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MULTIPLE FORMS OF HUMAN ADENOSINE DEAMINASE

I. PURIFICATION AND CHARACTERIZATION OF TWO MOLECULAR SPECIES

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SUMMARY

1. Gel filtration of human tissue extracts with Sephadex G-200 revealed the existence of two molecular species of adenosine deaminase (adenosine aminohydrolase, EC 3.5.4.4) differing markedly in their molecular sizes.

2. Tissue-specific distributions of these species were also revealed.

3. The larger and smaller enzymes were purified more than 1000-fold, nearly to homogeneity, from the lung and the stomach, respectively.

4. Determination of molecular weights of the enzymes using a calibrated Sephadex G-200 column resulted in the values of 230 000 and 47 000, which were in reasonable accordance with the values obtained by sucrose density gradient centrifugation.

5. No significant differences were observed enzymically or immunologically between the properties of the two enzymes except for their heat-stabilities and molecular activities.

6. A reversible conversion of the larger deaminase to the smaller was demonstrated when the former was treated with guanidine.

7. The smaller enzyme was apparently converted to the larger after incubating the former enzyme sample with crude lung supernatant.

8. The possible structural relationship between the two species of human adenosine deaminase is discussed.

INTRODUCTION

A considerable number of reports have been presented on the multiplicity of adenosine deaminase (adenosine aminohydrolase, EC 3.5.4.4), which catalyzes the hydrolytic deamination of adenosine to produce inosine and ammonia, from various sources. In these studies, successful separation of different molecular species was

achieved mostly by electrophoresis[1–4] or ion-exchange column chromatography[5–7] on the basis of differences in net charges of the protein molecules. The recent development of gel filtration techniques, however, has enabled us to estimate molecular sizes of proteins either in pure form or in the crude state[8,9] simultaneously, affording us a good system for investigating molecular species of an enzyme which differ in molecular size. When we applied various human tissue extracts to gel filtration with Sephadex G-200, two distinctly separated peaks of adenosine deaminase activity were found, suggesting the existence of two species of different molecular sizes. Moreover, whereas normal lung extracts showed essentially a single peak corresponding to the larger deaminase molecule, extracts from lung cancer tissues exhibited an additional peak corresponding to the smaller enzyme. Thus, the two species seemed to reflect physiological or pathological states of the cells in which they functioned. Since the physiological significance of multiple forms of adenosine deaminase has not yet been clarified despite the fact that the physicochemical nature of the deaminase isozymes of bovine source have been extensively studied[1,5,10], we attempted to purify the two molecular species of this particular enzyme in human tissues in order to characterize their properties in relation to their physiological roles. While this work was in progress, similar findings were reported by Ma and Fisher[11–13]: three molecular species of the enzyme having molecular weights of the order of 200 000, 100 000 and 35 000 were demonstrated by gel filtration with Sephadex in the livers of a wide variety of amphibians and mammals. The distribution patterns of these species in the liver were shown to represent phylogenetic classes of the organisms as well[13].

This paper presents the results of purification of human adenosine deaminases of larger molecular size (termed E_L) and of smaller size (E_S) from normal lung and stomach, respectively, as well as the results obtained in characterization and modification of the purified enzyme preparations. A brief outline of this work has already appeared[14].

MATERIALS AND METHODS

Tissue specimens and extraction of enzyme

Most human tissue specimens employed for extracting the enzyme were obtained by autopsy, but a small number of tissues, particularly lung and liver tissues, were obtained by surgery. These tissues were immediately stored at −20 °C. For extracting the enzyme, a tissue specimen was thawed overnight in the cold room and was then washed with cold distilled water to remove blood as thoroughly as possible. In a small-scale experiment, tissue was homogenized using a glass–teflon homogenizer with an appropriate amount of 20 mM phosphate buffer (pH 7.0) containing 2 mM 2-mercaptoethanol to make a 20% extract.

Chemicals

Adenosine, deoxyadenosine, inosine, guanosine, adenine, AMP, ATP, GTP, human albumin, bovine serum albumin, bovine hemoglobin, bovine pancreatic ribonuclease (EC 2.7.7.16), neuraminidase (EC 3.2.1.18) (from *Vibrio cholera*) and *p*-chloromercuriphenylsulfonate were obtained from Sigma Company. Crystalline cytochrome *c*, trypsinogen, catalase (EC 1.11.1.6) and phospholipase A (EC 3.1.1.4) and D (EC 3.1.4.4) were the products of Boehringer Mannheim Biochemicals; human gamma

globulin and guanidine hydrochloride were from Nutritional Biochemicals Corp. DEAE-cellulose was purchased from Serva Company and Sephadex G-100 and G-200 were obtained from Pharmacia.

Assay methods

Adenosine deaminase activity was measured ordinarily by the chromogenic method described by Martinek[15]. Reaction mixtures (0.6 ml) contained 25 μmoles of phosphate buffer (pH 7.0), 2.5 μmoles of adenosine and 0.1 ml of enzyme. Incubation was carried out at 37 °C for appropriate periods of time depending upon the degree of activity (usually 10–20 min). Tubes lacking enzyme or substrate, or tubes without incubation, served as blank. The reaction was terminated by adding successively 2.5-ml portions of phenol-color reagent and alkali-hypochlorite reagent, followed by an additional incubation at 37 °C for 20 min. The blue color resulting from ammonia formation was measured (using a Bausch and Lomb photometer) at 635 nm. In certain limited cases, particularly in kinetic studies, the deaminase activity was measured spectrophotometrically according to Kaplan *et al.*[16]. Protein concentrations were determined either by the biuret method[17] or by the method of Lowry *et al.*[18]. Bovine serum albumin was used as a standard.

Enzyme unit and specific activity

One unit of adenosine deaminase activity was defined as the amount which produces 1 μmole of ammonia per min under the assay conditions as described above. Specific activity was expressed as units per mg of protein.

Analytical gel electrophoresis

Thin-layer gel electrophoresis was performed with a 5% polyacrylamide gel plate (0.2 cm × 13.5 cm × 20 cm) in Tris–borate buffer (pH 8.4, I 0.05) at a constant current of 20 mA for 2 h. Disc gel electrophoresis was performed essentially according to Davis[19]. Protein bands were stained either with 1% Amido black 10B or with Coomassie blue by the method of Chrambach *et al.*[20].

Sucrose density gradient centrifugation

This technique was employed for determining molecular weight according to Martin and Ames[21]. Linear gradients of 5–20% sucrose dissolved in 30 mM phosphate buffer (pH 7.4) containing 5 mM 2-mercaptoethanol were prepared. Samples (0.2 ml) containing either a purified preparation of E_L (0.6 unit) or of E_S (0.8 unit) and crystalline bovine liver catalase (1000 units) as an internal standard were overlayered on the top of the gradients. Centrifugation was carried out at 38 000 rev./min for 16 h at 4 °C using a swinging bucket roter Hitachi RPS 40 and Hitachi Model 65 P centrifuge. Fractionation was made by punching the bottom of the tubes with a needle and collecting 10 drops each, followed by adding 0.3 ml portions of the phosphate buffer to each fraction. Adenosine deaminase activity was assayed by the chromogenic method with 0.1 to 0.2 ml of the fractions. Catalase activity was determined at 25 °C using the Cary spectrophotometer Model 14 by following the decrease in absorbancy at 240 nm of a 3-ml reaction mixture containing 0.06% H_2O_2 in 0.01 M phosphate buffer (pH 7.0) and 10 to 40 μl of enzyme fraction.

Immunochemical procedure

An E_L sample of maximum purity (2 mg of protein in 2 ml) was emulsified with 3 ml of Freund complete adjuvant (Difco) and then injected intramuscularly in a male rabbit of about 3 kg in body weight. Three weeks later, a booster injection was given intravenously through an ear vein with 1.5 mg of the purified sample. One week after the booster injection, blood was withdrawn by cutting an ear vein. Serum was separated and stored frozen until use. For preparing antiserum against E_S, a sample (2 mg of protein in 2 ml) emulsified with 3 ml of Freund complete adjuvant was injected intramuscularly in a guinea pig of about 500 g. Blood was collected by cardiac puncture 4 weeks after the injection. Immunochemical tests were performed by the precipitation reaction according to Ouchterlony gel diffusion techniques in agarose gel[22] and by inhibition of enzymic activity.

RESULTS

Gel filtration patterns of adenosine deaminase in various vuman tissues

When supernatants of human tissue extracts after centrifugation at 105 000 × *g* for 90 min were subjected to gel filtration with Sephadex G-200, two peaks of adenosine deaminase activity distinctly separated from each other were observed, as previously reported[14], suggesting the existence of two molecular species of the enzyme different in molecular size. A preliminary experiment for estimating molecular weights of the two species resulted in values of the order of 200 000 and 50 000 for the deaminases in the first (E_L) and the second (E_S) fractions, respectively. The relative ratio of the amounts of E_L and E_S in various tissue extracts varied with the sort of tissues employed for extraction but did not vary with storage of the extracts nor with increasing the ionic strength of the extracts. Distribution patterns of E_L and E_S in human tissues, so far as examined, are summarized in Table I. The liver and the lung tissues contain essentially only E_L. However, it is of interest that lung and liver cancer tissues contain considerable amounts of E_S though the amounts were variable in the individual tissues. On the other hand, fetal liver shows a similar pattern to normal adult liver. Thus E_L and E_S seemed to correspond to Types A and C, respectively, as described by Ma and Fisher[11–13].

TABLE I

DISTRIBUTION OF TWO TYPES OF ADENOSINE DEAMINASE IN HUMAN TISSUES

Tissue	E_L*	E_S**
Lung (normal)	++	
Liver (adult)	++	
Liver (fetus)	++	
Stomach	+	++
Leucocytes***		++
Lung (cancer)	+	+
Liver (cancer)	+	+

* Larger adenosine deaminase.
** Smaller adenosine deaminase.
*** Leucocytes from normal subjects and patients with myelocytic leukemia.

Purification of two molecular forms of adenosine deaminase from human tissues

E_L and E_S were purified from human lung and stomach extracts, respectively, by the following procedures. All steps were carried out at 0–4 °C, and all the buffers used in the purification contained 2 mM 2-mercaptoethanol. Frozen lung and stomach tissue samples were thawed overnight in the cold room and rinsed several times with cold distilled water to remove blood as thoroughly as possible. The tissue samples were minced using an electric meat grinder, and the minced tissues were then homogenized in 0.5 volume of 20 mM phosphate buffer (pH 7.0) using a Waring Blendor.

Purification of E_L

Step 1. To the homogenate of lung tissue (3 kg wet weight), an additional 0.5 volume of the same buffer was added and the homogenate was centrifuged at 40 000 $\times$ *g* for 60 min. The precipitate was discarded.

Step 2. Solid ammonium sulfate was added to the supernatant fluid until the salt concentration reached 30% saturation. The mixture was stirred for an additional 60 min and centrifuged at 20 000 $\times$ *g* for 20 min. The resulting supernatant fluid was brought to 55% saturation by adding solid ammonium sulfate slowly. The precipitate collected by centrifugation was dissolved in a minimal volume of 5 mM phosphate buffer (pH 7.0) and dialyzed against several changes of 3 l of the same buffer.

Step 3. The enzyme solution of *Step 2* was applied to a DEAE-cellulose column (3 cm $\times$ 40 cm) previously equilibrated with 5 mM phosphate buffer (pH 7.0). The column was first washed with 2 column volumes of the same phosphate buffer. Then a linear gradient elution was started with NaCl from 0 to 0.2 M in 5 mM phosphate buffer (pH 7.0). 5-ml fractions were collected. The enzyme emerged from the column at a NaCl concentration of approx. 0.09 to 0.1 M. The fractions of highest activity were pooled and brought to 80% saturation with ammonium sulfate by adding the solid salt. The precipitated protein obtained by centrifugation was dissolved in a minimal volume of 5 mM phosphate buffer (pH 7.0) and dialyzed for 20 h against three changes of 2 l of the same buffer.

Step 4. The resulting enzyme solution was again applied to a DEAE-cellulose column (1 cm $\times$ 30 cm) which had been equilibrated with 5 mM phosphate buffer (pH 7.0). After the column was washed with 2 column volumes of the same buffer, elution was performed by a linear gradient of phosphate buffer (pH 7.0) varying from 10 mM to 50 mM. Fractions of 2 ml were collected. The enzyme emerged at a phosphate concentration of approx. 30 mM. The fractions with the highest specific activity were pooled and then concentrated under reduced pressure to less than 2 ml using a collodion bag.

Step 5. The enzyme preparation of the previous step was finally subjected to horizontal zone electrophoresis using a 5% polyacrylamide gel (13.5 cm $\times$ 20 cm $\times$ 1 cm) in Tris–borate buffer (*I* 0.05, pH 8.4). The enzyme solution of approx. 2 ml was loaded in a sample trough (0.2 cm $\times$ 16 cm $\times$ 0.8 cm) and electrophoresis was carried out for 7 h at a constant current of 30 mA. Subsequently the gel was sliced in parallel with the direction of electrophoresis into one major and two minor segments. Each of the minor segments contained an end of the sample trough (0.5 cm in length). These two segments were then stained for protein for 10 to 15 min with 0.1% Amidoblack 10B (in 45% methanol, 9% acetic acid) followed by destaining in 5% acetic acid with constant stirring. Under these conditions, protein bands were visualized in 3 to 4 h

regardless of the insufficient destaining achieved meanwhile. One major, associated with the deaminase activity, and 2 to 3 minor protein bands were usually stainable at this stage of purification. Relative mobility of the major protein band was approx. 0.41 in comparison with human serum albumin as a standard. The major gel was then sliced in parallel with each protein band using the stained gel as a guide. The gel segments, each of which contained one protein band, were macerated using a spatula, followed by mixing with 10-ml portions of 50 mM phosphate buffer (pH 7.0). After standing overnight, each gel suspension was filtered through a glass-filter under reduced pressure. Each eluate obtained was tested for the deaminase activity. The gel segment corresponding to the major protein band was found to contain the enzyme activity, so that this was reextracted with an additional 5-ml portion of the buffer as above. Both eluates were combined and subsequently concentrated under vacuum to less than 2 ml using a collodion bag, and the concentrated enzyme solution was finally dialyzed overnight against 2 l of 50 mM phosphate buffer (pH 7.0). In Table II the results of the purification procedure were summarized.

The final preparation was stored at 0 °C. The deaminase activity was quite stable for at least one month.

TABLE II

SUMMARY OF PURIFICATION OF LARGER ADENOSINE DEAMINASE FROM HUMAN LUNG

	Volume (ml)	*Total protein (mg)*	*Total activity (units)*	*Specific activity (units/mg)*	*Yield (%)*
1. Crude extract	2240	159 000	190.4	0.012	100
2. $(NH_4)_2SO_4$, 30–55%	160	14 700	193.0	0.013	102
3. 1st DEAE-cellulose	166	274	86.6	0.31	44.5
4. 2nd DEAE-cellulose	62	21	15.5	0.74	8.2
5. Polyacrylamide gel electrophoresis	1.4	1.2	3.3	2.7	1.7

Purification of E_S

Step 1. To the homogenate of stomach tissue (540 g wet weight), an additional 1.5 volumes of 20 mM phosphate buffer (pH 7.0), was added and the homogenate was centrifuged at 40 000 × *g* for 60 min.

Step 2. The supernatant fluid was fractionated by ammonium sulfate at pH 7.0. The precipitate obtained between 40 to 60% saturation was dissolved in a minimum volume of 5 mM phosphate buffer (pH 7.0) and dialyzed against several changes of 3 l of the same buffer for 20 h.

Step 3. The dialyzed enzyme solution was applied to a DEAE-cellulose column (5.5 cm × 20 cm) which had been equilibrated with 5 mM phosphate buffer (pH 7.0). The column was first washed with 2 column volumes of the same buffer. Then elution was started by washing with 30 mM phosphate buffer (pH 7.0). 10-ml fractions were collected. The fractions of highest activity were collected and solid ammonium sulfate was added to the pooled effluents to bring the concentration to 75% saturation. The precipitate collected by spinning was redissolved in a small quantity of 5 mM phosphate buffer (pH 7.0), and the solution was dialyzed for 20 h against several changes of 2 l of the same buffer.

Step 4. The fraction of *Step 3* was loaded on a DEAE-cellulose column (2 cm × 36 cm) previously equilibrated with 5 mM phosphate buffer (pH 7.0), which was washed with 2 column volumes of the same buffer. The enzyme was eluted with 20 mM phosphate buffer (pH 7.0). 10-ml fractions were collected. The fractions of highest specific activity were combined and subjected to concentration under vacuum using a collodion bag to less than 2 ml.

Step 5. The concentrated enzyme solution was subsequently applied to preparative polyacrylamide gel electrophoresis. The experimental conditions were essentially the same as described in *Step 5* in the purification of E_L. Protein-staining with 0.1% Amidoblack 10B revealed three major and two minor bands. The protein band located in the most anodal side was associated with the deaminase activity. R_x value of the band was found as approx. 0.65 when human serum albumin served as a standard. The gel segment containing the activity was eluted with 15 ml of 50 mM phosphate buffer (pH 7.0), and the eluate was concentrated by ultrafiltration using a collodion bag.

The results of the purification procedure of E_S were summarized in Table III. The purified sample was found much more labile than the E_L preparation; more than 50% loss of activity was observed in 2 weeks by storage of the sample at 0 °C and almost complete inactivation in 24 h by freezing at −20 °C.

TABLE III

SUMMARY OF PURIFICATION OF SMALLER ADENOSINE DEAMINASE FROM HUMAN STOMACH

	Volume (ml)	*Total protein (mg)*	*Total activity (units)*	*Specific activity (units/mg)*	*Yield (%)*
1. Crude extract	1420	25 560	1075	0.042	100
2. $(NH_4)_2SO_4$, 40–60%	110	4 370	798	0.18	74
3. 1st DEAE-cellulose	605	929	414	0.44	38
4. 2nd DEAE-cellulose	156	32.6	157	4.8	14.5
5. Polyacrylamide gel electrophoresis	1.4	1.2	3.3	47.4	1.7

Purity

The degree of purity of the final purified preparations of E_L and E_S was first examined by thin-layer polyacrylamide gel electrophoresis. A single stainable protein band which was coincident with the enzymic activity was observed with the E_L preparation (Fig. 1A), but the E_S sample showed a major protein band associated with the activity and an additional two faintly stained minor bands (Fig. 1B).

Although no further examination for purity has been attainable with the E_S preparation, mainly because of its poor yield and instability, the purity of the E_L sample was further tested by the following two methods. Upon disc polyacrylamide gel electrophoresis, the sample again exhibited a single protein band (Fig. 2). On the other hand, application of the E_L preparation to analytical ultracentrifugation resulted in two schlieren peaks, $s_{20,w}$ values of which were calculated as $2.30 \cdot 10^{-13}$ s^{-1} for the slower moving component and $6.42 \cdot 10^{-13}$ s^{-1} for the faster moving component, respectively. The preparative ultracentrifugation for separation of the two components by prolonged centrifugation using a separation cell showed that the 6.42-S component demonstrated an intensive adenosine deaminase activity, whereas the 2.30-S component showed neither enzyme activity nor appreciable absorption at 280

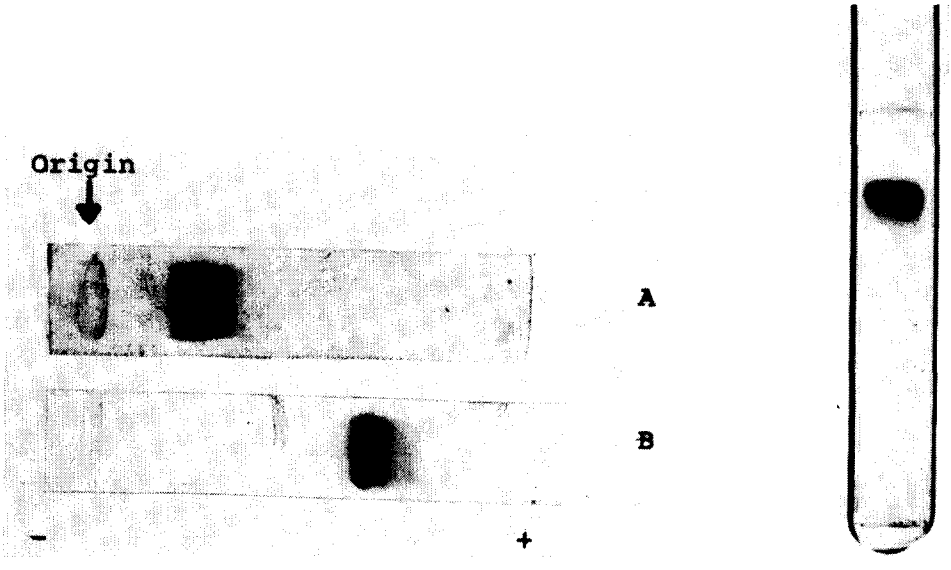


Fig. 1. Thin-layer polyacrylamide gel electrophoresis of two purified adenosine deaminases. Experimental details are given in Materials and Methods. A, the larger deaminase (120 μg protein); B, the smaller deaminase (30 μg protein).

Fig. 2. Disc-gel electrophoresis of purified larger adenosine deaminase. Electrophoresis was carried out with 7.5% polyacrylamide gel in Tris–glycine buffer (pH 8.9) for about 1.5 h at 3 mA per tube using 30 μg of protein of the finally purified sample.

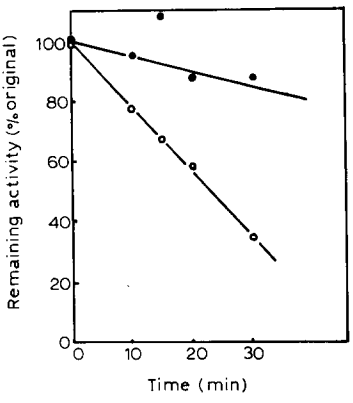
nm. The slower moving component was later found to be derived from polyacrylamide gel during the extraction procedure of the enzyme at the final step of the purification, presumably consisting of small fragments of polyacrylamide polymer, since the extract of macerated polyacrylamide gel without application of the enzyme demonstrated a single schlieren peak corresponding to the slower moving component. An attempt to remove the contaminant without appreciable loss of the enzyme activity has been unsuccessful.

Stability

As already mentioned above, the purified E_S preparation was more rapidly inactivated than the E_L preparation when stored at 0 °C in 50 mM phosphate buffer (pH 7.0), containing 2 mM 2-mercaptoethanol. Consistent results were obtained by heat treatment (Fig. 3). When heated at 55 °C for 30 min, the E_L sample lost approximately 17% of its original activity in contrast to approx. 66% loss of activity with E_S sample. Accordingly, it is obvious that E_L is much more stable than E_S.

Determination of molecular weight

The determination of molecular weights of E_L and E_S were first performed with a calibrated Sephadex G-200 column according to the method of Andrews[9]. Cytochrome *c*, trypsinogen, bovine hemoglobin, human albumin, human γ-globulin and catalase served as standard proteins. The molecular weights of E_L and E_S were estimated to be 230 000 and 47 000, respectively (Fig. 4).



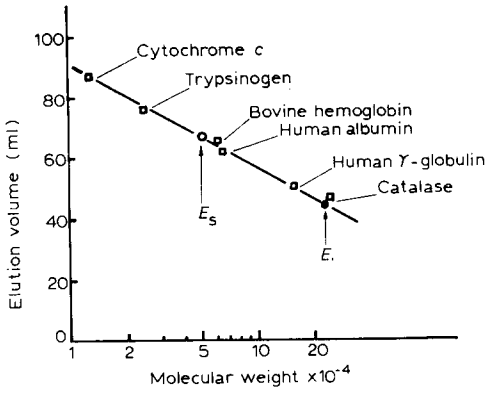


Fig. 3. Heat stabilities of the two adenosine deaminases. Purified enzymes (10 μg protein/ml) in 30 mM phosphate buffer (pH 7.4) *plus* 5 mM 2-mercaptoethanol were heated at 55 °C. At the time intervals indicated aliquots were taken for the activity assay. ●, the larger enzyme; ○, the smaller enzyme.

Fig. 4. Determination of molecular weights of two adenosine deaminases with a Sephadex G-200 column. The column (2 cm × 30 cm) equilibrated with 30 mM phosphate buffer (pH 7.0) containing 2 mM 2-mercaptoethanol was employed. 1-ml fractions were collected.

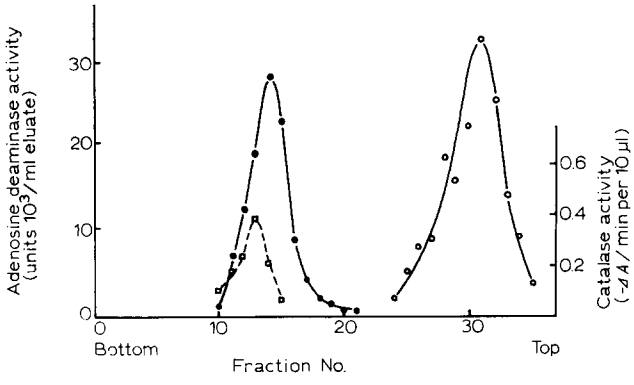


Fig. 5. Determination of molecular weights of two adenosine deaminases by sucrose density gradient centrifugation. Details are in Materials and Methods. ●, the larger enzyme; ○, the smaller enzyme; □, catalase.

Sucrose density gradient centrifugation was conducted in order to confirm the values obtained by gel filtration. When catalase served as a standard, the molecular weights of E_L and E_S were calculated to be 225 000 and 44 000, respectively, in agreement with the results of gel filtration experiments (Fig. 5).

pH optimum

Both enzymes showed a pH optimum around 7.0, similar to the deaminases from other mammalian sources (Fig. 6).

Substrate specificity and inhibition

Both enzymes, so far as tested, can catalize the hydrolytic deamination only of adenosine and deoxyadenosine. As in Table IV, no remarkable difference was observed

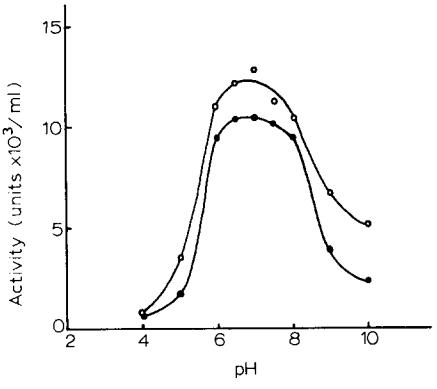


Fig. 6. pH activity profiles of two adenosine deaminases. ●, the larger enzyme; ○, the smaller enzyme. 0.05 M acetate buffer (pH 4–5), 0.05 M phosphate buffer (pH 6–8) and 0.05 M borate–NaOH buffer (pH 9–10) were used.

TABLE IV

SUBSTRATE SPECIFICITIES AND INHIBITIONS OF TWO ADENOSINE DEAMINASES

Substrate	*Inhibitor*	*Concentration*		*% Activity*	
		Substrate (mM)	*Inhibitor (mM)*	*(E_L)***	*(E_S)****
Adenosine	—	5.0	—	100	100
Deoxyadenosine	—	5.0	—	76.1	57.8
Guanosine	—	5.0	—	0	0
Adenosine	PCMS*	5.0	1.0	0	0
Adenosine		0.5		100	100
	Inosine	0.5	5.0	60.8	71.3
	Guanosine	0.5	5.0	43.3	43.9
	Adenine	0.5	5.0	73.9	66.6
	2-Aminopurine	0.5	5.0	71.0	61.0
	$Hg(NO_3)_2$	0.5	$2.0 \cdot 10^{-4}$	20.0	27.0
	$CuSO_4$	0.5	0.1	61.0	53.0
	$MgCl_2$	0.5	1.0	100	100

* *p*-Chloromercuriphenylsulfonate.
** Larger adenosine deaminase.
*** Smaller adenosine deaminase.

between the two enzymes in the relative substrate specificity (ratio of activity with deoxyadenosine to that with adenosine as substrate). Adenine, 2-aminopurine, AMP, ATP, guanosine, GMP and GTP were not decomposed by both enzymes.

Inhibitory effects of metals and substrate analogs on E_L and E_S are also shown in Table IV. The two deaminases were inhibited to similar extents by the compounds tested. Both enzymes were inactivated completely when preincubated with *p*-chloromercuriphenylsulfonate, a sulfhydryl blocker, at the concentration of 1 mM, suggesting that human adenosine deaminases also contain the essential sulfhydryl group(s). AMP, ATP, GMP and GTP showed no significant stimulatory nor inhibitory effect at the concentration of 10 mM on the deaminating activity of E_L as well as E_S.

Michaelis constant

The K_m values with respect to adenosine were estimated to be $(6.6 \pm 0.24) \cdot 10^{-5}$ M and $(7.4 \pm 0.40) \cdot 10^{-5}$ M for E_L and E_S, respectively, when the activities were measured colorimetrically at 37 °C in 50 mM phosphate buffer (pH 7.0). These values are of the same order of magnitude as those reported for this deaminase from calf. intestine[5], calf serum[2] and chicken liver[11]. Both of the enzymes showed the same K_m value of $4.0 \cdot 10^{-5}$ M with respect to deoxyadenosine in the colorimetric assay (37 °C, pH 7.0). The K_m values of E_S were also measured spectrophotometrically by following the decrease in the absorbance at 265 nm in 40 mM phosphate buffer (pH 7.0) at 25 °C; the K_m with respect to adenosine was $3.1 \cdot 10^{-5}$ M and that with respect to deoxyadenosine was $3.7 \cdot 10^{-5}$ M. Thus no significant differences between the two deaminases were noted in the affinities toward substrates.

Immunological characterization

Immunological distinction between E_L and E_S was examined by preparing antisera against the two purified preparations as described in Materials and Methods. In immunodiffusion tests with these antisera in every possible combination against the antigens, we failed to detect any apparent precipitation line. However, preincubation of E_L or E_S with either one of the antisera caused a definite loss of enzymic activity in both enzymes, as shown in Table V. Normal rabbit serum did not show any appreciable inhibition of the enzyme activities. Thus the deaminases are unable to be differentiated immunologically from each other.

TABLE V

NEUTRALIZATION OF THE ENZYME ACTIVITY BY ANTISERA AGAINST TWO ADENOSINE DEAMINASES

0.1-ml portions of antisera were mixed and preincubated at 37 °C for 10 min with 0.1-ml aliquots of E_L (0.011 unit) or E_S (0.016 unit) and then 0.1-ml aliquots of the mixtures were subjected to the activity assay. Tubes consisting of 0.1-ml portions of normal rabbit serum and enzyme served as controls representing 100% activity.

Enzyme sample	*% Loss of activity*	
	Incubated with antiserum against E_S	*Incubated with antiserum against* E_L
E_S*	83	33
E_L**	67	52

* Smaller adenosine deaminase.
** Larger adenosine deaminase.

Interconversion between the two deaminases

In order to clarify the relationship between the molecular structures of E_L and E_S, attempts to convert the larger enzyme to the smaller were performed by treating a purified E_L sample with various agents; *i.e.* acetone, phospholipase A and D, neuraminidase and ribonuclease. No apparent changes were observed in elution profiles of thus treated E_L samples upon gel filtration with Sephadex G-200. When the enzyme sample previously incubated at room temperature for 30 min with 2.7 M guanidine hydrochloride was loaded on a Sephadex G-200 column, however, more than 60% of the total deaminase activity in the effluents was found shifted to the fractions corre-

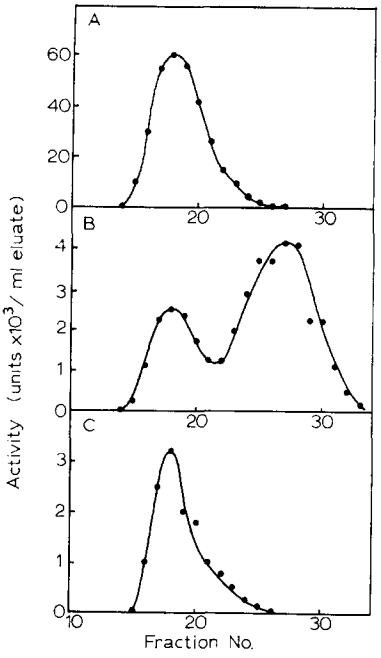


Fig. 7. Treatment of purified larger adenosine deaminase with guanidine hydrochloride. 0.34 unit of the enzyme was incubated at room temperature for 30 min in 2.7 M guanidine at pH 5.5 in a total volume of 0.56 ml. The mixture was then applied to a Sephadex G-200 column (1 cm × 54 cm) equilibrated with 20 mM phosphate buffer (pH 7.0) containing 2 mM 2-mercaptoethanol. 1-ml fractions were collected. The elution profile was as shown in B, where 0.041 unit of the activity were recovered. Tubes Nos 24–30 were combined, followed by concentration and exhaustive dialysis. The concentrated sample (0.018 unit of adenosine deaminase) was again loaded on the same Sephadex column. The elution pattern obtained was shown in C, where 0.013 unit of the activity were recovered. Untreated enzyme (0.34 unit) showed the elution pattern (0.31 unit recovered) on the Sephadex column as in A.

sponding to E_S, as shown in Fig. 7, though only about 10% of the original activity was recovered. The fractions comparable to E_S were then collected and were subjected to concentration and dialysis to remove guanidine under vacuum using a collodion bag. The concentrated sample was subsequently applied to the same Sephadex column as used previously. Consequently, the deaminase activity was recovered in the fractions where the E_L sample was originally present. Thus the larger deaminase is suggested to be converted to the smaller enzyme in the presence of guanidine salt. Low recovery of the activity after treatment with guanidine may raise an alternative possibility that E_L was converted to a less active subunit which happened to have a molecular weight near that of E_S, but could not be identical with E_S. The E_L sample treated with the same concentration of guanidine was applied to thin-layer polyacrylamide gel electrophoresis, followed by staining the activity of adenosine deaminase essentially according to the method of Spencer *et al.*[3] (detailed method will be described in the following paper). Two spots of enzyme activity were detected, one small spot corresponding to untreated E_L and the other large spot to untreated E_S. This is less favorable for the possibility of conversion of E_L to a less active subunit happened to be fractionated at the fraction of E_S with Sephadex column.

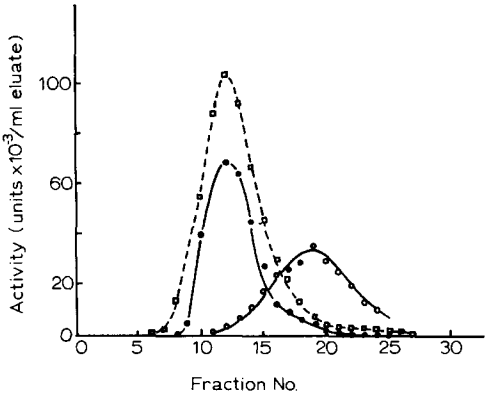


Fig. 8. Elution profile of smaller adenosine deaminase previously incubated with normal lung supernatant. 0.027 unit of a partially purified smaller deaminase sample (Step 3, Table III) was incubated at room temperature for 1 h with 1.5 ml of a supernatant fraction (105 000 × *g*, 90 min) of normal lung extract in a total volume of 1.65 ml. The mixture was applied to a Sephadex G-200 column (1 cm × 30 cm) equilibrated with 20 mM phosphate buffer (pH 7.0) containing 2 mM 2-mercaptoethanol. 1-ml fractions were collected. □---□, the mixture; ●—●, lung supernatant without the enzyme sample; ○—○, the enzyme sample *plus* 1.5 mg of bovine serum albumin which was added to stabilize the enzyme.

An attempt to convert E_S to E_L was made as follows: A crude normal lung extract after centrifugation at 105 000 × *g* for 60 min was mixed with the partially purified E_S sample, followed by incubation at room temperature for 60 min. The mixture was then applied to a Sephadex G-200 column. The E_S sample and the crude lung supernatant which contained a considerable amount of E_L were also applied separately to the same column. As shown in Fig. 8, the elution profile of the mixture showed a substantial decrease of the activity in the E_S fraction with a concomitant increase of the activity in the E_L fraction when compared with the elution profiles of the control samples. Similar results were obtained by incubating the mixture overnight in the cold room (4–6 °C), but no such changes were observed when the mixture was applied to the column without any incubation.

DISCUSSION

Through extensive studies on bovine intestinal adenosine deaminase by Brady and his collaborators[1,5,10], all isozymes of the deaminase were shown to have similar molecular weights of approx. 35 000. Cory *et al.*[2] first showed an adenosine deaminase of higher molecular weight in calf serum and then Fisher and Ma[11,13] found larger adenosine deaminases in the livers of various amphibians and mammals. They classified the enzymes into Types A, B and C, which have molecular weights in the order of 200 000, 100 000 and 35 000. Murphy *et al.*[23] have also found a large adenosine deaminase in rabbit duodenum having a molecular weight of 250 000.

We have demonstrated here the presence of two molecular species of this particular enzyme, E_L and E_S, in the soluble fractions of human tissue extracts. In view of their molecular sizes, which strikingly differed from each other, E_L and E_S seemed to correspond to Types A and C enzymes, respectively[11–13]. Tissue-specific distributions of E_L and E_S have also been shown. Normal human liver and lung tissues contain

almost exclusively only E_L, while similar patterns were reported by Ma and Fisher[13] in the livers of eastern cottontail and marsh rabbit. Each of the enzyme species has been successfully purified from the lung and the stomach to a nearly homogeneous state and their general enzymological as well as immunochemical properties have been investigated. Through these studies only a few remarkable differences between their properties have been clarified. Molecular weights of approximately 230 000 and 47 000 were obtained with a calibrated Sephadex column. Sucrose density gradient centrifugation carried out to confirm the above results gave fairly consistent values (225 000 and 44 000). The molecular weight of E_S was considerably greater than those of small adenosine deaminases from other mammalian sources (approx. 35 000). Further detailed study, such as amino acids analysis, should be made for determining the molecular weight of E_S conclusively, but the difference may be derived from the differences in the species of the enzyme sources. The specific activity of the most highly purified E_S preparation was found to be about 20 times higher than that of the purest E_L sample. The values of molecular activity (units per mole of enzyme) were about 4 times greater in E_S than in E_L. On the other hand, the purified E_L sample was obviously more stable than the E_S sample. These differences in the properties may represent the different physiological roles of the two adenosine deaminases in human tissues.

Ma and Fisher observed markedly different relative substrate specificities between Types A and C enzymes in the liver of chicken[11] but they did not find the same type of difference in the liver of higher mammals. Similarly in human adenosine deaminases we could not detect any significant change in the specificity (deoxyadenosine:adenosine ratio; 0.76 and 0.58 for E_L and E_S, respectively). They also reported that higher mammalian Type A enzyme was able to be dissociated into Type C enzyme in the presence of high concentrations of ammonium sulfate or sucrose, so that they assumed Type A enzyme as a polymeric form of Type C enzyme. In human adenosine deaminases, however, high concentrations of salts including ammonium sulfate or sucrose apparently did not affect the molecular size of E_L when judged by gel filtration. Treatments with enzymes involving ribonuclease, neuraminidase, and phospholipase A and D caused no apparent change on the size of E_L. However, when E_L was treated with guanidine salt a reversible shift of the elution position of E_L activity to that of E_S activity was observed upon Sephadex chromatography. Furthermore, E_S appeared to be converted to E_L by incubating E_S with normal lung supernatant. Although the products of these treatments have to be confirmed as identical with natively existing E_L or E_S, these findings suggest that E_L and E_S are interconvertible and afford us two possible interpretations of the structural relationship between E_L and E_S: (1) E_L is a polymeric form of E_S, (monomeric and tetrameric forms can be assumed due to their molecular weights). (2) E_L is a complex of E_S and some other macromolecule(s).

By the finding of the apparent conversion of E_S to E_L in the presence of normal lung sap, we supposed the existence of some unknown factor(s) capable of stimulating conversion of E_S to E_L and termed it tentatively the "conversion factor". We undertook to isolate conversion factor from lung extracts in order to clarify the physicochemical nature and role of the factor. Preliminary tests suggested the factor to be of protein nature. Recently we have succeeded in obtaining electrophoretically nearly homogeneous conversion factor preparations free of adenosine deaminase activity. The molecular weight appeared to be about 140 000 by gel filtration with Sephadex

G-200. Experiments so far conducted to elucidate the mechanism of function of conversion factor toward E_S seem to suggest that conversion factor is bound directly with E_S without exerting any catalytic activity upon polymerization of E_S, supporting the latter assumption. Detailed data concerning the conversion factor and the structural relationship between the two enzyme species will be presented in the following report.

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