Sensitive Fluorimetric Assay for Adenosine Deaminase with Formycin as Substrate; and Substrate and Inhibitor Properties of some Pyrazolopyrimidine and Related Analogues

Jacek Wierzchowski and David Shugar

Department of Physics, Academy of Agriculture, Wojska Polskiego 28, 60-637 Poznan, Poland; and Department of Biophysics, Institute of Experimental Physics, University of Warsaw, Zwirki Wigury 93, 02-089 Warszawa, Poland

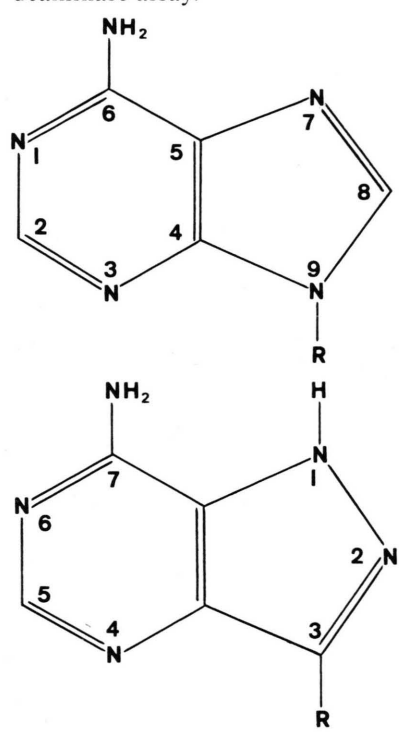
Z. Naturforsch. **38 c**, 67–73 (1983); received August 23, 1982

Formycin, Pyrazolopyrimidines, Adenosine Deaminase, Fluorimetric Assay, Inhibitors

The nucleoside antibiotic formycin, 7-amino-3-(β-D-ribofuranosyl)pyrazolo(4,3-d)pyrimidine, a structural analogue of adenosine, is deaminated about 10-fold faster by adenosine deaminase than adenosine itself, and is therefore a superior substrate for both routine assays and kinetic studies with the purified enzyme. The luminescence properties of formycin have been profited from to develop a fluorimetric assay for adenosine deaminase which is considerably more sensitive than the spectrophotometric procedure widely employed with adenosine as substrate. Examples are presented of its application to routine assays of adenosine deaminase levels in cellular extracts, as well as to kinetic studies with the purified enzyme, including the properties of some pyrazolopyrimidine and purine substrates and inhibitors.

Formycin, 7-amino-3-(β-D-ribofuranosyl)pyrazolo(4,3-d)pyrimidine, a naturally occurring nucleoside antibiotic and a formal analogue of adenosine (see Scheme 1), is capable of substituting for adenosine in a variety of enzymatic reactions [1].

In particular, it is a good substrate for adenosine deaminase from a variety of sources, with a rate of hydrolysis, in fact, several-fold higher than that for adenosine [2, 3]. It is also one of the rare natural nucleosides which exhibits appreciable fluorescence at room temperature; and the fluorescence properties of which, along with those of the related pyrazolo(4,3-d)pyrimidines, have now been extensively characterized [4, 5]. The use of formycin as a fluorescent probe in some enzymatic systems has been reported [4, 6]; and, since it is now commercially available, it occurred to us that it would be far superior to the parent adenosine as a substrate for assays of adenosine deaminase levels in various biochemical systems, as well as for kinetic studies, including the properties of substrate analogues and inhibitors, in *in vitro* systems. Preliminary investigations also demonstrated that the product of deamination of formycin, formycin B, neither fluoresces at room temperature, nor is it an inhibitor of the enzyme (see below), thus further enhancing the utility of formycin as a substrate. Furthermore, since formycin is a C–C nucleoside (see Scheme 1), it possesses additional utility for adenosine deaminase assays in bacterial systems, where adenosine itself is susceptible to the action of purine nucleoside phosphorylase, which may interfere with the deaminase assay.



Scheme 1. Showing structural analogy between adenosine (above, R = ribose) and formycin (R = ribose). With R = alkyl, we have the corresponding 9-alkyladenine and 3-alkylpyrazolo(4,3-d)pyrimidine.

Reprint requests to J. Wierzchowski or D. Shugar.

0341-0382/83/0100-0067 $ 01.30/0

Materials

Formycin (also known as formycin A) was a gift of Dr. R. K. Robins, and N_2,5′-anhydroformycin was kindly provided by Dr. J. Zemlicka. Formycin B was obtained from CalBiochem (Zurich, Switzerland), and deoxycoformycin was a gift from Dr. Robert A. Buchanan of Parke Davis & Co.

The synthesis of the methylated formycins and pyrazolopyrimidines has been described elsewhere [7, 8]. The 9-methyl and 9-ethyl adenines were products of Cyclo Chem. Corp. (Los Angeles, Calif., USA). The preparation of isoguanosine and 9-methylisoguanine has been elsewhere reported [9].

Adenosine deaminase (calf intestinal mucosa, Sigma type I, 2325 units/ml, ~ 200 U/mg) was a product of Sigma Chemical Co. (St. Louis, Missouri, USA).

Methods

Fluorescence measurements were carried out with an Aminco-Bowman SPF spectrofluorimeter, fitted with a Hanovia 901 xenon lamp and a Hamamatsu 1P28 photomultiplier, using 10-mm square cuvettes. The spectral width of the exciting beam was about 6 nm.

Spectrophotometric measurements were conducted with a Zeiss (Jena, GDR) UV-VIS instrument, using 10 mm pathlength cuvettes.

Enzyme assays

The incubation medium consisted of 0.02 M phosphate buffer pH 7.5, at a temperature of 25 ± 0.5 °C, in a thermostated 10-mm pathlength spectral cuvette. Enzyme concentrations employed were 0.008 to 1.5 U/ml.

With a suitable substrate concentration, the course of the reaction may be followed by continuous measurement of the decrease in fluorescence intensity of the substrate (since the product of deamination, formycin B, is virtually non-fluorescent at pH < 8). Excitation and emission wavelengths were selected according to the fluorescent background of the reaction mixture, and the optical density of the substrate. Excitation was most frequently at 295 or 305 nm, and emission measurements at 338 nm or 354 nm. For measurements of enzyme levels in cell extracts, excitation wavelengths were 305 nm or 318 nm, so that proteins in the extracts only minimally contributed to the fluorescence, if at all.

The initial substrate concentration should be such that its optical density at $\lambda = \lambda_{exc}$ does not exceed 0.3. With $\lambda_{exc} = 305$ nm, the substrate concentration may be in the range 4–40 μM. However, with $\lambda_{exc} = 318$ nm (where ε_{318} of formycin is low, 1.1×10^3), substrate concentration may be as high as 300 μM. For the higher substrate concentrations, which were conducted in test tubes, aliquots were withdrawn at given time intervals, diluted 20- to 100-fold with 0.05 M K_3PO_4 (pH ~ 11.5) to terminate the reaction, and the extent of deamination based on the fluorescence intensity of the formycin anion at 400 nm [4] with $\lambda_{exc} = 320$ nm, at which wavelength the anion of formycin B (the product of the reaction) is not excited and therefore exhibits no fluorescence.

When the optical density of the substrate at $\lambda = \lambda_{exc}$ does not exceed 0.1, the fluorescence intensity, following substraction of background, is proportional to the concentration of unchanged substrate, *i.e.*

$$c(t) = c(0)\frac{I(t) - I_b}{I(0) - I_b}$$

where $c(t)$ is the concentration at time t, $c(0)$ at time 0; $I(t)$, $I(0)$ the fluorescence intensities at times t and 0, and I_b is the background fluorescence (which, even for many cell extracts, following appropriate dilution, may be negligible). The foregoing is applicable because the deamination product of formycin (formycin B) exhibits negligible fluorescence; the same applies to the products of deamination of several other substrate analogues described below.

The rate of deamination during the course of the reaction, dc/dt, was determined by numerical differentiation of the curve of c *vs* t. Values of K_m and V_{max} were determined according to standard procedures [10].

With a substrate concentration $c < 50$ μM, the deamination of formycin is a pseudo first-order reaction (see below). Under these conditions, the rate constant is given by [E] V_{max}/K_m, where [E] is the enzyme concentration, or, in the presence of an inhibitor, by [E] $V_{max}/K_m\,(1 + [I]/K_i)$, where [I] is the inhibitor concentration. The rate constants for deamination were calculated from the relationship $k = 1/c \cdot dc/dt = 0.693/t_{1/2}$, where $t_{1/2}$ is the time corresponding to 50% deamination.

Correction at high substrate concentrations. With higher substrate concentrations, such as frequently employed for measurements of kinetic constants, the

resulting higher optical density requires introduction of a correction for the so-called inner filter effect (Parker, [11]). In such instances the corrected value of the fluorescence intensity, $I_c(t)$, and the actual optical density (or absorbance) of the sample, $A(t)$, were calculated according to the equations:

$$A_1(t) = [A(0) - A(\infty)] \frac{I(t) - I_b}{I(0) - I_b} + A(\infty)$$

$$I_1(t) = I(t) \cdot 10^{A_1(t)/2}$$

where $I(t)$ is the measured fluorescence intensity at time t, I_b is the background fluorescence intensity, and $A(0)$ and $A(\infty)$ are the absorbances of the sample prior to, and following completion of, the reaction. This procedure is fully adequate for samples with substrate optical densities higher than 0.1 and up to 0.2. For higher optical densities, the values obtained from the above equations are further corrected by an iterative procedure, as follows:

$$A_2(t) = [A(0) - A(\infty)] \frac{I_1(t) - I_b}{I_1(0) - I_b} + A(\infty)$$

$$I_2(t) = I(t) \cdot 10^{A_2(t)/2}.$$

With an optical density of 0.3, $I_2(t)$ is, following subtraction of background fluorescence, proportional to the substrate concentration to an accuracy of about 1%. Application of this procedure is shown in Fig. 1 A, below.

Results and Discussion

It had previously been noted, using the standard absorption spectroscopy procedure for adenosine deaminase activity, that the rate of deamination of

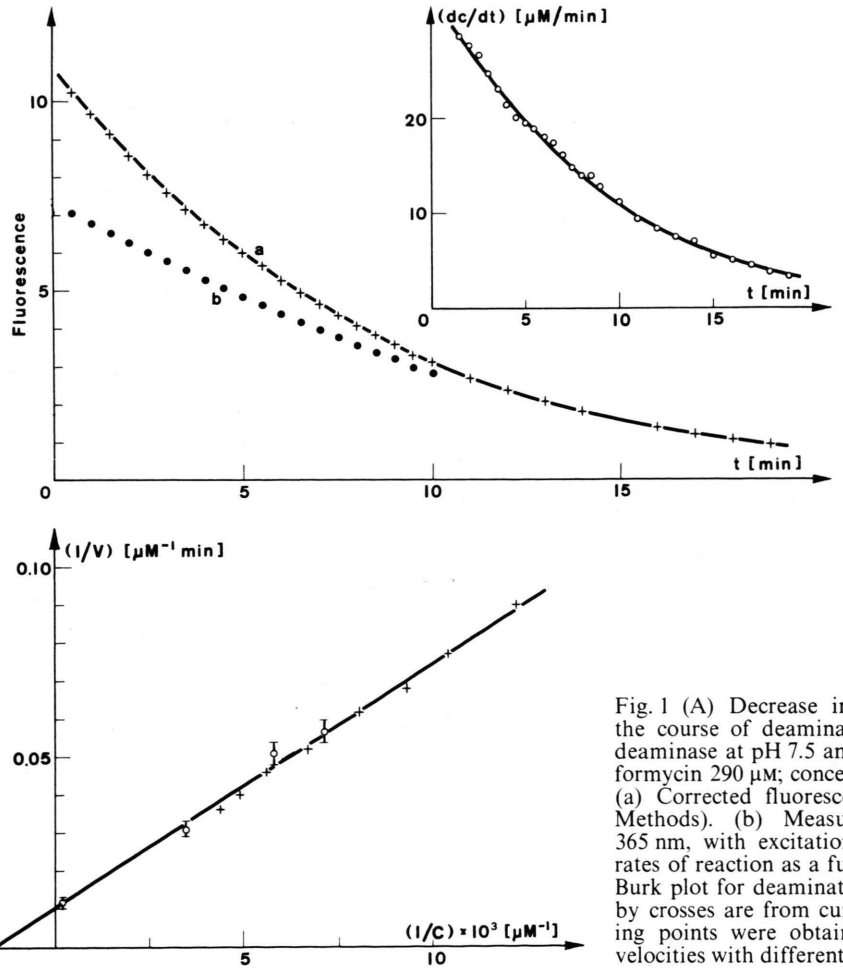


Fig. 1 (A) Decrease in fluorescence intensity during the course of deamination of formycin by adenosine deaminase at pH 7.5 and 25 °C. Initial concentration of formycin 290 μM; concentration of enzyme, 0.008 U/ml: (a) Corrected fluorescence intensity (as described in Methods). (b) Measured fluorescence intensity at 365 nm, with excitation at 318 nm. Insert: Calculated rates of reaction as a function of time. (B) Lineweaver-Burk plot for deamination of formycin. Points denoted by crosses are from curve (a) in Fig. 1 A. The remaining points were obtained by measurement of initial velocities with different substrate concentrations.

Table I. Substrate and inhibitor properties of purine and pyrazolopyrimidine analogues and some of their nucleosides, as determined fluorometrically (unless otherwise indicated) at 25 °C. Inhibitor properties were obtained with formycin as substrate.

Compound	K_m [μM]	K_i [a] [μM]	V_{max} [μmol/U/min]	K_m/V_{max} [b] [min]
Adenosine	25 [c]	27	1	25 [c]
Adenine	150 [d]	–	$\sim 10^{-5}$ [d]	$\sim 10^7$
Formycin	600	–	13	46
N_2-methyl-	6×10^3 [c]	–	4 [c]	1.6×10^3
N^7-methyl-	100 [c]	–	0.03 [c]	3×10^3
N_2,5′-anhydro-		$> 3 \times 10^3$ [f]	slow [e]	8×10^5
Isoguanosine (2-oxoadenosine)	–	45	–	–
Isoguanine, N_9-methyl-	–	2500	–	–
N_9-methyladenine		1000	$\sim 10^{-3}$	3×10^5
N_9-ethyladenine		400	$\sim 10^{-4}$	$\sim 10^6$
4-aminopyrazolo(3,4-d)pyrimidine	–	> 1000	–	–
N_1-methyl	–	800	–	–
N_1-(β-D-ribofuranosyl)-7-aminopyrazolo(4,3-d)pyrimidine	125 [g]	–	0.064 [g]	–
3-propyl-	55	49	0.017	3.2×10^3
N_1-methyl-3-propyl	–	> 500	–	–
N_2-methyl-3-propyl-		150	1.6×10^{-4}	9×10^5
N_2,3-dimethyl-		450	0.01	4.8×10^4
N^7-methyl-3-propyl-		~ 400		$\sim 6 \times 10^5$
7-oxo-3-propylpyrazolo(4,3-d)pyrimidine		> 500	–	–

[a] With a formycin concentration in the incubation medium of 10 μM.
[b] With an enzyme concentration of 0.001 units/ml.
[c] Data from Crabtree *et al.* [12] with human erythrocyte enzyme, assayed by absorption procedure.
[d] Data from Wolfenden [13].
[e] This compound is very slowly hydrolyzed (*cf.* [18, 19]).
[f] Because of the efficient fluorescence of this analogue, it was necessary in this case to use a formycin concentration of 10^{-3} M, and to dilute the incubation mixture 50-fold for measurements.
[g] Data from [16].

formycin by enzyme from different sources is 8- to 10-fold higher than for adenosine [2, 3].

With the use of the fluorescence procedure described above, and the calf intestinal enzyme, it was found that $K_m = 0.6$ mM and $V_{max} = 12$ μM/U/min at 25 °C, values close to those reported for the enzyme from human erythrocytes [3].

The kinetics of deamination, followed fluorimetrically, are consistent with the Michaelis-Menten equation (Fig. 1 B), and the product of deamination, formycin B, is not an inhibitor of the reaction, $K_i > 10^3$ μM.

The value of V_{max} for deamination of formycin is more than an order of magnitude higher than for adenosine (Table I). This fact, and the useful spectroscopic properties of formycin, make possible use of the latter as substrate for determination of the level of deaminase activity with a sensitivity much higher than with adenosine.

The high value of K_m for formycin is such that, in the range of substrate concentrations employed for measurements of enzyme levels (< 50 μM), the reaction is pseudo first-order, with a rate constant equal to V_{max}/K_m. We have applied this procedure to calculate K_m/V_{max} for N_2-methylformycin and N^7-methylformycin, 1.6×10^3 min and 3×10^3 min, respectively (Table I). The corresponding values, recalculated from the data of Crabtree *et al.* [12] obtained by absorption spectroscopy (values of K_m and V_{max} in Table I), are virtually identical.

Assay of deaminase levels in cell extracts

The foregoing fluorimetric procedure has been applied to measurements of adenosine deaminase levels in crude cell extracts, and during the course of purification of adenosine kinase from rat liver. Particularly with crude extracts, the absorption procedure with adenosine as substrate is rather inconvenient because of the relatively high absorption of the extracts at wavelength below 300 nm.

The sensitivity of the fluorimetric method is limited principally by the fluorescence background

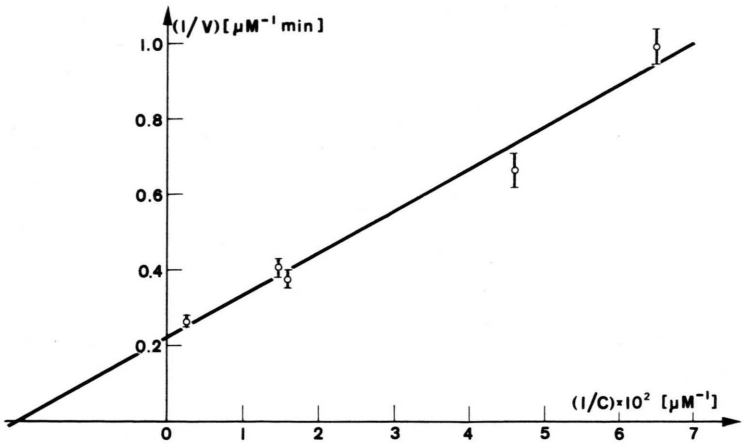


Fig. 2. Linweaver-Burk plot for deamination of 3-propyl-7-aminopyrazolo-(4,3-d)pyrimidine by adenosine deaminase (0.3 U/ml) at pH 7.5 and 25 °C. This gives $K_m = 55$ μM, $V_{max} = 1.7 \times 10^{-2}$ μmol/U/min.

of the extracts. However, this background may be reduced by appropriate dilution of the extracts and (as pointed out above), by appropriate selection of the excitation and fluorescence wavelengths (see Table II). From Table II it follows that the signal to background ratio is more favourable for the fluorimetric procedure.

Table II presents details associated with the measurement of adenosine deaminase levels during the course of purification of rat liver adenosine kinase. Fig. 4 exhibits details of a typical measurement, using a low concentration of substrate, so that no corrections for the inner filter effect were necessary. From the foregoing, it will be seen that the fluorimetric procedure permits of evaluation of enzyme levels as low as 10^{-3} units per ml with the use of less than 100 μl of crude extract.

Pyrazolopyrimidine and purine analogues as substrates and inhibitors

In an investigation on the influence of the ribose moiety of adenosine on the transition state affinity in the reactions catalyzed by adenosine deaminase, Wolfenden *et al.* [13, 14] observed that adenine is hydrolyzed at a limiting rate five orders of mangitude lower than that for adenosine, whereas the K_m was not far different, 150 μM for adenine as compared to 31 μM for adenosine. This prompted us to examine the substrate and inhibitor properties of some analogues of pyrazolo(4,3-d)pyrimidine (the aglycone of formycin) and related compounds. Some of the results are listed in Table I. These are being subjected to more detailed investigation and only a few pertinent points will be referred to here.

Table II. Use of formycin as fluorescent substrate for following the levels of adenosine deaminase in successive fractions of rat liver extracts during the course of isolation and purification of adenosine kinase.

Fraction	Dilution	λ_{exc} [nm]	Background				Formycin					$\frac{1}{c}\frac{dc}{dt}$ [min^{-1}]	Enzyme Activity [units/ml]
			O.D.	fluorescence 340	354	λ [nm] 365	Conc. [μM]	O.D.	fluorescence 340	354	λ [nm] 365		
1	50 ×	295	0.48	39	32	25	20	0.20	100	74	49	0.079±0.004	0.17±0.02
		306	0.25	9	8	7		0.15	78	63	44		
		318	0.14	–	3	4		0.02	–	18	15		
2	50 ×	305	0.14	–	6	–	9	0.07	–	35	–	0.075	0.16±0.02
4	20 × [a]	318	0.1	–	5	–	24	0.03	–	22	–	< 0.002	< 0.002
$1 + 10^{-7}$ M deoxycoformycin	25 × [b]	318	0.28	–	6	–	33	0.04	–	30	–	$< 5 \times 10^{-4}$	

[a] Absorbance at 280 nm was ~ 2.
[b] This is a control showing that, in the presence of deoxyformycin, which specifically inhibits the adenosine deaminase in the extract, the fluorescence of the substrate is unchanged.

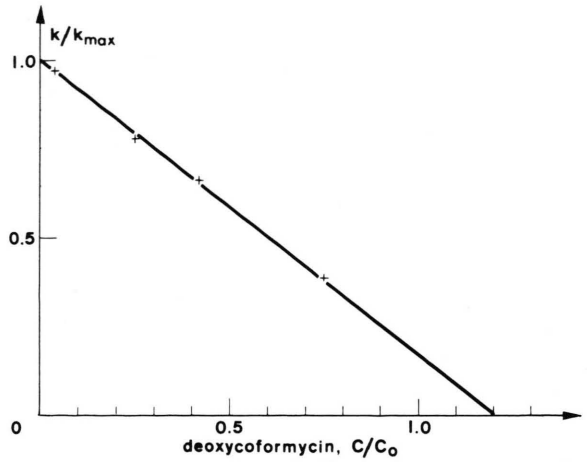


Fig. 3. Dependence of pseudo first-order rate constant for deamination of N_2-methylformycin on concentration of the adenosine deaminase inhibitor, deoxycoformycin. Enzyme concentration, 0.4 U/ml; initial concentration of N_2-methylformycin, 20 μM; concentration of deoxycoformycin, $c_0 = 1.56 \times 10^{-2}$ μM.

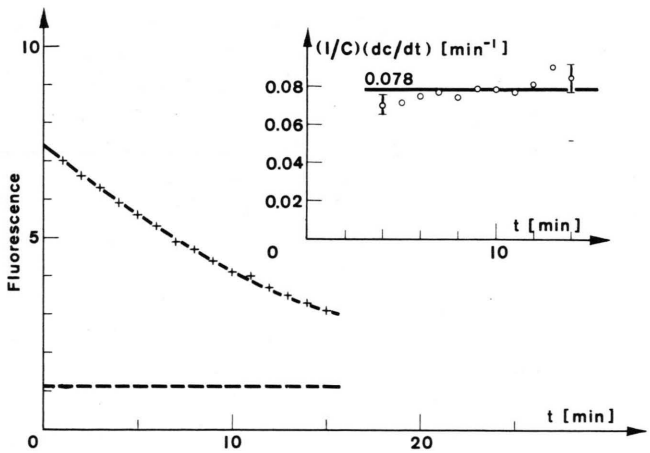


Fig. 4. Decrease of fluorescence intensity during the course of deamination of formycin by a 50-fold diluted extract of rat liver. Excitation was at 306 nm, and fluorescence was monitored at 354 nm. Initial concentration of formycin was 10 μM. The horizontal dashed line represents the background fluorescence of the extract. Insert shows the calculated rate constant for deamination during the course of the reaction (see Methods).

There are appreciable differences in the rates of deamination of adenine and its 9-alkyl derivatives, *e.g.* 9-methyladenine is hydrolyzed 100× faster than adenine, whereas 9-ethyladenine exhibits a V_{max} an order of magnitude lower than 9-methyladenine. By contrast, 9-ethyladenine is a more effective inhibitor than 9-methyladenine. Similarly, the 3-propyl derivative of N_2-methyl-7-aminopyrazole(4,3-d)pyrimidine is a more effective inhibitor than the 3-methyl analogue. Both of these are substrates, albeit poorer ones than the corresponding N_2-methylformycin, but it is of interest that the V_{max} for the 3-methyl derivative is 100-fold higher than for the 3-propyl.

The 3-propyl derivative of 7-aminopyrazolo-(4,3-d)pyrimidine, which is a formal analogue of 9-alkyladenine (Scheme 1), is almost as effective an inhibitor ($K_i = 49$ μM) as adenosine. It is also a substrate, but with a V_{max} about three orders of magnitude lower than for formycin, and less than two orders of magnitude lower than for adenosine. Furthermore, this analogue was found to be a competitive inhibitor of the enzyme, while its K_i as inhibitor is almost identical to its K_m as a substrate (55 μM).

In general nucleosides, by comparison with the corresponding base or N-alkylated base, exhibit a V_{max} 3–5 orders of magnitude higher. This applies to adenosine, the riboside of 4-aminopyrazolo(3,4-d)-pyrimidine, formycin and the N_2-methyl and N^7-methyl derivatives of the latter. By contrast, affinity for the enzyme (measured as K_m or K_i) of the N-nucleosides (adenosine and the riboside of 4-aminopyrazolo(3,4-d)pyrimidine) is 5- to 10-fold higher than for the bases; whereas for the C–C nucleoside formycin the reverse holds, *i.e.* the affinity of the bases is an order of magnitude higher than for the nucleosides, with the exception of N^7-methylformycin.

An interesting observation is that isoguanosine (2-oxoadenosine), a naturally occurring nucleoside with potent pharmacological activity, is not a substrate. This may be due to its unusual tautomeric form, which is the amino species like adenosine, but with a hydrogen on the ring N(1) [17]. Nonetheless, it inhibits deamination of formycin almost as effectively as adenosine, the K_i values being 45 μM and 27 μM, respectively.

Very low affinity for the enzyme ($K_i > 3$ mM) is exhibit by N_2,5′-anhydroformycin, which is an analogue of formycin fixed in the *anti* conformation about the glycosidic bond. Nonetheless, in agreement with previous reports [18, 19], this compound is deaminated very slowly, at a rate comparable to that for N_2-methyl-7-aminopyrazolo(4,3-d)pyrimidine (Table I). In view of the foregoing it is somewhat surprising that neither the *R*, or *S*, epimers of the corresponding 8,5′-cycloadenosines, both also in the fixed *anti* conformation, are detectably deaminated, in agreement with earlier results [20].

Titration of enzyme by deoxycoformycin. Particularly interesting is deoxycoformycin, known as one of the most potent inhibitors of adenosine deaminase [15]. Fig. 3 exhibits the decrease in rate constant for deamination of N_2-methylformycin in the presence of deoxycoformycin. The choice of N_2-methylformycin as substrate in this case was simply to enable the use of higher concentrations of enzyme ($> K_i$). As may be seen from the figure, the dependence of rate constant on deoxycoformycin concentration is linear with an enzyme concentration of 0.4 U/ml (unit = 4.3 μg protein) per ml., showing that the concentrations of both enzyme and deoxycoformycin are greater than K_i. It should also be noted that 19 nM of deoxycoformycin totally inhibits the enzyme; this corresponds to binding of 1 mol deoxycoformycin by about 90000 g protein. This is similar to the earlier finding of Wolfenden *et al.* [14] pointing to the binding of 2.5 mol of enzyme (with an adopted molecular weight of 33000) by 1 mol deoxycoformycin.

Concluding Remarks

The use of formycin as substrate for adenosine deaminase is clearly superior to adenosine, because of its more rapid rate of deamination, whether followed fluorimetrically or spectrophotometrically. But the fluorimetric procedure possesses the following advantages: (a) it requires a 6- to 10-fold lower concentration of substrate; (b) it may be more readily applied to cell extracts which, when added to the incubation medium, exhibit a high optical density (OD ~ 1); (c) with appropriate selection of the exciting wavelength, and monitoring fluorescence at a suitable wavelength, the ratio of signal to fluorescence background may be improved with concomitant increase in sensitivity.

The present investigation was carried out with a single-beam instrument, the only one at our disposal. With the use of modern commercial double-beam instruments, both the sensitivity and rapidity of measurements would be considerably enhanced. Furthermore, with the use of numerical data processing, calculations of the corrections required when using high substrate concentrations may be simplified (see Methods, above).

A fluorimetric procedure analogous to the above may be employed to follow the deamination of formycin-5′-phosphate, which is also a substrate for 5′-AMP deaminase. However, we have found the rates of deamination of 5′-AMP and formycin-5′-phosphate to be comparable, so that the fluorimetric procedure in this instance would be advantageous only in certain instances, *e.g.* with cell extracts which exhibit high optical density.

Acknowledgements

We should like to express our thanks to Dr. R. K. Robins, Dr. J. Zemlicka, and Dr. R. A. Buchanan for gifts of compounds. This investigation profited from the support of the Polish National Cancer Research Program (Project PR-6), Ministry of Higher Education, Science & Technology (MR.I.5), and the Wellcome Trust.

[1] R. J. Suhadolnik, Nucleoside Antibiotics, Chapt. 9, Wiley-Interscience, New York 1970.
[2] J. Giziewicz, E. de Clerq, M. Łuczak, and D. Shugar, Biochem. Pharmacol. **24,** 1813 (1975).
[3] R. P. Agarwal, S. M. Sagar, and R. E. Parks, Biochem. Pharmacol. **24,** 693 (1975).
[4] D. C. Ward, E. Reich, and L. Stryer, J. Biol. Chem. **244,** 1228 (1969).
[5] J. Wierzchowski and D. Shugar, Photochem. Photobiol. **35,** 445 (1982).
[6] a: D. C. Ward, T. Horn, and E. Reich, J. Biol. Chem. **247,** 4014 (1972);
b: S. J. D. Karlish, D. W. Yates, and I. M. Glynn, Nature **263,** 251 (1976);
c: J. L. Gabriel and G. W. E. Plaut, Photochem. Photobiol. **33,** 21 (1981);
d: E. F. Rossomando, J. H. Jahngen, and J. F. Eccleston, Proc. Nat. Acad. Sci. USA **78,** 2278 (1981).
[7] J. Giziewicz and D. Shugar, Acta Bioch. Pol. **24,** 231 (1977).
[8] J. Wierzchowski, Jarosław Kuśmierek, J. Giziewicz, D. Salvi, and D. Shugar, Acta Bioch. Pol. **27,** 35 (1980).
[9] Z. Kazimierczuk and D. Shugar, Acta Bioch. Pol. **21,** 455 (1974).
[10] L. Lineweaver and D. Burk, J. Amer. Chem. Soc. **56,** 658 (1934).
[11] C. A. Parker, Photoluminescence of solutions, Chapt. 3H, Elsevier, London 1968.
[12] G. W. Crabtree, R. P. Agarwal, R. E. Parks, A. F. Lewis, L. L. Worting, and L. B. Townsend, Biochem. Pharmacol. **26,** 359 (1977).
[13] R. Wolfenden, Biochemistry **8,** 2409 (1969).
[14] R. Wolfenden, D. F. Wentworth, and G. N. Mitchell, Biochemistry **16,** 5071 (1977).
[15] R. P. Agarwal, T. Spector, and R. E. Parks, Biochem. Pharmacol. **26,** 359 (1977).
[16] S. M. Hecht, R. B. Frye, D. Werner, T. Fukui, and S. D. Hawrelak, Biochemistry **15,** 1005 (1976).
[17] J. Sepioł, Z. Kazimierczuk, and D. Shugar, Z. Naturforsch. **31 c,** 361 (1976).
[18] J. Žemlička, J. Amer. Chem. Soc. **97,** 5896 (1975).
[19] O. Makabe, N. Nakamura, and S. Umezawa, J. Antibiot. **28,** 492 (1975).
[20] G. I. Birnbaum, M. Cygler, L. Dudycz, R. Stolarski, and D. Shugar, Biochemistry **20,** 3294 (1981).