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Preparative purification of adenosine deaminase from human erythrocytes by affinity chromatography

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ABSTRACT

The purification of adenosine deaminase from human erythrocytes is reported By means of classical procedures and by using affinity chromatography as the last step, the enzyme is purified 760 000-fold with a yield of 32%. The affinity resin is composed of purine riboside (nebularine) linked to Sepharose CL6B Since the compound has no leaving group at the C-6 position the affinity gel is stable and the chromatography can be repeated several times (up to fifteen times in eight months). Purine riboside was chosen because its potency as a reversible inhibitor of adenosine deaminase is greater than that of inosine (a low-affinity inhibitor), but lower than that of *erythro*-9-(2-hydroxy-3-nonyl)adenine (a high-affinity inhibitor)

INTRODUCTION

Adenosine deaminase (ADA, adenosine aminohydrolase, EC 3.5.4.4), an enzyme involved in the salvage of purine nucleosides, catalyses the irreversible hydrolytic deamination of adenosine and 2′-deoxyadenosine to the corresponding hypoxanthine derivatives, inosine and 2′-deoxyinosine.

Although this enzyme is present in all mammalian tissues, it appears to play a major role in the development and function of lymphoid tissue. It has been shown that abnormalities of the ADA activity are related to the development of certain diseases. Thus, the congenital defect of this enzyme is associated with the hereditary syndrome of severe combined immunodeficiency with impaired function of T and B lymphocytes [1–3]. Low levels of the enzyme have also been reported in chronic lymphocytic leukaemia [4]. On the other hand, in other diseases such as acute leukaemia [5] and lymphomas [6,7], myasthenia gravis [8], herpes simplex virus-induced corneal stromal disease [9] or acquired immunodeficiency syndrome (AIDS) [10], alterations of ADA activity and/or isozymic pattern have been described.

In fact, the measurement of ADA activity helps in the diagnosis of some lymphoid disfunctions, and thus the enzyme might be considered a marker for some diseases. Currently the methods of evaluation of the enzyme activity are biochemical on nature. However, the appearance of immunochemical methods to detect

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the enzyme in tissue slices and in cell extensions or in cell suspensions should be expected. The latter is a consequence of the association of ADA to the plasma membrane of blood cells, which has been demonstrated in our laboratory during work with cell suspensions [11,12].

The development of immunochemical methods requires the purification of the enzyme and the obtention of specific antibodies. Several methods of purification have been described in the literature, and those that achieve higher specific activity of the final preparation require the use of affinity chromatography. Thus, Daddona and Kelley [13] have purified 800 000-fold human erythrocyte ADA using an antibody affinity Sepharose 4B column. Schrader and Stacy [14] used a classical procedure combined with two immunoadsorbant columns to purify 10 000-fold the ADA from human kidney. The same laboratory [15] obtained ADA from human erythrocytes with a purification factor of 468 000, using an affinity column consisting of adenosine linked to Sepharose 6B.

This paper describes a purification procedure for ADA from human erythrocytes, using an affinity column resulting from the binding of an inhibitor of the enzyme, purine riboside (Fig. 1), to a Sepharose CL6B matrix. One of the advantages of the proposed method consists of the impossibility of the deamination of the purine riboside by the action of the enzyme (the compound has no amino group in the C-6 position). Thus the affinity column can be reused many times.

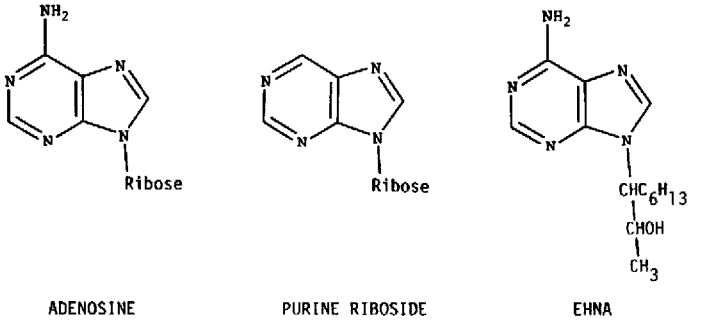

Fig 1 Structures of adenosine, nebularine and *erythro*-9-(2-hydroxy-3-nonyl)adenine (EHNA).

EXPERIMENTAL

Chemicals

Adenosine and DEAE fractogel TSK were purchased from Merck (Darmstadt, F.R.G.) and purine riboside (Nebularine) from Sigma (St. Louis, MO, U.S.A.). Sephadex G-100, Sephadex G-25, epoxy-activated Sepharose CL6B and DEAE-Sepharose CL6B were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). Adenosine deaminase from calf intestinal mucosa was purchased from Boehringer Mannheim (Barcelona, Spain). All other chemicals were of analyt-

ical-reagent grade. The water used throughout was deionized using a Milli-Q system from Millipore-Waters (Milford, MA, U.S.A.).

Adenosine deaminase assay

Enzyme activity was measured spectrophotometrically by using the method of Kalckar [16] following the decrease of absorbance at 265 nm. The standard reaction mixture contained 200 μl of 10^{-3} *M* adenosine, the enzyme solution, and 50 m*M* sodium phosphate buffer (pH 7 4) in a final volume of 3 ml. Assays were carried out at 30 ± 0.1°C in either a Pye Unicam SP-1700 or a Beckman K-25 spectrophotometer. One unit was defined as the amount of enzyme catalysing the deamination of 1 μmol adenosine per minute under the assay conditions.

Protein determination

Protein concentrations were measured by the method of Warburg and Christian [17], following spectrophotometric absorbance changes at 260 and 280 nm, or by the method of Read and Northcote [18] when the amount of protein was low. Bovine serum albumin was used as standard.

Affinity column preparation

The ligand purine riboside was coupled to epoxy-activated Sepharose CL6B by the following procedure: 5 g of epoxy-activated Sepharose 6B were washed with distilled water on a Büchner funnel and then suspended in 35 ml of a 26 m*M* solution of purine riboside (nebularine) prepared in 0.1 *M* $NaHCO_3$ (pH 11.2). The suspension was shaken for 24 h at 37°C. Gel was recovered and washed twice with 250 ml of distilled water and subsequently with 50 ml of the stopping solution, consisting of 1 *M* ethanolamine in 0.1 *M* sodium borate buffer (pH 9.1). The gel was then suspended in 100 ml of the latter solution and shaken for 4 h at 37°C The gel was then washed again with 250 ml of distilled water and three more times with 0.2 *M* $NaHCO_3$, 1 *M* NaCl and 0.2 *M* sodium acetate (pH 5 0). Finally it was suspended in the storage buffer, whose composition was 1 m*M* 2-mercaptoethanol, 0.02% NaN_3 and 0.1 *M* NaCl in 0.1 *M* potassium phosphate buffer (pH 7.4). Based on the amount of purine riboside that was removed from the solution after the 24-h coupling period, the yield of nebularine bound per gram of an epoxy-activated Sepharose CL6B powder was *ca.* 20 μmol. In essence, this method is similar to that employed by Schrader *et al.* [15] for the coupling of adenosine to the epoxy-activated resin. The affinity gel was stored at 4°C in 0 1 *M* potassium phosphate buffer (pH 7.4) containing 1 m*M* 2-mercaptoethanol, 0.02% (w/v) NaN_3 and 0.1 *M* NaCl. The purine riboside affinity column is stable for at least eight months under repeated chromatographic procedures.

Enzyme purification

As a source of human adenosine deaminase, erythrocytes were used (packed red blood cells from the blood bank of the University Hospital). Unless otherwise indicated all steps were carried out at 4°C.

Step 1: preparation of the hemolysate. Erythrocytes were washed twice with equal volumes of 0.15 *M* NaCl, and the packed cells obtained by centrifugation at 6000 *g* for 10 min were lysed by suspension in 50 m*M* sodium acetate buffer (pH 5.8) and sonication (18 μm during 5 min at intervals of 30 s). This procedure was carried out in an ice-bath while the suspension of erythrocytes was mixed by means of a magnetic stirrer. Disrupted cells were centrifuged at 16 000 *g* for 20 min to eliminate membrane debris.

Step 2: heat treatment. The hemolysate was rapidly heated at 55°C in a water-bath with continuous stirring. After 30 min the suspension was cooled by immersion in an ice-bath and centrifuged at 16 000 *g* for 25 min to eliminate denatured proteins.

Step 3: cation-exchange chromatography. The supernatant from step 2 was adjusted to pH 5.8 and to a conductivity of 5.3 mMHO by addition of diluted HCl or NaOH. It was subsequently applied to a column (19.5 cm × 5 cm I.D.) of CM-Sepharose CL6B "fast-flow" equilibrated in 50 m*M* sodium acetate buffer (pH 5.8). Elution was performed with the same buffer at a flux of 400 ml/h. Adenosine deaminase does not bind to the gel and elutes immediately, whereas haemoglobin remains attached to the column. The advantage of the method using a column is that the gel can be easily washed and reused for further purification procedures.

Step 4: ammonium sulphate fractionation. The first 500 ml eluted from the chromatographic process detailed in step 3 were saturated up to 60% with ammonium sulphate. The addition of the salt was slow and continuous, and the suspension placed in an ice-bath was continuously stirred. After 30 min of resting the final suspension was centrifuged at 16 000 *g* for 15 min. The precipitate, stored at −20°C retains 95% of ADA activity for at least three months.

Step 5. gel permeation chromatography. The residue from the centrifugation was dissolved in the minimum volume (15 ml) of 50 m*M* imidazole–HCl buffer (pH 6.2) and centrifuged at 20 000 *g* for 20 min. The supernatant was filtered through a Sephadex G-100 column (75 cm x 5 cm I.D) equilibrated with the imidazole buffer. Elution was performed at a rate of 100 ml/h, and fractions of 10 ml were taken. Fig. 2 shows a typical elution pattern. The protein peak detected by the absorbance at 280 nm does not coincide with the peak of adenosine deaminase activity. The peak of activity coincides with the single form C of the enzyme (M_r *ca.* 40 000) that is detected in human erythrocytes. Concentration of the fractions is necessary for further application to the affinity column (step 6). Thus, active fractions were pooled and concentrated at a rate of 250 ml/h by using a DEAE fractogel TSK column (3 cm × 1.5 cm I.D.) equilibrated with the imidazole buffer. Elution was performed at 100 ml/h in a 0.1 *M* sodium phosphate buffer (pH 7.4) containing 0.4 *M* NaCl. The first 10 ml of eluate were then chromatographed through a prepacked Sephadex G-25 gel for desalting and for changing the buffer to one suitable for the next step. The final volume was *ca.* 10 ml in a 0.1 *M* sodium phosphate buffer (pH 7.4) containing 1 m*M* mercaptoethanol and, unless otherwise indicated, 0.02% (w/v) NaN_3.

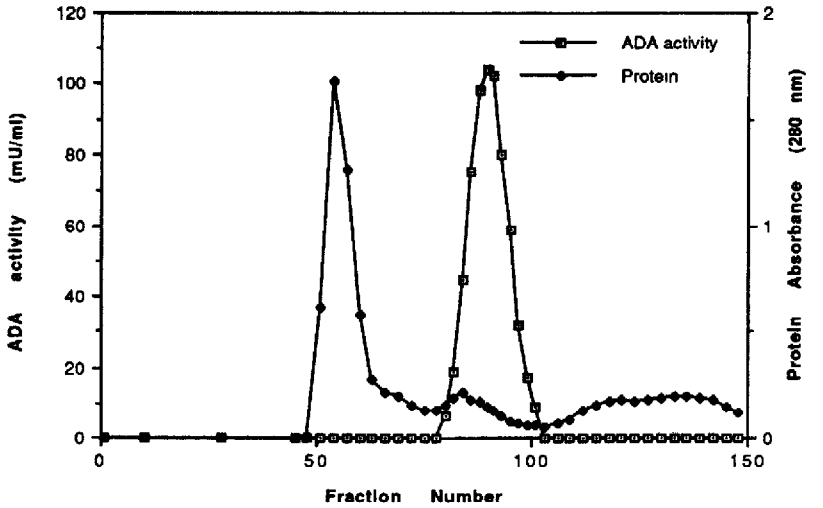

Fig 2 Elution pattern of adenosine deaminase from a Sephadex G-100 column

Step 6· affinity chromatography. The concentrated active solution from step 5 (15 ml) was applied to the purine riboside-Sepharose column prepared as described above. The gel was equilibrated with 0.1 *M* sodium phosphate buffer (pH 7.4) containing 1 m*M* mercaptoethanol and 0.02% (w/v) NaN_3. After entering the sample the column was washed with 2 volumes of the buffer. Elution of the retained enzyme was performed with an NaCl gradient (0–1 *M*) prepared in the same buffer. The flux along the chromatographic process was maintained at 2 ml/h, and aliquots of 2 ml were taken for analysis. A typical elution pattern is shown in Fig. 3.

DEAE-Sepharose chromatography

Pooled samples from the gel permeation chromatography were chromatographed through DEAE-Sepharose in order to separate the molecular forms of adenosine deaminase For this purpose it should be noted that NaN_3 has to be avoided in the preceding step because it interacts with the ion-exchange gel. The column (17.5 cm × 1.6 cm I.D.) was filled with DEAE-Sepharose fast-flow, and the two basic procedures described by Osborne and Spencer [19] and by Schrader *et al.* [15] were used. In the former the gel was equilibrated with 50 m*M* imidazole–HCl buffer (pH 6.2) and the elution was performed with a linear gradient of NaCl (between 0 and 0.2 *M*). In the latter the gel was equilibrated with 10 m*M* sodium acetate buffer (pH 4.9) and eluted with a linear gradient of the acetate buffer (between 10 and 70 m*M*). Since the gel is equilibrated with a low-ionic-strength buffer, the sample, prior to the application, was adjusted to pH 5.3 with acetic acid and to the conductivity of the equilibration buffer.

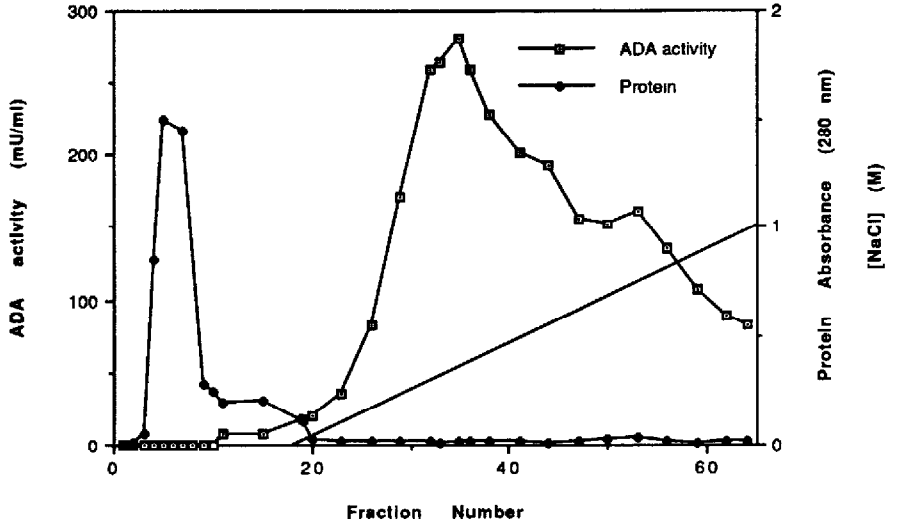

Fig 3 Chromatography of adenosine deaminase on nebularine-Sepharose. Purine riboside (nebularine) was linked to the epoxy-activated Sepharose as described in Experimental Pooled fractions from the Sephadex G-100 chromatography were applied to the column. The straight line corresponds to the NaCl gradient

Electrophoresis

Analytical electrophoresis of ADA isozymes was performed on 9% (w/v) polyacrylamide disc gels in Tris–HCl buffer (pH 8.9) by the method of Andrews [20]. A stacking gel prepared with Tris–H_3PO_4 buffer (pH 6 4) was used as concentrating gel. The upper chamber contained 40 m*M* Tris–glycine buffer (pH 8.3) and the lower 25 m*M* Tris–HCl buffer (pH 8.9). Electrophoresis was carried out at 75 V for 150 min. Adenosine deaminase activity was stained by the method of Spencer *et al.* [21] (see Fig 6).

The assay of purity as well as molecular mass determinations were carried out on polyacrylamide slab gels (7.5–37.5% gradient) containing 0.1% sodium dodecyl sulphate (SDS), as described by Weber and Osborn [22]. For SDS gel electrophoresis, protein samples were dialysed against 1000 volumes of 10 m*M* sodium phosphate buffer (pH 7.0) for 24 h and then denatured in 1% SDS and 1% 2-mercaptoethanol at 100°C for 5 min. Protein standards (molecular weight kit, Pharmacia) were prepared similarly. A silver-staining method was used for protein detection [23]. The molecular mass of adenosine deaminase was calculated on the basis of a linear relationship of the logarithm of molecular mass to the distance migrated in the SDS gel

RESULTS AND DISCUSSION

Purification of human erythrocyte adenosine deaminase

Table I summarizes the quick six-step purification procedure outlined in Experimental. The enzyme was purified up to 760 000-fold with a yield of 32%. Similar (very high) degrees of purification are achieved only by means of affinity chromatography. Thus Schrader *et al.* [15] purified 468 000-fold human erythrocyte adenosine deaminase with a yield of 26%; the affinity column used by these authors consisted of adenosine linked to Sepharose-6B. The enzyme was homogeneous by the SDS-electrophoretic criterion and had a molecular mass of 39 000 ± 1000. The method reported by Daddona and Kelley [13] also gave very high purification factors, up to 800 000, and a yield of 35%. This excellent method requires the obtention of specific antibodies against the enzyme, their binding to a suitable matrix polymer, and the performance of subsequent affinity chromatography. Since there is no commercially available antibody against adenosine deaminase it is necessary to raise them in a rabbit before their use for the affinity chromatography in the method proposed by Daddona and Kelley [13].

TABLE I

PURIFICATION OF ADENOSINE DEAMINASE FROM HUMAN ERYTHROCYTES

Step	Activity (U)	Protein (mg)	Specific activity (mU/mg protein)	Purification factor	Yield (%)
Hemolysate	19	56 225	0 34	1	100
Heat treatment	16	42 835	0 37	1.1	84
CM-Sepharose	15	900	17	50	79
Ammonium sulphate	8[a]	244	33	–	–
Sephadex G-100	10 5	25	420	1235	55
Nebularine-Sepharose	6	0 023	260 870	760 000	32

[a] The activity measured in presence of ammonium sulphate is artificial because the high salt concentration inhibits the enzyme.

Our method, employing an affinity column prepared by coupling nebularine to Sepharose-6B, has the advantage of the stability of the gel as well as fast preparation. The stability of nebularine against the enzyme was measured by following changes of absorbance due to the action of adenosine deaminase, either from human erythrocytes (Fig. 4) or from calf intestine (data not shown). The absorbance did not change during up to 8 h of incubation. In similar circumstances with the gel prepared by coupling adenosine to the matrix (method of Schrader *et al.* [15]), the absorbance in the UV region changes as the compound is deaminated to inosine.

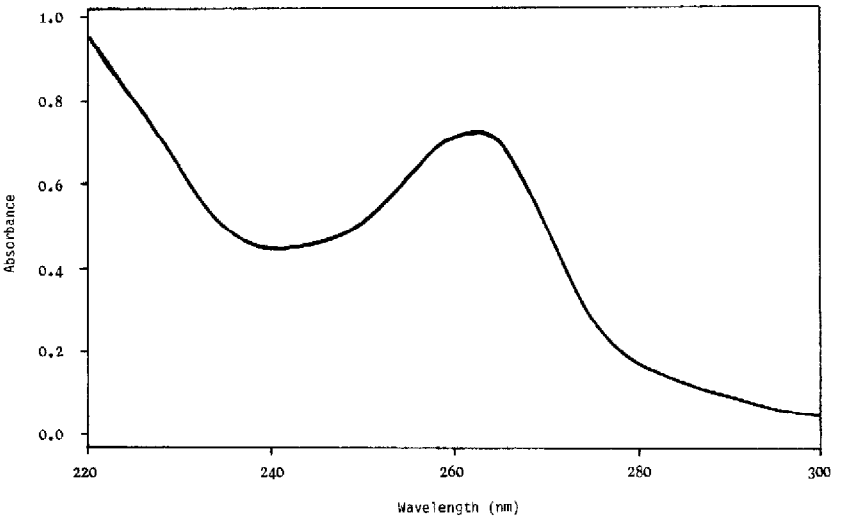

Fig. 4. Stability of nebularine in presence of adenosine deaminase Scan of nebularine (93 μM) in the UV region in presence of an aliquot of the pooled fractions obtained after the Sephadex G-100 chromatography The activity of the enzyme in the reaction mixture was 0 05 U/ml. The temperature of the assay was 37°C, and scans were performed every 10 min for 8 h

The stability of the gel that we prepared was tested by repeated chromatographic analysis. The elution pattern did not change after up to fifteen analyses in eight months. For chemical affinity chromatographies nebularine has two further advantages with respect to powerless inhibitors (inosine) and to stronger inhibitors (EHNA, see Fig. 1). Neither adenosine nor inosine is able efficiently to adsorb the enzyme within the column [13,15]; in contrast, other compounds that are potent inhibitors tested by Daddona and Kelley [13] do not permit the release of the enzyme from the column. The use of EHNA presumably would have prevented release of the enzyme from the column. Nebularine is a competitive inhibitor of the enzyme with a K_i of 5.8 μM, which is greater than the K_i for EHNA (1.5 nM).

Resolution of adenosine deaminase isozymes

Eluates from the Sephadex G-100 chromatography were subjected to a DEAE-Sepharose chromatography in order to separate the molecular forms of the enzyme that are present in the human erythrocyte (see Experimental). Three types of individual are expected on the basis of the existence of two allelic genes (1 and 2): type 1 (having only allele 1), type 2 (having only allele 2) and type 2-1 (having allele 1 and allele 2). Two chromatographic procedures were performed, which are essentially those described by Osborne and Spencer [19] and by Schrader *et al.* [15]. These methods differ in the buffer and the gradient used in the elution. The

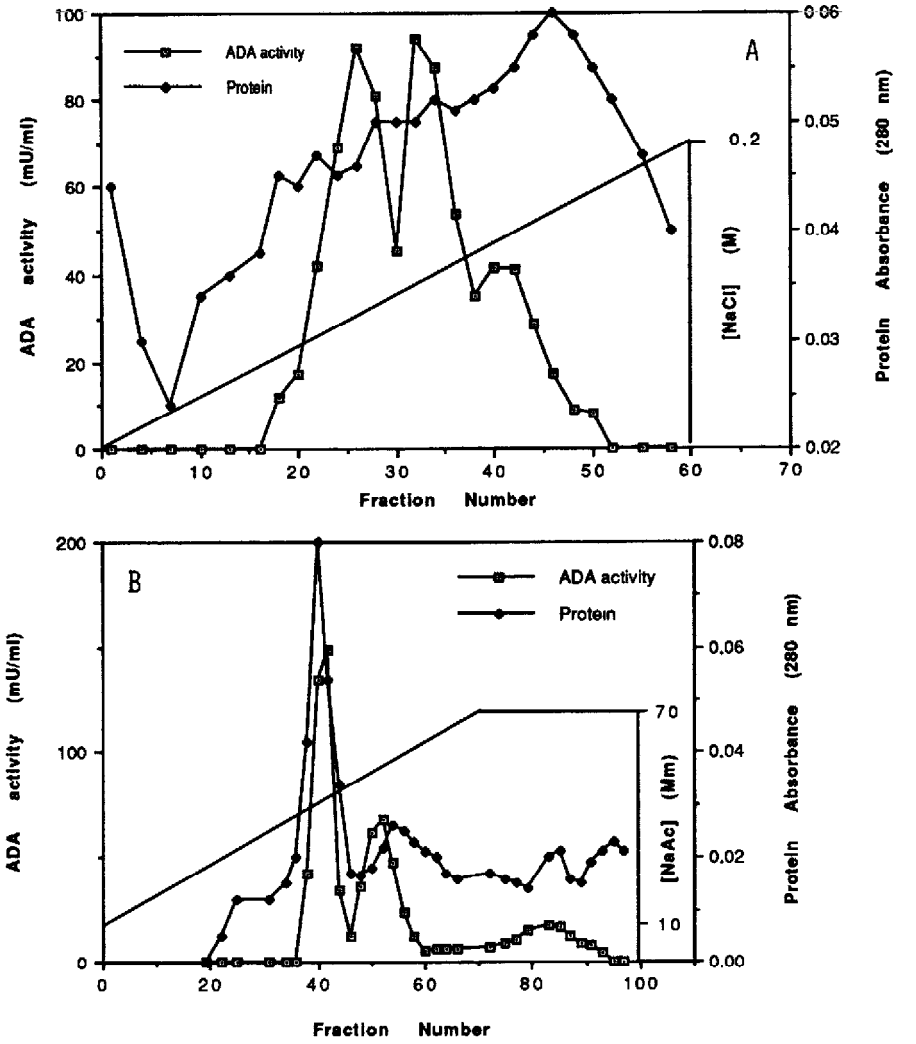

Fig 5 Resolution of adenosine deaminase of DEAE-Sepharose The two basic methods described in the literature were used for comparative purposes The straight line corresponds to the gradient. Fort further details see Experimental (A) Elution according the method of Osborne and Spencer [19] (B) Elution according to the method of Schrader *et al.* [15]

results are shown in Fig. 5A, which shows that three peaks of enzyme activity were found. Although the first method leads to a partial resolution of the two first peaks, the method of Schrader *et al.* [15] permits an almost complete resolution of the three peaks (Fig. 5B). Electrophoresis of these three peaks indicates the existence of a single band for each peak (Fig. 6) after specific staining for ADA activity (see Experimental). These results agree with the well described existence

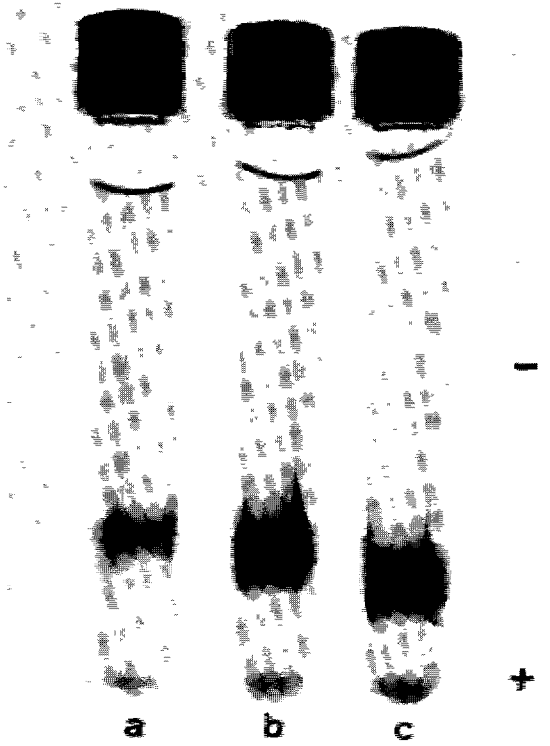

Fig. 6. Polyacrylamide gel electrophoresis of fractions resolved by chromatography on DEAE-Sepharose The three peaks resolved by the chromatography indicated in Fig 5B were analysed; 100 μl of each sample were applied. (a) First peak at a concentration of 0.06 U/ml, (b) second peak at a concentration of 0 26 U/ml, (c) third peak at a concentration of 0.21 U/ml These relative concentrations do not correspond to the relative concentrations found in erythrocytes or in the mixture prior to the resolution by DEAE-Sepharose, samples coming from the second and the third peak were concentrated prior to the electrophoretic procedure.

of three electrophoretic bands for the type 1 individual, which is the most common (95% of occurrence, Giblett *et al.* [24]). These three forms most likely correspond to the post-transcriptional processing of the protein product of the type 1 allele or to conformational isomers of the same molecule [25]. Schrader *et al.* [15] found four different peaks in the pattern of the DEAE chromatography, which correspond to four different bands encountered in agarose plates. The result is logical since the authors analysed the enzyme coming from pooled human erythrocytes, which contained a mixture of the three different enzyme patterns of all alleles: types 1, 2 and 2-1. On the other hand, Osborne and Spencer [19] analyzed, separately, the erythrocytes of the different types and found by isoelectric focusing three bands in the case of homozygosity for type 1 and four bands (by isoelectric focusing and by electrophoresis) in the case of heterozygosity 2-1.

NOTE ADDED IN PROOF

A similar affinity column was employed by Ogawa *et al.* [26] to study the differences among adenosine deaminases from different sources.

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