Rat Cerebral-Cortex Adenosine Deaminase Activity and its Subcellular Distribution

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A radioisotopic assay for adenosine deaminase (EC 3.5.4.4) is described together with its application in investigating the activity of the enzyme in rat cerebral cortex. Activity of the adenosine deaminase was determined to be 115 nmol/min per g of tissue, measured in iso-osmotic sucrose dispersions of the neocortex, and to be 170 nmol/min per g of tissue after treatment with Triton X-100. The enzyme was concluded to be largely cytoplasmic, with a K_m of 54–57 μ M for adenosine. Action of the deaminase, and other aspects of the metabolism of adenosine in intact neocortical tissue, were quantitatively appraised on the basis of the newly determined characteristics.

Electrical excitation of cerebral tissue is accompanied by an increase in its cyclic AMP content and by release from the tissue of purine compounds, including adenosine and inosine (Pull & McIlwain, 1972*b*; see also Shimizu *et al.*, 1970). Since adenosine, but not inosine, has the property of increasing the cyclic AMP of cerebral tissues (Sattin & Rall, 1970) one function of adenosine deaminase (EC 3.5.4.4) may be to limit the action of liberated adenosine. Added, exogenous, adenosine deaminase prevents the normal increase in cyclic AMP after excitation (Huang *et al.*, 1973), and in these circumstances also, adenosine is no longer found among the released purines (I. Pull & H. McIlwain, unpublished work).

These considerations have prompted re-examination of the activity and subcellular distribution of rat cerebral-cortex adenosine deaminase, for which divergent values have been reported. Activity of a soluble adenosine deaminase from rat brain has been given as 960 nmol/min per g of tissue (Jordan *et al.*, 1959) and as 55 nmol/min per g of tissue (Santos *et al.*, 1968), whereas Mustafa (1972) reported a total activity of 160 nmol/min per g of tissue, of which 33% occurred in a mitochondrial fraction and was revealed only after treatment with Triton X-100. Initial experiments using an existing spectrophotometric assay method suggested that the different substrate concentrations used by the investigators named above, were largely responsible for the divergent activities quoted (B. Rumble, personal communication). We confirm this suggestion (see below) but have found shortcomings in the assay method then used, and have accordingly developed the radioisotope assay method which is described first below.

Experimental

Subcellular fractionation

Male Wistar rats weighing 200–250 g were stunned by a blow to the neck, exsanguinated and the brain was rapidly removed. Cerebral cortex from both hemispheres was trimmed free from underlying structures and as much white matter as possible, and then homogenized in 9 vol. of ice-cold 0.32 M-sucrose, adjusted to pH 7.4 with Tris base, in a Perspex-and-glass homogenizer rotating at 840 rev./min (Whittaker & Dowe, 1965). Centrifugation was performed essentially as described by Gray & Whittaker (1962) and by Whittaker *et al.* (1964). The crude nuclear fraction (P_1) was deposited by centrifuging at 1000g for 10 min, the crude mitochondrial fraction (P_2) at 10000g for 20 min and a microsomal fraction (P_3) at 105000g for 60 min to yield the supernatant (S_3). Fraction P_2 was subfractionated by resuspending in water at 2 ml/g of original tissue and layered on a discontinuous sucrose gradient consisting of equal volumes of 0.4 M-, 0.6 M-, 0.8 M-, 1.0 M- and 1.2 M-sucrose. The gradient was centrifuged at 105000g for 60 min at 2°C and separated into seven fractions named O (cytoplasm), D (vesicles), E, F, G (membranes), H (undisrupted synaptosomes) and I (mitochondria).

Radioisotopic assay of adenosine deaminase

Adenosine deaminase was assayed by measuring the conversion of [8-¹⁴C]adenosine into [8-¹⁴C]inosine and [8-¹⁴C]hypoxanthine, separated by t.l.c. Duplicate tubes contained enzyme preparation (0.2–2 mg of protein) in 50 mM-potassium phosphate

buffer, pH 7, and were incubated at 37°C for 5 min before addition of 25 μ l of substrate solution to give a final concentration of 400 μ M-[8-¹⁴C]adenosine (1 mCi/mmol) in 0.25 ml. Reaction was terminated after 30 min by adding 50 μ l of 4 M-HClO₄ and centrifuging to deposit protein. Control tubes lacked enzyme preparation and were similarly treated; enzyme preparation was then added immediately after the HClO₄. Enzyme samples were also assayed after mixing with an equal volume of 2% (w/v) Triton X-100 in 100 mM-potassium phosphate, pH 7, and standing in ice for 2 h (Mustafa, 1972).

To 100 μ l samples of the protein-free supernatants obtained as above were added 40 μ g each of inosine, hypoxanthine and adenosine contained in a volume of 20 μ l, followed by 10 μ l of 5 M-K₂CO₃ to neutralize the acid. After centrifuging again, 10 μ l portions of these supernatants were applied to silica-gel G t.l.c. plates containing fluorescent indicator, and the plates developed in a solvent mixture of butan-1-ol-methanol-ethyl acetate-NH₃ (sp.gr. 0.88) (7:3:4:4, by vol.; Shimizu *et al.*, 1969). Areas absorbing in the u.v. after development were removed from the plates by suction and transferred to counting vials where the ¹⁴C-labelled compounds were eluted by shaking with 1 ml of 0.1 M-HCl for 1 h and then counted for radioactivity as described by Pull & McIlwain (1972a). Recovery of the compounds from the gel was determined and found to be the same for each (between 90–95%); samples were subsequently counted for radioactivity with a constant efficiency of 68–70%.

Other assays

In some cases adenosine deaminase activity was measured spectrophotometrically (Kalckar, 1947). Samples (100 μ l) of the protein-free supernatants obtained as described above were diluted with 2 ml of 0.2 M-potassium phosphate buffer, pH 7.0, and their decrease in absorbance from the controls was determined at 265 nm. An extinction coefficient of 8.1×10^3 litre $\cdot$ mol⁻¹ $\cdot$ cm⁻¹ (Mollering & Bergmeyer, 1963) was applied to determine the extent of adenosine deamination.

Lactate dehydrogenase activity was assayed spectrophotometrically in 0.15 M-Tris buffer, adjusted to pH 7.4 with HCl, as described by Johnston (1960) both before and after treatment of the enzyme preparation with 1% (w/v) Triton X-100 to disrupt synaptosomes (Marchbanks, 1967). Protein was determined by the method of Lowry *et al.* (1951) as modified by Miller (1959), with bovine plasma albumin as standard.

Materials

[8-¹⁴C]Adenosine (59 mCi/mmol) was obtained from The Radiochemical Centre, Amersham, Bucks.,

U.K. and the non-radioactive nucleosides were from Boehringer Corp. (London) Ltd., London W.5, U.K. Silica gel G used for preparing t.l.c. plates was from E. Merck, Darmstadt, Germany, and the fluorescent indicator (Woelm) was supplied by Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K., who also supplied Triton X-100 and the scintillation chemicals. Additional reagents and solvents were of analytical grade and were obtained from BDH Chemicals Ltd., Poole, Dorset, U.K. Crystalline bovine plasma albumin was from Sigma (London) Chemical Co. Ltd., London S.W.6, U.K.

Results

Enzyme assay

The radioisotopic assay for adenosine deaminase was validated by first scanning chromatograms of the reaction mixture for radioactivity. Only three peaks of radioactivity, corresponding to inosine, hypoxanthine and adenosine, were found, so that the extent of adenosine deamination could be calculated from the sum of the radioactivity present in [¹⁴C]-inosine and [¹⁴C]hypoxanthine and from the specific radioactivity of the added [¹⁴C]adenosine. Ischaemic rat brain tissue contains adenosine at a maximum of 340 nmol/g of tissue after 30 min (Deuticke *et al.*, 1966) and this value would give an endogenous adenosine content in the tissue homogenate being assayed of less than 2 nmol. This dilution of the 100 nmol of [¹⁴C]adenosine specific radioactivity was considered acceptable and no corrections for this were made. Product formation ([¹⁴C]inosine plus [¹⁴C]hypoxanthine) in this assay was linear both with time and with the amount of enzyme preparation, provided that no more than 40–50% of the substrate was consumed. The assays were normally conducted so that only 5–20% (5–20 nmol) of the adenosine was deaminated.

Dependence on substrate concentration

Adenosine deaminase activity of a homogenate of cerebral cortex dispersed in water was examined at various substrate concentrations and a concentration of 400 μ M-adenosine was found to be saturating. The *K_m* values of the enzyme, determined at two enzyme concentrations from double-reciprocal plots of activity versus substrate concentration, were found to be 54 and 57 μ M when substrate concentrations between 20 and 400 μ M were used and to be 56 μ M when substrate concentrations were 4–44 μ M (Fig. 1). In these experiments enzyme concentration and incubation time were adjusted so that no more than 20% of the substrate was deaminated and product formation remained linear with time. This was verified in separate determinations of the time-course of the reaction at different substrate

concentrations. In each case the K_m value was calculated by using a weighted regression method (Wilkinson, 1961).

Subcellular distribution

Adenosine deaminase activity of the rat cerebral cortex homogenized in 0.32M-sucrose was 115nmol/min per g of tissue, and on subcellular fractionation the greatest proportion was found in the high-speed supernatant fraction (Table 1). The specific activity of adenosine deaminase in this fraction was 3.7nmol/min per mg of protein, representing a fourfold enrichment over its specific activity in the homogenate. Lactate dehydrogenase activity was also measured and the distribution of this enzyme activity between the different fractions was similar to that of adenosine deaminase. The next highest proportion of each enzyme was present in the crude mitochondrial fraction, which contained 17% of the adenosine deaminase activity and 21% of the lactate dehydrogenase activity. Treatment with Triton X-100 before assay caused an apparent activation of both adenosine deaminase and lactate dehydrogenase which differed in the different fractions; the apparent activation of these enzymes was least in the supernatant fraction. The enhanced activity of both enzymes was similarly distributed among the subcellular fractions, with the crude mitochondrial fraction containing 51% of this adenosine deaminase activity and 47% of this lactate dehydrogenase activity.

The distribution of adenosine deaminase and lactate dehydrogenase in the crude mitochondrial fraction was examined after exposure to hypo-osmotic conditions and further fractionation by density-gradient centrifugation (Table 2). Almost 70% of the adenosine deaminase activity in the washed fraction P_2 pellet was now recovered in the supernatant fraction O and there was little activation of this by Triton X-100. There was no enrichment of

adenosine deaminase activity in these fractions greater than that found for the initial S3 fractions, the specific activity of adenosine deaminase in fraction O being 2.1nmol/min per mg of protein. Lactate dehydrogenase activity was also measured in the different subfractions and its distribution paralleled that of adenosine deaminase.

Fig. 1. Double-reciprocal plots of adenosine deaminase activity versus substrate concentration obtained for homogenates of the rat cerebral cortex

Substrate concentrations of 24–408 μ M-adenosine each containing 200 μ Ci of $[8-^{14}C]$ adenosine/ml were used and reactions were either for 15 min with 10mg of dispersed cortex (●) or for 10 min with 4.5mg of dispersed cortex (○). Units of reciprocal activity and reciprocal concentration are as shown on the diagram. Substrate concentrations of 4–44 μ M-adenosine each containing 200 μ Ci of $[8-^{14}C]$ adenosine/ml were also examined (----) by using a reaction time of 5 min and 2.25mg of dispersed cortex. For these results (■) the ordinate scale is $10^{-1}/v$ [(nmol/min) $^{-1}$] and the abscissa scale is $10^2/[adenosine]$ (μ M $^{-1}$). The lines were calculated by using a weighted regression method (Wilkinson, 1961).

Table 1. Adenosine deaminase and lactate dehydrogenase activities in subcellular fractions of rat cerebral cortex

Mean values from two experiments are given with the percentage distribution of the recovered totals in parentheses.

Fraction	Protein (mg/g of tissue)	Adenosine deaminase (nmol/min per g of tissue)		Lactate dehydrogenase (μ mol/min per g of tissue)	
		Without Triton X-100	With Triton X-100	Without Triton X-100	With Triton X-100
Homogenate	125.6	114.6	169.3	31.8	87.2
P ₁	35.9 (29)	13.5 (11)	23.8 (14)	2.7 (9)	15.5 (18)
P ₂	52.8 (43)	20.6 (17)	43.2 (26)	6.7 (21)	31.4 (37)
P ₃	11.8 (10)	5.5 (4)	7.6 (5)	1.0 (3)	7.5 (9)
S ₃	22.7 (18)	84.4 (68)	94.0 (55)	20.7 (67)	29.7 (36)

Table 2. Adenosine deaminase and lactate dehydrogenase activities in the P_2 fraction after hypo-osmotic treatment

The fraction P_2 pellet was resuspended in 0.32M-sucrose (5ml/g of original tissue) and deposited again by centrifuging at 10000g for 20min before resuspension in water and fractionation as described in the Experimental section. Single values from one experiment are given, with the percentage distribution of the recovered totals in parentheses; recoveries were within the range 95–101%.

Fraction	Protein (mg/g of tissue)	Adenosine deaminase (nmol/min per g of tissue)		Lactate dehydrogenase (μ mol/min per g of tissue)	
		Without Triton X-100	With Triton X-100	Without Triton X-100	With Triton X-100
O	4.8 (13)	10.1 (67)	10.4 (48)	6.8 (65)	7.6 (36)
D	1.9 (5)	1.2 (8)	1.5 (7)	1.1 (10)	1.2 (6)
E	3.4 (9)	0.4 (3)	1.2 (6)	0.6 (6)	1.6 (8)
F	4.3 (12)	0.5 (3)	1.6 (7)	0.4 (4)	2.1 (10)
G	5.3 (15)	0.7 (5)	2.2 (10)	0.3 (3)	2.8 (13)
H	7.8 (22)	1.0 (7)	3.1 (14)	0.6 (6)	4.0 (19)
I	8.2 (23)	1.2 (8)	1.7 (8)	0.6 (6)	1.7 (8)

Discussion

Assay and occurrence of the deaminase

The lower limit of sensitivity in the radioisotopic assay may be regarded as being equal to the 'blank' values of [14 C]inosine plus [14 C]hypoxanthine found in the control tubes, and these were typically equivalent to the deamination of 1 nmol of adenosine. In contrast, the spectrophotometric assay was found to be both less sensitive and less precise. Performed as described in the Experimental section the deamination of 10 nmol of adenosine would give an absorbance difference of 0.013, so that it was necessary to measure the enzyme activity after a considerable proportion of the substrate had been consumed. Also, the amount of tissue sample necessary for this assay contained u.v.-absorbing compounds giving an absorbance of 0.1–0.2, thus increasing the absorbance from which the decrease due to adenosine deamination was being measured. Nevertheless the spectrophotometric method was found to give results agreeing with those from the radioisotopic assay to within $\pm 5\%$ provided that more than 20% of the substrate was deaminated.

Activity of the adenosine deaminase of rat cerebral cortex dispersed in sucrose was found to be 115 nmol/min per g of tissue, which is understandably greater than the activities of 55 and 70 nmol/min per g of tissue measured by using lower substrate concentrations by Santos *et al.* (1968) and by Mustafa (1972). The K_m of 54–57 μ M is comparable with values reported for the adenosine deaminase of bovine placenta (40 μ M; Maguire & Sim, 1971) and of calf intestinal mucosa (50 μ M; Chassey & Suhadolnik, 1967), but is distinct from that of the mouse brain, which has been reported to show

two forms of adenosine deaminase with K_m values of 6.4 and 11 μ M (Mustafa & Tewari, 1970).

Similarity between the distribution of adenosine deaminase and lactate dehydrogenase in the sub-cellular fractions and in the subfractions of the crude mitochondrial pellet separated after hypo-osmotic shock suggest that the deaminase is cytoplasmic. Lactate dehydrogenase is a cytoplasmic enzyme in the brain and the additional activity which is revealed after hypo-osmotic shock or after treatment with Triton X-100 is contained within the cytoplasm of synaptosomes (Johnston & Whittaker, 1963; Fonnum, 1968). Present findings thus ascribe the particulate adenosine deaminase to synaptosomal and not mitochondrial subfractions, and the reported action of Triton X-100 on mitochondrial adenosine deaminase (Mustafa, 1972) we interpret as being due to lysis of synaptosomes present in the crude mitochondrial fraction. Adenosine deaminase of rat cerebral cortex is concluded to be contained within the cytoplasm of neurons, including nerve endings; we have no information on whether or not it occurs also in glial cells and interstitial fluid.

Adenosine and the deaminase in intact cerebral tissue

The concentration of adenosine in the rat brain is normally so low that its detection is difficult. Available values suggest a cerebral content of adenosine under normal conditions of approx. 0.5 nmol/g (Shimizu *et al.*, 1972) or, by assuming a uniform distribution in tissue water, a concentration of 0.7 μ M, which is considerably less than the K_m value of adenosine deaminase. Assuming also a uniform distribution of the deaminase and applying the Michaelis–Menten equation with K_m 55 μ M

and $V_{\max}$, 170nmol/min per g of tissue, a rate of deamination of 2.1nmol/min per g of tissue is calculated when the adenosine concentration is 0.7 μ M. We have found (Pull & McIlwain, 1972b) the output of inosine and hypoxanthine to be less than 1.5nmol/min per g of tissue on superfusing normal cortical tissue, and this rises to 10nmol/min per g of tissue on electrical stimulation of the tissue. To produce 10nmol of inosine/min per g of tissue by the deaminase is calculated to require adenosine at 3.5 μ M; inosine may also be produced from adenine nucleotides by AMP deaminase (EC 3.5.4.6) and 5'-nucleotidase (EC 3.1.3.5).

There can, nevertheless, be an output from cerebral tissue of adenosine as such (Pull & McIlwain, 1972b), and an accumulation of adenosine during ischaemia (Deuticke *et al.*, 1966). Added to fluids superfusing cerebral tissue, ATP yields adenosine with relatively little inosine and hypoxanthine (Pull & McIlwain, 1972a), findings consistent with the present observation of adenosine deaminase as a cytoplasmic enzyme. During electrical stimulation and superfusion *in vitro*, cerebral tissue was estimated to liberate adenosine at 6nmol/min per g of tissue, with adenosine plus its metabolites inosine and hypoxanthine appearing at a rate of approx. 16nmol/min per g of tissue (Pull & McIlwain, 1972b). These rates of production of adenosine do not exceed the activity of adenosine deaminase, yet could still, depending on considerations of compartmentation, give rise to adenosine concentrations sufficient to account for observed increases in cyclic AMP during electrical stimulation (Pull & McIlwain, 1972b). In increasing cyclic AMP, adenosine is presumed to be acting extracellularly and thus from a site in which it is little susceptible to deamination by the adenosine deaminase native to the tissue; action of adenosine would then be limited by processes of diffusion and cellular re-uptake.

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