Guanine Deaminase in Rat Liver and Mouse Liver and Brain

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1. The guanine deaminase in rat liver supernatant preparations was resolved into two fractions, *A* and *B*, on DEAE-cellulose columns. The two differed in electrophoretic mobility and in various properties. The most noteworthy distinction between *A* and *B* components was that the enzyme *A* activity showed a sigmoid dependence on substrate concentration whereas the enzyme *B* showed classical Michaelis–Menten kinetics. The K_m value of enzyme *A* for guanine was $5.3\ \mu\text{M}$ and that of enzyme *B* $20\ \mu\text{M}$. 2. The entire guanine deaminase activity of mouse liver was contained in the 15000g supernatant of iso-osmotic homogenates. 3. A reinvestigation of the behaviour of rat brain 15000g supernatant guanine deaminase isoenzymes revealed that one enzyme had sigmoidal kinetics and the other enzyme showed a hyperbolic response. 4. Of the guanine deaminase in mouse brain iso-osmotic sucrose homogenate 80% was recovered in the 15000g supernatant and the rest from the particles. The supernatant guanine deaminase was resolvable into two fractions on DEAE-cellulose columns. One enzyme showed sigmoidal kinetics whereas the other showed a hyperbolic response to increasing substrate concentration; the K_m values for the reaction with guanine were respectively 5 and $66\ \mu\text{M}$. 5. The particulate fractions of mouse liver and brain were devoid of any overt inhibitory activity.

The end product of guanine catabolism in rats and mice is allantoin. In anabolic reactions, guanine is known to be incorporated into nucleic acids in these animals (Abrams, 1951; Brown *et al.*, 1949). Xanthine formed by the action of guanine deaminase (guanine aminohydrolase, EC 3.5.4.3), or hypoxanthine formed from xanthine by the reversible action of xanthine dehydrogenase (Della Corte & Stirpe, 1970), may also enter into anabolic reactions leading to the formation of nucleotides or nucleic acids (Kelley *et al.*, 1967; Pomales *et al.*, 1965; Mager *et al.*, 1967; Abrams & Goldinger, 1952). The 'salvage' of the free purine bases for incorporation into nucleic acids (Kornberg, 1957) is initiated by the action of phosphoribosyl transferase (Korn *et al.*, 1955; Kornberg *et al.*, 1955) or nucleoside phosphorylase (Kalckar, 1947). These reactions involving guanine would compete with guanine deaminase for the utilization of intracellular guanine. Occupying as it does a pivotal position in the metabolism of guanine, the deamination reaction may be expected to be subject to intracellular regulation. This paper is concerned with the purification of guanine deaminase from rat and mouse liver and a study of its kinetic properties. A partial purification of mouse brain enzyme has been made and substrate saturation kinetics have been studied. Also, a reinvestigation has been made of the substrate-saturation kinetics of rat brain enzyme studied earlier (Kumar *et al.*, 1967). Preliminary accounts of the findings have already appeared (Josani & Krishnan, 1968; Sree Kumar & Krishnan, 1970a,b; Sitaramayya & Krishnan, 1970).

Materials and Methods

Animals

Albino rats and mice, originally procured from the Central Drug Research Institute, Lucknow, India, were bred in the departmental colony.

Special chemicals

The special reagents used in the studies were procured from Sigma Chemical Co., St. Louis, Mo., U.S.A.

Homogenates

Preparation. Adult male animals were killed by cervical dislocation and decapitation. After bleeding, the liver and the brain were excised. The chilled tissues were blotted and the optic lobes and olfactory regions and the medulla oblongata cut away from the rest of the brain and discarded.

A 10% (w/v) homogenate of brain and 20% (w/v) homogenate of liver were prepared in 250 mM-sucrose in a Potter–Elvehjem homogenizer with a mechanically driven Teflon pestle operated for 2–3 min at 0–2°C.

Differential centrifugation. Differential centrifugation was carried out at 0–2°C.

The liver homogenates were centrifuged according to the method described earlier (Kumar *et al.*, 1965) and the 'nuclear' (700g, 10 min), 'heavy mitochondrial' (5000g, 20 min), 'light mitochondrial'

(15000g, 1h) and the post-mitochondrial supernatants were collected.

For brain, the post-nuclear fraction of homogenate was directly centrifuged at 15000g for 1h.

Enzyme assay

The enzyme activity was determined spectrophotometrically by measuring the extinction change due to the conversion of guanine into xanthine. All assays were at the optimum pH of 8.0 and rates were proportional to enzyme concentration. The following two assay systems were employed.

(a) A final volume of 2.0 ml containing 50mM-tris-HCl, pH 8.0, and 0.3mM-guanine was used except with mouse liver; for mouse liver the volume was increased to 3.0ml, since the enzyme activity on fresh-tissue-weight basis was low and a larger volume of enzyme preparation had to be employed. After incubation for 15 min at 30°C the enzyme was inactivated by the addition of 1 ml of 10% (w/v) HClO₄, and the substrate was added to controls. After 10min the mixture was centrifuged at 700g for 10min at 28–30°C. The supernatant was diluted tenfold with water and the extinction measured at 245nm.

(b) The volume of enzyme preparation was decreased, and 0.03mM-guanine was used in a volume of 2.0ml; after acidification *E* at 245nm was measured without dilution.

Assay method (a) was employed to determine the activity in the homogenate, subcellular fractions and crude enzyme preparation. The measurements of activity in DEAE-cellulose eluates and in the investigation of kinetic properties were by assay method (b).

One unit of guanine deaminase activity was defined as the transformation of 1μmol of guanine into xanthine under the conditions specified above. Specific activity was expressed as units/mg of protein.

Determination of protein

Protein concentration was usually measured by the colorimetric method of Lowry *et al.* (1951), with bovine albumin (crystallized and freeze-dried) as reference standard. Protein concentration in the fractions eluted from DEAE-cellulose columns was determined spectrophotometrically by measuring *E* at 280 and 260nm and calculating the protein concentration by the equation employed by Kalckar (1947).

Enzyme purification from 15000g supernatant

All operations were conducted at 2–5°C. Fractionation was carried out with enzyme-grade (NH₄)₂SO₄ at pH 7.0. Dialysis was against frequent changes of water during a period of 8h. Phosphate buffers were prepared from KH₂PO₄ and K₂HPO₄.

Rat liver. The pooled liver tissue from two rats was used in the experiments.

(a) Acidification. The pH of the 15000g supernatant was gradually lowered to 5.0–5.5 by the addition of 100mM-acetic acid and the precipitate, allowed to flocculate for 10min, collected by centrifugation at 3000g for 30min and discarded.

(b) Ammonium sulphate fractionation. To the supernatant resulting from step (a), powdered (NH₄)₂SO₄ was added (39g/100ml) to give 60% saturation. After 1h the precipitated protein was centrifuged at 3000g for 30min and discarded. The concn. of (NH₄)₂SO₄ in the supernatant was increased to 75% saturation by adding a further 10.5g/100ml and the protein fraction was collected as above and suspended in a minimum volume of water.

(c) Dialysis and gel treatment. The solution was dialysed for 8h, the resulting suspension centrifuged at 15000g for 20min and the supernatant used. The enzyme was adsorbed on alumina-Cγ gel (gel:protein ratio, 1:1, w/w). The adsorbed enzyme was eluted in 10ml of 50mM-phosphate buffer, pH 7.0, after washing the gel residue with 10ml each of 10- and 20mM-phosphate buffer, pH 6.5, and 20mM-phosphate buffer, pH 7.0.

(d) DEAE-cellulose column chromatography. The enzyme eluted from alumina was diluted 2.5-fold with water and loaded on a DEAE-cellulose column (1.5cm × 15cm) equilibrated with 20mM-phosphate buffer, pH 7.0. The column was washed with 60ml of the starting buffer, followed by 30ml each of 50-, 75-, 100- and 200mM-phosphate buffer, pH 7.0.

Rat brain. The purification was according to the method described earlier (Kumar *et al.*, 1967), with the differences that the treatment with alumina step was left out and the DEAE-cellulose column was not washed with 100mM-phosphate, pH 6.5.

Mouse liver. The pooled liver tissue from nine mice was used in each experiment.

(a) Acidification. This was carried out in the same manner as for rat liver.

(b) Ammonium sulphate fractionation. The protein fraction precipitating between 55 and 80% saturation with (NH₄)₂SO₄ (35.1g/100ml to increase the saturation of the supernatant to 55% and 17.9g/100ml to increase it further to 80%) was collected by centrifugation at 3000g for 30min and dissolved in water.

(c) Heat treatment and dialysis. The solution was mixed with an equal volume of 200mM-phosphate buffer, pH 6.0, and kept at 60°C for 1min and then rapidly chilled in ice. The precipitate obtained by centrifuging at 15000g for 20min was discarded, the supernatant adjusted to pH 7.0 and dialysed against glass-distilled water, and the small amount of precipitate that formed removed by centrifugation at 15000g for 20min.

(d) DEAE-cellulose column chromatography. The concentration of phosphate in the supernatant was adjusted to 20mM by dilution with water and the proteins were loaded on a column of DEAE-cellulose (1cm×25cm) equilibrated with 20mM-phosphate buffer, pH 7.0. The column was washed with 100ml of starting buffer, followed by 60ml each of 50- and 85mM-phosphate buffer and 50ml each of 125- and 200mM-phosphate buffer, all at pH 7.0.

Mouse brain. The major part of the enzyme activity in the 15000g supernatant derived from pooled whole brain from 15 mice was precipitated between 35 and 65% saturation with $(\text{NH}_4)_2\text{SO}_4$ (20.9g of $(\text{NH}_4)_2\text{SO}_4$ /100ml to increase the saturation to 35% and 20g/100ml to increase it further to 65%). The precipitate was spun down at 15000g for 30min, dialysed and the suspension centrifuged. The supernatant was adjusted to 20mM-phosphate, pH 7.0, by

addition of concentrated buffer and loaded on a DEAE-cellulose column (1cm×15cm) equilibrated with 20mM-phosphate buffer, pH 7.0. After washing of the column with 60ml of starting buffer, proteins were eluted stepwise with 40ml of 50mM-phosphate buffer and 50ml of 100- and 200mM-phosphate buffer, all at pH 7.0.

Starch-gel electrophoresis of rat liver enzyme

Starch-gel electrophoresis was carried out as described by Smithies (1955), with a constant voltage of 250V and an average current of 7–8mA for 20h inside a refrigerator at 5°C. At the end of the run the starch block was cut horizontally; the lower half was used for detection of protein by staining with Amido Black and the upper half cut transversely into 0.5cm pieces, frozen for 2h at –18°C and thawed.

Table 1. *Purification of guanine deaminase from rat liver*

The starting material was the 15000g liver supernatant in 250mM-sucrose (livers from two adult rats were used). The purification method was as described in the text. Volumes (30ml each) of 100- and 200mM-phosphate buffer, pH 7.0, were used for enzyme elution from DEAE-cellulose columns. Protein was estimated by a colorimetric method in all fractions except in eluates from DEAE-cellulose columns, in which the estimation was by spectrophotometry as cited in the text. Enzyme assay in fractions (I)–(V) was by assay method (a) and in DEAE-cellulose eluates by assay method (b) (see the Materials and Methods section).

Fraction	Vol. (ml)	Total activity (units)	Total protein (mg)	Sp. activity (units/mg of protein)	Recovery (%)	Purification
(I) Supernatant (15000g for 1 h)	120	180	852	0.21	(100)	(1)
(II) Acidified to pH 5.5, centrifuged, supernatant adjusted to pH 7.0	142	122	284	0.43	67.7	2
(III) 60–75% satd. $(\text{NH}_4)_2\text{SO}_4$ ppt.	9.1	82	63	1.30	45.6	6
(IV) Dialysed, centrifuged, supernatant adjusted to pH 7.0	18.0	73.8	54	1.36	41.0	6.5
(V) Adsorbed on alumina gel and eluted with 50mM-phosphate, pH 7.0	10	43	6.2	7.0	24.0	33
(VI) DEAE-cellulose column chromatography: eluate (4ml fractions) with phosphate buffer, pH 7.0						
100mM-buffer: no. 1		1.8	0.044	40.0		190
2		1.6	0.036	45.0		214
3		1.0	0.032	32.8	3.0*	157
4		1.0	0.022	43.6		207
200mM-buffer: no. 4		1.63	0.040	40.3	2.8*	192
5		1.63	0.036	45.3		216
6		1.70	0.022	75.3		359

* Recovery calculated as the sum of four and three fractions eluted respectively with 100mM- and 200mM-phosphate buffer.

Each piece was ground with 1.0ml of 200mM-phosphate buffer, pH7.0, for 15min in a hand-operated Potter-Elvehjem homogenizer; the suspension was centrifuged at 700g for 15 min at 0–2°C and the supernatant assayed for guanine deaminase.

Results

Purification of guanine deaminase from 15000g supernatants of rat liver and rat brain

Rat liver guanine deaminase. As reported earlier (Kumar *et al.*, 1965), the major part of the guanine deaminase activity of rat liver was present in the 15000g supernatant of iso-osmotic sucrose homogenates, with the rest of the activity associated with particulate fractions. In the present experiments, only the supernatant enzyme will be described. The analytical results are recorded in Table 1 and the elution pattern is shown in Fig. 1.

The enzyme activity was eluted in two distinct peaks, one each in 100- and 200mM-phosphate buffer, pH7.0. The enzyme eluted in 100mM-phosphate is termed enzyme *A* and that in 200mM-phosphate enzyme *B*. The enzymes *A* and *B* were purified 214-fold and 359-fold in the most active of the respective fractions. In a number of trials, the range of enrichment was 150–215-fold for enzyme *A* and 280–400-fold for enzyme *B*. The overall yields were very low (only about 3% of the initial activity).

The elution pattern of enzyme *A* and enzyme *B*

Fig. 1. Elution pattern from a DEAE-cellulose column of rat liver 15000g supernatant guanine deaminase

The details of the purification procedure are described in the text. ●, Guanine deaminase activity; ○, protein, estimated by spectrophotometry.

respectively with 100- and 200mM-phosphate buffer, pH7.0, was reproducible on mixing the two and rechromatographing on DEAE-cellulose.

Rat brain guanine deaminase. As reported by Kumar *et al.* (1967), the enzyme from rat brain was resolved into two peaks, one eluted in 100mM- (enzyme *A*) and the other in 200mM- (enzyme *B*) phosphate buffer, pH7.5. The enzyme used in the following studies had specific activities of 9.9 and 33.6 units/mg of protein respectively for isoenzymes *A* and *B*.

Influence of detergent on 15000g supernatant fraction of rat liver

In a final concentration of 1% (v/v) and with an exposure period of 1 h at 0–2°C, Triton X-100 caused 25–50% activation of the guanine deaminase activity of a freshly prepared rat liver 15000g supernatant fraction. Similar activation was not observed when the detergent was incorporated into the assay system of the supernatant fraction. Neither MgSO₄ nor NaCl, tested at 100mM or 1M, influenced the degree of activation by Triton X-100, nor did either salt activate the enzyme in the absence of detergent. The pH optimum of the Triton-activated enzyme (pH8.0) was the same as that of the control. The activation by Triton X-100 progressively decreased when the supernatant was 'aged' at 0–5°C before treatment with detergent. On the fourth or fifth day of storage the activation of the supernatant was 10% or less.

Tween-80, 0.1 and 0.4% (v/v), lauryl sulphate, 0.1% (w/v) and cetyltrimethylammonium bromide, 0.1% (w/v), did not affect the enzyme activity of rat liver supernatant. At 0.4%, cetyltrimethylammonium bromide inhibited the enzyme by 80–90%.

The enzyme activity in mouse liver supernatant was not affected by Triton X-100 under conditions similar to those employed for the rat liver fraction.

The reason for the activation of guanine deaminase in rat liver 15000g supernatant by Triton X-100 is not clear. After centrifugation of a freshly prepared 15000g supernatant at 104000g for 1 h the supernatant contained all the activity.

Properties of guanine deaminase isoenzymes of rat liver

Electrophoretic mobility of proteins of the 15000g supernatant. When the (NH₄)₂SO₄-precipitated fraction of the 15000g supernatant (fraction IV, Table 1) was subjected to starch-gel electrophoresis, the proteins were resolved into three anodic bands, the most anodic being enzymically inactive. When the freeze-dried preparations of enzymes *A* and *B* (no loss of activity occurred on freeze-drying) were subjected to electrophoresis, simultaneously with the crude enzyme used in the above experiments, enzyme *B* appeared homogeneous, showing only one

band on staining and this corresponded with the slowest-moving band of the crude enzyme. Enzyme *A* separated into two bands: the more anodic was enzymically inert and had the same mobility as the inert band seen with the crude enzyme, whereas the less anodic carried the enzyme activity and corresponded in mobility to the middle protein band of the crude enzyme.

Properties of the rat liver enzymes eluted from the DEAE-cellulose column. (a) Storage stability. At a protein concentration of 10–20 µg/ml the activity of

enzyme *A* was completely lost in 16 days at 0–4°C, whereas enzyme *B* lost only 30% of activity. Enzyme *A* lost about 60% of activity on thawing after 7 days in the frozen state at –18°C, whereas enzyme *B* was practically unaltered in activity under these conditions.

Addition of bovine serum albumin stabilized the two isoenzymes in the cold as well as enzyme *A* in the frozen state.

(b) Effect of heat. After heat-treatment at 50°C for 30 min, both enzymes retained full activity. Heated at 60°C, there was 33% residual activity of enzyme *A* and enzyme *B* suffered 60% loss. In these experiments the protein and phosphate concentrations of the enzyme preparations were made equal by the addition of crystallized bovine albumin and phosphate.

(c) Effect of metal ions. The salts, at the specified concentration, were added to the assay systems before the addition of guanine to start the reaction. The results are reported in Table 2.

Both isoenzymes were activated by Mg²⁺ and inhibited by Fe²⁺ and Fe³⁺. Zn²⁺ and Cu²⁺ activated enzyme *B* marginally, while inhibiting enzyme *A*. Ca²⁺ feebly activated enzyme *A*, but was without effect on enzyme *B*.

(d) Effect of substrate concentration on reaction velocity. The response to substrate concentration of enzyme *A* was sigmoidal and that of enzyme *B* hyperbolic, as shown in Fig. 2. The *K_m* for enzyme *A*, calculated as *s*_{0.5} from Hill plots (cf. Atkinson, 1966), was 5.3 µM, and for enzyme *B*, calculated from Lineweaver–Burk plots, was 20 µM.

Table 2. Effect of metal ions on rat liver isoenzymes

Assay system (b) was employed. Substrate was added last after mixing of enzyme and buffer with the specified salt. +, Activation; –, inhibition.

Metal ion	Concn. (mM)	Activation or inhibition (%)	
		Enzyme <i>A</i>	Enzyme <i>B</i>
Nil, control		Nil	Nil
Mg ²⁺ (as MgSO ₄)	1.0	+27	+22
	0.5	+18	+11
Mg ²⁺ (as MgCl ₂)	1.0	+45	+33
	0.5	Nil	+11
Zn ²⁺ (as ZnSO ₄)	0.1	–27	+11
Ca ²⁺ (as CaCl ₂)	0.1	+18	Nil
Cu ²⁺ (as CuSO ₄)	0.05	–45	+11
Fe ²⁺ (as FeSO ₄)	0.1	–82	–66
Fe ³⁺ (as FeCl ₃)	0.25	–82	–78

Fig. 2. Substrate concentration–reaction rate relationship of rat liver 15000g supernatant guanine deaminase isoenzymes

Curves for the isoenzymes *A* (●) and *B* (○) are shown. (a) Double-reciprocal plot; (b) Hill plot for enzyme *A*.

(e) Relative affinity for guanine and 8-azaguanine. The saturation curve for 8-azaguanine, with assays at the pH optimum of 5.5, with 100mM-sodium acetate buffer, was hyperbolic for isoenzymes *A* and *B* (Fig. 3). Calculated from Lineweaver-Burk plots (Fig. 3), the two isoenzymes had nearly the same K_m values, $18\mu\text{M}$ for enzyme *A* and $8.3\mu\text{M}$ for *B*.

(f) Response to thiol reagents. The enzyme was inhibited by HgCl_2 , *p*-chloromercuribenzoate and *N*-ethylmaleimide. HgCl_2 (0.2mM) caused 90% inhibition when included in the standard assay

Fig. 3. Double-reciprocal plot showing the substrate concentration-reaction rate relationship of rat liver 15000g supernatant guanine deaminase

Results were obtained with isoenzymes *A* (●) and *B* (○) with 8-azaguanine as substrate.

(method *b*). Inhibition by *p*-chloromercuribenzoate was progressive; enzyme *B* was rather more rapidly inactivated, and the inhibition was less effectively reversed by thiol supplements, than was enzyme *A*.

(g) Reversal of inactivation by guanidine. Tests were carried out for reversal of inactivation by guanidine hydrochloride (Table 3). Dilution by itself reversed by 33% the inhibition of enzyme *B* (90%), which occurred on contact with 2M-guanidine hydrochloride for 30min, but there was no change in the inhibition (60%) of enzyme *A*. Of the thiol supplements tested, cysteine had a marginal effect, but 2-mercaptoethanol and dithiothreitol reversed the inhibition either completely or partly. The reactivation of enzyme *A* was more prominent than that of enzyme *B*. By using 2-mercaptoethanol for reversal of inhibition of enzyme *A*, there was actually a 20% increase in the activity over that of the control sample of enzyme. The presence of 100 μM -GTP caused considerable reactivation of enzyme *A*, but had no effect on enzyme *B*. As briefly reported (Sree Kumar & Krishnan, 1970b), GTP is a positive effector of enzyme *A* and is without influence on the activity of enzyme *B*.

Under similar conditions, the inactivation of the enzymes by 8M-urea could not be reversed.

Localization of guanine deaminase in mouse liver and brain

The guanine deaminase activity in mouse liver was located in the 15000g supernatant of iso-osmotic homogenates. The particulate fractions were devoid of any enzymic activity even after treatment with 1% (v/v) Triton X-100.

When the post-nuclear supernatant of mouse brain

Table 3. Tests for reversal of inhibition of rat liver isoenzymes by guanidine hydrochloride

The enzyme preparation was incubated for 30min with 2M-guanidine hydrochloride in 100mM-tris-HCl buffer, pH8.0. Enzyme *A* was diluted 20-fold and enzyme *B* 10-fold; the enzymes were assayed after storage for 30min at 30°C either at these dilutions or with specified supplements. Assay was by method (*b*).

Addition	Activity (%)	
	Enzyme <i>A</i> (100)	Enzyme <i>B</i> (100)
Nil, control	100	100
Enzyme* + guanidine (0min)	40	10
Enzyme* + guanidine (30 min)	40	40
Treated enzyme diluted for 30min	50	50
Diluted enzyme + 10mM-cysteine	120	100
+1mM-2-mercaptoethanol	80	90
+5mM-dithiothreitol	70	70
+1mM-Mg ²⁺	90	40
+100 μM -GTP		

* Enzyme activity was determined immediately after dilution.

was centrifuged at 15000g for 1 h, about 80% of the activity was recovered from the supernatant and the rest from the particles. Whether latent activity was associated with the particles was not tested.

Test for inhibitor in mouse liver and brain. The occurrence of a particle-associated endogenous inhibitor of guanine deaminase in rat liver and brain was reported earlier (Kumar *et al.*, 1967). The heavy mitochondrial fraction of mouse liver, tested as a suspension or after treatment with 1% (v/v) TritonX-100, was devoid of any inhibitory activity towards mouse liver supernatant. Post-nuclear mouse brain particles, used as a suspension, did not influence the enzymic activity of the 15000g supernatant of brain homogenate.

Purification and properties of guanine deaminase from 15000g supernatant of mouse liver and brain

Purification. Protocols for this are given in Tables 4 and 5 and the elution patterns in Figs. 4 and 5. The enzyme from mouse liver was eluted in a single peak (125mm-phosphate buffer, pH 7.0), purified 195-fold and in yield of 8.8% in the most active fraction. This fraction was mixed with the highly active preceding and succeeding fractions, when the total recovery was 25.5%.

The enzyme from mouse brain was resolved into two fractions on DEAE-cellulose columns, enzyme *A* being eluted with 100mm- and enzyme *B* with 200mm-phosphate buffer, pH 7.0, with respective 15- and 5.5-fold purification factors and yields of 6.8 and 2.2% in the most active fractions.

Properties of liver enzyme. (a) Storage stability. The enzyme eluted from DEAE-cellulose columns retained its activity unaltered during storage for a month at 0°C.

(b) pH optimum. The standard assay was at pH 8.0, with 1.0ml of 100mm-tris-HCl buffer, but it was found that the enzyme had a broad plateau for optimum activity in the range pH 7-8.

(c) Effect of metal ions. Mg²⁺, Ca²⁺, Fe²⁺, Fe³⁺, Cu²⁺, Zn²⁺ and Ba²⁺, tested at 10μM, 100μM and 1mm concentration, did not influence the enzyme activity, unlike the situation with the rat enzymes.

(d) Response to thiol reagent. *p*-Chloromercuribenzoate (1mm) inhibited guanine deaminase progressively at 30°C. The inhibition was 33% in 10min and complete in 30min. HgCl₂ (20μM) caused 69% inhibition in 5min. Activity was not restored by treatment with 10mm-cysteine, 10mm-2-mercaptoethanol or 10mm-dithiothreitol for 30min at 30°C. By themselves, none of the reducing agents influenced the activity of a control sample of enzyme.

Table 4. Purification of guanine deaminase from mouse liver

The starting material was the 15000g liver supernatant in 250mm-sucrose (livers from nine adult mice were used). Phosphate buffer (60ml) was used for enzyme elution. Details are the same as those given in Table 1.

Fraction	Vol. (ml)	Total activity (units)	Total protein (mg)	Sp. activity (units/mg of protein)	Recovery (%)	Purification
(I) Supernatant (15000g for 1 h)	85	36.4	1300	0.028	(100)	(1)
(II) Acidified to pH 5.5, centrifuged, supernatant adjusted to pH 7.0	95	36.4	513	0.071	100	2.5
(III) 55-80% satd. (NH ₄) ₂ SO ₄ ppt., heat-treated for 1 min at 60°C, in 100mm-phosphate buffer, pH 6.0, supernatant adjusted to pH 7.0	28	27.0	153	0.176	74	6.3
(IV) Dialysed, centrifuged, supernatant adjusted to pH 7.0	43	25.8	144	0.179	71	6.4
(V) DEAE-cellulose column chromatography: eluate (5ml fractions) with 125mm-phosphate buffer, pH 7.0						
no. 2		3.43	0.731	4.69	9.4	167
3		3.20	0.587	5.45	8.8	195
4		2.64	0.517	5.10	7.3	182

Table 5. *Purification of guanine deaminase from mouse brain*

The starting material was the 15000g whole-brain supernatant in 250mM-sucrose (brains from 15 adult mice were used). Volumes (50ml each) of 100mM- and 200mM-phosphate buffer, pH 7.0, were used for enzyme elution. Details are the same as those given in Table 1.

Fraction	Vol. (ml)	Enzyme activity (units)	Protein (mg)	Sp. activity (units/mg of protein)	Recovery (%)	Purification
(I) Supernatant (15000g for 1 h)	50	34.29	216.6	0.158	(100)	(1)
(II) 35–65% satd. $(\text{NH}_4)_2\text{SO}_4$ ppt.	10	17.14	106.6	0.160	50	1.01
(III) Dialysed, centrifuged, supernatant adjusted to pH 7.0	11.2	15.36	70.93	0.216	44.8	1.37
(IV) DEAE-cellulose column chromatography: eluate (5ml fractions) with phosphate buffer, pH 7.0						
100mM-buffer: no. 7		2.35	0.97	2.423	6.8	14.1
200mM-buffer: no. 14		0.75	0.86	0.872	2.2	5.52

Fig. 4. *Elution pattern from a DEAE-cellulose column of mouse liver 15000g supernatant guanine deaminase*

The details of the purification procedure are described in the text. $\blacktriangle$, Guanine deaminase activity; $\triangle$, protein, estimated by spectrophotometry.

Substrate saturation curves of mouse liver and rat and mouse brain enzymes

Mouse liver. Mouse liver enzyme yielded a substrate saturation curve which was almost linear up to the highest concentration tested ($16\mu\text{M}$).

Rat brain. The two isoenzymes differed in their velocity response to increasing substrate concentration (Fig. 6). Enzyme *A* responded sigmoidally, with a K_m value $6.3\mu\text{M}$ calculated as $s_{0.5}$ from a Hill plot, whereas enzyme *B* apparently behaved in a typical hyperbolic manner. In earlier work (Kumar *et al.*, 1967) the allosteric behaviour of enzyme *A* was not

Fig. 5. *Elution pattern from a DEAE-cellulose column of mouse brain 15000g supernatant guanine deaminase*

The details are given in the text. $\blacksquare$, Guanine deaminase activity; $\square$, protein, estimated by spectrophotometry.

seen, probably because high concentrations of guanine ($50\mu\text{M}$ – 2.6mM) were used in the assay; it was also possible that the enzyme may have been desensitized (cf. Stadtman, 1966).

Mouse brain. In mouse brain, enzyme *A* yielded a rectangular hyperbola and enzyme *B* a sigmoid curve (Fig. 7). The K_m value for enzyme *A*, calculated from a double-reciprocal plot, was $66\mu\text{M}$; for enzyme

Fig. 6. Substrate concentration-reaction rate relationship of rat brain 15000g supernatant guanine deaminase. Results were obtained with isoenzymes A (●) and B (○). (a) Double-reciprocal plot; (b) $S-v$ plot; (c) Hill plot for enzyme A.

Fig. 7. Substrate concentration-reaction rate relationship of mouse brain 15000g supernatant guanine deaminase. Results were obtained with isoenzymes A (□) and B (■). (a) Double-reciprocal plot; (b) $s-v$ plot; (c) Hill plot for enzyme B.

B the K_m value was $5.0 \mu\text{M}$, determined as $s_{0.5}$ from the Hill plot (Fig. 7).

Discussion

Rat liver 15000g supernatant guanine deaminase was isolated in two isoenzymic forms. One of the forms, B, was obtained in a highly purified state, but the second, A, was clearly non-homogeneous. The two forms differed in their electrophoretic mobility on starch gel at pH 8.0, were separable on DEAE-cellulose columns at pH 7.0, and differed in such properties as (a) storage stability, (b) effect of heat, (c) influence of metal ions, (d) K_m value for guanine and

(e) relative affinity for guanine and 8-azaguanine as substrates. They differed in their response to treatment with thiol reagents and structure-disrupting agents and subsequent reversal by appropriate treatments. Isoenzyme A was an allosteric protein, with sigmoid substrate-saturation characteristics, whereas isoenzyme B showed typical Michaelis kinetics.

It is recognized that isoenzymes can arise as artifacts of enzyme fractionation *in vitro*. The facts that the crude enzyme, obtained on $(\text{NH}_4)_2\text{SO}_4$ fractionation of the 15000g supernatant fraction of homogenates, separated into two enzymically active components on starch-gel electrophoresis (one of which had the same mobility as that of fraction B from the

DEAE-cellulose column and the other the same mobility as the enzymically active component of A), that the elution pattern from the DEAE-cellulose column was unaltered on rechromatography of a mixture of the two components and that only one of them was an allosteric protein, constituted evidence against the isoenzymes arising as artifacts of isolation.

Alteration of kinetic properties on enzyme purification is known (Murphey & Kaplan, 1967). The possibility that enzyme B, obtained in a highly purified form, also was allosteric but had lost the property during isolation could not be excluded. This was, however, not probable since the two enzyme fractions were subjected to the same treatment.

Detailed studies were not conducted with the two enzyme components separable on DEAE-cellulose columns from the 15000g supernatant fraction of rat and mouse brain after $(\text{NH}_4)_2\text{SO}_4$ fractionation. But the finding that only one form in each case was regulated by substrate suggested that rat brain and mouse brain 15000g supernatant fractions also contained isoenzymes. The enzyme from mouse liver 15000g supernatant that had been fractionated with $(\text{NH}_4)_2\text{SO}_4$ behaved as a single component when eluted from DEAE-cellulose at pH 7.0. At the present time it appears that there may be some differences between organs of mice and rats with respect to guanine deaminase activity. Thus in rat liver some of the enzyme appears to be mitochondrial (Kumar *et al.*, 1967).

As briefly communicated (Josan & Krishnan, 1968; Sree Kumar & Krishnan, 1970b; Sitaramayya & Krishnan, 1970), guanine deaminase activity can be modulated by positive and negative effectors.

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