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Purification, characterization and radioimmunoassay of adenosine deaminase from human leukaemic granulocytes

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Adenosine deaminase was purified 3038-fold to apparent homogeneity from human leukaemic granulocytes by adenosine affinity chromatography. The purified enzyme has a specific activity of 486 μmol/min per mg of protein at 35°C. It exhibits a single band when subjected to sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, non-denaturing polyacrylamide-gel electrophoresis and isoelectric focusing. The pI is 4.4. The enzyme is a monomeric protein of molecular weight 44000. Both electrophoretic behaviour and molecular weight differ from those of the low-molecular-weight adenosine deaminase purified from human erythrocytes. Its amino acid composition is reported. Tests with periodic acid–Schiff reagent for associated carbohydrate are negative. Of the large group of physiological compounds tested as potential effectors, none has a significant effect. The enzyme is specific for adenosine and deoxyadenosine, with K_m values of 48 μM and 34 μM respectively. There are no significant differences in enzyme function on the two substrates. *erythro*-9-(2-Hydroxy-non-3-yl)adenine is a competitive inhibitor, with K_i 15 nM. Deoxycoformycin inhibits deamination of both adenosine and deoxyadenosine, with an apparent K_i of 60–90 pM. A specific antibody was developed against the purified enzyme, and a sensitive radioimmunoassay for adenosine deaminase protein is described.

Adenosine deaminase (adenosine aminohydrolase, EC 3.5.4.4) catalyses the irreversible hydrolytic deamination of adenosine to inosine and ammonia. Hereditary absence of adenosine deaminase activity in man causes lymphopenia and severe immunodeficiency disease (Giblett *et al.*, 1972; Polmar, 1980). Potent inhibitors of the enzyme are lymphocytotoxic and may have a role in the chemotherapy of human lymphoid malignancies (Smyth, 1979; Siaw *et al.*, 1980). The relationship between hereditary or pharmacologically induced absence of adenosine deaminase activity and selective lymphopenia is not entirely understood, but abnormal metabolism of deoxynucleosides and deoxynucleotides has been implicated (Coleman *et al.*, 1978; Cohen *et al.*, 1978; Donofrio *et al.*, 1978; Polmar, 1980). Large amounts of dATP accumulate in erythrocytes and lymphocytes of adenosine deaminase-deficient patients. The gross perturbation of deoxynucleoside metabolism was unexpected, when first discovered, because adenosine deaminase had generally been thought to participate largely in ribonucleoside salvage and catabolism.

Adenosine deaminase from human tissues exhibits heterogeneity in size of molecular species and in electrophoretic mobility of species of the same size. The enzyme has been purified to apparent homogeneity from human erythrocytes and found to be a monomeric protein with a molecular weight of 36000–38000 (Schrader *et al.*, 1976; Dadonna & Kelley, 1977). The purified proteins migrated as a single band when subjected to SDS/polyacrylamide-gel electrophoresis, but exhibited three or four bands when subjected to non-denaturing polyacrylamide-gel electrophoresis, agarose-gel electrophoresis, or ion-exchange chromatography. The catalytic activity of all forms of human adenosine deaminase appears to reside in a polypeptide coded for at a single genetic locus (Hirschhorn *et al.*, 1973) and the multiple forms arise through complex-formation or secondary modification (Harris, 1975; Hirschhorn, 1975; Schrader & Stacy, 1977; Swallow *et al.*, 1977).

In the present paper, we describe the purification

Abbreviation used: SDS, sodium dodecyl sulphate.

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of adenosine deaminase from human leukaemic granulocytes to apparent homogeneity by affinity chromatography. The purified protein differs from forms found in the erythrocyte. It has a molecular weight of 44000 and exhibits a single band when subjected to SDS/polyacrylamide-gel electrophoresis, non-denaturing polyacrylamide-gel electrophoresis and isoelectric focusing. This purified enzyme has been used to examine the kinetics and specificity for substrates and inhibitors. These have been examined previously with partially purified enzyme of low specific activity from erythrocytes (Osborne & Spencer, 1973; Agarwal *et al.*, 1975). Special care was taken to examine enzyme functions with deoxyadenosine as substrate, because hereditary absence of adenosine deaminase affects deoxynucleoside metabolism more than ribonucleoside metabolism in man. A specific antibody was prepared against the purified enzyme and used to develop a sensitive generalized radioimmunoassay for the adenosine deaminase protein.

Materials and methods

Chemicals

erythro-9-(2-Hydroxynon-3-yl)adenine was purchased from Burroughs Wellcome Co., Research Triangle Park, NC, U.S.A. Deoxycoformycin was generously given by Parke, Davis and Co., Detroit, MI, U.S.A. Protein standards, purine and pyrimidine bases, nucleosides and nucleotides were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Enzymobead radioiodination reagent, Bio-Gel P-6 (200–400 mesh) and gel-electrophoresis materials were obtained from Bio-Rad Laboratories, Richmond, CA, U.S.A. IgGsorb was purchased from The Enzyme Center, Boston, MA, U.S.A. DEAE-Sephadex A-50 anion-exchange resin, Sephadex G-100 and epoxy-activated Sepharose 6B were purchased from Pharmacia Fine Chemicals, Piscataway, NJ, U.S.A. [8-^{14}C]Adenosine, deoxy-[8-^{14}C]adenosine, [U-^{14}C]AMP and [8-^{14}C]adenine were purchased from New England Nuclear, Boston, MA, U.S.A. [U-^{14}C]Guanosine, deoxy[U-^{3}H]-guanosine, [U-^{14}C]cytidine, deoxy[U-^{14}C]cytidine and Na^{125}I (carrier free) were purchased from Amersham, Chicago, IL, U.S.A.

Adenosine deaminase assay

The assay for adenosine deaminase activity was the radiochemical assay described previously (Coleman & Hutton, 1975) with either [^{14}C]adenosine or deoxy[^{14}C]adenosine as substrate.

During purification and characterization, bovine serum albumin was included at 0.5 mg/ml to stabilize the enzyme. One unit of adenosine deaminase activity is defined as the amount of enzyme required to produce 1 μmol of deaminated product/min at 35°C. Several other compounds were examined as potential substrates by substitution in the normal assay for adenosine or deoxyadenosine. When chromatographed on Whatman DE-81 paper with 1 mM-ammonium formate as solvent, adenosine, inosine, cytidine, uridine, guanosine, xanthosine, adenine and hypoxanthine had R_F values of 0.55, 0.14, 0.82, 0.31 0.12, 0.01, 0.04 and 0.12 respectively. Deoxyribonucleosides had R_F values very similar to those of the corresponding ribonucleosides. When AMP was tested as substrate, the reaction samples were subjected to ascending chromatography on Whatman no. 1 paper with a solvent mixture of propan-2-ol/water/conc. HCl (325:92:83, by vol.). AMP and IMP had R_F values of 0.53 and 0.41 in this system.

Protein determination

This was done by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard.

Enzyme purification

The purification scheme is based on the method of Schrader *et al.* (1976), with significant alterations because of the difference in cell type chosen as crude starting material. The specific activity of our initial crude extract was 100 times higher and the volume 10 times lower than those of their crude haemolysate. Consequently their initial batch-binding to and elution from DEAE-Sephadex was omitted from our procedure. A single DEAE-Sephadex column step with elution by a linear NaCl gradient was introduced. The procedure was carried out at 4°C.

A 650ml pellet of frozen leukaemic cells, obtained by leukapheresis of a patient with chronic myelogenous leukaemia in blast crisis (76% blasts), was suspended in 500ml of 50mM-potassium phosphate (pH 7.2)/0.1M-β-mercaptoethanol (buffer I). The suspension was divided into 40ml batches, which were sonicated in three 20s bursts with an Artek Sonic Dismembrator through a micro tip at 40W setting with cooling between bursts. The samples were then centrifuged at 27000 *g* for 1h. The supernatants were decanted and the pellets discarded. The 940ml of combined supernatant was dialysed against 14 litres of 1mM-β-mercaptoethanol twice for 8h and against 14 litres of 10mM-sodium acetate (pH 6.4)/1mM-β-mercaptoethanol (buffer II) for 3h. The flocculent precipitate that appeared during dialysis was removed by centrifugation at 30000 *g* for 30min. The extract was then combined with 1800ml of DEAE-Sephadex A-50 suspended in 1200ml of buffer II (the gel had been previously swollen in buffer II). The gel was stirred with the dialysed extract for 5h. The supernatant was removed by suction filtration in a Buchner funnel (fraction I) and the gel was washed with 3 litres of buffer II giving fraction II. The gel

was suspended in buffer II, poured into a column (9cm × 30cm), and the excess buffer was then removed (fraction III). The adenosine deaminase activity was eluted with a linear gradient (4 litres) of 0–0.2M-NaCl in buffer II (gradient I) and the eluate was collected as 200 20ml fractions. The activity was eluted as a broad peak in fractions 100–190. A second linear gradient (1.6 litres) of 0.2–1.0M-NaCl in buffer II (gradient II) eluted only a small amount of additional enzyme activity. Of the adenosine deaminase activity in the initial extract 3% was recovered in fraction I, 1% in fraction II, 0.1% in fraction III, 64% in gradient I and 2% in gradient II. Column fractions 115–185 (representing 62% of the initial activity) from gradient I were combined and concentrated to 160ml by ultrafiltration through an Amicon PM-10 membrane.

Chromatography on Sephadex G-100 and the adenosine–Sepharose affinity column were as described by Schrader *et al.* (1976). The steps of enzyme purification are shown in Table 1. The enzyme was stable when stored at 3mg/ml at −20°C for at least 18 months.

Gel electrophoresis and isoelectric focusing

The basic methods for SDS/polyacrylamide-gel electrophoresis (Weber & Osborn, 1969; Laemmli, 1970), non-denaturing polyacrylamide-gel electrophoresis (Hedrick & Smith, 1968) and isoelectric focusing in our laboratories have been described (Deibel & Coleman, 1979). Enzyme activity in gel slices was determined by elution with 0.4ml of buffer I containing 50mM-NaCl and 20% glycerol (v/v) at 4°C for 16h and then assaying the eluate.

Kinetic analysis

Michaelis constants and maximum velocities in the kinetic analysis of purified adenosine deaminase were determined by the curve-fitting methods of Wilkinson (1961) and Cleland (1967). All reactions examined in the kinetic analyses were linear in the time span studied. The analysis of deoxycoformycin inhibition of adenosine deaminase was performed by the method for examining tight-binding inhibitors described by Agarwal *et al.* (1979). This procedure allows for lowering of the inhibitor concentration owing to formation of enzyme–inhibitor complex.

Amino acid analysis

These were performed on a Beckman model 119C amino acid analyser and on a Durrum Analyzer model D-500. Identical protein samples were hydrolysed in 6M-HCl at 110°C for either 24h (two samples) or 48h (one sample), and the results were averaged. Phenol was added to each sample before acid hydrolysis. Tryptophan and cysteine were not determined.

Radioimmunoassay

Antiserum to purified human adenosine deaminase was raised in a goat and immunoglobulin G was prepared (Heide & Schwick, 1978). Radioiodination of purified human adenosine deaminase was carried out by enzymic iodination with lactoperoxidase and glucose oxidase (Marchalonis, 1969; Hubbard & Cohn, 1972) in the form of Enzymobead Reagent. The iodinated protein was separated from ^{125}I on a Bio-Gel P-6 column (1cm × 25cm). The labelled enzyme was stable for at least 3 weeks and had a minimum specific radioactivity of 25Ci/g. The radioimmunoassay was carried out at 4°C in a 1.5ml polypropylene micro centrifuge tube. All components of the assay were diluted with 50mM-potassium phosphate, pH7.2, containing bovine serum albumin (1mg/ml) and 1mM-β-mercaptoethanol (buffer A). Immunoglobulin G was diluted to precipitate 30–40% of the ^{125}I-labelled adenosine deaminase in the assay in the absence of unlabelled enzyme. In the normal assay, 25μl of diluted immunoglobulin G was mixed with 50μl of buffer A containing 0.03–100ng of unlabelled purified human adenosine deaminase (for standard curve), an appropriate dilution of sample cell extract, or buffer A with no addition (control). The samples were mixed and incubated at 4°C for 16h. ^{125}I-labelled adenosine deaminase (25μl; 15000c.p.m.) was added to each tube and samples were further incubated at 4°C for 16h. Then 100μl of a 10% suspension of IgGsorb (heat-killed freeze-dried *Staphylococcus aureus* containing Protein A) was added to each sample, and they were incubated at room temperature for 15min and centrifuged at 8000*g* for 2min. The resulting pellets were washed twice with buffer A and precipitated ^{125}I radioactivity was determined by gamma counting. The IgGsorb bound 2–3% of the ^{125}I radioactivity in the assay in the absence of immunoglobulin G. The standard curve for binding competition was determined between unlabelled purified human enzyme and ^{125}I-labelled enzyme. Data for both the standard curve and cell extracts were analysed by the modified logit–log transformation (Rodbard *et al.*, 1969). The least detectable dose (that sample amount statistically different from zero) was taken where the lower 95% confidence limit of the radioactivity bound in the absence of unlabelled enzyme crosses the extrapolated standard line. The least detectable dose determined in this manner was 0.1ng in a 50μl sample, or 2ng/ml.

Cell extracts for the radioimmunoassay were prepared at 0.4×10^8–1.0×10^8 cells/ml in 0.1–0.5ml of buffer A. Cell samples were subjected to sonic disruption, as described under 'Enzyme purification', and cell debris was pelleted by centrifugation at 165000*g* for 10min. Samples were normally assayed at a number of dilutions.

Results

The purification of adenosine deaminase from human leukaemic granulocytes is summarized in Table 1. After greater than 3000-fold purification with an overall yield of 52%, 7.3 mg of purified enzyme was obtained. The three-step purification process resulted in an enzyme with a specific catalytic activity (with adenosine as substrate) of 486 units (μmol/min) per mg of protein at 35°C. The final affinity-chromatography step resulted in an 84-fold purification with 91% yield. In this step the enzyme was not immobilized by the column, but was retarded sufficiently to allow good separation from non-absorbed proteins. The adenosine deaminase activity was eluted as a single peak from each of the three columns (DEAE-Sephadex, Sephadex G-100 and adenosine–Sepharose). During each step of enzyme purification, column chromatography was monitored carefully to make certain that no secondary or minor peaks of enzyme activity were overlooked or discarded. The ratio of enzyme specific activity with adenosine as substrate to that with deoxyadenosine as substrate was monitored throughout the purification process (Table 1, 'Activity ratio') and did not vary significantly. This result is consistent with purification of a single activity responsible for deamination of both substrates in the initial extract.

The purified enzyme exhibited a single band of protein when subjected to SDS/polyacrylamide-gel electrophoresis, non-denaturing polyacrylamide-gel electrophoresis and isoelectric focusing. A single sharp peak of adenosine deaminase activity was found after isoelectric focusing, which corresponded to the single band of protein seen on the stained gel. The pI value for this peak was 4.4. Determination of enzyme activity in a non-denaturing gel identical with that stained for protein also exhibited a single peak of activity aligning with the single band of protein. A 10μl sample of the crude extract which was used as starting material for the enzyme purification also exhibited a single peak of enzyme activity on non-denaturing polyacrylamide-gel electrophoresis, with an R_F value comparable with that of the purified enzyme.

A 30μg sample of purified enzyme was subjected to SDS/polyacrylamide-gel electrophoresis and was stained with periodic acid–Schiff reagent to test for the presence of carbohydrate (Zacharius *et al.*, 1969). No carbohydrate was detected. A 20μg sample of ovalbumin, which contains 3.5% carbohydrate by weight, was included as a positive control and gave an easily detected positively staining band.

The molecular weight of native purified adenosine deaminase was examined by the method of Hedrick & Smith (1968) in six non-denaturing gels with acrylamide contents of 7–12%. The estimated molecular weight of the native enzyme was 43600 (Fig. 1*a*). This molecular weight was confirmed by SDS/polyacrylamide-gel electrophoresis performed by the methods of Weber & Osborn (1969) and Laemmli (1970), giving values of 45300 ± 1100 (Fig. 1*b*) and 43900 ± 900 respectively. These determinations indicate that the purified adenosine deaminase is a monomeric protein with a molecular weight of approx. 44000.

The substrate specificity of purified adenosine deaminase was examined. The purified enzyme catalyses the deamination of adenosine and deoxyadenosine, but does not deaminate adenine, AMP, guanosine, deoxyguanosine, cytidine or deoxycytidine. Kinetic constants were determined for deamination of adenosine and deoxyadenosine by the purified enzyme: K_m values were 48μM and 34μM respectively. Maximal-velocity values, in pmol/min per ng of protein, were 416 for adenosine and 312 for deoxyadenosine.

A large number of purines, pyrimidines and their derivatives were tested as possible physiological effectors of adenosine deaminase activity with adenosine and deoxyadenosine as substrates. These compounds are listed in the legend of Table 2. Potential effectors were present at 0.1 mM, with substrates at 0.5 mM. All compounds were tested

Table 1. *Purification of adenosine deaminase from human leukaemic granulocytes*

A unit of adenosine deaminase activity is the amount required to deaminate 1μmol of adenosine/min at 35°C. The 'Activity ratio' is defined as the ratio of the specific activity with adenosine (Ado) as substrate to that with deoxyadenosine (dAdo) as substrate at each step of purification.

Fraction	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Purification (fold)	Recovery (%)	Activity ratio (Ado/dAdo)
Crude extract	6867	42300	0.16	1	100	1.53
DEAE-Sephadex A-50	4242	4080	1.04	6.5	62	1.54
Sephadex G-100	3883	683	5.69	36	57	1.20
Adenosine–Sepharose affinity chromatography	3550	7.3	486	3038	52	1.55

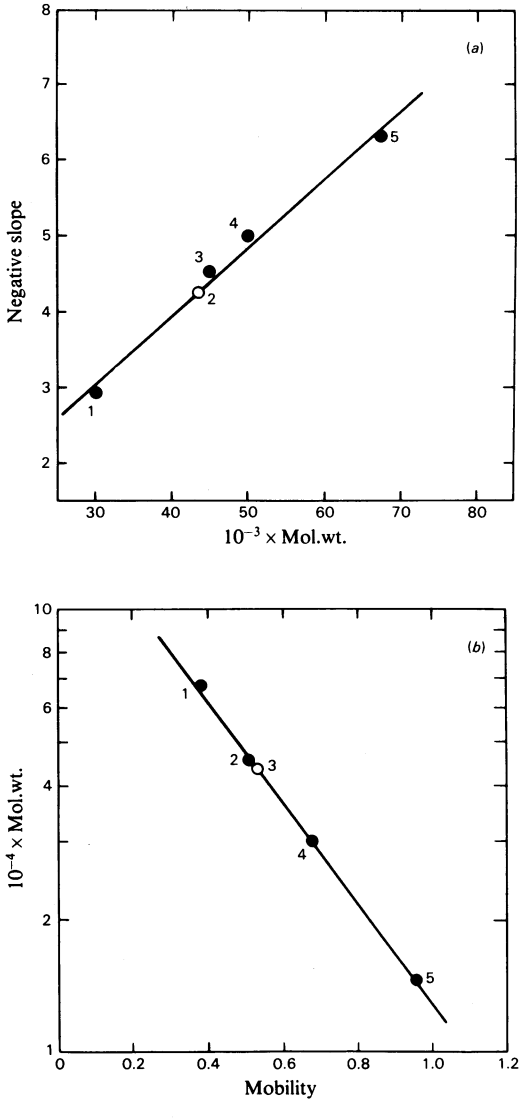

Fig. 1. *Molecular-weight determination of purified adenosine deaminase: (a) non-denaturing polyacrylamide-gel electrophoresis [Hedrick & Smith (1968) method] and (b) SDS/polyacrylamide-gel electrophoresis [Weber & Osborn (1969) method]*

(*a*) The proteins examined were (1) carbonic anhydrase (mol.wt. 30000), (2) adenosine deaminase, (3) ovalbumin (47000), (4) hexokinase (50000) and (5) bovine serum albumin (67000). The R_m (migration relative to Bromophenol Blue tracking dye) was determined and plotted as a function of acrylamide concentration for each protein [as a plot of $100\log(R_m \times 100)$ versus acrylamide-gel concentration]. The negative slope of each such plot was determined by linear regression and subsequently a standard curve of negative slopes versus molecular weights was calculated by linear regression. The slope for the plot for adenosine deaminase was −4.28, which corresponds to a molecular weight of 43600. (*b*) The electrophoresis was performed by the method of Weber & Osborn (1969). A 10μg sample of each protein was used. The samples were (1) bovine serum albumin (67000), (2) ovalbumin (47000), (3) adenosine deaminase, (4) carbonic anhydrase (30000) and (5) lysozyme (14500). Mobilities are expressed relative to the tracking dye, Bromophenol Blue (=1). The apparent molecular weight for adenosine deaminase is 45300 ± 1100.

against both substrates. None of the compounds had a significant effect (greater than ±5%) on adenosine deaminase activity with either adenosine or deoxyadenosine as substrate.

The kinetics of inhibition of adenosine deaminase by two known inhibitors, deoxycoformycin (Woo *et al.*, 1974) and *erythro*-9-(2-hydroxynon-3-yl)-adenine (Schaeffer & Schwender, 1974) were examined with purified enzyme with both adenosine and deoxyadenosine as substrate. The results are shown in Table 2. *erythro*-9-(2-Hydroxynon-3-yl)adenine is a competitive inhibitor of the enzyme, with a K_i of 13–16nM with either substrate. Inhibition of adenosine deaminase by deoxycoformycin was examined by the method for analysis of tight-binding inhibitors described by Agarwal *et al.* (1979). This method was used because Michaelis–Menten methods give inaccurate or misleading results for tight-binding inhibitors. Inhibition with adenosine as substrate is shown in Fig. 2. Similar results were obtained with deoxyadenosine as substrate; K_i values of 92pM and 66pM respectively were found. The type of inhibition caused by deoxycoformycin could not be determined because this method for K_i determination does not give an indication of inhibition type. Thus the K_i values for deoxycoformycin are apparent K_i values.

The amino acid composition of the purified adenosine deaminase was determined in three independent analyses. The mean results are shown in Table 3.

Antiserum directed against purified human adenosine deaminase was raised in a goat. This antiserum was both precipitating and neutralizing in tests against the purified human enzyme. The antiserum also cross-reacted with adenosine deaminase in crude extracts of a number of human cell types, including peripheral lymphocytes, granulocytes, erythrocytes, fibroblasts and lymphoid cells from human bone marrow and lymph node and with purified adenosine deaminase from calf intestine. Immunoglobulin G isolated from the goat antiserum was used to develop a radioimmunoassay for adenosine deaminase protein. The radioimmunoassay was used to quantify adenosine deaminase protein in human cell extracts. Samples were assayed at a number of dilutions. The slope for

Table 2. *Inhibitors of adenosine deaminase: kinetic constants*

Tested non-effectors: uracil, uridine, cytosine, cytidine, deoxycytidine, thymine, thymidine, adenine, inosine, deoxyinosine, hypoxanthine, guanine, guanosine, deoxyguanosine, GMP, GDP, GTP, dGMP, dGDP, dGTP, AMP, ADP, ATP, dAMP, dADP, dATP.

Compound	Substrate	K_i	Apparent type
Deoxycoformycin	Adenosine	92 pM	
	Deoxyadenosine	66 pM	
erythro-9-(2-Hydroxynon-3-yl)adenine	Adenosine	13 nM	Competitive
	Deoxyadenosine	16 nM	Competitive

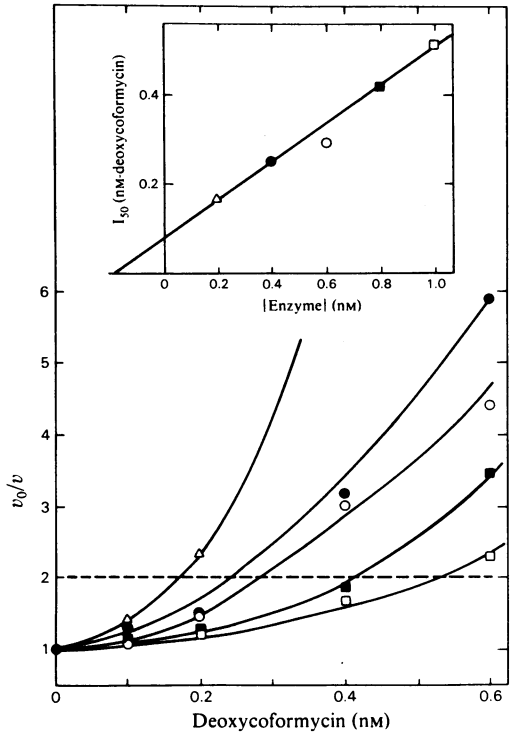

Fig. 2. *Inhibition of purified adenosine deaminase by the tight-binding inhibitor deoxycoformycin*

The effects of various concentrations of deoxycoformycin on the activity of various concentrations of adenosine deaminase (△, 0.2 nM; ●, 0.4 nM; ○, 0.6 nM; ■, 0.8 nM; □, 1.0 nN) are expressed as ratios v_0/v, where v_0 is the activity of the appropriate amount of enzyme in the absence of deoxycoformycin. In this manner the amount of deoxycoformycin necessary to inhibit enzyme activity to half of v_0 (I_{50}) was determined for each concentration of enzyme. These I_{50} values were plotted as a function of enzyme concentration (insert) and this plot was used to estimate a K_i of 92 pM.

Table 3. *Amino acid composition of adenosine deaminase from human granulocytes*

Results are based on three determinations; 50 μg of adenosine deaminase was used for each determination. Cysteine and tryptophan were not determined.

Amino acid	Content (mol/44 000 g)	Content (mol%; ± S.D.)
Alanine	30	8.3 ± 0.4
Arginine	18	4.9 ± 0.2
Aspartic acid	34	9.2 ± 0.6
Glutamic acid	52	14.2 ± 1.0
Glycine	30	8.2 ± 0.8
Histidine	12	3.6 ± 0.6
Isoleucine	17	4.5 ± 0.1
Leucine	36	9.9 ± 0.4
Lysine	24	6.5 ± 0.4
Methionine	6	1.6 ± 0.3
Phenylalanine	11	3.1 ± 0.2
Proline	22	5.9 ± 0.3
Serine	21	5.7 ± 1.1
Threonine	18	4.9 ± 0.2
Tyrosine	11	3.0 ± 0.9
Valine	25	6.7 ± 0.3

various dilutions of the sample was the same as the slope for the purified enzyme in the standard curve. This indicates that adenosine deaminase protein in the cell extracts has the same affinity for the antibody as does the purified enzyme (Hunter, 1978). Assay of adenosine deaminase from calf intestine gave a significantly different slope, consistent with a very low affinity between the calf enzyme and antibody to the human protein.

A comparison of adenosine deaminase amounts in a number of human lymphocyte samples, as determined by enzymic assay and radioimmunoassay, is shown in Table 4. Enzymic assays were carried out in the presence and absence of the inhibitor deoxycoformycin. A relatively constant low adenosine-deaminating activity not sensitive to deoxycoformycin was observed in all extracts. The existence of an adenosine-deaminating activity distinct from the major adenosine deaminase has been well documented (Schrader *et al.*, 1978). This minor activity appears to be the product of a genetic locus separate from adenosine deaminase and does not cross-react with antiserum to the major enzyme. The two activities can be distinguished by assay in the

Table 4. *Determination of adenosine deaminase in normal human lymphocytes by radioimmunoassay and enzymic assay*
Samples 1–3 were lymphocytes from separate adult human peripheral-blood samples. Samples 4–6 were lymphocytes from separate human cord-blood samples. Enzyme activity was determined in the presence and absence of deoxycoformycin (100μM). Enzyme activity was converted into protein concentration on the basis of a specific activity of 486μmol/min per mg for purified human adenosine deaminase.

	Enzyme activity (nmol/min per 10^8 cells)		Adenosine deaminase protein concn. (ng/10^8 cells)	
Sample	+Deoxycoformycin	−Deoxycoformycin	By activity	By radioimmunoassay
1	3	127	249	295
2	6	121	229	270
3	5	109	208	264
4	5	167	325	365
5	4	206	404	465
6	7	227	441	466

presence of adenosine deaminase inhibitors (Schrader *et al.*, 1978). The major activity is inhibited by deoxycoformycin, whereas the minor activity is not. Amounts of deoxycoformycin-sensitive adenosine deaminase activity were converted into apparent protein concentrations on the basis of specific activity of the purified human enzyme. Consistently higher amounts of adenosine deaminase were observed in lymphocytes from human cord blood than in lymphocytes from adult peripheral blood. The slope observed in radioimmunoassay of adenosine deaminase in the lymphocytes from both sources was the same, indicating an enzyme of the same cross-reactivity in each case.

Discussion

Human leukaemic granulocytes are excellent starting material for purification of adenosine deaminase. Several hundred grams of fresh cells can be obtained from a single patient and processed immediately. The specific activity of our initial cell extract was 0.16 unit/mg of protein, compared with 0.0002–0.0011 unit/mg of protein in extracts of human erythrocytes (Schrader *et al.*, 1976; Agarwal *et al.*, 1975) and 0.006 unit/mg of protein in extracts of human kidney (Schrader & Stacy, 1977). Our purification scheme was based on that used by Schrader *et al.* (1976) to purify adenosine deaminase from human erythrocytes, but was altered in several ways to take advantage of the higher specific activity and small amount of haemoglobin in the starting material. The purified enzyme was shown to be a homogeneous monomeric protein by several electrophoretic methods. The monomer is similar to the only other low-molecular-weight or 'small form' adenosine deaminase purified from human tissues, that from erythrocytes (Schrader *et al.*, 1976; Dadonna & Kelley, 1977). Like the enzyme from leukaemic granulocytes, adenosine deaminase from erythrocytes demonstrates a single protein band under denaturing conditions on SDS/polyacrylamide gels. The specific activity of the enzyme from leukaemic cells (486 units/mg) is similar to values of 538 and 515 units/mg reported for the purified enzyme from erythrocytes (Schrader *et al.*, 1976; Dadonna & Kelley, 1977). However, there are significant differences between the two. Purified adenosine deaminase from erythrocytes is electrophoretically heterogeneous, demonstrating three or four bands on non-denaturing gel electrophoresis, whereas the granulocyte enzyme gives a single band on non-denaturing gel electrophoresis. Multiple forms of erythrocyte adenosine deaminase are seen in crude haemolysate or partially purified preparations after starch-gel electrophoresis (Spencer *et al.*, 1968), ion-exchange chromatography (Osborne & Spencer, 1973; Agarwal *et al.*, 1975) and isoelectric focusing (Agarwal *et al.*, 1975). Isoelectric focusing of partially purified erythrocyte enzyme demonstrated four components, with pI values of 4.70, 4.83, 4.94 and 5.06. Isoelectric focusing of the purified granulocyte enzyme gave a single band with pI 4.4.

The molecular weight of adenosine deaminase from granulocytes is 44000, compared with a value of 36000–38000 for the enzyme from erythrocytes (Schrader *et al.*, 1976; Dadonna & Kelley, 1977). The amino acid composition of the enzyme from granulocytes is similar to that of the enzyme from erythrocytes (Dadonna & Kelley, 1977), but small differences in mole percentages of most amino acids were noted.

Adenosine deaminase from some human tissues other than erythrocytes is heterogeneous in size and electrophoretic mobility (Van der Weyden & Kelley, 1976; Hirschhorn, 1975; Edwards *et al.*, 1971). The variation in molecular size has been explained, at least in part, by complexing protein which can convert 'small form' adenosine deaminase into 'large form', of molecular weight 250000–300000 (Van der Weyden & Kelley, 1976; Hirschhorn, 1975;

Schrader & Stacy, 1977). Electrophoretic variants of the 'large form' adenosine deaminase have been attributed to variation in the type and amount of carbohydrate in complexing protein (Swallow *et al.*, 1977). The 'tissue isozymes' that were originally observed by Edwards *et al.* (1971) are a reflection of heterogeneity in both the low-molecular-weight adenosine deaminase (as in erythrocytes) and the high-molecular-weight complex (as in kidney).

The kinetics and specificity of substrates and inhibitors of adenosine deaminase have been previously examined most extensively with partially purified human enzyme of low specific activity. These specific activities have ranged from 0.71 unit/mg (Agarwal *et al.*, 1975) to 5.3 units/mg (Osborne & Spencer, 1973), compared with a specific activity of approx. 500 units/mg for homogeneous enzyme. Purified granulocyte adenosine deaminase is specific for adenosine and deoxyadenosine among the potential substrates tested, with K_m values of 48 μM and 34 μM respectively. K_m values of 25–30 μM for adenosine (Osborne & Spencer, 1973; Agarwal *et al.*, 1975) and 7 μM for deoxyadenosine (Agarwal *et al.*, 1975) had previously been determined for partially purified preparations of erythrocyte enzyme. A K_m value of 52 μM has been reported for homogeneous erythrocyte adenosine deaminase, but a value for deoxyadenosine was not reported (Dadonna & Kelley, 1977). No significant differences in metabolism of adenosine and deoxyadenosine by purified granulocyte adenosine deaminase were noted. Comparison of these two substrates was important because deficiency of adenosine deaminase appears to have a much greater impact on metabolism of deoxynucleosides and deoxynucleotides than on the corresponding ribonucleosides and ribonucleotides (Coleman *et al.*, 1978; Cohen *et al.*, 1978). No significant effect on adenosine deaminase was noted with any of the large group of compounds surveyed as potential physiological effectors with either adenosine or deoxyadenosine as substrate. The basis for specific perturbation of deoxynucleoside and deoxynucleotide metabolism in adenosine deaminase deficiency cannot be attributed to a difference in normal metabolism of adenosine and deoxyadenosine by the enzyme.

Both of the structural analogues, deoxycoformycin and *erythro*-9-(2-hydroxynon-3-yl)adenine, are potent inhibitors of purified granulocyte adenosine deaminase with either adenosine or deoxyadenosine as substrate. *erythro*-9-(2-Hydroxynon-3-yl)adenine has a K_i of 13 nM with adenosine as substrate and 16 nM with deoxyadenosine as substrate, and is a competitive inhibitor. Deoxycoformycin has a K_i of 92 pM with adenosine as substrate and 66 pM with deoxyadenosine as substrate. K_i values of 1.6 nM and 2.5–15 pM have been reported for *erythro*-9-(2-hydroxynon-3-yl)adenine and deoxycoformycin respectively when partially purified enzyme from human erythrocyte was examined with adenosine as substrate. K_i values for these inhibitors with deoxyadenosine as substrate have not been previously reported. Deoxycoformycin is being tested as a specific cytotoxic treatment of human patients with lymphoid malignancies (Smyth, 1979; Prentice *et al.*, 1980; Siaw *et al.*, 1980). The rationale is that inhibition of adenosine deaminase by deoxycoformycin will mimic hereditary deficiency of the enzyme, which is associated with severe lymphopenia.

The radioimmunoassay described here is a sensitive method for detecting adenosine deaminase protein. Radioimmunoassay is not only a convenient method for adenosine deaminase determination in normal cells, but also a method for detecting inactive protein in cells deficient in activity of the enzyme (Dadonna *et al.*, 1980). The radioimmunoassay may prove useful in monitoring the type and extent of disease in patients with certain lymphoid malignancies. Human thymus/leukaemia-associated antigen, a previously described marker protein in human thymocytes and certain leukaemias, has been identified as a form of adenosine deaminase (Chechik *et al.*, 1979). The antigen has a molecular weight of 43000 (Chechik *et al.*, 1978) and was thought to play a role in the differentiation of T lymphocytes and other haematopoietic cells (Chechik *et al.*, 1976). Since very large amounts of adenosine deaminase are found in thymocytes and non-B 'marked' lymphoid precursors, the amount of circulating enzyme in plasma could be related to the total body mass and turnover of these cells.

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