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LATENT ADENOSINE DEAMINASE IN MOUSE BRAIN

I EXPOSURE AND SOLUBILIZATION OF THE MITOCHONDRIAL ENZYME

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SUMMARY

1 Adenosine deaminase (adenosine aminohydrolase, EC 3 5 4 4), activity in cerebral hemispheres and cerebellum of mouse brain was more or less of the same order, expressed per gram fresh weight

2 The mitochondrial fraction had negligible adenosine deaminase activity However, significant activity was demonstrable following treatment with Triton X-100, pointing to the latent particle-associated enzyme

3 Optimum Triton X-100 concentration and time for exposure of the latent mitochondrial enzyme have been determined

4 Detergents other than Triton X-100 were ineffective although they solubilized considerable protein

5 Salts and mechanical means for exposing the latent enzymic activity were also ineffective

6 Triton X-100 treatment resulted in true solubilization of the enzyme

7 Reconstitution experiment showed absence of inhibitor or activator of the enzyme

INTRODUCTION

Brain tissue has been reported to have a somewhat diffuse pattern of localization of adenosine deaminase (adenosine aminohydrolase, EC 3 5 4 4) JORDAN *et al* [1] found that 65 3% of adenosine deaminase activity of rabbit brain was in the 23 000 × *g*, 30 min, supernatant fraction, a total of 32 2% of the activity was recovered from the cell debris, the nuclear, the mitochondrial and the microsomal fractions The mitochondrial fraction contained 9 5% of the activity in the homogenate but the specific activity was least in this fraction The nuclear fraction, with a recovery of 10 1% of the total activity, had a specific activity much higher than that of the other particular fractions JORDAN *et al* [1] further found that when the 23 000 × *g* supernatant of rabbit brain was centrifuged at 140 000 × *g* for either 1 or 1 5 h, 96–98% of the adenosine deaminase activity remained in the soluble fraction The intracellular distribution of

adenosine deaminase in rat brain paralleled that in rabbit brain, the intracellular distribution of guanosine and guanine deaminases closely followed that of adenosine deaminase POLYAKOVA AND MALISHEVA[2] reported that adenosine deaminase activity of rabbit brain was highest in the soluble fraction and considerably lower in the structural components These authors also studied the activity of adenosine deaminase in various parts of nervous system The highest enzyme activity was found in cerebral white matter, followed by the spinal cord, cerebellum, cerebral grey matter and the sciatic nerve

In all the experiments reported above, the particulate fractions, whether inactive or active, were not subjected to special treatment for exposing latent activity, if any The present authors have been able to show that significant adenosine deaminase activity exists in a latent form bound to the mitochondrial fraction of mouse brain

MATERIALS AND METHODS

Animals Adult male mice (about 6 months old), raised on laboratory stock diet, were used in the investigation The animals were sacrificed by cervical dislocation, followed by decapitation Brain consisting of olfactory lobes, cerebral hemispheres, corpus callosum, optic lobes and cerebellum was excised as quickly as possible, freed from blood and extraneous material and used while fresh Enzyme activity was determined in whole brain, cerebral hemispheres and cerebellum

Homogenization 20% (w/v) homogenate of brain tissue was prepared in 0 25 M sucrose with the aid of Potter–Elvehjem-type homogenizer with Teflon pestle, type A, supplied by Arthur H Thomas Co (Philadelphia, Pa)

Subcellular fractionation The nuclear fraction was sedimented by centrifugation of mouse brain (whole) homogenate at $700 \times g$ for 10 min, washed thrice by suspending in fresh medium with the aid of the tissue homogenizer and centrifugation, the washings being added to the main supernatant The nuclear fraction was suspended in sucrose medium to give 20% tissue equivalent of suspension The combined supernatant was centrifuged at 15 000 × *g* for 60 min to sediment the total mitochondrial fraction (consisting of both the "heavy" and the "light" fractions) The pellet was washed twice with sucrose solution and suspended in fresh medium to yield a concentration equivalent to 20% tissue homogenate The final supernatant was equivalent to a 10% homogenate All the operations were carried out at 0–2° in a model PR-2 centrifuge

Adenosine deaminase assay The assay system for enzyme activity contained 1 0 ml of buffer (0 20 M Tris–HCl or phosphate (pH 7 0)) and a suitable aliquot (0 1 ml) of enzyme preparation, the volume was adjusted to 1 9 ml with water The reaction was started by the addition of 0 05 μmole adenosine in 0 1 ml At the end of 30-min incubation at 37°, 1 0 ml of 10% perchloric acid was added The solution was clarified by centrifugation, and the absorbance was read at 265 mμ after a 2-fold dilution with water A control was simultaneously run, the substrate being added after enzyme inactivation The absorption of the control was subtracted from that of the experimental tube to give the absorbance change due to the enzyme

In a few experiments, the assay of enzyme was based on estimation of NH_3 formed The method employed, using Conway units, was similar to that described by KUMAR *et al* [3] for guanine deaminase The activity of enzyme preparation determined by this method was found to be comparable with that determined by the above method

0 9-ml portion of the mitochondrial suspension was added to 0 1 ml of inorganic salt and/or detergent solution After an appropriate time at 0°, the suspension was centrifuged at 15 000 × *g* for 15 min Activity and protein were estimated in the supernatant In a few experiments, the residue was suspended and assayed for enzymic activity

Protein was determined by the method of LOWRY *et al* [4], using bovine serum albumin as standard The protein was precipitated by 10% trichloroacetic acid

Enzyme unit A unit of enzyme was the transformation of 1 μmole of adenosine to inosine under the assay conditions

RESULTS

Characterization of cell fractions

The use of the terms "nuclear", "mitochondrial" and "supernatant" fractions in the preceding section was based on generally accepted pattern of sedimentation on differential centrifugation of homogenates In view of the fact that brain tissue is less characterized, the brain fractions obtained were subjected in separate experiments to biochemical characterization, involving the determination of DNA, RNA, succinoxidase and total protein DNA and RNA were estimated by the method of SANTEN AND AGRANOFF[5] on the basis of total phosphorus determination Succinoxidase was estimated manometrically by the method of SCHNEIDER AND POTTER[6] The results recorded in Table I justify the terminology used in the present investigation

TABLE I

DISTRIBUTION OF PROTEIN, RNA, DNA AND SUCCINOXIDASE ACTIVITY IN SUBCELLULAR FRACTIONS FROM MOUSE BRAIN

The centrifugal forces used to separate the fractions were as indicated in the text Separate homogenates were employed for the determination of protein and nucleic acids and for succinoxidase The whole homogenate per g of brain tissue contained 105 0 mg protein, 2 4 mg of RNA, 1 7 mg DNA and utilized 290 8 μl of O_2 per h during the oxidation of succinate

Fractions	*Distribution (% of total in homogenate)*			
	Protein	*RNA*	*DNA*	*Succinoxidase*
Whole homogenate	(100)	(100)	(100)	(100)
Nuclear	25	3 3	88	4 2
Mitochondrial	61	43	Nil	83
Supernatant	25	43	Nil	Nil

Distribution of adenosine deaminase in cerebellum and cerebral hemispheres of mouse brain

The analytical data obtained in 5 experiments with whole brain, 3 experiments with cerebral hemispheres pooled from two animals and 3 with cerebellum (pooled from two animals) are recorded in Table II as mean values with standard deviation

Unlike guanine deaminase[3], adenosine deaminase was present not only in cerebral hemispheres but also in cerebellum The activity in the two regions was more or less of the same order, expressed per g of fresh weight Whole brain contained a significantly higher enzyme activity per g than either of the separated parts, although it should have been expected that its activity would not be higher than that of the hemispheres

TABLE II

DISTRIBUTION OF ADENOSINE DEAMINASE IN CEREBELLUM AND CEREBERAL HEMISPHERES OF MOUSE BRAIN

The units of adenosine deaminase activity in μmoles of NH_3 formed in 30 min/g of tissue or tissue equivalent at 37° are expressed as mean values ± S D , The results are from 5 different experiments with whole brain ,and three each with cerebellum and cerebral hemispheres

Tissue	*Enzyme activity (units/g fresh weight)*
Whole brain	3 59 ± 1 089
Cerebellum	2 61 ± 0 115
Cerebral hemispheres	2 7 ± 0 126

The reason for this is not clear, but it may be pointed out that S D of determinations on whole brain was of much higher magnitude than in those of either of the separated parts

Since the cerebral hemispheres constitute the greater bulk of whole brain (two thirds), it follows that the hemispheres have more of enzyme than cerebellum

Effect of varying Triton X-100 concentration on exposure and solubilization of the mitochondrial enzyme

The effect of varying Triton X-100 concentration on the exposure of adenosine deaminase activity (90-min contact) is shown in Fig 1 Under these conditions, all the activity was found in the supernatant, the residue obtained did not have any activity 1% final concentration of Triton X-100 was found to be optimum for exposure and subsequent solubilization The values for protein solubilized at various concentrations of the Triton X-100 tested are also given

Optimum time of Triton X-100 treatment

Incubation of the mitochondrial fraction with 1% (v/v) Triton X-100 released no activity in 15 min (Fig 2) Further there was gradual release of activity up to 90 min

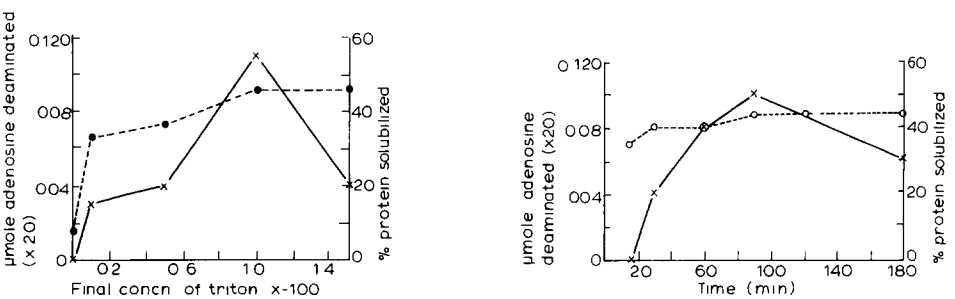

Fig 1 The effect of varying concentrations of Triton X-100 on the exposure of mitochondrial adenosine deaminase activity (90 min contact) ●— — —●, percentage protein solubilized, — , enzyme activity

Fig 2 Incubation of mitochondrial fraction with 1% (v/v) Triton X-100 for different times (min) ○— — —○ percentage protein solubilized, ×— , enzyme activity

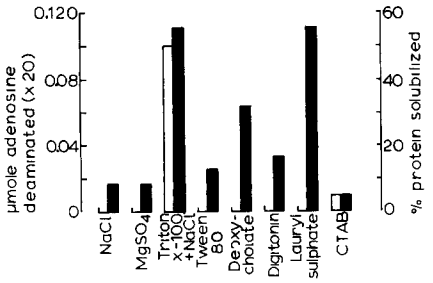

Fig 3 The effect of detergents (1 % in case of Triton X-100 and Tween-80 and 0 1 % in all other cases) and salts (0 4 M) on exposure of the mitochondrial adenosine deaminase activity The activity is shown by white columns and the % protein solubilized is shown by black columns Other details are same as in Fig 1 CTAB, cetyl trimethyl ammonium bromide

when the maximum was reached After this period there was inactivation of the enzymic activity, which was 20 and 40% after further contact of 30 and 60 min, respectively The data further show that 35% protein is solubilized within 15 min, with little change up to 90 min when the values become almost constant

Effect of salts and detergents on exposure and solubilization of the mitochondrial enzyme

The effect of solubilization by salts and detergents is shown in Fig 3 Concentrations of salts (0 4 M) and detergents (1% in case of Triton X-100 and Tween-80 and 0 1% in all other detergents) were chosen arbitrarily Under the experimental conditions, no activity could be exposed by 0 4 M NaCl or 0 4 M $MgSO_4$ although 8 4% protein is solubilized by the action of salts Triton X-100, a nonionic detergent, produced the maximum activation whereas other detergents were ineffective, although they solubilized considerable amounts of protein Lauryl sulfate, an anionic detergent solubilized almost the same amount of protein (55%) as Triton X-100 + NaCl but released no enzyme activity Deoxycholate, which is anionic in nature, solubilized 32% protein but no activity could be demonstrated Digitonin and Tween-80, the nonionic detergents, solubilized 13% and 16% protein, respectively, which had no enzymic activity Cetyl trimethyl ammonium bromide, a cationic detergent, solubilized the least protein (5%) but brought about 10% exposure of the activity as compared to that of Triton X-100 When the salt (NaCl) was also incorporated along with the Triton X-100, the activation was exactly the same as with Triton X-100 alone Inclusion of salt brought higher solubilization of mitochondrial protein

VirTis treatment of the mitochondrial suspension

VirTis homogenization of mitochondrial fraction did not lead to any exposure of the activity of adenosine deaminase When this treatment was followed by Triton X-100, the activity of the final preparation was the same as that resulting from treatment of fresh mitochondria with Triton X-100 alone

Test for true solubilization

That true solubilization of the enzyme from mitochondria occurred was shown by the fact that on centrifugation of the Triton X-100 suspension at 140 000 × *g* for 1 h, only the supernatant was active

TABLE III

PARTICULATE ASSOCIATION OF ADENOSINE DEAMINASE IN MOUSE BRAIN

Details are the same as in Table II The % distribution has been calculated from the mean values taking whole homogenate as 100 The results are from 5 different experiments

Fractions	*Enzyme activity*			
	Without Triton X-100		*With Triton X-100 added to fraction*	
	Units/g tissue equivalent	*Distribution (%)*	*Units/g tissue equivalent*	*Distribution (%)*
Whole homogenate	3 71 ± 0 85	(100)	6 61 ± 1 3	(100)
Supernatant (15 000 × *g*)	2 59 ± 0 07	69 8	2 59 ± 0 07	39 8
Residue (15 000 × *g*)	0 65 ± 0 14	18	2 92 ± 0 78	44 2
Reconstituted	3 73 ± 0 73	100	6 92 ± 0 75	104

Localization of adenosine deaminase

Experiments were performed with sucrose–0 2 M Tris–HCl buffer (pH 7 0) homogenates of mouse brain The total particles sedimenting at 15 000 × *g* had 18% of the activity of homogenate Treatment of this particulate fraction resulted in an increase of over 4-fold of the activity Reconstitution experiments showed that mixing the sediment with the supernatant did not lead to either activation or inhibition, whether after treatment with Triton X-100 or not The results are reported in Table III

Typical results obtained on analysis of brain homogenates and isolated cell fractions left in contact with 1% Triton X-100 for 90 min in the cold are given in Table IV

The results obtained show that the supernatant fraction contained the greater part of the activity of brain homogenate The mitochondrial and nuclear fractions had either no activity or very little adenosine deaminase activity when tested as such

However, on treatment with Triton X-100, significant enzymic activity was

TABLE IV

SUBCELLULAR DISTRIBUTION OF ADENOSINE DEAMINASE AND EFFECT OF TRITON X-100

Details are same as in Tables II and III

Fractions	*Enzyme activity*			
	Without Triton X-100		*With Triton X-100 added to fraction*	
	Units/g fresh wt equivalent	*Distribution (%)*	*Units/g fresh wt equivalent*	*Distribution (%)*
Whole homogenate	3 4	(100)	5 7	(100)
Nuclear (700 × *g*)	0 42	12	0 71	12
Mitochondrial (15 000 × *g*)	0 25	7	1 08	19
Supernatant	3 08	90	2 8	50

found to be associated with the particles, the nuclear fraction showed 12–19% adenosine deaminase activity of the brain homogenate Treatment of the homogenate with the detergent resulted in 46–48% increase in activity The treated mitochondrial fraction contained about 16–19% of the activity of Triton X-100-treated whole homogenate The activity of the 15 000 × *g* supernatant was not affected by treatment with Triton X-100

DISCUSSION

The general concept of the exclusive location of adenosine deaminase in the supernatant fraction of tissue homogenate needs revision The existence of latent enzymes and their exposure by detergent action is a well-recognized phenomenon A number of enzymes have been shown to be associated with mitochondria derived from liver[7–12], heart[13] or brain[12,14,15] which are latent and elicit maximal activity only on treatments which disrupt the structural integrity of mitochondria Triton X-100 is one of the most effective nonionic detergents employed in exposure of latent enzymic activity or in solubilization of enzymes[14,16–18] Our studies have clearly indicated that adenosine deaminase in quantities of 15–20% of the activity in mouse brain homogenate resides in the mitochondrial fraction essentially in a latent form The mitochondrial enzyme could not be exposed by the use of mechanical means, salts and detergents other than Triton X-100 However, these treatments cause the solubilization of protein to a different extent, but the enzyme is not exposed This may be attributed to the selective disruption or removal of subcellular membranous structures which results in the exposure of enzyme The possibility that other detergents may have an inactivating or inhibitory effect on the particle-bound enzyme cannot be ruled out Thus, Triton X-100 treatment by itself was able to expose and to solubilize the mitochondrial-bound adenosine deaminase of mouse brain

In mouse brain there are at least two forms of adenosine deaminases, one is the soluble enzyme present in cell sap and the other is particle-bound essentially in a latent form and associated with the mitochondria Added support was forthcoming from the observation that the enzymes purified from the mitochondrial and cytoplasmic fractions reveal distinction in many respects, including electrophoretic and immunochemical characterization, pointing to distinct molecular forms (results to be reported elsewhere[19])

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