Biochemical Pharmacology, Vol. 32, No. 1, pp. 115–122, 1983.
Printed in Great Britain.

0006-2952/83/010115-08 $03.00/0

CHARACTERIZATION OF ADENOSINE DEAMINASE FROM THE MALARIAL PARASITE, *PLASMODIUM LOPHURAE*, AND ITS HOST CELL, THE DUCKLING ERYTHROCYTE

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(*Received* 5 *December* 1981; *accepted* 17 *May* 1982)

Abstract—Adenosine deaminase (adenosine aminohydrolase, EC 3.5.4.4) was purified and characterized from the malarial parasite, *Plasmodium lophurae*, and its host cell, the duck (*Anas domesticus*) erythrocyte, using chromatofocusing (Pharmacia) and adenosine affinity columns. Gel filtration of the enzymes gave molecular weights of 33,800 (*P. lophurae*) and 36,500 (duck erythrocyte); both enzymes had broad pH optima (pH 6.8 to 8.0), similar stabilities when stored as crude lysates, and like K_m values with adenosine: $2.74 \pm 0.88 \times 10^{-5}$ M (parasite) and $1.74 \pm 0.27 \times 10^{-5}$ M (erythrocyte). The *P. lophurae* adenosine deaminase had a pI of 5.37 ± 0.09, and the duck erythrocyte enzyme had a pI of 4.72 ± 0.09, as determined by chromatofocusing. The parasite enzyme exhibited a specific activity in the crude lysate that was an average 60-fold higher than that of the erythrocyte enzyme. The pattern of elution from the adenosine affinity column, as well as kinetic studies with three adenosine analogs, revealed distinct differences in the binding characteristics of the two enzymes. The *P. lophurae* adenosine deaminase was weakly retarded by the affinity column, whereas the duck erythrocyte enzyme was strongly retarded. With 9-β-D-arabinosyladenine as substrate, the K_m values were similar ($2.29 \pm 0.98 \times 10^{-4}$ M for *P. lophurae* and $1.10 \pm 0.21 \times 10^{-4}$ M for the duck erythrocyte). Erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA) was a potent inhibitor of the duck erythrocyte enzyme with 100% inhibition at 1.3 μM, whereas the parasite adenosine deaminase was not inhibited at 422 μM even when incubated for 24 hr. Inhibitor studies with coformycin, a tight-binding inhibitor, resulted in K_i values of 7.14×10^{-11} M for *P. lophurae* and 1.86×10^{-10} M for the duck erythrocyte. The molar equivalencies, E_t, and catalytic numbers, k_3, were slightly different for both enzymes. The E_t values were 2.80×10^{-10} M (*P. lophurae*) and 3.13×10^{-10} M (duck erythrocyte); the k_3 values were 5.18×10^3 min^{-1} and 4.36×10^3 min^{-1} respectively.

Intraerythrocytic malarial parasites are incapable of *de novo* purine biosynthesis [1–3] and, thus, are dependent on preformed purines [4, 5]. Mature avian and mammalian erythrocytes also lack a *de novo* purine biosynthetic pathway [6–10] and depend on exogenously supplied purines primarily derived from the liver [6]. Therefore, both the malarial parasite and its host cell, the erythrocyte, depend on purine salvage and interconversion of preformed purines.

Thus far, studies of the purine salvage pathway in a variety of species of *Plasmodium* have described only the basic kinetics of the enzymes involved in purine salvage [7–11]. A comprehensive study involving several substrate analogs and inhibitors of the parasite and host enzymes has not been carried out previously, and therefore such investigations were considered to be an essential first step toward determining differences that might be exploited for drug development. Adenosine deaminase (ADA†, adenosine aminohydrolase, EC 3.5.4.4) was examined in the present work because: (a) it is the most active of the purine salvage enzymes studied [11], (b) an extensive number of adenosine analogs are available [12–15], and (c) the enzyme plays an important role in the regulation of adenine nucleotide levels since adenosine accumulation leads to substrate inhibition of adenosine kinase [16, 17] and lowering of phosphoribosyl pyrophosphate levels [18].

MATERIALS AND METHODS

Chemicals

Adenosine, epoxy-activated Sepharose 6B, Sephadex G-100-120, Sephadex G-25-80, bovine serum albumin, blue dextran, ovalbumin, carbonic anhydrase, α-chymotrypsinogen, cytochrome *c*, imidazole, TRIZMA base, EDTA, and ara-A were obtained from the Sigma Chemical Co. (St. Louis, MO). Polybuffer 74 and Polybuffer exchanger 94 were obtained from Pharmacia Fine Chemicals (Piscataway, NJ). Coformycin was a gift from Dr. J. D. Douros, National Cancer Institute, Washington, DC. EHNA was a gift from Dr. T. Krenitsky, Burroughs Wellcome, Triangle Park, NC.

Enzyme assay

ADA was assayed by the method of Kalckar [19]. Enzyme activity was routinely assayed spectrophotometrically at 265 nm using 0.1 M Tris·HCl/0.5 mM EDTA, pH 8, with 80 μM adenosine. The

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† Abbreviations: ADA, adenosine deaminase; coformycin, 3-β-D-ribofuranosyl-6,7,8-trihydroimidazo(4,5-*d*)(1,3)diazepin-8(*R*)-ol; EHNA, erythro-9-(2-hydroxy-3-nonyl)adenine; and ara-A, 9-β-D-arabinofuranosyladenine.

assay volume was 1.0 ml. All assays were carried out at 37°. Specific activity is expressed as μmoles adenosine converted per min per mg protein. Kinetic studies were carried out using 80, 40, 20 and 10 μM adenosine. Inhibitors were used at the concentrations indicated.

Protein

Protein concentration was estimated by the method of Bradford [20] with bovine serum albumin as the standard. Protein eluting from the columns during purification was monitored as absorbance at 280 nm.

pH Optimum

Enzymatic activity was determined in the pH range 6.8 to 8.2 using 0.1 M Tris·HCl/0.5 mM EDTA buffer.

Molecular weight determination

Molecular weight was determined by gel filtration on a Sephadex G-100 column (1.5 cm × 56.3 cm) standardized with blue dextran, bovine serum albumin, ovalbumin, carbonic anhydrase, α-chymotrypsinogen and cytochrome *c*.

Adenosine affinity column preparation

An adenosine affinity column was prepared according to the method of Schrader *et al.* [21] with the following modification. The epoxy-activated Sepharose 6B (15 g) was incubated in 40 mM adenosine/0.1 M Na_2CO_3, pH 11.2 (100 ml), at 37° for 42 hr. This incubation resulted in the maximum binding of 0.115 mmole adenosine/g Sepharose 6B.

Enzyme preparation

Normal duck erythrocyte adenosine deaminase. Whole blood (10 ml) was obtained, in a heparinized syringe, by venipuncture of the jugular vein of a white Pekin duckling (Ward Duck Farm, La Puente, CA). The whole blood was then centrifuged at 550 *g* for 10 min. Plasma and buffy coat were removed by aspiration. The packed erythrocytes were washed twice with 0.85% NaCl by centrifugation (550 *g*, 10 min). The washed packed erythrocytes were lysed by diluting to 20% (v/v) with hypotonic PBS (1 mM sodium phosphate/0.085% NaCl, pH 7.4). The resulting suspension was centrifuged at 20,000 *g* for 20 min. The lysate (2.5 to 5.0 ml) was then loaded onto a Pharmacia chromatofocusing column (1.0 cm × 30.5 cm) equilibrated to pH 7.4 with 0.025 M imidazole buffer, pH 7.4. The column was developed with pH 4.0 Polybuffer 74, and 2.5 ml fractions were collected at a flow rate of 40 ml/hr. The eluate was concentrated, and the pH was adjusted to 7.4 by diafiltration with 0.1 M potassium phosphate/0.1 M NaCl, pH 7.4, in an Amicon ultrafiltration cell with a PM 10 membrane. The concentrated sample was then loaded onto an adenosine affinity column (1.0 cm × 20 cm) equilibrated with 0.1 M potassium phosphate/0.1 M NaCl, pH 7.4. The enzyme was eluted from the column with 10 mM adenosine/0.1 M potassium phosphate/0.1 M NaCl, pH 7.4. Fractions (1.0 ml) were collected at a flow rate of 26 ml/hr. The eluate was then loaded onto a Sephadex G-25 column (2.3 cm × 4.0 cm) equilibrated with 0.1 M potassium phosphate/0.1 M NaCl, pH 7.4. Fractions (1.0 ml) were collected at a flow rate of 80 ml/hr.

P. lophurae *adenosine deaminase.* On day 5 or 7 of the malaria infection with *P. lophurae* in white Pekin ducklings, heavily infected blood (parasitemia > 70%), with most parasites at the schizont stage, was obtained by venipuncture of the jugular vein into heparinized syringes. The parasites were freed from the erythrocytes by the immune lysis method of Trager *et al.* [22]. The free parasites were then stored at −70° until needed. Free parasites were lysed by freeze-thawing twice after diluting the parasites with an equal volume of distilled, deionized H_2O. The parasites were freeze-thawed by alternating between −70° and 37° for 5–10 min each. The resulting suspension was centrifuged at 20,000 *g* for 20 min. Then 1.0 to 8.0 ml of lysate was loaded onto a chromatofucusing column which was equilibrated as with the duck erythrocyte enzyme. The *P. lophurae* enzyme was purified in the same manner as the duck erythrocyte enzyme except when the enzyme was minimally retarded on the affinity column. Since 10 mM adenosine was not necessary to elute the enzyme, the Sephadex G-25 column was omitted.

Kinetic studies

The duck erythrocyte enzyme used in the present studies was purified at least 3000-fold. The *P. lophurae* enzyme was purified at least 50-fold. Kinetic studies were performed with the inhibitors, EHNA and coformycin, and the substrate analog, ara-A (Fig. 1). A 24-hr incubation at 4° of the parasite ADA with EHNA was carried out by mixing equal amounts of the enzyme (0.028 units) with EHNA (0.17 μmole) or distilled, deionized H_2O. Forty microliters of the incubation mixture was used for the assay in a final volume of 1.0 ml with 80 μM adenosine. For the coformycin study, *P. lophurae* ADA, which was not retarded by the adenosine affinity column, was further purified on a Sephadex G-100 column to ensure that all Polybuffer was

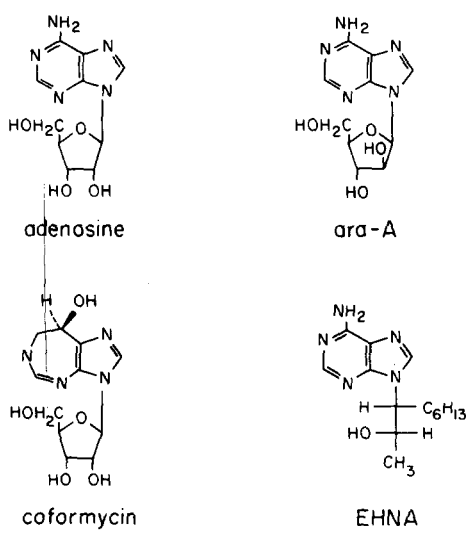

Fig. 1. Structures of some adenosine analogs.

Table 1. Purification of the adenosine deaminases of the duckling erythrocyte and the malarial parasite, *Plasmodium lophurae*

Purification step	Volume (ml)	Protein (mg)	Enzyme units (μmoles/min)	Specific activity (units/mg)	Purification factor	Yield (%)
		Duck erythrocyte				
(1) Crude lysate	2.5	278	3.2	0.011		100
(2) Chromatofocusing column	2.8	0.12	2.1	18	1500	68
(3) Adenosine affinity column	3.8	0.014	0.83	59	5100	26
(4) G-25 column	6.6	0.014	1.5	110	9200	47
		P. lophurae				
(1) Crude lysate	2.5	23.7	36	1.5		100
(2) Chromatofocusing column	2.1	0.55	11	20	13	36
(3) Adenosine affinity column						
(a) Peak 1	2.2	0.15	6.8	45	29	22
(b) Peak 2	4.7	0.012	1.8	150	98	6
(4) G-25 column	7.6	0.012	1.6	130	84	5

removed. Coformycin, a tight-binding inhibitor, was incubated at 37° with the duck erythrocyte enzyme or the parasite enzyme for 1 hr to ensure equilibrium binding. The molar absorbancy change (ΔA) for ara-A was 8600 $M^{-1}cm^{-1}$ at A_{265}. Concentrations of coformycin ($\varepsilon_{282\,nm}$ in $H_2O = 8.25 \times 10^3$), EHNA ($\varepsilon_{261\,nm}$ in $H_2O = 14.2 \times 10^3$) and ara-A ($\varepsilon_{259\,nm}$, pH 7 = 13.4×10^3) were determined spectrophotometrically [23–25].

RESULTS

Purification

The results of a typical purification are summarized in Table 1. Purification of duck erythrocyte ADA resulted in overall yields of 15–50% and purifications from 1,000 to 10,000. Occasionally the duck erythrocyte enzyme eluted with a shoulder on the peak from the chromatofocusing column (Fig. 2). This shoulder suggested the presence of another isozyme, but two distinct isozymes were never isolated. Also, the eluate from the Sephadex G-25 column occasionally showed a greater than 100% yield (Table 1). This result, which was inconsistent from purification to purification, suggested inhibition by high concentrations of adenosine and/or inosine. Both adenosine and inosine completely inhibited both the erythrocyte and *P. lophurae* ADA at a concentration of 8×10^{-4} M. Purification of *P. lophurae* ADA resulted in overall yields of 21–64% for Peak 1 and

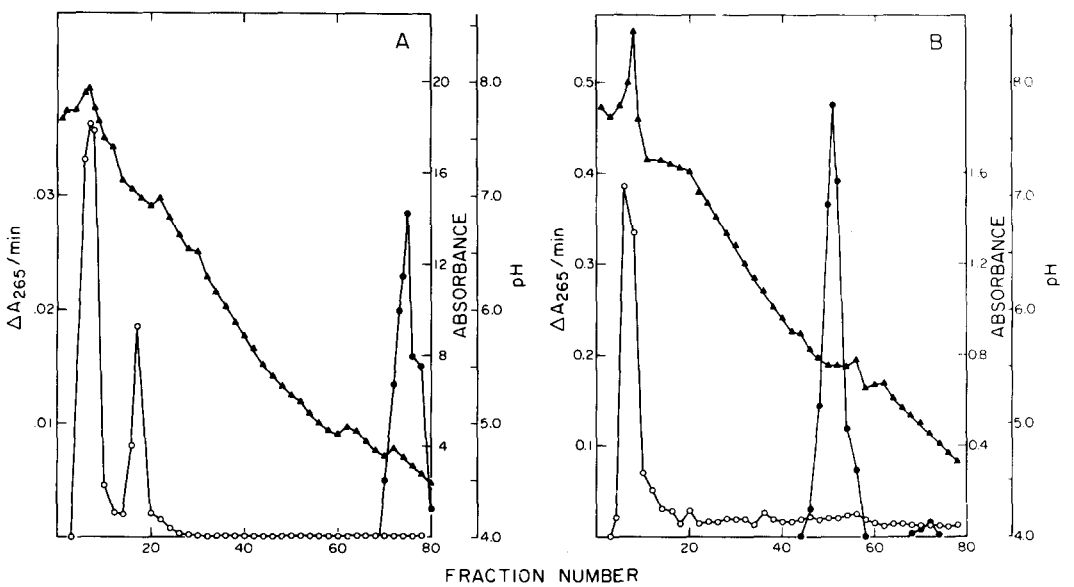

Fig. 2. Chromatofocusing of: (A) duck erythrocyte adenosine deaminase and (B) *P. lophurae* adenosine deaminase. Enzyme activity (●——●) was measured as ΔA/min at 265 nm. Protein (○——○) was measured at 280 nm, and the pH (▲——▲) was determined. The enzyme solution was loaded onto a column (1.0 cm × 30.5 cm) equilibrated with 0.025 M imidazole, pH 7.4. The column was eluted with Polybutter 74, pH 4. Fractions (2.5 ml) were collected at 40 ml/hr.

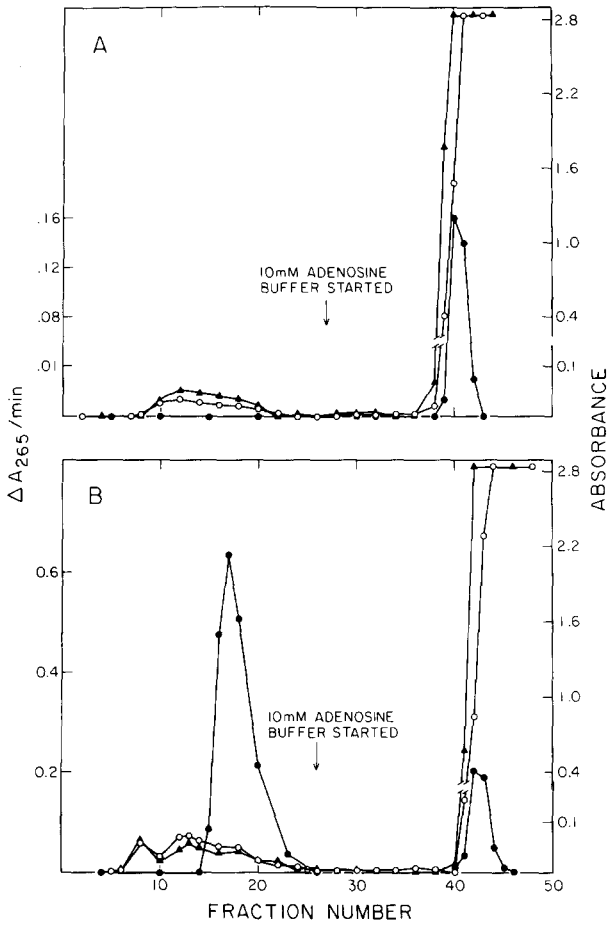

Fig. 3. Adenosine affinity of: (A) duck erythrocyte adenosine deaminase and (B) *P. lophurae* adenosine deaminase. Enzyme activity (●——●) was measured as ΔA/min at 265 nm; protein (○——○) was measured at 280 nm; and adenosine (▲——▲) was measured at 260 nm. The diafiltered enzyme was loaded onto a column (1.0 cm × 20 cm) equilibrated with 0.1 M potassium phosphate/0.1 M NaCl, pH 7.4. Unbound protein was eluted with this buffer. Bound adenosine deaminse was eluted with 0.1 M potassium phosphate/0.1 M NaCl, pH 7.4, containing 10 mM adenosine. Fractions (1.0 ml) were collected at 26 ml/hr.

2–27% for Peak 2 (Fig. 3). Purifications ranged from 29 to 140 for Peak 1 and 84 to 270 for Peak 2. The parasite enzyme also occasionally eluted from the chromatofocusing column with a shoulder on the peak (Fig. 2). However, a second isozyme was never isolated.

Differences in the erythrocyte enzyme and *P. lophurae* enzyme first became evident during purification. The parasite ADA exhibited an average 60-fold greater specific activity in the crude lysate than the erythrocyte enzyme. The enzymes eluted at different pH values on the chromatofocusing column, allowing total removal of any contaminating erythrocyte adenosine deaminase from the parasite enzyme (Fig. 2). Even more striking were the binding characteristics of the two enzymes with the adenosine affinity column (Fig. 3). The adenosine affinity column was developed by Schrader *et al.* [21] for use in the purification of human erythrocyte ADA. The human enzyme was not tightly bound to the column but eluted in a broad peak with a 3:1 partition coefficient. The duck erythrocyte enzyme exhibited a similar behavior but, in order to elute the enzyme in a minimal volume, it was found expedient to add 10 mM adenosine to the elution buffer. This buffer resulted in elution of the ADA with the adenosine front. The parasite enzyme behaved in a similar manner on an affinity column which had not been used and contained at least 0.085 mmole adenosine bound/g gel, i.e. the *P. lophurae* ADA eluted with the adenosine front. The affinity column could be used repeatedly with no apparent change in binding characteristics with the erythrocyte enzyme. If, however, the affinity gel bound less than 0.085 mmole adenosine/g gel or had been used for purification of an enzyme sample, either parasite or duck eythrocyte, only part or none of the parasite enzyme was retarded by the affinity gel. In Fig. 3B, 21% of the recovered parasite ADA was retarded by the affinity gel and eluted with the adenosine front in Peak 2. The remaining 79% of the ADA (Peak 1) eluted near the end of the unbound proteins. Further use of the same affinity gel with the parasite enzyme resulted in elution of all of the ADA near the end

Table 2. Kinetic parameters of the adenosine deaminases of the duck red cell and malarial parasite, *P. lophurae*

	N	K_m (μM)	Relative V_{max}
	Adenosine		
Duck erythrocyte	6	17.4 ± 2.7	1.0
P. lophurae	8	27.4 ± 8.8	1.0
	9-β-D-Arabinofuranosyladenine		
Duck erythrocyte	2	110 ± 21	0.35
P. lophurae	4	229 ± 98	0.20

of the unbound proteins with an approximate 2-fold purification on the column.

pH Optimum

Both the duck erythrocyte enzyme and the *P. lophurae* enzyme exhibited a broad pH optimum over the pH range 6.8 to 8.0.

Stability

Both enzymes were stable for more than 1 month when stored as crude lysates at 4° and −20° with or without 37% glycerol. The purified duck erythrocyte and parasite enzymes were stable for more than 2 months when stored with 0.5 mg/ml BSA/30% glycerol at −20°. The purified *P. lophurae* adenosine deaminase but not the erythrocyte enzyme, was unstable at 37°, losing 25% of its activity after a 4-hr incubation. The purified parasite enzyme was also unstable at 4°.

pI Determination

pI Values were determined from the chromatofocusing column. *P. lophurae* adenosine deaminase had a pI of 5.37 ± 0.09 (seven determinations) and duck erythrocyte adenosine deaminase had a pI of 4.72 ± 0.09 (six determinations).

Molecular weight determination

The duck erythrocyte enzyme and parasite enzyme were found to have similar molecular weights of 36,500 and 33,800 respectively.

Kinetic studies

Both the duck erythrocyte and *P. lophurae* enzymes were found to follow Michaelis–Menten kinetics with adenosine with K_m values of 1.74×10^{-5} M and 2.74×10^{-5} M respectively (Table 2). Ara-A was utilized as a substrate by both enzymes. K_m values were 2.29×10^{-4} M for the parasite enzyme and 1.10×10^{-4} M for duck erythrocyte adenosine deaminase. V_{max} values were 0.20 and 0.35 respectively. EHNA totally inhibited duck erythrocyte adenosine deaminase at a concentration of 1.35×10^{-6} M (Table 3). The *P. lophurae* enzyme was not inhibited after a 24-hr incubation at 4° during which the control lost 10% activity by concentrations of EHNA as high as 4.22×10^{-4} M, a concentration over 300-fold higher than was necessary to totally inhibit the erythrocyte enzyme. Kinetic data from inhibitor studies with coformycin, shown in Figs. 4 and 5, were analyzed by the methods of Ackermann and Potter [26] and Cha *et al.* [15]. From Fig. 4B, it was determined that 1 unit of adenosine deaminase/liter corresponded approximately to a concentration of 2.80×10^{-10} M for *P. lophurae* and 3.13×10^{-10} M for the duck erythrocyte since $E_t = I_t$ when $V = 0$. From the slope of Fig. 4C, $\left[\text{slope} = k_3 \frac{s}{(K_m + s)}\right]$, the value of the catalytic number, k_3, was calculated to be 5.18×10^3 min^{-1} for *P. lophurae* and 4.36×10^3 min^{-1} for the duck erythrocyte. From Fig. 5 the K_i value of coformycin was calculated to be 7.14×10^{-11} M with *P. lophurae* and 1.86×10^{-10} M with the duck erythrocyte adenosine deaminase. From this plot, 1 unit of adenosine deaminase/liter corresponded approximately to a concentration of 2.75×10^{-10} M for *P. lophurae* and 2.91×10^{-10} M for the duck erythrocyte.

DISCUSSION

EHNA (Fig. 1) was designed specifically to bind to ADA due to the characterstics of the substituent at the 9-position of the purine ring. The binding site was found to have a close spatial relationship with a methyl binding site, a hydrophilic binding site, and a large hydrophobic region [14]. In the case of the

Table 3. Inhibition parameters of the adenosine deaminase of the duck red cell and malarial parasite, *P. lophurae*

	Coformycin		
	K_i (pM)	E_t (pM)	k_3 (10^3 min^{-1})
Duck erythrocyte	186	313	4.36
P. lophurae	71.4	280	5.18
	EHNA		
	Concn (μM)	Incubation (hr)	Inhibition (%)
Duck erythrocyte	1.35	0	100
P. lophurae	13.5	0.33	0
	422	24	0

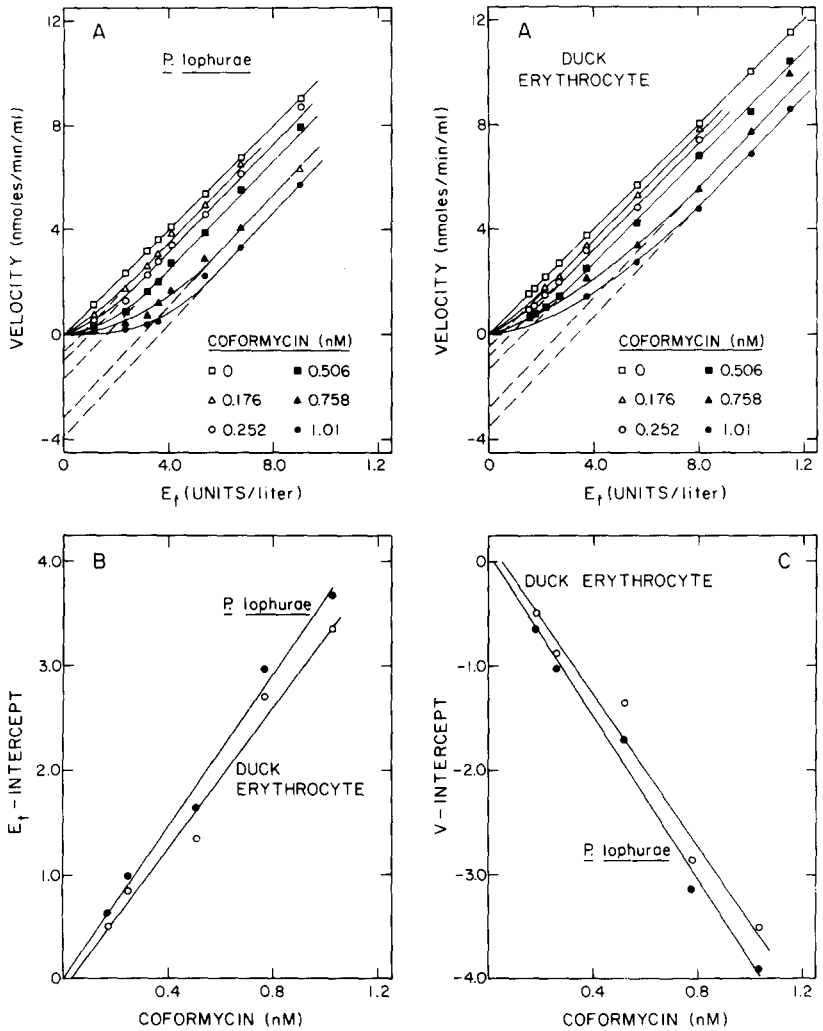

Fig. 4. Ackermann–Potter plot of adenosine deaminase from *P. lophurae* and duck erythrocyte with coformycin. Various amounts of adenosine deaminase were incubated in a total volume of 0.98 ml containing 0.1 M Tris·HCl/0.5 mM EDTA, pH 8 and various concentrations of coformycin. After incubation for 1 hr at 37°, the enzymic reaction was started by addition of 20 μl of 4 mM adenosine (final concentration, 80 μM). The reaction was followed by measuring the decrease in absorbance at 265 nm at 37° (A) plot of enzymic velocity, V (nmoles/min/ml), vs enzymic concentration (units/liter). (B) Plot of E_t (units/liter) intercept (from A) vs coformycin concentration. (C) Plot of V-intercept (from A) vs coformycin concentration.

duck erythrocyte enzyme, the binding site appears to be similar to that of calf intestinal mucosal adenosine deaminase for which EHNA was synthesized. The parasite enzyme, however, appears not to depend on this close spatial relationship of methyl, hydrophobic and hydrophilic binding sites as evidenced by the lack of inhibition with EHNA.

This difference in binding was also evident during enzyme purification. The inability of the adenosine affinity column to retard the parasite enzyme even when the duck erythrocyte ADA continued to be retarded suggested a significant difference in the characteristics of the two enzyme preparations. This unusual behavior of the parasite ADA could have been due to: (a) specific differences in the binding properties of the plasmodial and duck erythrocyte enzymes, (b) a low capacity affinity column (0.085 mmole adenosine/g gel), or (c) reduced capacity of the column resulting from the conversion of adenosine to inosine as a result of previous use of the column. Since the erythrocyte enzyme was retarded by the affinity gel even after the column was used repeatedly with both the parasite and erythrocyte ADA, it appeared that the lack of retardation with the parasite enzyme was due to its unique properties and was not a column effect.

Slight differences were also found with the substrates, adenosine and ara-A, and the tight-binding inhibitor, coformycin. The parasite enzyme bound coformycin more tightly as shown by its approximate 2.5-fold lower K_i. Since the degree of inhibition of the duck erythrocyte enzyme was constant over a 48-hr incubation period, an attempt was made to determine the K_i value after a 19-hr incubation.

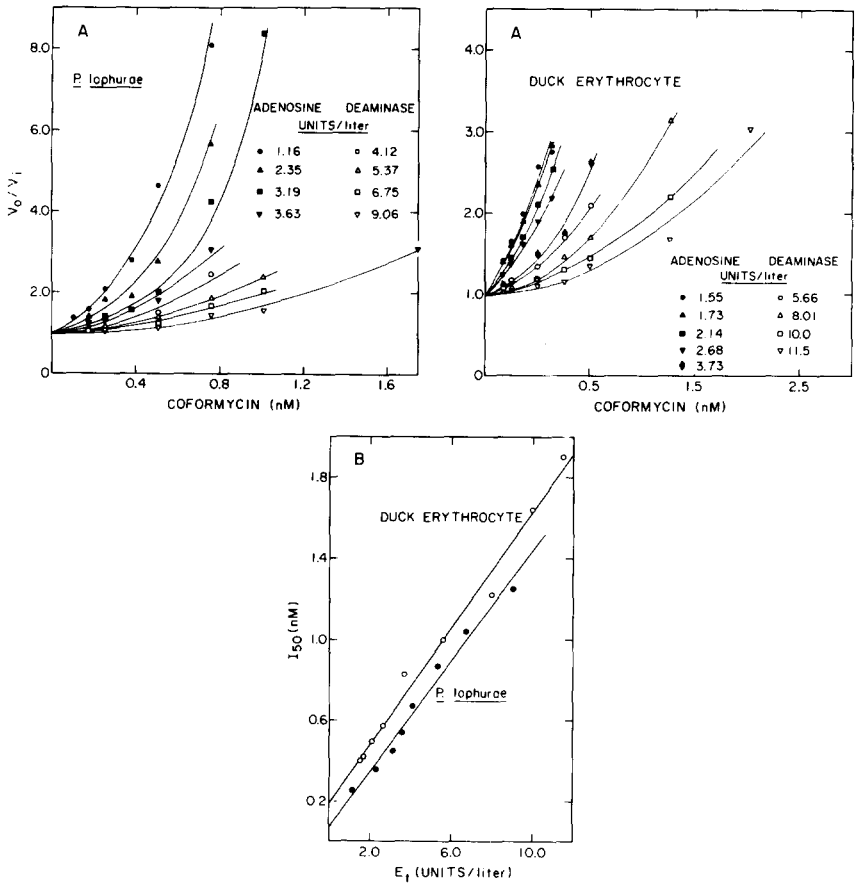

Fig. 5. Determination of I_{50} and K_i for coformycin. (A) The data of Fig. 4A were replotted as V_o/V_i vs coformycin concentration for *P. lophurae* and the duck erythrocyte adenosine deaminase. (B) Plot of I_{50} vs enzyme concentration ($I_{50} = 1/2\ E_t + K_i$).

However, analysis of the data using the I_{50} value gave a negative K_i. The observed differences in K_i values with longer incubation times could be due to slow binding of the inhibitor to the enzyme or a slow conformational change after initial binding as mentioned by Cha *et al.* [15] in their study with human erythrocyte ADA. The same behavior may occur with the parasite ADA; however, the instability of the enzyme prevented determination of the K_i value after longer periods of incubation.

Comparison of the results obtained in this study, especially (a) the 60-fold higher specific activity of the parasite ADA, (b) the inability of EHNA to inhibit the plasmodial enzyme, (c) the higher pI for *P. lophurae* ADA, and (d) the inability of the adenosine affinity column to retard the parasite ADA, point to a definitive difference between *P. lophurae* and duck erythrocyte ADA. This difference could be due to differences in the structures of the binding sites and/or the structures of the enzymes. Further study of inhibitors which are specific for the parasite enzyme could provide a basis for development of an effective antimalarial drug.

Acknowledgements—This investigation was supported by a research grant (AI 05226) from the National Institutes of Health, Allergy and Infectious Diseases. C. M. S. received fellowship support from the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases.

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