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LATENT ADENOSINE DEAMINASE IN MOUSE BRAIN

II. PURIFICATION AND PROPERTIES OF MITOCHONDRIAL AND SUPERNATANT ADENOSINE DEAMINASES

SYED JAMAL MUSTAFA* AND CHANDRA PRAKASH TEWARI
Department of Biochemistry, Lucknow University, Lucknow U.P. (India)

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SUMMARY

1. Adenosine deaminase was purified 160-fold using the mitochondrial extracts and 200-fold from the 15 000 × *g* supernatant of mouse brain homogenates by a combination of ammonium sulfate precipitation, dialysis and DEAE-cellulose column chromatography. The enzymes emerged as a single peak from DEAE-cellulose column using buffers of different molarity.

2. There was selective inhibition of the supernatant enzyme by Ba^{2+}, Ca^{2+} and Mg^{2+}, whereas the mitochondrial enzyme was selectively inhibited by Cu^{2+} and Molybdenum.

3. A comparison of properties (such as K_m value, heat inactivation behaviour towards PCMB, immunochemical and electrophoretic properties) of the enzyme purified from the mitochondrial fraction with those of the cytoplasmic fraction revealed the two forms as distinct molecular species.

INTRODUCTION

The distribution of adenosine deaminase (adenosine aminohydrolase, EC 3.5.4.4) in subcellular fractions of adult mouse brain and its presence in latent form were reported in our earlier communication[1]. The major part of the activity of the homogenate was recovered in the 15 000 × *g* supernatant. The mitochondrial fraction had negligible activity; however, on treatment with Triton X-100, this fraction showed considerable adenosine deaminase activity.

The present communication deals with the purification of mitochondrial and supernatant enzymes of adult mouse brain and a comparison of their properties. The results reported here reveal many differences between the two fractions, pointing to distinct molecular forms of the enzyme.

* Address of the author to whom reprint requests should be addressed: Industrial Toxicology Research Centre, Chattar Manzil Palace, Lucknow (India).

MATERIALS AND METHODS

Animals

Adult male mice were sacrificed by cervical dislocation, followed by decapitation. The brain was excised as a whole as quickly as possible, cleaned free of blood and extraneous material and used fresh.

The homogenate and various subcellular fractions from adult mouse brain were prepared by the method of MUSTAFA AND TEWARI[1].

Adenosine deaminase assay

Assay of the enzymic activity was done by direct and indirect spectrophotometry. The direct method of spectrophotometry was employed only in a limited number of experiments with purified enzyme preparations. The indirect method of spectrophotometry was usually preferred because of temperature control.

(a) *Direct:* To an appropriate quantity of enzyme preparation contained in 3.0 ml quartz cells, 1.0 ml 0.2 M Tris–HCl or phosphate buffer (pH 7.0) was added, followed by the addition of water to 2.90 ml, and the reaction was started by the addition of 0.05 μmole adenosine in 0.10 ml. The absorption was read initially and at 5-min intervals over a 15-min period at 265 nm.

(b) *Indirect:* This procedure differed slightly from that reported earlier[1] in which the time of incubation was 30 min and the supernatant after centrifugal clarification was diluted 2-fold. In this case the time of incubation was 15 min and the supernatant after centrifugal clarification was not diluted further.

Enzyme unit and specific activity

A unit of enzyme was the deamination of 1 μmole of adenosine in 15 min under the assay conditions. The specific activity was the activity in units per mg protein.

Protein estimation

Protein was usually estimated by the method of LOWRY *et al.*[2]. In eluate fractions from DEAE-cellulose column, the estimation was made by the spectrophotometric method of WARBURG AND CHRISTIAN[3], using the equation of KALCKAR[4].

Purification of adenosine deaminase

(a) *Supernatant enzyme:* The starting material was the 15 000 $\times$ *g* supernatant, which corresponded to a 10% homogenate obtained from whole brain tissue of adult male mice. The supernatant was fractionated with ammonium sulfate, and the precipitate, separating in the saturation range 0.4–0.8, was dialysed against repeated changes of water in the cold over a period of 6–8 h. The resulting suspension was centrifuged at 15 000 $\times$ *g* to separate the inactive bulky and protein-rich residue. The clear supernatant containing the enzyme activity was adjusted to 0.02 M phosphate concentration (pH 7.0) by mixing the required amount of 0.2 M phosphate buffer.

An aliquot of the sample was applied on the DEAE-cellulose column equilibrated with 0.02 M phosphate buffer (pH 7.0). The column was washed down with the equilibrating buffer. Elution was attempted by gradual increase in the molarity of the phosphate buffer (pH 7.0).

(b) *Mitochondrial enzyme:* The mitochondrial fraction was treated with Triton X-100 under optimum conditions[1], and the resulting supernatant obtained on centrifugation at 15 000 × *g* for 30 min was fractionated with ammonium sulfate. The precipitate, separating in the saturation range 0.4–0.8, was dialysed against water and adjusted to 0.02 M phosphate concentration with 0.2 M phosphate buffer (pH 7.0). The sample passed through a DEAE-cellulose column equilibrated with 0.02 M phosphate buffer (pH 7.0) and eluted with increasing molar concentrations of phosphate buffer (pH 7.0).

Immunochemical characterization

The general techniques employed were as described by KABAT AND MAYER[5]. Male rabbits, about 9 months old, were used for raising antibodies against a partially purified preparation of the supernatant enzyme given intravenously. Starting with an initial dose of 0.5 mg protein and gradually increasing the amount, a total of 28.5 mg was injected into each animal through an ear vein over a period of about 30 days. About 15 ml blood was withdrawn from each rabbit by cardiac puncture, the serum separated and stored frozen. The immunochemical test was carried out by the precipitin reaction and by enzyme inhibition.

Starch gel electrophoresis

The two enzyme preparations were subjected simultaneously to electrophoresis on starch gel. For determining the enzyme activity in the gel regions, 1-cm wide cut gel pieces were suspended in 0.02 M phosphate buffer and used as such for the enzyme assays. As a control a cut gel piece without any enzyme was also suspended and assayed.

RESULTS

Purification of adenosine deaminase

(a) *Supernatant enzyme:* The results obtained in a typical experiment are given in Table I and Fig. 1.

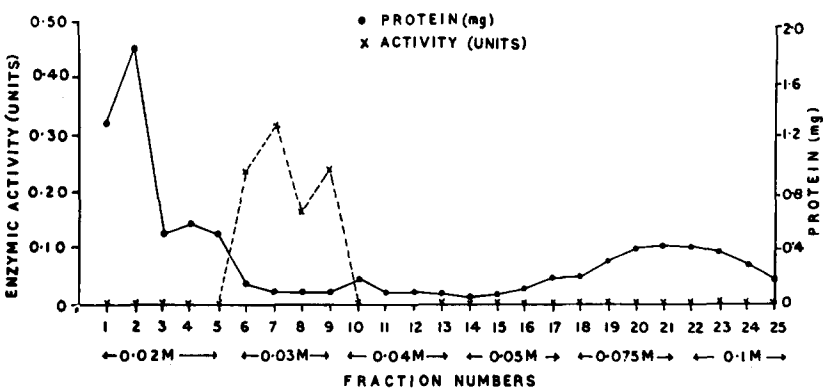

Fig. 1. Elution pattern of mouse brain supernatant adenosine deaminase from DEAE-cellulose column. Deaminase activity and protein are expressed as total content per 4-ml fraction collected.

TABLE I

ENRICHMENT OF ADENOSINE DEAMINASE FROM MOUSE BRAIN SUPERNATANT (15 000 × *g*)

Only a part of the partially purified enzyme fraction III was applied on DEAE-cellulose column equilibrated with 0.02 M phosphate buffer, pH 7.0; the % recovery data were recalculated to correspond to the whole enzyme solution.

Fractions	*Volume (ml)*	*Total activity (units)*	*Total protein (mg)*	*Specific activity*	*Fold enrich-ment*	*Yield (%)*
I. 15 000 × *g* supernatant	36	3.2	114.3	0.026	(1)	(100)
II. Fractionation with $(NH_4)_2SO_4$						
(a) 0–40% saturation	4	Nil	70.4	—	—	—
(b) 40–60% saturation	3	1.4	15.7	0.092	4	43
(c) 60–80% saturation	3	1.9	12.0	0.16	6.2	59
III. Fraction II (b) and (c) were mixed, dialysed and clarified	10	3.2	16.8	0.2	8	100
IV. DEAE-cellulose column chromatography 5.0 ml of fraction III loaded on column and enzyme eluted with 0.03 M phosphate buffer, pH 7.0						
Fraction No. 6	4	0.24	0.14	1.7	85	15
7	4	0.32	0.08	4.0	200	20
8	4	0.16	0.08	2.0	100	10
9	4	0.24	0.07	3.4	170	15

The enzyme was eluted with 0.03 M phosphate buffer in four successive fractions with a total recovery of 80% of the enzyme sample applied on the column. All the other fractions were devoid of the enzymic activity. These results indicated the presence of only one type of adenosine deaminase in the supernatant.

TABLE II

ENRICHMENT OF ADENOSINE DEAMINASE FROM MOUSE BRAIN MITOCHONDRIA

Only a part of the partially purified enzyme fraction III was applied on DEAE-cellulose column equilibrated with 0.02 M phosphate buffer, pH 7.0; the % recovery data were recalculated for the whole enzyme solution.

Fractions	*Volume (ml)*	*Total activity (units)*	*Total protein (mg)*	*Specific activity*	*Fold enrich-ment*	*Yield (%)*
I. Extract after exposure	30	1.2	231	0.005	(1)	(100)
II. Fractionation with $(NH_4)_2SO_4$						
(a) 0–40% saturation	15	Nil	207	—	—	—
(b) 40–90% saturation	6	1.2	39	0.03	6	100
III. Dialysed and clarified	11	1.3	22	0.06	12	108
IV. DEAE-cellulose column chromatography 4.5 ml of fraction III loaded on column and enzyme eluted with 0.05 M phosphate buffer, pH 7.0	(4.5)	(0.53)	(9)	(0.06)	(12)	(108)
Fraction No. 7	4	0.44	0.55	0.8	160	83
Fraction No. 8	4	0.06	0.27	0.22	44	11

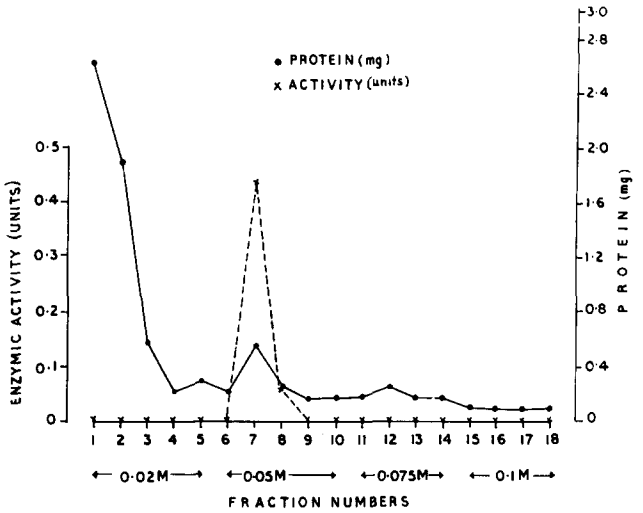

Fig. 2. Elution pattern of mouse brain mitochondrial adenosine deaminase from DEAE-cellulose column. Deaminase activity and protein are expressed as total content per 4-ml fraction collected.

(b) *Mitochondrial enzyme:* The results obtained in a typical experiment are given in Table II and Fig. 2.

The enzyme activity was recovered in two successive fractions with 0.05 M phosphate buffer (pH 7.0). It was clear that a single type of enzyme protein was present. The enzyme activity recovered in two successive fractions, 7 and 8, represented 94% of the enzyme applied on the column. Of the total protein applied on the column, 78% was recovered in the eluates with various buffers.

As the activity was not eluted with 0.03 M phosphate buffer, which was tested in a separate experiment (not reported here), we passed on directly to 0.05 M phosphate buffer.

Properties of the supernatant and the mitochondrial adenosine deaminases

In the majority of the experiments reported here, fraction IV of the supernatant and the mitochondrial enzymes were employed. Mention has been made in the text wherever Fraction III was used. The aliquots of the mitochondrial and the supernatant enzyme preparations taken in the assay system for the study of a particular effect had nearly the same enzymic activities; however, the amounts of protein were different.

pH–activity relationship: The pH–activity relationship with Tris (Universal) buffer is given in Fig. 3.

Both enzymes showed a pH optimum at 7.0; however, shape of the curves in the two cases was different. The supernatant enzyme retained 55% and 33% of its activity at pH 9.0 and 9.5, respectively, in comparison to the activity at pH 7.0;the mitochondrial enzyme showed no activity at pH values 9.0 and 9.5. It should be mentioned here that the two enzymes showed some difference in their behaviour at different pH values, although they had the same optimum pH.

Inhibition by p-chloromercuribenzoate (PCMB) and reversal by cysteine: An

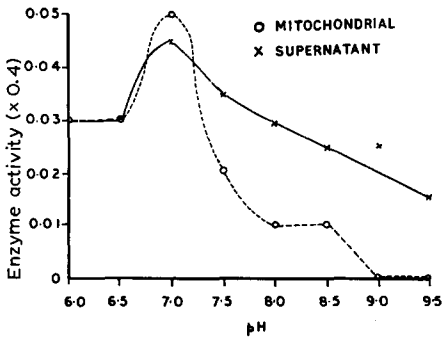

Fig. 3. pH–activity relationship of the adenosine deaminases from mouse brain.

aliquot of fraction III with a protein content of 134 μg and 168 μg in the mitochondrial and the supernatant enzyme, respectively, was employed in the assay system. PCMB at $1 \cdot 10^{-5}$ M concentration inhibited 50% of the mitochondrial enzyme activity. There occurred 72% inhibition at $1 \cdot 10^{-4}$ M and complete inhibition at $2 \cdot 10^{-4}$ M. The inhibition which resulted with $1 \cdot 10^{-5}$ M PCMB was reversed by the subsequent addition of cysteine at a concentration of $3.3 \cdot 10^{-5}$ M. The supernatant enzyme, on the other hand, showed no inhibition at $1 \cdot 10^{-5}$ M but resulted in 45% and 50% inhibition at $2 \cdot 10^{-5}$ and $4 \cdot 10^{-5}$ M concentrations, respectively. No complete inhibition could be achieved even at much higher concentrations, namely $6 \cdot 10^{-5}$ M, 1 and $3 \cdot 10^{-4}$ M. The inhibition in the case of the supernatant enzyme which resulted at $2 \cdot 10^{-5}$ M was reversed by subsequent addition of $6.6 \cdot 10^{-5}$ M cysteine.

Michaelis constant: The K_m value was determined by Lineweaver–Burk[6] double reciprocal plot. The results of a typical experiment with both mitochondrial and supernatant enzymes for the K_m values and interaction coefficients are given in the Figs. 4 and 5.

The K_m values of the supernatant and mitochondrial enzymes for adenosine were found to be $6.4 \cdot 10^{-6}$ M and $11 \cdot 10^{-6}$ M, respectively. The interaction coefficients, obtained from the HILL[7] plot, were found to be 1.08 and 1.0 for the supernatant and

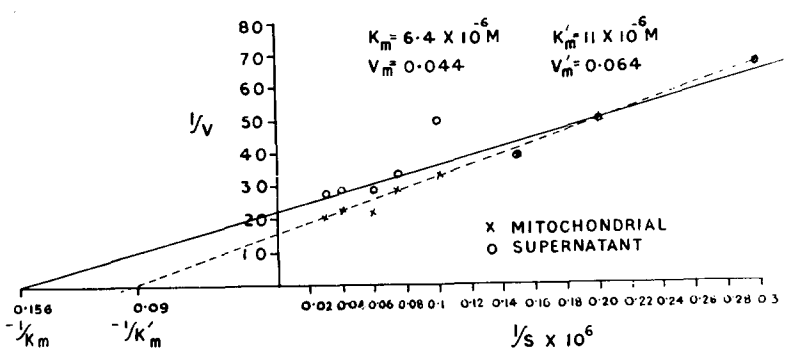

Fig. 4. Lineweaver–Burk plots of mouse brain adenosine deaminases. The assay system contained 20.4 μg protein in the case of the mitochondrial enzyme and 8.0 μg in the case of the supernatant enzyme of the Fraction IV, and adenosine added was in the range 0.01–0.10 μmoles in a total volume of 3.0 ml.

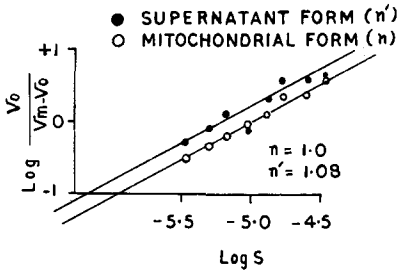

Fig. 5. Hill plots of mouse brain adenosine deaminases.

mitochondrial enzymes, respectively, which ruled out the possibility of the enzymes being allosteric in nature.

Inorganic ions have been shown to act as an allosteric effector in the case of AMP deaminase[8,9]. As the enzyme obtained after DEAE-cellulose column chromatography was in phosphate buffer, the authors thought it worthwhile to investigate whether phosphate ions acted as an allosteric effector for adenosine deaminase. Thus, the purified fraction, obtained after column chromatography, was dialysed against distilled water to remove phosphate ions. Experiments were carried out with this enzyme preparation using Tris–HCl buffer instead of phosphate buffer, as used above. This experiment was done only with the mitochondrial enzyme. There was no indication that the phosphate ions acted as an effector molecule for the enzyme.

The possibility of the allosteric nature of these enzymes was further tested by drawing a number of curves, viz. v and s; s/v and $s;v$ and -log$s$[10]. All these curves finally excluded the possibility of these two forms of the enzyme having an allostric nature.

It was, therefore, clear that the two forms of adenosine deaminases differed in their affinity towards the substrate. The mitochondrial enzyme had a low affinity for adenosine, as compared with the supernatant enzyme.

Heat inactivation: Equal amounts of the supernatant and mitochondrial enzyme proteins were taken in a number of tubes, and the volume adjusted to 0.50 ml with water; the final pH was 6.1. Tubes were heated in water baths of different temperatures for 5 min each, immediately cooled in an icebath for 30 min and then assayed. A control was run simultaneously.

Both the enzymes retained their activity up to 50°. The supernatant enzyme retained its full activity up to 60°, and any further rise in temperature resulted in loss of almost all enzyme activity. The mitochondrial enzyme was somewhat more stable up to 70° with a loss of 22% at 60 and 70°; any further rise in temperature resulted in complete loss of the enzymic activity. The mitochondrial and the supernatant enzymes were, therefore, different in their stability towards heat treatment.

Effect of metallic ions: The enzyme and the metal supplements were preincubated before starting the reaction.

Whereas Mn^{2+} and Zn^{2+} inhibited both the enzymes, though in varying degrees, Mg^{2+}, Ba^{2+} and Ca^{2+} did not inhibit the mitochondrial enzyme but totally inhibited the supernatant enzyme when used in final concentrations of $5.0 \cdot 10^{-3}$, $7.5 \cdot 10^{-3}$ and $5.0 \cdot 10^{-3}$ M, respectively. Cu^{2+} and Molybdate showed a reverse pattern; there occurred a 50% and 66% inhibition in the activity of mitochondrial enzyme at $3.3 \cdot 10^{-5}$

M and $0.66 \cdot 10^{-5}$ M concentrations of Molybdenum and Cu^{2+}, respectively.

Effect of EDTA and fluoride: Fluoride, used in final concentration up to $10 \cdot 10^{-3}$ M and EDTA in final concentration up to $5 \cdot 10^{-3}$ M in the assay system, were found to be without effect on either enzyme.

Substrate specificity: Adenosine triphosphate, cytosine, guanine and guanosine-mono-phosphate were tested as substrates. The assays were carried out employing 17 μg and 15.6 μg protein of the mitochondrial and the supernatant enzymes, respectively.

None of the substrates were acted upon, as shown by the constancy of absorption at 265 nm and at the respective absorption maxima for the various compounds.

Immunochemical tests. The reaction between the antiserum of the supernatant enzyme and the two forms of adenosine deaminases were followed visually by precipitate formation and quantitatively by centrifugation to sediment any precipitate and carry out enzymic activity determination with the supernatant. In the control tubes, a sample of each of the enzymes was made to 0.90 ml volume with normal saline and stored for the same period in the cold to test the storage stability. The results are reported in Table III.

The supernatant obtained on centrifugation of the reaction system, containing the antiserum of the supernatant enzyme and the purified supernatant enzyme, was

TABLE III

REACTION OF THE ANTISERUM OF THE SOLUBLE ENZYME WITH THE "MITOCHONDRIAL" AND THE "SOLUBLE" FORMS OF ADENOSINE DEAMINASES

Enzyme sample/amount of protein (μg)		*Enzymic activity without reaction with the antiserum*			*Enzymic activity after antigen–antibody reaction*		
		Units in whole of sample		*Loss*	*Units in total supernatant of sample*	*Inhibition (%)*	
		Initial	*Stored*	*(%)*			
Mitochondrial							
(a) 100-fold purified							
(i)	29.25	0.010	0.010	nil	0.008	nil	No precipitate
(ii)	39.0	0.014	0.014	nil	0.015	nil	id.
(iii)	58.5	0.020	0.020	nil	0.020	nil	id.
control	58.5	0.020	0.020	nil	—	—	id.
(b) 300-fold purified							
(i)	8.75	0.009	0.009	nil	0.010	nil	No precipitate
(ii)	14.0	0.015	0.015	nil	0.016	nil	id.
(iii)	27.0	0.022	0.022	nil	0.022	nil	id.
control	27.0	0.022	0.022	nil	—	—	id.
Soluble							
(i)	10	0.016	0.016	nil	0.00	100	Precipitate
(ii)	15	0.024	0.024	nil	0.012	50	id.
(iii)	20	0.032	0.032	nil	0.012	63	id.
control	10	0.016	0.016	nil	—	—	No precipitate

The antiserum was diluted 3-fold with 0.9% NaCl, and 0.15-ml aliquots of the diluted preparations were mixed with the 100-fold purified mitochondrial enzyme protein in one experiment, the 300-fold purified mitochondrial enzyme in the second experiment and 100-fold purified supernatant enzyme in the third experiment. The final volume was adjusted to 0.90 ml with normal saline in all the tubes. After thorough mixing, the tubes were stored in the cold for 60–64 h with occasional agitation.

devoid of enzymic activity at the lowest enzymic concentration tested, i.e. 10 μg of protein. This shows that the homologous antibody was able to remove the entire enzyme protein from the solution. In higher concentrations of enzyme, i.e. 15 and 20 μg of enzyme protein, as much as 37–50% activity was left in the supernatant. It is assumed that under these conditions, the precipitation was incomplete, leaving a marked amount of antigen in solution. No attempt was made for complete recovery of activity.

At comparable levels of enzymic activity the supernatant form of enzyme was precipitated totally or in part; the mitochondrial form of enzyme was neither precipitated nor inhibited by the antiserum. All the initial enzyme activity could be recovered in the solution following centrifugation at the end of 48 h incubation with the antiserum. These results were confirmed with a number of samples of the purified mitochondrial enzyme.

Normal rabbit serum, under similar conditions, did not show any effect on the activities of the mitochondrial and of the supernatant enzymes, nor was there any precipitation. The antiserum by itself, under the above-mentioned conditions of dilution and concentration and assayed separately, did not exhibit any adenosine deaminase activity. Hence, it could be used directly in the tests without heat treatment.

Electrophoretic characterization

The results of a typical experiment are reported in Fig. 6.

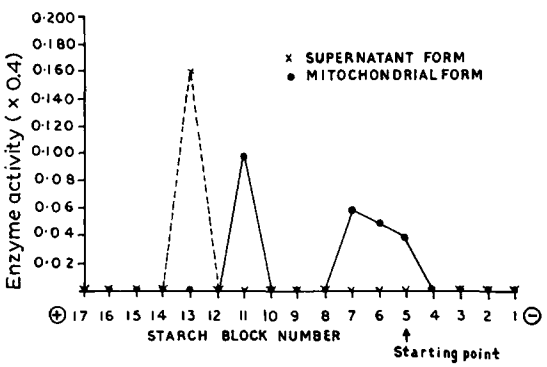

Fig. 6. Starch gel electrophoresis of mouse brain adenosine deaminases. The run was made with a phosphate buffer, pH 7.0 (0.02 M), 28 h at 250 V. The 'mitochondrial' enzyme preparation contained 1.4 mg protein with 0.10 unit of activity in 0.50 ml. The supernatant enzyme was applied in an amount of 0.76 mg protein with 0.10 unit of activity in 0.10 ml. The enzyme preparations used were ammonium sulfate-fractionated and dialysed supernatants.

With the 'mitochondrial' enzyme sample, the pattern of distribution of the activity revealed that the preparation was nonhomogenous with respect to the enzymic activity, thus showing two distinct enzymically active and electrophoretically separable forms of mitochondrial adenosine deaminase. One which was slow moving travelled a distance of 2 cm, whereas the fast-moving one travelled a distance of 6 cm. Almost all the activity applied was recovered from the four gel blocks.

In experiments with the 'soluble' enzyme sample, the pattern of activity in the gel blocks revealed that the preparation was homogenous and moved as a single

enzymically active band. This spot was just 2 cm ahead of the fast-moving band of the mitochondrial enzyme.

In every experiment a starch gel control of same dimensions, suspended under similar conditions, was assayed. This was found to be without effect on the activity or recovery of the enzyme from the gel.

Test against artifact formation

(a) *Chromatography of a mixture of supernatant and mitochondrial enzyme:* Artifacts arising out of the isolation procedures appeared to be unlikely. Not only was the original pattern of chromatography on the DEAE-cellulose column reproducible, but the mixture of the two yielded the same elution pattern as the original on chromatography.

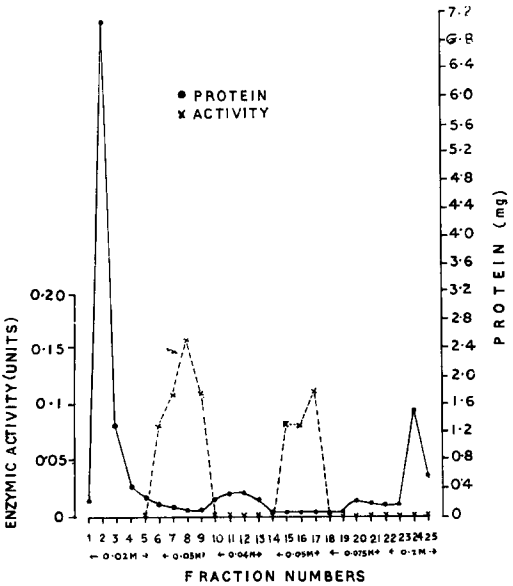

Fig. 7. Chromatography of a mixture of mitochondrial and supernatant forms of mouse brain adenosine deaminases on DEAE-cellulose column. Each contained 1.0 unit of enzyme activity. 7.62 mg protein in 1.0 ml in the case of the supernatant enzyme and 14.0 mg protein in 5.0 ml in the case of the mitochondrial enzyme were mixed with 0.2 M phosphate buffer, pH 7.0, to give a final phosphate ion concentration of 0.02 M.

The distinction between the two peaks was made on the basis of the distinctive properties of the supernatant and mitochondrial enzyme. This provided a further proof for the distinctiveness of the two forms of adenosine deaminase.

(b) *Action of Triton X-100:* The experiments of SOPHIANOPOULOS AND VESTLING[11] had demonstrated that butanol treatment effected a marked change in the molecular properties of malate dehydrogenase. It was considered desirable to establish whether the detergent treatment was, in any way, responsible for the isolation of adenosine deaminase from the mitochondria in a form distinct in molecular properties from the supernatant enzyme. There was no easy means of testing it directly

with the mitochondrial fraction, but it appeared that the results obtained with the 15 000 × *g* supernatant might be valid for the mitochondrial fraction. With this aim the 15 000 × *g* supernatant was treated with Triton X-100 under conditions simulating those for the mitochondria. The resulting solution was processed for the isolation of the enzyme. Tests were performed on the enzyme isolated in this manner to establish whether any properties of the soluble enzyme in the original supernatant were affected by prior treatment of the supernatant with detergent.

It was concluded that the detergent treatment did not alter the properties of the enzyme, such as the responses towards metal ions (effect of Molybdenum, Ba^{2+}, Ca^{2+}, Cu^{2+} and Mg^{2+}), heat inactivation and immunochemical characteristics.

The results lend support to the belief that the enzyme isolated from the mitochondrial fraction is in a form molecularly different from the supernatant enzyme. This may be taken as indirect evidence that the distinctiveness of the mitochondrial adenosine deaminase is not an artifact, but is representative of conditions *in vivo*.

DISCUSSION

Differences in heat stability, catalytic properties such as K_m value, metal ion sensitivity, heat inactivation, immunochemical reaction and electrophoretic mobility have established that the enzyme isolated from mitochondria is a distinct molecular form of the supernatant enzyme.

MA AND FISHER[12] showed the presence of adenosine deaminases of different sizes. Generally, they fall into three sizes, most have molecular weights in the order of 30 000 (Type C), numerous examples are in the order of 100 000 (Type B) and one in the range of 150 000–200 000 (Type A). Studies of tissue from a wide variety of vertebrates have shown that all of these enzyme types are widespread among vertebrates, including mammals. The differences are due to variation in the properties of low-molecular-weight adenosine deaminases. They have further shown that heating at 68° for 10 min destroys most of the Type C enzyme, much of the Type A enzyme and some of Type B enzyme. The difference in heat stability of Types A, B and C adenosine deaminases is likely to be due to the differences in apolar residues in the various enzyme protein, especially the active site. The greater stability of adenosine deaminases to heat treatment (up to 50°) was attributed to the presence of a great number of apolar residues in the protein molecule, as shown by the amino acid analysis[13]. This strongly supports the assumption that the hydrophobic residues have an important role in terms of conformation of an active site for adenosine deaminase[14,15]. The same may be true in our studies also. The supernatant enzyme has been shown to be of a single-peak nature, which may be considered to correspond to Type C enzyme, since 80–90% of the enzyme activity was lost on heating for 5 min at 70°. The mitochondrial form has been found to consist of two enzymically active peaks on starch gel electrophoresis; heating in this case results only in 22% loss in enzymic activity. This suggests the presence of Types B and A.

There has been a number of instances where the multiple molecular forms of adenosine deaminase occur, but there is not even a single report showing that the multiple forms are made up of particulate and supernatant forms of enzyme. Evidence for the distinction between the two mitochondrial enzymes was not as strong as between the soluble and particulate forms of enzyme. The presence of two adenosine

deaminases in the mitochondrial fraction could be demonstrated only by starch gel electrophoresis experiments. It may be pointed out that the preparations used in electrophoretic experiments were partially purified. It is therefore, quite possible that one of the forms constituting the minor portion was lost during further purification by DEAE-cellulose chromatography, and the one constituting the major proportion being purified and emerged as a single peak after column chromatography. Alternatively the other possibility is that the two enzymes are not resolved under the conditions employed in DEAE-cellulose column chromatography. It was clear from electrophoresis that the soluble enzyme did not reveal any contamination with the mitochondrial form of enzyme.

Thus, the identification of isozymes of adenosine deaminase in mouse brain adds one more example to the short list of enzymes occurring in distinct molecular forms in the soluble and the mitochondrial fractions.

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