constants will be considered in the discussion.

Effect of D_2O. Substitution of 95% D_2O in place of water had no effect on the values of K_m and V_m for adenosine. Although Wolfenden (1969) showed that the fungal enzyme behaved in a similar manner he did detect a 30–40% decrease in both V_m and K_m for the calf duodenal enzyme in the presence of D_2O.

Effect of pH. The effect of pH on the two kinetic constants V_m and V_m/K_m is shown in Figure 4. For the binding of the substrate (log V_m/K_m plot) two groups appear to be essential. One, with a pK_a of 5.7 and required in the deprotonated form, is possibly a histidine residue. The second, with a pK_a of 8.7 required in the protonated form, is probably a cysteine residue. Chilson and Fisher (1963), working with the calf enzyme, demonstrated the presence of a group with a pK_a

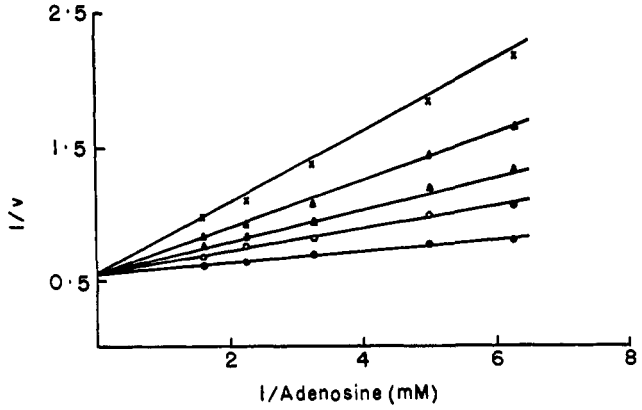


FIGURE 1: Inosine inhibition of adenosine aminohydrolase. The initial velocities (v_{NH_3} = μmoles of NH_3/min per mg of protein) were measured at 37° in potassium phosphate buffer (pH 7.5). The concentrations of inosine were: 0 (●), 2.7 mM (○), 5.4 mM (▲), 8.0 mM (△), and 13.3 mM (×).

of about 5.5, and in the same work the chicken duodenal enzyme was shown to contain a group with a pK_a of 8.8.

The rate-limiting step (log V_m plot) appears to be governed by a single ionizing group with a pK_a of 4.8. The nature of this group is unknown but the shift in the pK_a (Table III) with solvent changes in two different buffer series (Findlay *et al.*, 1962) suggest it is cationic, possibly a histidine residue, and required in the deprotonated form.

Inhibition by p-Chloromercuribenzoate. The rate of inactivation of adenosine aminohydrolase by *p*-chloromercuribenzoate was found to follow first-order kinetics. The apparent first-order rate constant (k_1) was shown to vary with the hydrogen ion concentration and the data obtained were fitted to eq 5 where K_a is the dissociation constant of the thiol residue in the enzyme and k_1^0 is the apparent first-order rate constant for the reaction of the thiol anion.

$$1/k_1 = \frac{(H^+)}{k_1^0 K_a} + 1/k_1^0 \qquad (5)$$

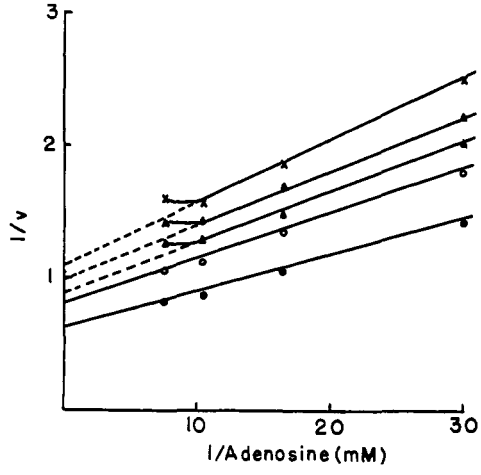


FIGURE 2: Ammonia inhibition of adenosine aminohydrolase. The initial velocities (v_{in} = μmoles of inosine/min per mg of protein) were measured at 25° in glycine–HCl buffer (pH 9.2). The concentrations of ammonia were: 0 (●), 0.5 M (○), 0.75 M (▲), 1.0 M (△), and 1.5 M (×).

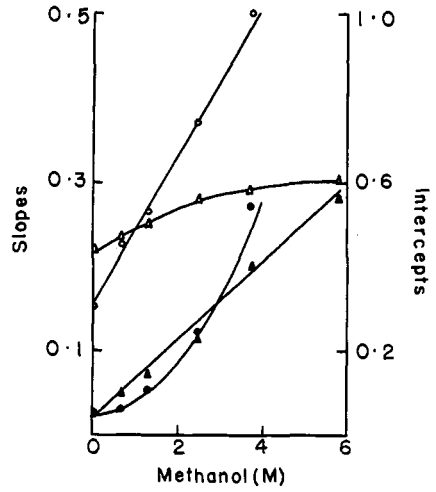


FIGURE 3: The variation of slopes (●, ▲) and intercepts (○, △) from double-reciprocal plots as a function of the methanol concentration. Reaction rate measured by ammonia formation (▲, △); reaction rate measured by inosine formation (●, ○). Reactions were carried out with adenosine (0.03–0.6 mM) in Tris-HCl buffer (pH 7.5) at 37°. Maximum velocities (1/intercept) are recorded as μmoles/min per mg of protein. Michaelis constants (slope/intercept) are recorded in mM. In the case of the intercepts from the ammonia determinations (△) the maximum velocities have been multiplied by a scale factor of 1.5.

The results (Figure 5) showed the expected linear relationship, and calculation of the pK_a from the slope and intercept gives a value of 8.6. This agrees well with the value of 8.7 obtained from kinetic studies (Figure 4). Methanol, a product, had no protective effect. In contrast the substrate 6-methylaminopurine ribonucleoside gave considerable protection and appeared to raise to pK_a of the reactive thiol to 9.9 (Figure 5).

These results fully substantiate previous work on this enzyme (Ronca *et al.*, 1967; Pfrogher, 1967), that, in common with most aminohydrolases, a thiol residue is essential for activity.

Effect of Diisopropyl Fluorophosphate. No inactivation of adenosine aminohydrolase could be detected even on prolonged incubation (2 hr at 37°) with high concentrations (2 mM) of diisopropyl fluorophosphate. This result contrasts with that obtained by Baer and Drummond (1966) and suggests that an activated serine hydroxyl is not involved in the reaction.

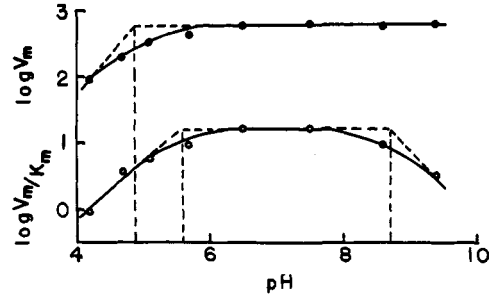


FIGURE 4: pH dependence of the kinetic constants of adenosine aminohydrolase V_m (●) (ΔOD 265 nm/9 min) and V_m/K_m (○) (K_m in mM) The kinetic constants were obtained at 37° using buffers of 0.05 M. The buffers used were: acetate (pH 4.2–5.7), phosphate (pH 6.5–7.5), Tris (pH 8.7), and glycine (pH 9.4). For V_m, the data were fitted to eq 5; for V_m/K_m the data were fitted to an equation of the type $1/k = 1/k_0(1 + (H^+)/K_{a_1} + K_{a_2}/(H^+))$.

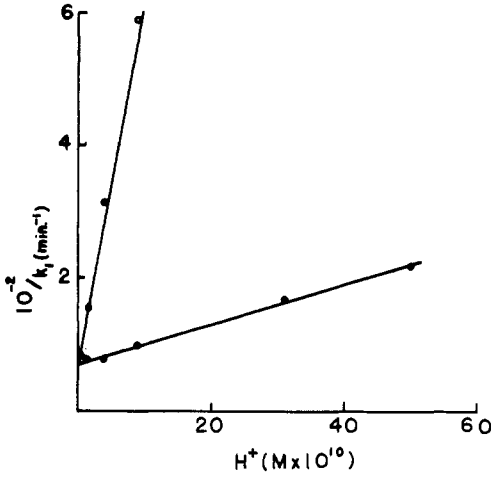


FIGURE 5: The variation of the apparent first-order rate constant for inactivation of adenosine aminohydrolase by *p*-chloromercuribenzoate. The data were obtained in the absence (●) and presence (○) of 0.5 mM 6-methylaminopurine ribonucleoside.

Discussion

The linearity of the reciprocal plots show that the enzyme follows the simple rate law described by eq 1. The product inhibition patterns of one linear noncompetitive (ammonia) and one linear competitive (inosine) suggest an ordered uni-bi (Cleland, 1963b) as the simplest kinetic mechanism (Scheme I).

In this scheme A is adenosine, P is ammonia, and Q is inosine. The reciprocal rate equation (eq 6) for this mechanism in the presence of either (but not both) product is

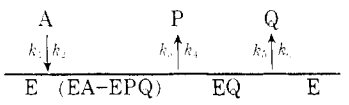
$$1/v = \frac{1}{(A)}\frac{K_a}{V}\left(1 + \frac{K_{ia}(P)}{K_{ip}K_a} + \frac{(Q)}{K_{iq}}\right) + \frac{1}{V}\left(1 + \frac{(P)}{K_{ip}}\right) \quad (6)$$

where K_{ia} ($=k_2/k_1$) is the dissociation constant of A, K_{ip} [$= (k_3 + k_5)/k_4$], and K_{iq} ($=k_5/k_6$) are product inhibition constants for P and Q, respectively.

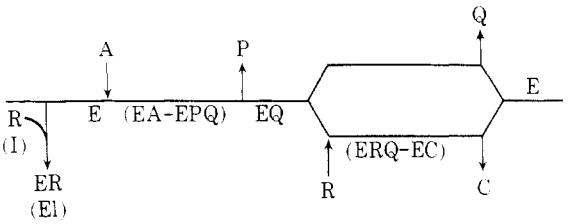
Possible alternative mechanisms occur where there is a random release of products. This would result in two noncompetitive inhibition patterns in which both slopes and intercepts would be nonlinear functions of the inhibitor concentrations. However, there are two random mechanisms that would give linear secondary plots. Firstly if the release of one product is much faster than the other then one product gives noncompetitive and the other competitive inhibition. Secondly if the rate-limiting step is the conversion of EA ↔ EPQ (rapid equilibrium random) linear replots are obtained, but here both products give competitive inhibition. However, the formation of a dead-end complex by either product would change these patterns.

In view of the fact that 6-methoxypurine ribonucleoside is a substrate then the effects of methanol as an inhibitor can readily be interpreted in terms of alternate product inhibition (Scheme II), where R is methanol, C is 6-methoxypurine ribonucleoside, and I is either 1-propanol or isopropyl alcohol (see later).

SCHEME I



SCHEME II



If methanol was acting purely as an alternate first product then it would be a linear noncompetitive inhibitor when v_{in} (release of inosine) was measured and a hyperbolic uncompetitive inhibitor when v_{NH_3} (release of ammonia) was measured. The results actually obtained, *S*-parabolic *I*-linear noncompetitive for v_{in} and *S*-linear *I*-hyperbolic noncompetitive for v_{NH_3}, suggest that methanol can also act as a dead-end inhibitor for the free enzyme in addition to its role as an alternate product. This would not be an unexpected phenomenon. The reciprocal rate equations for methanol inhibition in Scheme II measuring either v_{in} or v_{NH_3} are given in eq 7 and 8.

$$1/v_{in} = \frac{1}{(A)}\frac{K_a}{V}\left(1 + \frac{K_{ic}(R)}{K_cK_{ir}} + \frac{K_{ic}(R)^2}{K_cK_{ir}K_{IR}}\right) + \frac{1}{V}\left(1 + \frac{(R)}{K_{irr}}\right) \quad (7)$$

$$1/v_{NH_3} = \frac{1}{(A)}\frac{K_a}{V}\left(1 + \frac{(R)}{K_{IR}}\right) + \frac{1}{V}\left(\frac{1 + (R)/K_{irr}}{1 + \dfrac{K_{ic}(R)}{K_cK_{ir}}}\right) \quad (8)$$

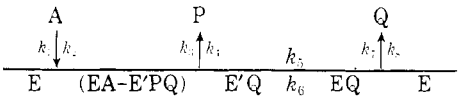
In these equations K_{irr} is a new kinetic constant, K_c and K_{ic} are the Michaelis and inhibition constants for 6-methoxypurine ribonucleoside, K_{ir} is the product inhibition constant and K_{IR} is the dead-end inhibition constant for methanol. The important thing about these equations is that the same constants or collection of constants appear in both. Consideration of Table II shows reasonably good agreement between the determinations of these constants in the two systems thus giving considerable weight to our interpretation.

The fact that ethanol behaves like methanol suggests that 6-ethoxypurine ribonucleoside would also be a substrate although this has not yet been tried. Both 1-propanol and isopropyl alcohol behaved as simple linear competitive inhibitors with inhibition constants very similar to the dead-end inhibition constants of both methanol and ethanol (Table II).

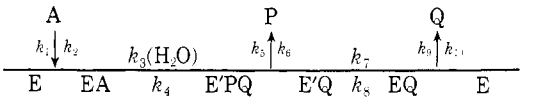
These results also confirm that the order of release is first ammonia and then inosine. If ammonia was the last product methanol, now an alternative second product, would give linear competitive inhibition in either system.

If ammonia behaves like methanol in forming a dead-end complex with free enzyme in addition to its effect as a product inhibitor then one might expect ammonia to give *S*-parabolic *I*-linear noncompetitive inhibition in place of the usual linear noncompetitive. No evidence was obtained that the slope variation with ammonia concentration was other than linear (Figure 2). The parabolic nature of the slope replot would however, be difficult to detect if the slope inhibition constant for normal product inhibition K_aK_{ip}/K_{ia} was considerably less than the inhibition constant for dead-end inhibition (K_{IP}). As this is quite the opposite of what is actually observed with

SCHEME III



SCHEME V



methanol ($K_cK_{ir}/K_{ic}:K_{IR} \simeq 6$) it seems unlikely that ammonia forms an additional dead-end complex. This point is important as the formation of such a complex would render invalid the estimation of K_{ia} from the ratio of the slope and intercept inhibition constants of ammonia.

The equation for the simple ordered uni-bi mechanism is changed if isomerization of free enzyme occurs but in this case both products should give linear noncompetitive inhibition. Although isomerization of EA–EPQ or EQ does not change the form of rate equation (eq 6), it is possible to detect the isomerization of EQ.

If the mechanism of Scheme I holds then $K_{eq} = k_1k_3k_5/k_2k_4k_6$ and the K_{eq} and rate constants are related to the kinetic constants as follows

$$K_{eq} \leq \frac{K_{ip}K_{iq}}{K_{ia}} = \frac{k_1(k_3 + k_5)k_5}{k_2k_4k_6}$$

Assuming K_{ia} behaves like K_a as a function of pH then the estimated value of K_{ia} at pH 7.5 is 4×10^{-5} M. Further if K_{ip} is independent of pH then calculation gives $K_{eq} = 1.26 \times 10^2$. In fact the experimentally determined value at pH 7.5 is 2.1×10^3 (Wolfenden, 1967) and this suggests that the simple ordered uni-bi mechanism is inadequate. Postulating an EA–EPQ isomerization step does not solve the problem whereas isomerization of EQ does (Scheme III).

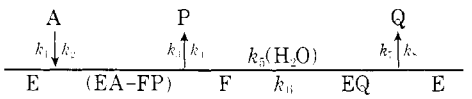
With this mechanism K_{eq} can be equal to, less than, or greater than $K_{ip}K_{iq}/K_{ia}$. Thus the mechanism shown in Scheme III represents the minimal acceptable one. A similar phenomenon has been observed with potato phosphomonoesterase (Hsu *et al.*, 1966) and with a K^+-dependent ATP phosphohydrolase from rat brain (Robinson, 1970).

In the strict sense the kinetics of adenosine aminohydrolase are "apparent" ordered uni-bi with water as a second substrate. The apparent requirement for an isomerization of EQ could be interpreted in terms of a Ping-Pong (substitution) mechanism (Scheme IV).

In this mechanism F is the modified enzyme which in this case would represent a purinyl ribonucleoside–enzyme covalent compound. Unfortunately this mechanism, which might be termed crypto Ping-Pong uni-bi (Findlater *et al.*, 1970) is indistinguishable as it stands from the true ordered uni-bi (Scheme III) or from a crypto sequential uni-bi (Scheme V).

That a crypto Ping-Pong mechanism is operating is rendered more than likely by again considering the results of alternate first product inhibition (or alternate second substrate). Such inhibition as can occur in a crypto sequential-type mechanism is represented in Scheme VI, where R is acting as the alternate second substrate. In this mechanism R would give linear noncompetitive inhibition if the release of Q was measured but if the release of P was measured both slopes and intercepts would be hyperbolic functions of the concentration of R. This result is to be contrasted with the results actually obtained and those predicted on the basis of the mechanism in Scheme II which represents the equivalent situation with a crypto Ping-Pong mechanism. Many similar studies with alternate products (substrates) have been carried out in an attempt to solve the kinetics of hydrolytic enzymes (Hsu *et al.*, 1966; Chung *et al.*, 1970; Ehrman *et al.*, 1971). In particular Greenzaid and Jencks (1971) working with pig liver esterase considered this problem in detail. Analysis of the methanol inhibition by their method shows that adenosine aminohydrolase probably acts by the hydrolysis of a purinyl ribonucleoside–enzyme compound which is formed in the rate-limiting step.

SCHEME IV



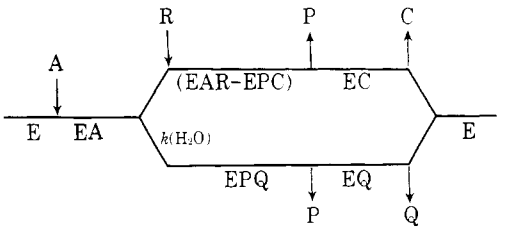
One might also speculate that with a crypto sequential uni-bi mechanism (Scheme V) the product P would give uncompetitive product inhibition even if water was saturating whereas with the crypto Ping-Pong uni-bi (Scheme IV) saturation with water would eliminate all inhibition by P. The fact that with adenosine aminohydrolase P (ammonia) was a very poor inhibitor in terms of both slope and intercept effects suggests that a crypto Ping-Pong mechanism may be operating.

If we consider Scheme IV as a likely mechanism then the limiting maximum velocity is probably governed primarily by the EA $\leftrightarrow$ FP isomerization; this is borne out by the fact that the V for various C_6-substituted purine ribonucleosides differ by a factor of 300 (Table I). However it seems likely that the maximum velocity is not governed by any single step but probably also includes k_3 and to a lesser extent k_5 and k_7.

There is little doubt as to the nature of one of the groups required for activity by adenosine aminohydrolase. The pK_a of 8.7 from kinetic studies agrees well with the value of 8.6 obtained from *p*-chloromercuribenzoate inactivation, indicating that this is indeed a thiol residue. Protection by 6-methylaminopurine ribonucleoside also confirms the importance of this group in the enzyme mechanism.

The nature of the second group ($pK_a = 5.7$) required for binding is unknown but possibly it is a histidine residue. Preliminary results in this laboratory have shown that photooxidation by Methylene Blue at pH 8 results in the loss in activity with an apparent first-order rate constant of 0.035 min^{-1} compared to a value of 0.025 min^{-1} for the loss of histidine residues.

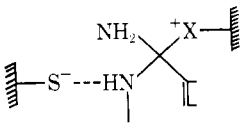
SCHEME VI



In spite of its low pK_a (4.8) the residue controlling the rate-limiting step also appears to be a histidine or rather a cationic type. It is probable that this group is the same as that necessary for binding (pK_a = 5.7). The presence of the substrate probably induces a hydrophobic environment thus causing the shift in the pK_a. It is possible that the pK_a shift accompanying the protection by 6-methylaminopurine ribonucleoside (Figure 5) is due to a similar phenomenon.

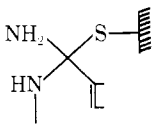
Interest in the molecular mechanism of action centers around the role played by the essential thiol. Wolfenden *et al.* (1966) have produced evidence that the mechanism does not require the formation of a Schiff base. The two main alternatives are: (a) the thiol residue acts as a general acid assisting nucleophilic displacement by some other group resulting in a tetrahedral intermediate (Scheme VII); and (b) the thiol

SCHEME VII



residue acts as a nucleophile to form a tetrahedral intermediate (Scheme VIII).

SCHEME VIII



The requirement for a tetrahedral intermediate seems well established both on mechanistic grounds and by the finding that the possible transition-state analog 1,6-dihydro-6-hydroxymethylpurine ribonucleoside is an extremely effective competitive inhibitor with a K_i of 7.6×10^{-7} M (Evans and Wolfenden, 1970).

The main objection advanced against protonation of the purine ring N_1 (Scheme VII) is that both isoadenosine and 1-methyladenosine are substrates for the enzyme (Wolfenden *et al.*, 1966). However as both of these are very poor substrates, their V being 0.5% or less than that for adenosine (Wolfenden *et al.*, 1969), it is perhaps not a major objection and such a mechanism remains a possibility.

The special effectiveness of thiol anions in aromatic substitutions (Bunnett and Merritt, 1957), and the observation that thiols, such as glutathione, even at pH 7, were 20,000 times as effective as imidazole or phenol in the nucleophilic substitution on 6-chloropurine ribonucleoside (Walsh and Wolfenden, 1967) leads one to conclude that Scheme VIII is the most likely mechanism. Possibly the histidine or cationic group acts as a general base during the formation and hydrolysis of the thiopurinyl ribonucleoside–enzyme compound by interacting with the N_1 of the purine ring.

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