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ADENOSINE DEAMINASE FROM HUMAN ERYTHROCYTES:

PURIFICATION AND EFFECTS OF ADENOSINE ANALOGS*

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Abstract—Adenosine deaminase (adenosine aminohydrolase, EC 3.5.4.4) has been purified about 3000-fold from human erythrocytes. The molecular weight of the enzyme was estimated to be 33,000. With the partially purified erythrocytic adenosine deaminase, K_m and V_{max} values relative to adenosine were: adenosine, 25 μM, 100 per cent; formycin A, 1000 μM, 753–850 per cent; 8-aza-adenosine, 130 μM, 310 per cent; 6-chloropurine ribonucleoside, 1000 μM, 91 per cent; 2,6-diaminopurine ribonucleoside, 74 μM, 91 per cent; 2′-deoxyadenosine, 7 μM, 60 per cent; xylosyladenine, 33 μM, 62 per cent; arabinosyl adenine, 100 μM, 47 per cent; 3′-deoxyadenosine (cordycepin), 41 μM, 100 per cent; 3′-amino-3′-deoxyadenosine, 133 μM, 89 per cent; 4′-thioadenosine, 13 μM, 43 per cent; and 6-methylselenopurine ribonucleoside, 27 μM, 88 per cent. Apparent K_i values of reaction products and some adenosine analogs using adenosine as a substrate were as follows: inosine, 116 μM; 2′-deoxyinosine, 60 μM; guanosine, 140 μM; 2-fluoroadenosine, 60 μM; 2-fluorodeoxyadenosine, 19 μM; N^6-methyladenosine, 17 μM; N^1-methyladenosine, 275 μM; 6-thioguanosine, 92 μM; 6-thioinosine, 330 μM; 6-methylthioinosine, 270 μM; arabinosyl 6-thiopurine, 360 μM; and coformycin, 0·01 μM. Tubercidin (7-deaza-adenosine) and toyocamycin were devoid both of substrate and inhibitor activity. Also, N^7-methylinosine, N^7-methylguanosine and dipyridamole (Persantin®) did not inhibit the enzymic activity.

The enzyme, adenosine deaminase (adenosine aminohydrolase, EC 3.5.4.4), was first identified in human erythrocytes by Conway and Cooke in 1939 [1], and since that time it has been studied in a wide variety of microorganisms, plants and animals. Apparently homogeneous preparations of the enzyme have been obtained from a number of tissues, such as calf intestine and spleen [2–4]. An excellent review of prior investigations of the enzyme has appeared recently [5]. However, despite the importance of this enzyme in purine metabolism and its influence on the behavior of certain chemotherapeutic agents in man, adenosine deaminase from human tissues has not received due attention until recently [6–8].

A recent investigation in this laboratory [9] on the incorporation of adenosine analogs into the nucleotide pools of human erythrocytes suggested an important regulatory role for adenosine deaminase in the control of adenine nucleotide levels. Analogs, such as F-Ado‡ and tubercidin, that are not substrates for adenosine deaminase, are phosphorylated in the erythrocyte and form large amounts of the corresponding triphosphate nucleotides. On the other hand, analogs such as formycin A, that are active substrates for adenosine deaminase, do not form the nucleotides in human erythrocytes. Although a number of publications have described the interactions of a variety of purine nucleoside analogs with preparations of adenosine deaminase from calf intestine and other sources [10–25], to date no reports have appeared on the reaction of purified human erythrocytic adenosine deaminase with nucleoside analogs. For these and other reasons, a detailed examination of purified human erythrocytic adenosine deaminase was undertaken. Preliminary reports of

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‡ Abbreviations used: Ado, Adenosine; dAdo, 2′-deoxyadenosine; ara-Ade, arabinosyladenine or 9-β-D-arabinofuranosyladenine; cordycepin, 3′-deoxyadenosine; amino-3′-dAdo, 3′-amino-3′-deoxyadenosine; DAPR, 2,6-diaminopurine ribonucleoside; F-Ado, 2-fluoroadenosine; F-dAdo, 2-fluorodeoxyadenosine; formycin A, 7-amino-3-β-D-ribofuranosylpyrazolo-(4,3d) pyrimidine; PNPase, purine nucleoside phosphorylase (purine nucleoside: orthophosphate ribosyltransferase, EC 2.4.2.1); toyocamycin, 4-amino-5-cyano-7-β-D-ribofuranosylpyrrolo-(2,3d) pyrimidine; tubercidin, 7-deaza-adenosine or 4-amino-7-β-D-ribofuranosylpyrrolo-(2,3d) pyrimidine; xylo-Ade, xylosyladenine or 9-β-D-xylofuranosyl adenine; MMPR, 6-methylmercaptopurine ribonucleoside; arabinosyl-6-thiopurine, 9-β-D-arabinofuranosyl-6-thiopurine.

portions of this work have been presented [6, 7]. Subsequent to these reports, a publication appeared that describes the partial purification and some of the molecular properties of adenosine deaminase from human erythrocytes [8].

MATERIALS AND METHODS

Materials

Lactate dehydrogenase (L-lactate: NAD oxidoreductase, EC 1.1.1.27) (rabbit muscle), α-ketoglutarate, adenosine, deoxyinosine, 6-methylmercaptopurine ribonucleoside, α-chymotrypsinogen A (bovine pancreas), cytochrome c (horse heart), and cordycepin were purchased from Sigma Chemical Co.; guanosine was from P–L Biochemicals, Inc., inosine and 6-thioguanosine from Calbiochem, and 2′-deoxyadenosine from California Biochemical Research. Beef liver glutamate dehydrogenase (L-glutamate: NAD oxidoreductase, EC 1.4.1.2) in 50% glycerol was obtained from Boehringer-Mannheim Corp., and ovalbumin and creatine phosphokinase (ATP: creatine phosphotransferase, EC 2.7.3.2) (rabbit muscle) from BioRad Co. Arabinosyl 6-thiopurine and 6-thioinosine were purchased from Nutritional Biochemical Corp., Cleveland, Ohio. 2-Fluoroadenosine and 2′-deoxy-2-fluoroadenosine were gifts from Dr. J. Montgomery of the Southern Research Institute. N^6-methyladenosine was a gift from Dr. M. H. Fleysher of Roswell Park Memorial Institute and N^1-methyladenosine, N^7-methyladenosine and N^7-methylinosine were from Terra Marine Bioresearch. The tubercidin, xylo-Ade, ara-Ade, 3′-amino-3′-dAdo, 4′-thioadenosine and 8-aza-adenosine were obtained through the courtesy of Dr. Harry Wood of the Cancer Chemotherapy National Service Center. 2,6-Diaminopurine ribonucleoside and 6-methylselenopurine ribonucleoside were gifts from Dr. S.-H. Chu of this Department. Toyocamycin was kindly supplied by Royal Netherland Fermentation Industries, Ltd. and formycin A and coformycin ($C_{11}H_{16}N_4O_5$; F.W., 284) were gifts from Dr. Hamao Umezawa of the Institute of Microbial Chemistry, Tokyo. Human erythrocytes from blood from which platelets and white blood cells were removed were supplied by Dr. M. Baldini of the Memorial Hospital, Pawtucket, R. I. Calcium phosphate gel was prepared by the method of Tsuboi and Hudson [26]. Dipyridamole (Persantin®) was a product of Geigy Pharmaceuticals (Ardsley, N.Y.).

Direct spectrophotometric assay

The activity of adenosine deaminase was followed by measuring the change of optical density at 265 nm resulting from the conversion of adenosine to inosine [27]. The assay mixture in 1 ml contained potassium phosphate buffer, pH 7·4, 50 μmoles, adenosine, 0·1 μmole, and adenosine deaminase in appropriate amounts. The reaction was carried out at 30° in a Gilford recording spectrophotometer.

The molar absorbancy changes (ΔA) for the various compounds employed in these studies are as follows: adenine glycosides, $\Delta A_{265} = -8600\ M^{-1}\ cm^{-1}$ [11, 28]; 8-aza-adenosine, $\Delta A_{280} = -6800\ M^{-1}\ cm^{-1}$ [29]; Formycin A, $\Delta A_{305} = -6000\ M^{-1}\ cm^{-1}$; 2,6′-diaminopurine ribonucleoside, $\Delta A_{256} = +3900\ M^{-1}\ cm^{-1}$ [11]; dechlorination of 6-chloropurine ribonucleoside to form inosine, $\Delta A_{246} = +6300\ M^{-1}\ cm^{-1}$ [14].

In addition to the assay method described above, which was employed in all of the experiments described in this paper, a number of preliminary studies were carried out with a coupled enzymic assay similar to the method employed for the measurement of glutaminase activity [30]. In this procedure, the ammonia liberated by the adenosine deaminase reaction is measured in a coupled reaction with glutamate dehydrogenase, α-ketoglutarate and NADH. The reaction is followed by measuring the oxidation of NADH, continually, at 340 nm. This permits the direct measurement of rates of enzymic activity with inhibitors or substrates that have high absorbancy at or near 260 nm. However, the major disadvantage of this assay is that the K_m value of NH_4^+ for glutamic dehydrogenase is relatively high, and a lag period of several min is usually encountered before a constant reaction rate is achieved. Although this procedure has proved useful in the comparison of reaction velocities with a variety of substrates and inhibitors, it has limited reliability for the determination of kinetic parameters. When compared with the direct assay, this method usually gave apparent K_m values that were several-fold higher [7].

The activity of purine nucleoside phosphorylase was measured as described by Kim *et al.* [31]. Protein concentration was measured by ultraviolet absorption at 280 nm by the method of Warburg and Christian [32].

One unit of adenosine deaminase is the amount of enzyme that catalyzes the deamination of 1 μmole adenosine per min under the conditions of the direct assay. Specific activity is expressed as units/mg of protein.

Estimation of molecular weight

The molecular sieving method of Andrews [33] with the use of a Sephadex G-200 column was employed to estimate the molecular weight of erythrocytic adenosine deaminase. Lactate dehydrogenase (mol. wt 135,000), creatine phosphokinase (mol. wt 81,000), peroxidase (mol. wt 40,000), chymotrypsinogen A (mol. wt 25,000) and cytochrome c (mol. wt 12,400) were used as marker proteins. The column (2 × 40 cm) was equilibrated and eluted with 50 mM potassium phosphate buffer, pH 7·4, containing 0·1 M NaCl.

RESULTS

Purification of adenosine deaminase from human erythrocytes

Method I. In a typical purification procedure, the human erythrocytes were freed of buffy coat by wash-

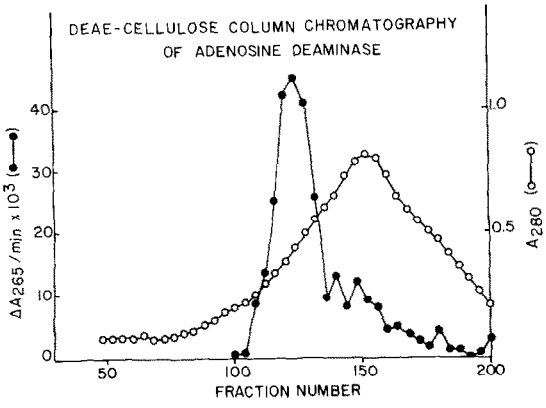


Fig. 1. DEAE-cellulose column chromatography of adenosine deaminase. The enzyme solution (about 800 ml) was loaded on a column (4·5 × 36 cm) of DEAE-cellulose (phosphate form) which had been equilibrated with 0·05 M NaCl dissolved in 0·01 M potassium phosphate buffer, pH 7·5. The enzyme was eluted with a linear gradient of 0·05–0·2 M NaCl solution (1 l. each) in 0·01 M potassium phosphate buffer. Ten-ml fractions were collected. Tubes 105 through 140 were pooled.

ing three times with 0·9% NaCl solution. The packed cells (100 ml) were hemolyzed by addition of an equal volume of distilled water. The mixture was frozen and thawed once and then was diluted with 2·5 vol. of distilled water. Cell debris and unbroken cells were removed by centrifugation. Calcium phosphate gel was added to the crude hemolysate at a concentration of 1 mg gel/5 mg of protein. The suspension was stirred for about 1 hr and the gel was removed by centrifugation at 2000 *g* for 5 min. This process removes a bulk of purine nucleoside phosphorylase from the hemolysate [34]. The supernatant fluid (about 300 ml) was poured onto a column (4·5 × 36 cm) of DEAE-cellulose (phosphate form) which had been equilibrated with 0·05 M NaCl solution in 0·01 M potassium phosphate buffer, pH 7·5. The elution was carried out with a linear gradient of NaCl solution (0·05 to 0·2 M) in 0·01 M potassium phosphate buffer, pH 7·5 (Fig. 1). The major peak of adenosine deaminase activity emerged in tubes 105 through 140, which were pooled at 4°. The minor peaks of adenosine deaminase activity (Fig. 1) were not examined further and were discarded. Solid ammonium sulfate (231 g/l.) was added to the pooled fraction to bring the concentration to 40% of saturation. The mixture was stirred overnight at 4° and the precipitate was removed by centrifugation at 16,000 *g* for 60 min. Then ammonium sulfate (186 g/l.) was added to the supernatant fluid to bring the saturation to 70%. After stirring for 6 hr at 4°, the precipitate was collected by centrifugation at 16,000 *g* for 60 min. The precipitate was dissolved in a minimal quantity (3–5 ml) of 0·1 M Tris–Cl buffer, pH 7·0. The solution was dialyzed overnight against the same buffer. The dialyzed enzyme was added to a Bio-Gel P-60 column (2 × 60 cm) equilibrated with 0·1 M potassium phosphate buffer, pH 7·4, containing 10 mM EDTA. The enzyme was eluted with the same buffer at the rate of 2·5 ml/hr (Fig. 2). Five-ml fractions were collected and tubes 7 through 15, which contained the peak of enzymic activity, were pooled. The pooled enzyme was concentrated by ultrafiltration in an Amicon cell using an XM50 membrane under nitrogen pressure (50 psi). This method yielded an approximately 3000-fold purification of adenosine deaminase activity with a recovery of about 15 per cent. The results of this procedure are summarized in Table 1.

Method II. Recently a method for the large-scale separation of several erythrocytic enzymes has been developed by this laboratory [34]. This method has been successfully used at the New England Enzyme Center, Tufts University Medical School, Boston, Mass., for the initial steps in the isolation of several enzymes from quantities of human erythrocytes in the order of 15 l. by the use of calcium phosphate gel adsorption and DEAE-cellulose chromatography. The adenosine deaminase was found in the fractions from the DEAE-cellulose column [34] that contained the guanylate kinase (ATP:GMP phosphotransferase, EC 2.7.4.8) activity and was purified further as described below.

The DEAE-cellulose column eluate that contained the adenosine deaminase was dialyzed overnight against 0·03 M Tris–acetate, pH 7·5, and then the dialyzed solution was heated at 55° ± 1° for 10 min in a water bath and cooled rapidly by immersion in an ice bath. Denatured proteins were removed by centrifugation at 16,000 *g* for 20 min. The clear supernatant fluid was loaded onto a DEAE-cellulose column (acetate form; 2 × 12 cm) equilibrated with 0·03 M Tris–acetate, pH 7·5. The column was washed with the same

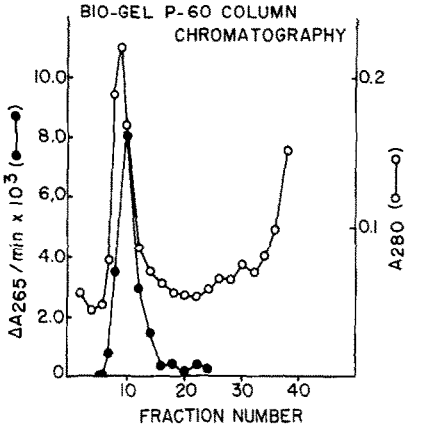


Fig. 2. Bio-Gel P-60 column chromatography of human erythrocytic adenosine deaminase. The dialyzed enzyme solution (about 5 ml) was loaded onto a Bio-Gel P-60 column (2 × 60 cm) equilibrated with 0·1 M potassium phosphate buffer, pH 7·5. The enzyme was eluted with the same buffer and 5-ml fractions were collected at the rate of 2·5 ml/hr. Fractions 7 through 15 were pooled.

Table 1. Purification of adenosine deaminase from human erythrocytes—Method I*

Fractions	Total activity (units)	Specific activity (units/mg protein)	Recovery (%)	Purification (fold)
Hemolysate (1:5)	21·25	$2·3 \times 10^{-4}$	100	1
Calcium phosphate gel treatment	17·0	$1·4 \times 10^{-4}$	80	
DEAE-cellulose column	14·0	$3·7 \times 10^{-2}$	66	161
Ammonium sulfate fractionation (40–70% saturation)	7·4	$2·7 \times 10^{-1}$	35	1174
Bio-Gel filtration and concentration	3·2	$7·1 \times 10^{-1}$	15	3087

* A typical small-scale purification of adenosine deaminase starting from about 100 ml of washed, packed human erythrocytes.

buffer until a band of red protein was completely removed. This fraction (Fraction 1, Table 2) contained about 30 per cent of the adenosine deaminase activity but did not exhibit a substantial increase in specific activity. The remaining enzyme was then eluted with 0·1 M Tris–acetate, pH 7·5, and emerged in the first 65 ml of eluent (Fraction 2, Table 2). This fraction, which contained about 40 per cent of the adenosine deaminase activity (sp. act., 0·25 unit/mg), was loaded onto a calcium phosphate gel cellulose column (2 × 12 cm) equilibrated with 0·1 M Tris–acetate, pH 7·5, in order to remove the remaining contamination of purine nucleoside phosphorylase. The adenosine deaminase which was not adsorbed on the calcium phosphate gel was collected in the first 85 ml of eluent. This adenosine deaminase preparation, which was essentially free of purine nucleoside phosphorylase activity, had a specific activity of 0·36 unit/mg of protein and was used in the kinetic studies described below. The purification procedure increased the specific activity about 7-fold above that of the fraction obtained from the New England Enzyme Center and is summarized in Table 2. About two working days are required for completion of this procedure. Since the enzyme is relatively stable, all steps may be performed at room temperature.

Properties of partially purified human erythrocytic adenosine deaminase

Molecular weight. The molecular weight of partially purified erythrocytic adenosine deaminase was estimated to be about 33,000 by use of the molecular sieving method of Andrews [33]. This value is in good agreement with the value of 30,000–35,000 reported recently by Osborne and Spencer [8]. There are reports indicating that adenosine deaminase from erythrocytes and other tissues may occur as aggregates of higher molecular weight [35, 36]. In preliminary experiments we have also observed apparent aggregation resulting in enzyme species with molecular weights of 60,000 and 180,000. However, the factors responsible

Table 2. Purification of adenosine deaminase from human erythrocytes—Method II

	Vol. (ml)	Total activity (units)	Specific activity (units/mg)	Recovery (%)	$\frac{\text{ADase*}}{\text{PNPase}}$
Fraction from enzyme center†	44	88	0·05	100	0·76
Heat denaturation (60%)	155	85	0·08	97	0·76
DEAE-cellulose column					
Fraction 1	35	23	0·11	27	2·1
Fraction 2‡	65	33	0·25	39	0·9
Calcium phosphate gel cellulose column	85	31	0·36	94§	>1000‖

* Ratio of units of adenosine deaminase (ADase) to the units of purine nucleoside phosphorylase (PNPase).
† Guanylate kinase fraction containing adenosine deaminase as described in the text.
‡ This fraction was carried through the next step.
§ On the basis of Fraction 2 from the preceding step.
‖ No PNPase activity detected with 50 times the quantity of adenosine deaminase employed in routine assays.

Table 3. Kinetic constants of substrates for adenosine deaminase

	Human erythrocytic enzyme		Calf intestinal enzyme*	
	K_m (μM)	Relative V_{max}	K_m (μM)	Relative V_{max}
Modification of ring structure				
Adenosine	25	100	35	100 [11–15]
Formycin A	1,000†	750–850	57,000†	[37]
8-Aza-adenosine	130	310	96–125	217–457 [10, 14]
Tubercidin	‡	‡		
Toyocamycin	‡	‡		
Substitutions on purine ring				
6-Chloropurine ribonucleoside	1000	91	250–700	21–40 [11, 13, 14]
2,6-Diaminopurine ribonucleoside	74	91	34	25 [13]
2-Fluoroadenosine	‡	‡		
2-Fluorodeoxyadenosine	‡	‡		
6-Methylselenopurine ribonucleoside	27	88		
9-Substituted adenine derivatives				
2′-Deoxyadenosine	7	60	22	93 [14]
Xylosyladenine	33	62	19–100	17–57 [14, 15]
Arabinosyladenine	100	47	77–140	18–25 [14, 15]
3′-Deoxyadenosine	41	110	25–55	52 [13, 15]
3′-Amino-3′-deoxyadenosine	133	89	167	101 [14]
4′-Thioadenosine	13	43	33	73 [14]

* Literature values; references are given in brackets.

† K_m values are so high that an exact evaluation becomes difficult.

‡ No activity detected during 10 min of incubation with 50 times the quantity of adenosine deaminase employed normally in the glutamate dehydrogenase coupled assay.

for the aggregation phenomenon have not yet been evaluated.

The erythrocytic enzyme is stable to storage in the frozen state and has a broad pH optimum ranging from pH 6 to 8, a property shared by adenosine deaminase from other sources [2, 4, 11, 23].

Substrate specificity. Table 3 presents kinetic parameters of adenosine and a number of purine nucleoside analogs with partially purified human erythrocytic adenosine deaminase. Figure 3 shows the structures of some of these compounds. Among the compounds included are those with modifications in the structure of the purine ring, in the substituents on the purine ring and in the carbohydrate moiety. Alterations in the purine ring resulted in striking changes in substrate activity. The compounds, formycin A and 8-aza-adenosine, which are modified in the imidazole portion of the purine ring, have about 40- and 4-fold higher K_m values with the enzyme but were deaminated about eight and three times faster than Ado respectively. On the other hand, compounds such as tubercidin and toyocamycin, in which the N^7 of the imidazole ring is replaced by a carbon atom, are totally devoid of activity either as substrates or inhibitors of the enzyme.

The analogs, 6-chloropurine ribonucleoside and DAPR, are both substrates for the erythrocytic enzyme with V_{max} values comparable to that of Ado, but with higher K_m values. The fact that 6-chloropurine ribonucleoside has a similar V_{max} but a 4-fold higher K_m than Ado suggests that the amino group of Ado plays a significant role in the binding of the substrate to the enzyme. Of interest is the observation that F-Ado and F-dAdo, although inhibitors of the enzyme (Table 4), were devoid of substrate activity under the conditions of assay.

Although changes in the carbohydrate moiety of Ado have moderate effects on the V_{max} of the enzymic reaction, more striking effects are observed in the K_m values. Of particular interest is the observation that the K_m of dAdo is 3- to 5-fold lower than that of Ado. On the other hand, the K_m value of ara-Ade is about four

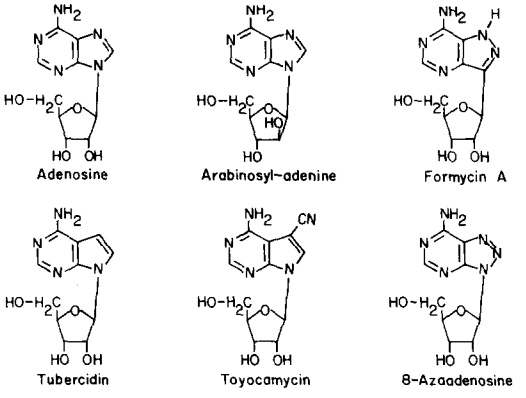


Fig. 3. Structures of some adenosine analogs.

Table 4. Inhibitors of human erythrocytic adenosine deaminase

Inhibitors	K_i (μM)
Inosine	116
2'-Deoxyinosine	60
Guanosine	140
2-Fluoroadenosine	60
2-Fluorodeoxyadenosine	19
N^6-methyladenosine*	17
N^1-methyladenosine*	275
N^7-methylinosine	†
N^7-methylguanosine	†
6-Thioguanosine	92
6-Thioinosine	330
6-Methylthioinosine*	270
Arabinosyl 6-thiopurine	360
Tubercidin	†
Toyocamycin	†
Dipyridamole (Persantin®)	†
Coformycin	0·01‡

* Weak substrate activity was detected with N^6-methyladenosine (about 0·5% of the reaction velocity seen with adenosine) in a special shift assay (ΔA = approx. -12200 M^{-1} cm^{-1}). N^1-methyl adenosine was not tested for its substrate activity. No spectral shift was detected when methylthioinosine was incubated with a 50-fold excess of adenosine deaminase.

† No inhibition was observed when these compounds were used at 1000 μM concentrations. Dipyridamole (Persantin®) was used at the concentration of 100 μM.

‡ Nature of inhibition is under study.

times greater than that of Ado, which suggests a significant role for the 2'-carbon and its substituents in substrate binding. A surprising finding is that 6-methylselenopurine ribonucleoside is a substrate with kinetic parameters comparable to those of Ado. By contrast, no substrate activity was detected with the related analog, MMPR. This unexpected observation is being studied further. Also presented in Table 3, for purposes of comparison, are literature values of K_m and V_{max} for certain of these substrates with the calf intestinal enzyme.

Inhibitors of adenosine deaminase. Table 4 presents the apparent inhibition constants (K_i) for certain adenosine analogs. All of the compounds described yielded patterns of competitive inhibition in double reciprocal plots when Ado was used as a substrate. The substitution of a fluorine atom in the 2-position of Ado or dAdo caused a complete loss of substrate activity (Table 3), while the analogs became good inhibitors of the enzyme. Of interest is the finding that the K_i value of F-dAdo is about 3-fold lower than that of F-Ado, which is in agreement with the observation (Table 3) that the K_m value of dAdo is about 3-fold lower than that of Ado. Of the other analogs presented in Table 4, N^6-methyladenosine was a relatively potent competitive inhibitor, with a K_i value of 17 μM. Only weak substrate activity, about 0·5 per cent of the reaction velocity with Ado, occurred with N^6-methyladenosine. It is also of interest that the 7-deaza-adenosine analogs, tubercidin and toyocamycin, were devoid of inhibitory activity. Also, the coronary vasodilator, dipyridamole (Persantin®), which is believed to act by increasing Ado concentrations, did not inhibit erythrocytic adenosine deaminase at a concentration of 100 μM.

In contrast to prior reports [8, 38], which failed to detect inhibition by inosine of adenosine deaminase from human erythrocytes and calf intestine, clear-cut competitive inhibition was seen with the reaction products, inosine, deoxyinosine and guanosine, in the present study.

The antibiotic, coformycin, a potent inhibitor of adenosine deaminase [39, 40] whose chemical structure has not yet been described*, was also tested as an inhibitor of the partially purified human erythrocytic enzyme. As with the enzymes from other sources, coformycin was found to be a powerful inhibitor of erythrocytic adenosine deaminase. Because of the unusually strong inhibition caused by this compound, it has not been possible by the application of standard procedures for the study of enzyme inhibitors to determine with precision the K_i value. However, a K_i value of about 0·01 μM was estimated. The inhibitory behavior of coformycin will be subjected to further detailed study with the application of new methods for the examination of tight-binding enzyme inhibitors currently under development in this laboratory.

DISCUSSION

With the application of relatively simple isolation procedures and the assistance of the Enzyme Center of Tufts University School of Medicine, it has been possible to purify human erythrocytic adenosine deaminase several thousand-fold in quantities that were sufficient for the present study. However, the specific activities of the best preparations were still below 1 unit/mg of protein, which suggests that further purification of 50-fold or greater may be necessary to achieve homogeneity. Although the enzyme was only partially purified, no evidence was obtained of contamination by other enzymes that would interfere with the adenosine deaminase assay. Since the activity of purine nucleoside phosphorylase in human erythrocytes is approximately 100-fold greater than that of adenosine deaminase, and since the presence of this enzyme might significantly affect kinetic studies by the removal of products and by phosphorolysis of potential inhibitors etc., it was especially important to eliminate all traces of this enzyme. This was readily accomplished by passage of the final preparation of adenosine deaminase through a calcium phosphate gel column. Since the activity of adenosine deaminase is relatively low in human erythrocytes, this cell does not provide an ideal source for the isolation of this enzyme. However, if, in the future, it becomes desirable to isolate homo-

* See Addendum.

geneous preparations of adenosine deaminase from a human source, erythrocytes and the methods described in this paper may prove useful for the initial stages of purification.

No qualitative differences were seen when the reactivities of various purine analog nucleosides with human erythrocytic and calf intestinal adenosine deaminases were compared, i.e. similar substrate and inhibitory activities were observed with both enzymes. However, quantitative differences occur in the kinetic parameters of the two enzymes. With both enzymes, the substituents on the 2'-carbon of the pentose moiety played a significant role. When the hydroxyl group of Ado was replaced by a hydrogen atom, as in dAdo, a decrease in the K_m values was seen. On the other hand, inversion of the 2'-hydroxyl group, as in ara-Ade, resulted in an approximately 4-fold increase in the K_m values. Especially intriguing is the influence of the imidazole ring on the deamination reaction as seen when one compares Ado with 8-aza-adenosine, formycin A, tubercidin (7-deaza-adenosine) and toyocamycin. When N-7 is replaced by a carbon atom, as in tubercidin, the molecule loses its capacity to bind with the active site of the enzyme. On the other hand, replacement of C-8 by a nitrogen atom, as in 8-aza-adenosine and formycin A, markedly enhances the deamination rate, which suggests that the electron-withdrawing properties of the diazo bond between N-7 and N-8 makes the C-6 more electro-positive, thereby potentiating a nucleophilic displacement reaction. It has been proposed by Bär and Drummond [41] and by Wolfenden [42] that the adenosine deaminase reaction involves the formation of a tetrahedral intermediate at the C-6 position of the purine ring involving the enzyme or enzyme-bound water. This mechanism suggests the formation of a transient covalent bond between the enzyme and the substrate. Our observations with human erythrocytic adenosine deaminase are consistent with this proposed mechanism. However, still unexplained is the finding that when the hydrogen on C-2 of adenosine is replaced by a fluorine atom, as in F-Ado and F-dAdo, enzyme-catalyzed deamination reactions were not detected, despite the fact that these analogs are capable of binding to the active site since they are competitive inhibitors with K_i values of the same order of magnitude as the K_m values of adenosine and 2'-deoxyadenosine. This suggests that substitution on C-2 by a fluorine atom strengthens the bond between the amino group and C-6. Since the van der Waals radii of fluorine (1·35 Å) and hydrogen atoms (1·20 Å) are similar, it is not surprising that the presence of fluorine on C-2 does not impede binding. The fact that F-Ado is not a substrate for erythrocytic adenosine deaminase has made it a useful tool for the study of Ado metabolism in animal tissues [9, 43, 44].

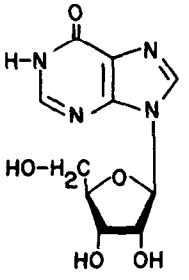
Recent investigations in this laboratory of the incorporation of Ado and its analogs into the nucleotide pools of human erythrocytes [9] have suggested an important physiological role for adenosine deaminase in the regulation of adenosine metabolism. Incubation of erythrocytes with adenosine or analogs that are good substrates for adenosine deaminase did not significantly affect the size or the pattern of the adenine nucleotide pools. For example, IMP, rather than adenine nucleotides, was formed from Ado, and no new nucleotides were detected when erythrocytes were incubated with formycin A. On the other hand, analogs such as tubercidin, toyocamycin and F-Ado, which are not substrates for adenosine deaminase, were phosphorylated, presumably by the enzyme adenosine kinase, leading ultimately to the formation of the analog triphosphate nucleotides. The generation of 2F-ATP from F-Ado was especially marked, and during 2 hr of incubation, concentrations of 2F-ATP accumulated that exceeded the normal ATP level of the erythrocyte by several-fold [9]. The hypothesis was offered that the relative activities and K_m values of adenosine deaminase and adenosine kinase play a key role in determining whether the Ado that enters the erythrocyte will be salvaged by phosphorylation to adenine nucleotides or degraded by the formation of inosine. Although the activity and K_m values of human erythrocytic adenosine kinase have not been determined precisely, it appears that the activity of adenosine deaminase (0·2 unit/ml of cells) is about 8-fold greater than that of adenosine kinase (0·027 unit/ml of cells) [9]. The K_m of Ado with adenosine deaminase is about 25 μM and, by comparison with adenosine kinases in other animal tissues, it seems likely that the K_m value of erythrocytic adenosine kinase is of the order of 1 μM, i.e. about 25-fold lower. If these assumptions are correct, the degradation reaction (deaminase) would predominate when Ado enters the erythrocyte in relatively high concentration, as would occur during tissue breakdown. However, when Ado enters the erythrocyte in low concentrations, as might be expected under normal physiological conditions, it would be preferentially salvaged through the kinase reaction. The observation that coformycin is an extremely powerful inhibitor of erythrocytic adenosine deaminase offers an opportunity to test the hypothesis. Thus, in the presence of coformycin, Ado should form AMP rather than inosine, and should enter readily and increase substantially the adenine nucleotide pools. However, one cannot yet rule out possible intervention by the enzyme, 5'-AMP deaminase. Also, in the presence of coformycin, analogs such as formycin A might be converted to nucleotide by erythrocytes. Recently the usefulness of coformycin as a biochemical tool was demonstrated in a study of the metabolism of dAdo in several animal tissues [40].

The importance of erythrocytic adenosine deaminase in whole body purine metabolism and in modifying the action of purine nucleoside antimetabolites of potential chemotherapeutic interest may be appreciated when one considers that the activity is approximately 0·2 unit/ml of human erythrocytes. If one assumes that the adult human has an erythrocytic mass of about 2·5 l., there is sufficient adenosine deaminase in the erythrocytes of the body to deaminate ap-

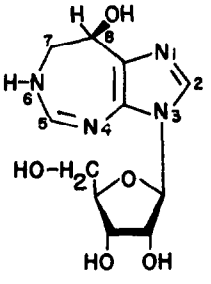
proximately 135 mg Ado/min or about 190 g/day. The coronary vasodilator, dipyridamole (Persantin®), is believed to act by inhibiting the transport of Ado into cells, thus causing an increase in Ado levels in the blood [45]. It seems possible that inhibitors of adenosine deaminase might also elevate blood Ado levels, resulting in clinically useful vasodilatation. A number of adenosine analogs, such as ara-Ade, tubercidin, cordycepin, have shown promise as antitumor, antiviral or antiparasitic agents [44, 46–49]. Since deamination of adenosine analogs often renders them inactive, the possibility that they might be deaminated in the erythrocyte before reaching the desired site of action is of obvious chemotherapeutic interest and importance. Some studies of this question have been performed with intact erythrocytes [50], but not previously with the isolated human erythrocytic enzyme. A recent discovery that brings adenosine deaminase to the forefront of interest in immunology and human genetics is the finding that several patients with a combined immunological deficiency syndrome are lacking in adenosine deaminase in their erythrocytes and leukocytes [51]. These patients, in addition to bony abnormalities, display marked defects in the functioning of both T and B lymphocytes. This discovery suggests that adenosine deaminase plays a crucial role in the behavior of lymphocytes and that the enzyme might represent a logical target for the design of new immunosuppressive agents. Of course, a highly intriguing and as yet unanswered question is: Why is a genetic deficit in adenosine deaminase associated with such a profound and life-threatening defect in the normal functioning of lymphocytes?

ADDENDUM

Subsequent to the submission of this manuscript, two papers appeared in which the synthesis and structure of coformycin were described [M. Ohno, N. Yagisawa, S. Shibahara, S. Kondo, K. Maeda and H. Umezawa, *J. Am. chem. Soc.*, **96**, 4326 (1974); H. Nakamura, G. Koyama, Y. Iitaka, M. Ohno, N. Yagisawa, S. Kondo, K. Maeda and H. Umezawa, *J. Am. chem. Soc.*, **96**, 4327 (1974)]. The structure has been identified as 3-β-D-ribofuranosyl-6,7,8-trihydroimidazo[4,5-d][1,3]diazepin-8(R)-ol. Interestingly, coformycin is a structural analog of inosine that has a 7-membered ring in place of the 6-membered pyrimidine ring of the purine base. The normal keto-enol tautomerism of inosine is interrupted and the 7-membered ring takes a puckered form. The structures of coformycin and of inosine are as follows:



Inosine



Coformycin

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