Design of Substrate-Site-Directed Irreversible Inhibitors of Adenosine 5'-Phosphate Aminohydrolase. Effect of Substrate Substituents on Affinity for the Substrate Site

Alexander Hampton,* Takuma Sasaki, Florian Perini, Lewis A. Slotin, and Francis Kappler

The Institute for Cancer Research, The Fox Chase Cancer Center, Philadelphia, Pennsylvania 19111.
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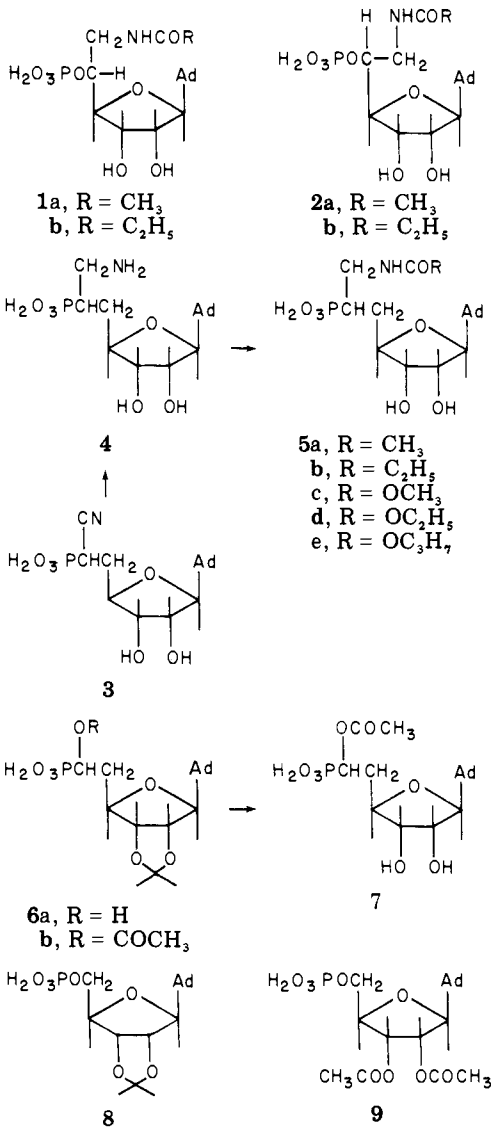
Derivatives of adenosine 5'-phosphate (AMP) have been synthesized in which the phosphoester ($POCH_2$) grouping of AMP is replaced by $PCH(R)CH_2$ where R is OC(O)Me, $CH_2NHCOMe$, $CH_2NHCOEt$, and $CH_2NHCOOR'$ (R' = Me, Et, and Pr). The 2',3'-*O*-isopropylidene and 2',3'-di-*O*-acetyl derivatives of AMP were also prepared. All compounds were competitive inhibitors of rabbit muscle AMP aminohydrolase with enzyme–inhibitor dissociation constants (K_i values) of 330, 20, 17, 19, 16, 14, 260, and 105 μM, respectively. All compounds were substrates except those in which R was $CH_2NHCOEt$ and $CH_2NHCOOR'$ (R' = Me, Et, and Pr). The previously described allo and talo epimers of 5'-*C*-acetylaminomethyl-AMP and the allo epimer of 5'-*C*-propionylaminomethyl-AMP were substrates and competitive inhibitors with K_i values of 18, 47, and 42 μM, respectively. The talo epimer of 5'-*C*-propionylaminomethyl-AMP was not a substrate and was a noncompetitive inhibitor, K_i = 205 μM. 8-Bromo-AMP was a substrate (V_{max} 0.03% that of AMP). The results indicate that affinity for the AMP site of the aminohydrolase is retained when the above substituents (except 5'-*C*-propionylaminomethyl in the talo configuration) are attached to AMP and that it might therefore be possible to design substrate-site-directed irreversible inhibitors for this enzyme by suitable modification of these substituents.

Adenosine 5'-phosphate (AMP) aminohydrolase, which catalyzes replacement of the 6-amino group of AMP by a hydroxyl group, occurs in many tissues and among many species and has been postulated to play important roles in metabolism as a control factor in the interconversions of adenine and guanine nucleotides and in glycolysis and gluconeogenesis.[1] Inhibition of AMP aminohydrolase might therefore interfere profoundly with cellular survival and/or multiplication, and in this sense the enzyme can be considered a potential chemotherapeutic target. It is of interest to determine whether it might be possible to design substrate-site-directed irreversible inhibitors which act by alkylating the enzyme outside its substrate site; inhibitors of this type were devised by Baker who showed that the approach could generate powerful inhibitors of many of the enzymes examined.[2-7] To design inhibitors of that type it is necessary firstly to determine at which atoms (if any) of AMP it is possible to introduce a substituent without preventing specific adsorption of the derivative to part or all of the AMP recognition site. Earlier studies of the substrate activity of phosphonate analogues of AMP showed that adsorption to the AMP site still occurs when a hydroxyl[8] or cyano[9] group (structure **3**) is introduced α to the phosphorus, while other studies[10] showed that it likewise occurs when a methyl group is substituted on C-5' of AMP. In the present work longer substituents (acylaminomethyl and acetoxy) have been inserted α to phosphorus (compounds **5** and **7**) and at C-5' (compounds **1** and **2**) and their effect on affinity for the AMP site has been assessed by a study of substrate and inhibitor properties. The effect of groups substituted on O-2', O-3', and C-8 of AMP has been investigated in the same manner.

Chemical Syntheses. Synthesis of the β-D-allo (**1**) and α-L-talo (**2**) epimers of 5'-*C*-acylaminomethyl derivatives of AMP was reported previously.[11] The α-aminomethyl phosphonate analogue of AMP (compound **4**) was obtained in 85% yield by hydrogenation of the corresponding α-cyano phosphonate **3**[9] with rhodium–alumina.[12] Selective N-acetylation of nucleosides has been accomplished by the use of acetic anhydride in the presence of tri-*n*-butylamine[13] or with methanol[14] or pyridine[15] as solvents, but application of these methods to **4** appeared to give principally several unidentified derivatives of the acid anhydride of **4** as judged by paper electrophoretic analysis, and only trace amounts of the desired **5a** were obtained.



A tenfold excess of acylating agent in pyridine was then employed in order to minimize formation from **4** of phosphoric anhydrides;[16] upon treatment of the products with aqueous NH_4OH to remove *O*-acyl groups, the *N*-

Table I. Properties of Compounds 3, 4, and 5

Compd	R_f system A	R_f system B	Electrophoresis[a] (pH 7.5)	Uv max (H_2O), λ max, nm ($\epsilon \times 10^{-3}$)	Yield, %
3	0.27	0.71	1.00	259	
4	0.21	0.49	0.45	258	85
5a	0.29	0.73	0.84	259 (14.9)	87[b]
5b	0.39	0.83	0.80	259 (14.7)	67[b]
5c	0.33	0.75	0.85	259	23[c]
5d	0.41	0.78	0.83	259	25[c]
5e	0.55	0.81	0.81	259	28[c]

[a] Adenosine 5′-phosphate is assigned a mobility of 1.00. [b] Prepared by the *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline method; the acyl anhydride–pyridine method gave 35% of 5a and 29% of 5b. [c] Prepared by the action of an alkoxycarbonyl chloride in pyridine solution.

acetyl, propionyl, and methoxy-, ethoxy-, and propoxycarbonyl derivatives **5a–e** were obtained in purified form in 23–35% yield (Table I). Each of these derivatives is presumably a mixture of approximately equal proportions of its two 6′ epimers as is their precursor **3**.[9] The amide bond of **5a** was unaffected by proteases but was cleaved with concentrated NH_4OH to regenerate **4**. Compounds **5a** and **5b** were obtained in higher yields (87 and 67%, respectively) by treatment of **4** with acetic or propionic acids which had been converted in situ to mixed carbonic anhydrides by the action of *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline.[17] This reagent was studied because of its ability to form *N*-acetyl derivatives under conditions in which adenosine remains unchanged.[18]

The α-acetoxy phosphonate derivative, **7**, of AMP was obtained in 38% overall yield by treatment of the previously described α-hydroxy phosphonate analogue of 2′,3′-*O*-isopropylidene-AMP (**6a**)[8] with acetic anhydride in pyridine to give **6b**, followed by removal of the isopropylidene group with trifluoroacetic acid.[19] Compound **6a** is a 1:1 mixture of its two 6′ epimers,[8] and **7** is presumed to consist of a similar proportion of 6′ epimers (Table II).

2′,3′-*O*-Isopropylideneadenosine 5′-phosphate (**8**) was prepared by phosphorylation of 2′,3′-*O*-isopropylideneadenosine with phosphorus oxychloride in trimethyl phosphate solution,[20] and 2′,3′-di-*O*-acetyladenosine 5′-phosphate (**9**) was obtained by subjecting adenosine 5′-phosphate to the method of Bredereck[21,22] for the selective O-acetylation of adenosine.

Enzyme Studies. Data on the substrate specificity of AMP aminohydrolase[23,24] indicate that during the catalytic process important interactions take place between the enzyme and at least three locations of AMP, namely the phosphate moiety, the 3′-OH, and the adenine ring. The substrate and inhibitor properties of the present AMP derivatives are given in Table III. Introduction of an isopropylidene residue at O-2′ and O-3′ does not prevent binding to the adenine-binding portion of the AMP site because **8** is a substrate. The relative V_{max} value of **8** is intermediate between the value (0.22) of 3′-deoxy-AMP[23] and the value (1.0) of adenosine[24] and it is possible, therefore, that the isopropylidene group may interfere with the normal interactions between the enzyme and the 3′ hydroxyl and/or the phosphoryl group of the substrate. Compound **8** was a competitive inhibitor, indicating that it combines preferentially with the free enzyme rather than with the enzyme–AMP complex; the dissociation constant (K_i value) obtained is most probably, though not necessarily, a measure of the affinity of **8** for the AMP site of

Table II. Properties of Compounds 6, 7, 8, and 9

Compd	R_f system A	R_f system C	R_f system D	R_f system E	R_f system F	Electrophoresis (pH 7.5)[a]	Uv max, nm ($\epsilon \times 10^{-3}$) pH 6	Uv max, nm ($\epsilon \times 10^{-3}$) pH 1
6b	0.65		0.50	0.39	0.49	0.69	260	257
7	0.31		0.18	0.17	0.27	0.87	259 (15.4)	257 (15.0)
8	0.59	0.34	0.40	0.30		0.92	259 (15.2)	257 (14.9)
9	0.56		0.34		0.41	0.96	259 (15.4)	257 (15.0)

[a] Mobilities are relative to adenosine 5′-phosphate.

Table III. Substrate and Inhibition Constants of AMP Derivatives with AMP Aminohydrolase

Compd	Substrate constants[a] Protein per assay, μg	K_m, μM	V_{max}, rel %	Inhibition properties Type[b]	K_i,[c] μM	Inhibitor concn, μM
AMP	0.1	600	100[d]			
1a	100.0		>0[e]	L, C	18	11, 23
1b	>100.0[f]		>0[f]	L, C	42	14, 28
2a	>100.0[f]		>0[f]	NL, C	47, 30[g]	23, 46
2b	>100.0		0[h]	L, NC	205	19, 38
4	0.8	140	0.65			
5a	0.8	80	0.61	L, C	20	26, 52
5b	60.0		0	L, C	17	21, 42
5c	60.0		0	L, C	19	27, 54
5d	60.0		0	L, C	16	11, 22
5e	60.0		0	L, C	14	12, 24
7	1.0	120	7.8	L, C	330	64, 128
8	5.0	130	0.50	L, C	260	49, 148
9	9.1	670	0.07	L, C	105	49, 99
8-Br-AMP	100.0	330	0.03[i]			

[a] K_m (Michaelis constant) = concentration of substrate for half-maximal velocity; V_{max} = maximal velocity. [b] L = linear, NL = nonlinear, C = competitive with respect to AMP, NC = noncompetitive. [c] Enzyme–inhibitor dissociation constant. [d] V_{max} was 294 μmol of AMP transformed per minute per milligram of protein. [e] The decrease in absorbancy at 265 nm of a 100 μM solution was complete within 24 h and the resulting uv spectrum was the same as that of inosine 5′-phosphate produced from AMP by the enzyme. [f] The conversion was incomplete under the conditions used for **1a**; an additonal 100 μg of enzyme was then added whereafter the conversion was complete in a further 24 h. [g] The values are calculated from the two inhibitor levels used (23 and 46 μM, respectively). [h] No significant substrate activity was detected under the conditions used for **1b** and **2a**. [i] The conversion was monitored at 275 nm ($\Delta\epsilon$ 7500).

the enzyme or for an overlapping site which includes the adenine site. The K_i value of AMP itself has not been reported. The V_{max} value of the 2',3'-di-*O*-acetyl derivative **9** was one-seventh that of **8**, possibly as a result of the increased bulk of the combined O-2' and O-3' substituents and of limited bulk tolerance near the O-3' region of the AMP–enzyme complex resulting from a postulated interaction of the 3'-OH with the enzyme.[23] The K_i value of **9** indicates an affinity for the AMP site slightly higher than that of **8**.

The α-acetoxy phosphonate analogue **7** had a relative V_{max} value eightfold higher than that of adenosine, indicating that **7** might interact with the enzyme in the manner described above for AMP. On the other hand, the α-acetylaminomethyl phosphonate **5a**, like **8**, had a V_{max} intermediate between those of adenosine and 3'-deoxy-AMP. Compound **5a** was a strong competitive inhibitor (K_i = 20 μM) and the longer chain α-acylaminomethyl phosphonates **5b–e**, though not substrates, were also competitive inhibitors and had K_i values of the same order or less than **5a**. Since **5a** is a substrate, the K_i values most likely represent dissociation of compounds **5a–e** from the AMP site. These compounds bind three to four times more strongly to the enzyme than their parent unsubstituted phosphonate[9] and ca. 20 times more strongly than **7** and the enhanced binding might therefore result from hydrogen bonding between the enzyme and the amide systems in the substituents of **5**. The remaining portions of these substituents appear to interact weakly if at all with the enzyme as judged by the similarity of the K_i values of **5a–e** but when sufficiently long, as with **5b–e**, the substituents apparently block the catalytic process.

The 5'-*C*-acylaminomethyl derivatives **1a,b** and **2a** exhibited weak substrate activity (Table III) and were competitive inhibitors with K_i values similar to those of the contiguously substituted acylaminomethyl derivatives **5**. This suggests that **1a,b** and **2a** may adsorb to the AMP site or to portions of that site in a manner similar to compounds **5** and that the amide systems of **1a,b** and **2a** may be hydrogen bonded to a hydrophilic region of the enzyme adjacent to or identical with a hydrophilic region which is hydrogen bonded to the amide systems of **5**. The view that **1a,b** and **2a** may be hydrogen bonded to the enzyme in this manner is supported by the effect of replacing their 5' substituents with a methyl group[10] which lowers affinity for the AMP site (as indicated by K_i values) 5.5-, 2.5-, and 5.9-fold, respectively. Introduction of a methylene group into the 5' substituent of **2a**, giving **2b**, led to a loss of detectable substrate activity, to a fourfold or greater increase in the K_i value, and to a change in the type of inhibition from competitive to noncompetitive. These effects suggest that **2b** is hindered from interacting with AMP site in a manner similar to **2a** by steric and/or electronic properties of its terminal ethyl group.

8-Bromo-AMP was a weak substrate; the low relative V_{max} could be due to lack of bulk tolerance at C-8 of AMP or to the probability that in aqueous solution the ribose–adenine relationship of 8-bromo-AMP is mainly syn in character[25] whereas aminohydrolase-bound AMP appears to possess an anti type conformation.[23,26] In regard to bulk tolerance at other atoms of the adenine ring of AMP, an ethyl group at N^6 is tolerated as shown by the substrate activity of N^6-ethyl-AMP,[24] and a methyl group at N-1 is probably not tolerated because 1-methyl-AMP lacks substrate or effective inhibitor properties.[24] In the case of AMP aminohydrolase from rat muscle, the presence of one- or two-atom substituents at C-2 abolishes substrate and inhibitor properties.[27]

The present studies have thus indicated that affinity of AMP for its site on AMP aminohydrolase can be retained when several types of aliphatic substituents are introduced into AMP at the ribose–phosphate bridge (5' and O-5' positions) or at the ribose moiety (O-2', O-3'), or when bromine is introduced at C-8. By attachment of suitable leaving groups on the above or similar substituents it may therefore be possible to obtain substrate-site-directed irreversible inhibitors of the type devised by Baker.[2]

Experimental Section

Chemical Syntheses. General. Triethylamine, acetic anhydride, propionyl chloride, methyl chloroformate, ethyl chloroformate, and *n*-propyl chloroformate were distilled prior to use. Pyridine and *N,N*-dimethylformamide were distilled from calcium hydride. Paper chromatography was performed on Whatman No. 1 or 3 MM paper in (A) 1-butanol–acetic acid–water (5:2:3), (B) 2-propanol–0.25 M triethylammonium bicarbonate (1:1), (C) 2-propanol–concentrated ammonia–water (7:1:2), (D) 1-butanol–acetic acid–water (4:1:5, upper phase), (E) 95% ethanol–1 M ammonium acetate (7:3, pH 7.5), (F) 1-propanol–water (7:3). Electrophoresis was performed with Whatman No. 1 paper in 0.05 M triethylammonium bicarbonate (pH 7.5). Spots on chromatograms were detected by their ultraviolet absorption. Ultraviolet spectra were determined with a Cary Model 15 spectrophotometer and infrared spectra with a Perkin-Elmer spectrophotometer Model 137. ^{1}H NMR spectra were obtained with Varian XL-100-15 and Jeolco MH 60 spectrometers and are recorded as parts per million (ppm) downfield from an external standard (concentric capillary) of $SiMe_4$; use of the external standard caused a downfield shift of 0.4–0.5 ppm for all protons. Evaporations were carried out in vacuo at bath temperatures below 30°. Elemental analyses were performed by Atlantic Microlabs, Atlanta, Ga., and Midwest Microlab, Ltd., Indianapolis, Ind.

5'-Deoxy-5'-(*C*-aminomethyl)phosphonylmethyladenosine (4). Compound **3**[9] (250 μmol) was dissolved in 10 ml of 15% ammonium hydroxide; 5% rhodium on alumina (500 mg) was added and the mixture was hydrogenated under 50 atm of pressure. Hydrogen consumption ceased within 5 h. The solution was filtered and evaporated to dryness in vacuo and the residue was purified by downward chromatography in system B on sheets of Whatman 3 MM paper. Compound **4** was obtained in 85% yield as a white solid by lyophilization of the aqueous eluate and repeated coevaporation with methanol in vacuo; it was homogeneous on paper chromatography and electrophoresis in the systems of Table I and on TLC on silica gel in solvent B (R_f 0.19): ^{1}H NMR (D_2O) δ 8.76 (br s, 1, H-8), 8.60 (s, 1, H-2), 6.49 (d, 1, J = 5 Hz, H-1'), 5.07 (H-2', overlap of HDO and H-2' bands caused uncertain location of the H-2' signal), 4.74 (br s, 2, H-3' and H-4'), 3.61 (m, 2, H-7'), 2.40 (br m, 2, H-5'), 1.74 (m, 1, H-6').

Attempted reduction of **3** in aqueous HCl, pH 3–4, was unsuccessful using 5% Pd on charcoal and gave low yields with Pt or with PdO–$BaSO_4$.

5'-Deoxy-5'-(*C*-acetylaminomethyl)phosphonylmethyladenosine (5a). Compound **4** (0.26 mmol, determined spectrophotometrically) was dissolved in ethanol–water (3:2, 15 ml). To this was added *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (0.54 mmol) and acetic acid (0.54 mmol) and the solution was stirred at 37 °C (bath temperature) for 5 h. The mixture was concentrated in vacuo below 30° and the residue was washed with ether (3 × 50 ml), then dissolved in water, and applied to paper chromatograms. After development in solvent B the band at R_f 0.60 was eluted with water and the product was isolated as its disodium salt by addition of NaI in acetone to a solution in MeOH of the triethylammonium salt:[28] ^{1}H NMR (D_2O) δ 8.77 (br s, 1, H-8), 8.62 (s, 1, H-2), 6.49 (d, 1, J = 5 Hz, H-1'), 5.10 (overlap of HDO and H-2' bands caused uncertainty in location of H-2' signal), 4.78 (br s, 2, H-3', H-4'), 3.89 (m, 2, H-7'), 3.04 (d, 2, J = 18 Hz, H-6'), 2.42 (br s, 2, H-5'), 2.27 (s, 3, acetamido CH_3). Anal. ($C_{14}H_{19}N_6O_7PNa_2 \cdot 4H_2O$) C, H, N, P. Compound **5a** was also obtained by the action of acetic anhydride on **4** in pyridine solution by the general procedure described below.

General Method for the Conversion of 4 to the 5'-Deoxy-5'-(*C*-acylaminomethyl)phosphonylmethyladenosines 5. Compound **4** (65 μmol of the pyridinium salt) was dried by

repeated coevaporation with anhydrous pyridine and dissolved in anhydrous pyridine (1.5 ml) and anhydrous *N,N*-dimethylformamide (0.5 ml). The acylating agent (acetic anhydride, propionyl chloride, or methyl, ethyl, or propyl chloroformate) (650 μmol) was added to the cooled (5°) solution with stirring during 45 min. The mixture was stirred at 5° for 14 h and at 22° for 4 h and was then evaporated to dryness in vacuo. The residue was treated with 2 ml of concentrated NH_4OH at 5° for 15 h. The solvent was removed in vacuo, and the residue was subjected to paper electrophoresis at pH 7.5. The products were eluted with water and subjected to paper chromatography in system A. The products were eluted with water and after lyophilization obtained as white solids which were homogeneous in the systems of Table I. The products had uv spectra and paper chromatographic and electrophoretic properties which confirmed their structures and, as expected, reacted as *cis*-α-glycols toward the periodate–benzidine spray test[29] on paper chromatograms and electrophoretograms. The yields, determined spectrophotometrically, are given in Table I. Compounds **5a** and **5b** could be prepared in higher yield (Table I) by the *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline method described above for the synthesis of **5a**.

When **5a** (5 μmol) was treated with 2 ml of concentrated NH_4OH at 90–100° for 18 h in a sealed tube, the principal ultraviolet-absorbing component of the mixture was found by paper chromatography to correspond to 4.

5′-Deoxy-5′-(*C*-acetoxy)phosphonylmethyladenosine (7). The blocked α-hydroxyphosphonate **6a**[8] (0.53 mmol) was converted to its pyridinium salt by passage of its aqueous solution through Dowex 50 (pyridinium) ion-exchange resin. The water was removed in vacuo and the salt dried by evaporation of pyridine (3 × 30 ml) from it. Acetic anhydride (4.8 mmol) was added to a solution at 2° of **6a** pyridinium salt in dry pyridine (40 ml). The mixture was stirred at 5° for 36 h with exclusion of moisture. Volatiles were removed in vacuo and methanol (3 × 50 ml) was evaporated from the residue. The residue was extracted with ether (20 ml) and then dissolved in pyridine (20 ml) containing 2% water. Paper electrophoresis at pH 7.6 showed that **6b** was progressively formed from a uv-absorbing component of lower mobility and that the yield was optimum after 36 h at 5° (longer hydrolysis times regenerated **6a**). The solvent was removed in vacuo and the residue was subjected to downward chromatography for 22 h on Whatman No. 17 paper in solvent F. The band at R_f 0.5 was eluted into H_2O at 5° and the eluate lyophilized to give **6b** as a pale yellow powder (68% yield determined spectrophotometrically) which contained a trace of **6a**: ir (KBr) 5.78 (OAc C=O), 8.15 μ (OAc COC); ^1H NMR (D_2O, 60 MHz) δ 6.77 (s, 1, H-1′), 3.32 (s, 3, OCOMe), 1.94, 1.70 (2 s, 6, CMe_2); for other properties see Table II. A solution of **6b** in 4 ml of 90% aqueous trifluoroacetic acid was stirred at 22° for 9 min after which volatiles were removed in vacuo and benzene (2 × 20 ml) and then pyridine (5 ml) were evaporated from the residue. This was subjected twice to paper chromatography in solvent F and **7** was obtained as its disodium salt (0.20 mmol; 38% yield from **6a**) by the procedure described for **5a**. The product was homogeneous in the paper electrophoresis and chromatography systems of Table II: ir (KBr) 5.78 (C=O), 8.15 μ (OAc COC); ^1H NMR (D_2O, 100 MHz) δ 8.70 (s, 1, H-8), 8.54 (s, 1, H-2), 6.50 (d, 1, $J_{1',2'}$ = 4.0 Hz, H-1′), 6.44 (dd, 1, $J_{2',3'}$ = 5.0 Hz, H-2′), 5.57 (m, 1, H-3′), 4.79 (m, 1, H-4′), 2.14 (m, 2, H-5′), 4.67 (m, 1, H-6′), 2.48 (s, 3, OAc CH_3). Anal. ($C_{13}H_{16}N_5O_8PNa_2 \cdot 3H_2O$) C, H, N, P.

Solutions of **7** in Tris buffer pH 8.5 were stable for at least 16 h at 22° as shown by chromatography in solvents D–F.

2′,3′-*O*-Isopropylideneadenosine 5′-Phosphate (8). To a solution of 2′,3′-*O*-isopropylideneadenosine (6.50 mmol) in dry trimethyl phosphate (20 ml) cooled in an ice bath was added $POCl_3$ (13.0 mmol). The mixture was stirred in the bath for 15 min and stored at −15° for 24 h and then poured into 100 g of ice and the pH was adjusted to 9.0 with LiOH. The solution was extracted with chloroform (2 × 100 ml) and 3.075 g of barium acetate was added. The precipitate was centrifuged off and the supernatant concentrated in vacuo to 50 ml and 100 ml of acetone was added. The precipitated barium salt of **8** was centrifuged and washed with acetone. An aqueous solution of this product was passed through a Dowex 50 (pyridinium) column (5 × 15 cm) which was washed free of **8** with water. The eluates were adjusted to pH 3.5 with aqueous HCl and stirred with 25 g of DARCO G 60 charcoal and 25 g of Celite filter aid for 2 h. The charcoal was filtered and washed with 1 l. of water. Compound **8** was desorbed from the charcoal with 250 ml of 50% aqueous ethanol containing 1 ml of concentrated NH_4OH and the solution was concentrated to small volume and chromatographed on Whatman 3 MM paper by descending elution with solvent C over 1 day. Compound **8** (71% yield) was obtained as a white solid disodium salt in the usual way.[28] This was homogeneous in the systems of Table II: uv max (pH 12.0) 259 nm (ε 15 000); ^1H NMR (D_2O, 60 MHz) δ 8.94 (s, 1, H-8), 8.62 (s, 1, H-2), 6.73 (d, 1, $J_{1',2'}$ = 3.5 Hz, H-1′), 5.90 (m, 1, H-2′), 5.66 (m, 1, H-3′), 5.12 (m, 1, H-4′), 4.59 (m, 2, H-5′), 2.23, 1.99 (2 s, 6, CMe_2). Anal. ($C_{13}H_{16}N_5O_7PNa_2 \cdot 1.25H_2O$) C, H, N, P.

2′,3′-Di-*O*-acetyladenosine 5′-Phosphate (9). Adenosine 5′-phosphate (0.288 mmol) was allowed to react with acetic anhydride (8.18 mmol) in anhydrous pyridine (1.5 ml) solution at 25° for 2 h, with exclusion of moisture. The mixture was evaporated to dryness in vacuo and the gummy powder was coevaporated with 95% aqueous ethanol (2 × 2 ml). The product was dissolved in the minimum of warm methanol and ethyl acetate was added to turbidity. The resulting colorless microcrystalline precipitate of **9** was centrifuged and washed with ethyl acetate and ether: yield, 62%; mp 175–177° dec; ir 5.73 (OAc C=O), 8.20 μ (OAc COC); uv max (pH 12) 259 nm (ε 15 300); ^1H NMR (D_2O, 60 MHz) δ 9.12 (s, 1, H-8), 8.84 (s, 1, H-2), 6.90 (d, 1, $J_{1',2'}$ = 4.0 Hz, H-1′), 6.33 (m, 1, H-2′), 6.27 (m, 1, H-3′), 5.17 (m, 1, H-4′, partially obscured by the HDO signal), 4.77 (m, 2, H-5′), 2.75 and 2.61 (2 s, 6, –OAc Me). Anal. ($C_{14}H_{18}N_5O_9P \cdot 2.5H_2O$) C, H, N, P.

Enzymatic Stability of the Amide Bonds of 5a and 5b. These compounds were incubated at 37° for 24 h in 0.05 M Tris buffer, pH 7.6 (2 ml), with the enzymes below and the solutions were analyzed by paper electrophoresis (pH 7.6, 4kV, 30 min); in no case was cleavage of the amide linkage detected. The enzymes (all obtained from Sigma Chemical Co.) were protease type I (pancreatic, 20 mg), protease type II (fungal, 20 mg), protease type III (papaya, 10 mg), protease type IV (bacterial, 200 mg), protease type VI (bacterial, 30 mg), protease type VII (bacterial, 30 mg), protease type VIII (bacterial, 15 mg), protease type X (bacterial, 5 mg), acylase (20 mg), and peptidase (hog intestine, Grade III, 20 mg).

Enzyme Kinetic Studies. Adenylate aminohydrolase (Sigma, Grade IV, from rabbit muscle) activity was measured at 22° in 1-cm cells in a Cary Model 15 spectrophotometer by following the decrease in absorbance at 265 nm in a solution containing 0.01 M citrate buffer (pH 6.5) and 25 mM KCl. AMP aminohydrolase was diluted into 1 M KCl prior to use; Δε for the conversion was taken as 8400 for all compounds except 8-bromo-AMP (see Table III). In all measurements the initial velocity was linear and proportional to the concentration of enzyme. Each substrate or inhibitor study employed at least four substrate levels and two inhibitor levels and the kinetic parameters were obtained from Lineweaver–Burk plots,[30] all of which were linear. In studies of inhibitors, the level of AMP ranged between 20 and 200 μM; K_i values (dissociation constants) were obtained from replots of inhibitor level against slope of the Lineweaver–Burk plot. For studies of their substrate properties the AMP derivatives were employed at levels between 20 and 200 μM.

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Theoretical Model-Based Equations for the Linear Free Energy Relationships of the Biological Activity of Ionizable Substances. 1. Equilibrium-Controlled Potency

Yvonne C. Martin* and James J. Hackbarth

Drug Metabolism Department, Abbott Laboratories, North Chicago, Illinois 60064. *Received October 20, 1975*

Because of the ambiguities of how to treat ionization in empirical equations which relate biological activity to partition coefficient by use of a $(\log P)^2$ term, a theoretical approach to the problem is proposed. Based on a simplified view of assays of potency following in vitro or continuous infusion administration of drugs, equations have been derived from a combination of mass law, equilibrium, and extrathermodynamic assumptions. In general form the equations which relate potency to partition coefficient (P) and degree of ionization (α) are the following. If the neutral form reacts with the receptor, $\log (1/C) = -\log [1 + \Sigma^m(d_i P^{c_i}) + \Sigma^n[a_j/P^b(1 - \alpha_j)]] + X$. If the ionic form reacts with the receptor, $\log (1/C) = -\log [1 + (1 - \alpha_n)/(\alpha_n)[\Sigma^m(d_i P^{c_i}) + \Sigma^n[a_j/P^b(1 - \alpha_j)]]] + X$. In this generalized model there are m nonaqueous compartments and n aqueous compartments of different pH. The parameters a, b, c, and d can be interpreted in terms of the model. The shape of the $\log (1/C)$ vs. $\log P$ curve may be asymptotic, linear, or composed of two portions of unequal slope which meet at an optimum or a bend. With the use of these equations it is possible to examine whether the ion or the neutral form is the active species and whether there is hydrophobic bonding to the receptor and/or an inert compartment. The models may be further extended to include terms other than $\log P$ and α.

During the past 10 years a great deal of progress has been made in the study of quantitative structure–activity relationships. In particular, the extrathermodynamic or linear free energy approach has been used to analyze the relationships in many series of compounds.[1,2] However, very little attention has been paid to the nature of these relationships when the drugs involved are partially ionized at the pH of the biological system.[1,3,4] This report deals with the derivation of equations for the relationship between potency, partition coefficient, and degree of ionization for closed, i.e., equilibrium, systems. Such equations may be useful in the correlation of potency in certain in vitro and continuous infusion assays.

The main problem to be discussed is how to correctly account for the effect of ionization in the modeling of biological partitioning. It is known that the partition coefficient (P) of the ionic species of a compound is approximately 15000× lower than that of the neutral form of the same compound.[5] Thus for all practical purposes, if the concentration of the neutral form is at least 0.001 that of the ionized form, then the ion does not contribute to the observed partition coefficient. This may be expressed in equation form

$$\log P \text{ (obsd, solvent X)} = \log P \text{ (neutral, solvent X)} + \log (1 - \alpha) \quad (1)$$

in which α is the fraction of drug ionized at the pH of measurement. Some workers have assumed that one should therefore use the partition coefficient between buffer of biological pH and an organic solvent to model the hydrophobic effect of the compound in the biological system.[6] Others have divided the observed potency ($1/C$) by the fraction un-ionized ($1 - \alpha$) in order to "correct" the potency to be that of the neutral form.[3,4] The following discussion will evaluate the merits of each approach as well as suggest the possible complications.

The problem with using the extrathermodynamic approach with ionizable substances in biological systems is that in general there is not a simple linear relationship between the $\log P$ of a substance and its biological potency. This nonlinear relationship has traditionally been fit by