Studies on Guanine Deaminase and its Inhibitors in Rat Tissue

By S. KUMAR, V. JOSAN, K. C. S. SANGER, K. K. TEWARI* AND P. S. KRISHNAN
Department of Biochemistry, Lucknow University, Lucknow, U.P., India

(Received 4 July 1966)

1. In kidney, but not in rat whole brain and liver, guanine-deaminase activity was localized almost exclusively in the 15000g supernatant fraction of iso-osmotic sucrose homogenates. However, as in brain and liver, the enzymic activity recovered in the supernatant was higher than that in the whole homogenate. The particulate fractions of kidney, especially the heavy mitochondria, brought about powerful inhibition of the supernatant guanine-deaminase activity.
2. In spleen, as in kidney, guanine-deaminase activity was localized in the 15000g supernatant fraction of iso-osmotic sucrose homogenates. However, the particulate fractions did not inhibit the activity of the supernatant.
3. Guanine-deaminase activity in rat brain was absent from the cerebellum and present only in the cerebral hemispheres. The inhibitor of guanine deaminase was located exclusively in the cerebellum, where it was associated with the particles sedimenting at 5000g from sucrose homogenates.
4. Homogenates of cerebral hemispheres, the separated cortex or the remaining portion of the hemispheres had significantly higher guanine-deaminase activity than homogenates of whole brain. The enzymic activity of the subcellular particulate fractions was nearly the same.
5. Guanine deaminase was purified from the 15000g supernatant of sucrose homogenates of whole brain. The enzyme separated as two distinct fractions, A and B, on DEAE-cellulose columns.
6. The guanine-deaminase activity of the light-mitochondrial fraction of whole brain was fully exposed and solubilized by treatment with Triton X-100, and partially purified.
7. Tested in the form of crude preparations, the inhibitor from kidney did not act on the brain and liver supernatant enzymes and the inhibitor from cerebellum did not act on kidney enzyme, but the inhibitor from liver acted on both brain and kidney enzyme.
8. The inhibitor of guanine deaminase was purified from the heavy mitochondria of whole brain and liver and the 5000g residue of cerebellum, isolated from iso-osmotic homogenates. The inhibitor appeared to be protein in nature and was heat-labile. The inhibition of the enzyme was non-competitive.
9. Kinetic, immunochemical and electrophoretic studies with the preparations purified from brain revealed that the enzyme from light mitochondria was distinct from enzyme B from the supernatant. A distinction between the two forms of supernatant enzyme was less certain.
10. Guanine deaminase isolated from light mitochondria of brain did not react with 8-azaguanine or with the inhibitor isolated from heavy mitochondria.

In a previous paper from this Laboratory, some findings on the intracellular distribution pattern of guanine deaminase in rat whole brain and in liver were reported (Kumar, Tewari & Krishnan, 1965a). It was also shown that an inhibitor of the enzyme was associated with the heavy mitochondria sedimenting between 700 and 5000g. The intracellular distribution studies have since been extended to rat kidneys and spleen. The inhibitor in brain is located exclusively in the cerebellum,

* Present address: Department of Botany and Plant Biochemistry, University of California, Los Angeles, Calif., U.S.A.

where it is particle-associated. Guanine deaminase from brain has been purified and its properties studied. Guanine deaminase of the supernatant fraction exists in two separable forms; the enzyme isolated from light mitochondria is a distinct molecular form. The inhibitor from brain and liver has been partially purified and some properties studied.

MATERIALS AND METHODS

Tissue samples. Adult male albino rats were killed by cervical dislocation and decapitation and the tissues excised, cleaned and chilled in ice. The cerebellum was separated

from the cerebral hemispheres by blunt dissection. The cortex was sliced from the cerebral hemispheres with a Stadie-Riggs apparatus. The region of the cerebral hemispheres, after slicing off the cortical layer, is referred to below as the 'rest of the brain'.

Preparation of homogenates. Whole brain and brain parts, liver, kidneys (whole) and spleen of rat were examined for guanine-deaminase activity and for its inhibitor. Homogenates (10%, w/v, in 250 mM-sucrose) were prepared in a Potter-Elvehjem type of homogenizer with Teflon pestle (size A, supplied by Arthur H. Thomas Co., Philadelphia, Pa., U.S.A.), the grinding being effected for a total period of 2 min. with an interval of 1 min. after the first minute of preparation.

Differential centrifugation. The preparation of the different subcellular fractions from homogenates, their recombination and the special treatment of the particle fractions were essentially as described by Kumar *et al.* (1965a).

Determination of guanine-deaminase activity. Guanine-deaminase activity was determined spectrophotometrically by the disappearance of substrate, by the method of Roush & Norris (1950). The assay system was made up of the following components, added in the order given: 100 μ moles of tris-HCl buffer, pH 8.0 or 8.2, in 1.0 ml.; 0.50 ml. of enzyme preparation, or a smaller amount diluted with water to 0.50 ml.; 1.5 μ moles of guanine in 0.50 ml. A pH of 8.0 was chosen for brain and liver and 8.2 for kidneys and spleen. The reaction was started by the addition of the substrate. After 15 min. incubation at 30°, the reaction was stopped by the addition of 1.0 ml. of 10% (v/v) HClO₄. In the control experiments, guanine was added after deproteinization. After centrifugal clarification, the extinction of a 30-fold-diluted solution was measured at 245 m μ in a Beckman model DU spectrophotometer, with silica cells of light-path 1 cm. The control samples, diluted 30-fold, had an extinction in the range 0.18–0.25. The net decrease in extinction in the experimental samples was in the range 0.02–0.10.

Under the above conditions, enzyme concentration and activity were proportional. For determining the K_m values for the different enzyme preparations, guanine was employed in the concentration range 0.10–5.28 μ moles/2.0 ml. In every case enzyme concentration and activity were approximately proportional.

Separate determinations of the extinction of standard guanine and xanthine under the conditions of acidity, dilution and other factors prevailing at the end of enzyme assay gave an extinction difference of 0.0766 as equivalent to the deamination of 1 μ mole of guanine. This value was employed in calculating the activity of enzyme.

One unit of guanine-deaminase activity was defined as the transformation of 1 μ mole of guanine into xanthine under the conditions specified above. The specific activity was expressed as units/mg. of protein. In the Tables on the subcellular distribution of the enzymic activity, the units are reported as mean values $\pm$ S.D.

In tests with guanine analogues, carried out with the purified enzyme preparations, the determination of enzymic activity was by the liberation of ammonia. The reaction system, contained in the outer chamber of Conway units (Conway, 1933), comprised 0.90 ml. of tris universal buffer (containing 22.5 μ moles of tris, 22.5 μ moles of citric acid, 20.7 μ moles of borax, 22.5 μ moles of KH₂PO₄ and 22.5

μ moles of KCl) of appropriate pH (Davies, 1959), 0.10 ml. of enzyme preparation (15, 5 and 10 μ g. of protein respectively of supernatant enzyme preparations A and B and enzyme isolated from light mitochondria) and 1.5 μ moles of guanine, 8-azaguanine, guanosine or guanylic acid. The central well contained 300 μ moles of boric acid in 0.20 ml. At the end of 30 min. incubation at 37°, the reaction was stopped by adding 0.30 ml. of saturated K₂CO₃ to the outer well. After allowing 90 min. at 37° for complete distillation, the ammonia was determined by nesslerization. Colour matching was in a Klett-Summerson colorimeter. The blank readings were in the range 15–25. Where a positive reaction was obtained, the colorimeter registered an increase of 35–190 in the readings.

In experiments with 8-azaguanine, the enzymic activity was followed also by direct spectrophotometry.

Assay of the activity of guanine-deaminase inhibitor. Tris-HCl buffer (100 μ moles) in 1.0 ml., pH 8.0 or 8.2, guanine deaminase and the inhibitor were mixed together and preincubated for 5 min. at 30°. The volumes of enzyme and inhibitor were adjusted to make a total of 0.50 ml. In the control experiments, water replaced the inhibitor solution. At the end of preincubation, 1.5 μ moles of guanine in 0.50 ml. were added. The subsequent procedure was the same as for enzyme activity determination.

The guanine deaminase used as standard in inhibitor isolation and comparison studies was the 15000g supernatant of whole brain, cerebral hemispheres, liver or kidneys; the comparisons were between the inhibitor and enzyme of the same tissue, except with the inhibitor of cerebellum, when the supernatant from cerebral hemispheres was used as enzyme source. Stored frozen, these enzyme solutions were stable for about a week. In experiments on the differential inhibition of the forms of guanine deaminase, the purified inhibitor was mixed with the purified preparations of the enzyme isolated from the supernatant and the light mitochondria.

The extent of inhibition was calculated in terms exclusively of the exogenously added guanine deaminase, after making allowance for any enzymic activity endogenously present in the inhibitor preparation. Inhibition in the range 10–60% was directly proportional to the concentration of inhibitor. One unit of the inhibitor was the amount that caused 50% inhibition of a unit of guanine deaminase under the conditions of the experiment. The specific activity of the inhibitor was expressed as units/mg. of protein.

Estimation of protein. Protein was usually estimated by the method of Lowry, Rosebrough, Farr & Randall (1951). In eluate fractions from DEAE-cellulose columns, the estimation was by the spectrophotometric method of Warburg & Christian (1941), with the equation of Kalckar (1947).

Purification of guanine deaminase from whole brain. (1) Supernatant enzyme. The 15000g supernatant was fractionated with ammonium sulphate and the precipitate separating in the saturation range 0.45–0.70 dialysed against water. After centrifugal clarification, the enzymic activity was adsorbed on alumina C_γ gel (protein/gel ratio 1:2) and protein eluted from gel successively with 20 mM-, 50 mM-, 100 mM- and 200 mM-phosphate buffer, pH 6.5, followed by 200 mM-phosphate buffer, pH 7.5. The enzyme eluted in 200 mM-phosphate buffer, pH 7.5, was dialysed against water, clarified by centrifugation, adjusted to 20 mM concentration of phosphate, pH 6.5, and passed through a

DEAE-cellulose column (5 cm. $\times$ 1.5 cm.) equilibrated with 20 mM-phosphate buffer, pH 6.5. The column was eluted in succession with 40 ml. of 50 mM- and 5 ml. of 100 mM-phosphate buffer, pH 6.5, and 10 ml. each of 100 mM- and 200 mM-phosphate buffer, pH 7.5. The alumina-gel step was attended with a fall in yield with no appreciable purification, but was a routine since the preparations resulting from this treatment were more stable.

(2) Enzyme from light mitochondria. Frozen-and-thawed light mitochondria were treated with Triton and the resulting suspension was fractionated with ammonium sulphate. The precipitate separating in the saturation range 0.30–0.70 was dialysed against water. The supernatant on centrifugation was treated with calcium phosphate gel to adsorb the enzymic activity (protein/gel ratio 1:5) and protein eluted from gel with increasing molarities of phosphate buffer, pH 6.5, commencing with 20 mM. The gel eluate with 200 mM-phosphate buffer was treated with alumina C γ gel (protein/gel ratio 1:6) and the supernatant, containing the enzymic activity, dialysed against water. The solution was adjusted to 50 mM-phosphate, pH 6.5, and passed through a DEAE-cellulose column (5 cm. $\times$ 1.5 cm.) equilibrated with 50 mM-phosphate buffer, pH 6.5, and the column eluted with increasing concentrations of phosphate buffer, pH 6.5, starting with 50 mM. The enzyme was eluted with 100 mM-phosphate buffer.

Immunochemical characterization. The general techniques were as described by Kabat & Mayer (1964). Male rabbits (8–12 months old) were used for raising antibody against a partially purified preparation of the light-mitochondrial enzyme. Starting with an initial dose of 0.5 mg. of protein and gradually increasing the amount, a total of 25–30 mg. was injected into the ear over a period of about 40 days. About 20 ml. of blood was withdrawn from each rabbit by cardiac puncture, and the serum was separated and stored frozen. The immunochemical test was carried out by the precipitin reaction in tubes and by enzyme inhibition.

Starch-gel electrophoresis. The enzyme preparations were subjected singly and in mixture to electrophoresis on starch gel in the cold. For determining the enzyme activity in the gel regions, spots were marked to correspond to the protein stains with Amido Black 10B in control runs, the cut gel pieces were frozen and thawed and ground with tris buffer, pH 8.0, in a homogenizer and portions of the suspension were used in the assay system. As control, an identical portion of enzyme was stored in contact with gel in a centrifuge tube.

RESULTS

Guanine-deaminase activity in brain parts and cell fractions

Results reported in Table 1 show that the guanine-deaminase activity was significantly higher in the whole homogenate of the cerebral hemispheres and 'rest of the brain' than in that of the whole brain. The activity of the different subcellular fractions, however, was nearly the same in whole brain, cerebral hemispheres and 'rest of the brain'. There was no enzymic activity in whole homogenate or subcellular fractions of the cerebellum.

Assays on reconstituted mixtures revealed that whereas the heavy-mitochondrial fraction and, to some extent, the nuclear fraction of whole brain inhibited the activity of the corresponding 15 000g supernatant fraction (S₂), mixing of the corresponding fractions of the cerebral hemispheres or the 'rest of the brain' elicited no inhibition. Treatment with detergents did not expose any inhibitory activity in the particles from cerebral hemispheres.

The values for the percentage subcellular distribution of guanine deaminase of whole brain reported in the present experiment were substantially different from those reported by Kumar *et al.* (1965a), although the general trend remained unaltered. There was a lapse of over 2 years between the two sets of experiments and it was likely that altered environmental temperature of the animals, food and related factors might have contributed to the variation.

Homogenates of cortex slices had more or less the same enzymic activity as the homogenates of cerebral hemispheres; these homogenates were not fractionated.

Localization of brain inhibitor activity in cerebellum

The inhibitory activity of cerebellar subcellular fractions was tested against the 15 000g supernatant of cerebral hemispheres. The inhibitor was almost equally distributed between the nuclear fraction and the heavy mitochondria, the former containing 40–50% and the latter 50–60% of the total inhibitory activity of the homogenate. The light mitochondria and the 5000g and the 15 000g supernatant fractions of the cerebellum did not have any inhibitory activity (even after Triton treatment, or grinding of the particles in a VirTis homogenizer followed by Triton action).

Guanine deaminase and inhibitor in kidney

The results of the determination of guanine-deaminase activity in three different experiments, with six animals in each, are recorded in Table 2.

The nuclei and the heavy and the light mitochondria were feebly active, each fraction containing only about 5% of the enzymic activity of the whole homogenate. The 5000g and 15 000g supernatants contained about 50% more activity than the whole homogenate. Triton treatment of the whole homogenate and the 5000g and the 15 000g supernatants resulted in a negligible change in guanine-deaminase activity.

On mixing the nuclei or the heavy mitochondria with the 15 000g supernatant, about 80% inhibition of enzymic activity occurred. Light mitochondria

Table 1. *Subcellular distribution of guanine-deaminase activity in regions of the rat brain*

A 10% (w/v) homogenate of whole brain or separated regions from adult male rats was prepared in 250 mM-sucrose and subjected to differential centrifugation. The volume of the supernatant fractions was adjusted to twice the volume of homogenate and that of the suspensions of the particles to one half. The mixing of components was in the ratio in which they were present in the homogenate. The incubation mixture was as given in the text. The units of guanine-deaminase activity, in μ moles of guanine degraded in 15 min./g. of tissue or tissue equivalent at 30°, are expressed as mean values $\pm$ S.D.; the percentage distribution has been calculated from the mean values, taking whole homogenate as 100. H stands for the whole homogenate, N the nuclear fraction, M₁ and M₂ the 'heavy-mitochondrial' and 'light-mitochondrial' fractions sedimented at 5000g and 15000g respectively and S₁ and S₂ the supernatant fractions obtained at these forces. In this and all following Tables, the activity has been reported as 'Nil' when the change in the extinction was 0.01 or less, even on using the enzyme preparation in high concentration.

Fraction	Whole brain		Cerebral hemispheres		Rest of the brain		Cerebellum
	No. of experiments ... 3		3		4		3
	Units (standard mean value)	Distribution (%)	Units (standard mean value)	Distribution (%)	Units (standard mean value)	Distribution (%)	Units (standard mean value)
H	17.3 $\pm$ 1.7	(100)	29.1 $\pm$ 4.5	(100)	24.8 $\pm$ 1.6	(100)	Nil
N	2.2 $\pm$ 1.0	13	2.6 $\pm$ 1.5	9	1.2 $\pm$ 0.4	5	Nil
M ₁	3.6 $\pm$ 0.6	21	5.5 $\pm$ 3.8	19	4.5 $\pm$ 1.6	18	Nil
S ₁	21.1 $\pm$ 4.3	122	23.3 $\pm$ 4.6	80	18.8 $\pm$ 1.7	76	Nil
M ₂	5.0 $\pm$ 3.2	29	4.9 $\pm$ 2.8	17	4.8 $\pm$ 2.5	19	Nil
S ₂	16.0 $\pm$ 5.1	92	18.2 $\pm$ 3.2	63	13.4 $\pm$ 4.2	54	Nil
S ₂ + N	13.3 $\pm$ 6.1	77	18.5 $\pm$ 1.9	64	15.0 $\pm$ 5.2	61	
S ₂ + M ₁	12.0 $\pm$ 3.1	69	22.5 $\pm$ 5.0	77	19.8 $\pm$ 5.1	80	
S ₂ + M ₂	20.6 $\pm$ 7.6	122	22.1 $\pm$ 4.3	76	20.1 $\pm$ 5.9	81	
S ₂ + N + M ₁ + M ₂	21.5 $\pm$ 9.8	124	29.4 $\pm$ 7.8	101	22.7 $\pm$ 4.7	92	

Table 2. *Subcellular distribution of guanine-deaminase activity in rat whole kidney*

Details are the same as in Table 1. The whole homogenate and the isolated subcellular fractions were also analysed after detergent treatment. Three different experiments were performed, each with the pooled kidneys from six rats.

Fraction	Control		Triton added after cell fractionation	
	Units (standard mean value)	Distribution (%)	Units (standard mean value)	Distribution (%)
H	12.7 $\pm$ 2.8	(100)	13.7 $\pm$ 4.9	(100)
N	0.5 $\pm$ 0.6	3.6	0.7 $\pm$ 1.1	5
M ₁	0.7 $\pm$ 0.5	5	Nil	Nil
S ₁	18.9 $\pm$ 3.4	148	19.5 $\pm$ 1.0	142
M ₂	0.8 $\pm$ 0.6	6	0.3 $\pm$ 0.6	2
S ₂	19.2 $\pm$ 3.5	151	19.5 $\pm$ 1.0	142
S ₂ + N	4.0 $\pm$ 2.6	31	6.3 $\pm$ 1.9	46
S ₂ + M ₁	3.6 $\pm$ 2.4	28	1.2 $\pm$ 2.1	9
S ₂ + M ₂	14.9 $\pm$ 2.9	118	10.2 $\pm$ 2.2	74
S ₂ + N + M ₁ + M ₂	14.1 $\pm$ 2.6	110	22.3 $\pm$ 2.7	163

inhibited the activity of the 15000g supernatant by about 20%. When the light mitochondria were added along with the nuclei and heavy mitochondria, the strong inhibition of the 15000g supernatant by the two latter fractions was abolished.

When the tests were performed with Triton-treated fractions, similar results were obtained, and the completely reconstituted system was appreciably more active than the corresponding whole homogenate.

Table 3. *Subcellular distribution of guanine-deaminase activity in rat spleen*

Details are the same as in Tables 1 and 2. The results are from three different experiments.

Fraction	Control		Triton added after cell fractionation	
	Units (standard mean value)	Distribution (%)	Units (standard mean value)	Distribution (%)
H	22.7 ± 10.5	(100)	28.8 ± 7.3	(100)
N	0.9 ± 0.9	4	0.8 ± 0.7	3
M ₁	0.2 ± 0.4	1	0.7 ± 0.6	2
S ₁	20.3 ± 5.6	89	24.9 ± 7.8	86
M ₂	1.6 ± 0.5	7	0.7 ± 0.6	2
S ₂	20.3 ± 5.6	89	24.9 ± 7.8	86
S ₂ + N	19.9 ± 9.4	87	24.9 ± 7.8	86
S ₂ + M ₁	22.8 ± 8.6	100	26.9 ± 8.1	93
S ₂ + M ₂	23.9 ± 7.5	105	26.3 ± 8.5	91
S ₂ + N + M ₁ + M ₂	22.8 ± 10.5	100	25.6 ± 8.6	89

Table 4. *Purification of supernatant guanine deaminase*

The starting material was the 15000g supernatant from ten adult rat whole brains in 250mm-sucrose. The purification method was as described in the text. Protein was estimated by colorimetric method in all fractions except the eluates from DEAE-cellulose column, in which case the estimation was by spectrophotometry. For column chromatography, the solution was applied in two equal batches. The fraction volume was 2.5 ml.

Fraction	Vol. (ml.)	Total activity (units)	Total protein (mg.)	Recovery (%)		Sp. activity (units/mg. of protein)	Puri- fication
				Activity	Protein		
I. Supernatant, 15000g	200	143.3	291.5	(100)	(100)	0.49	(1)
II. 0.45–0.70 saturation-(NH ₄) ₂ SO ₄ precipitate	25	130.0	105.7	90.7	36.2	1.22	2.5
III. Dialysed and clarified by centri- fugation (15000g, 20min.)	55	122.4	38.1	85.4	13.1	3.2	6.5
IV. Alumina-gel adsorption (protein/gel ratio 1:2) and elution with 200mm-phosphate buffer, pH 7.5	10	91.5	3.8	63.8	1.3	24.1	49.1
V. Eluate dialysed and clarified by centrifugation (15000g, 20min.)	15	84.4	3.8	58.8	1.3	22.2	45.3
VI. DEAE-cellulose chromatography: elution with phosphate buffer, pH 7.5							
(a) 100mm-Phosphate							
Fraction no. 7	5	15.96	0.42	11.1	0.144	38.0	77.5
Fraction no. 8	5	17.50	0.605	12.2	0.21	28.9	59.0
(b) 200mm-Phosphate							
Fraction no. 12	5	29.0	0.1	20.2	0.034	290	590
Fraction no. 13	5	16.1	0.09	11.2	0.03	180	365

Guanine deaminase in spleen

The results of three fractionation studies of guanine-deaminase activity in rat spleen, with pooled tissue from six animals each, are recorded in Table 3.

The nuclei and the heavy and the light mitochondria contained negligible enzymic activity. The activity in the supernatant fraction was nearly the same as in whole homogenate. To determine the contribution by erythrocytes to the enzymic activity of spleen supernatant, the haemoglobin

content of the solution was determined according to Cohen & Smith (1919). Simultaneously, the haemoglobin content and guanine-deaminase activity of lysed and 'ghost' cell-free rat erythrocytes were determined. Calculating on the basis of the relationship between haemoglobin content and guanine-deaminase activity in erythrocytes, about 20% of the guanine-deaminase activity of spleen supernatant originated from erythrocytes.

The nuclei and the heavy and the light mitochondria, with or without Triton treatment, did not have any effect on the 15 000g supernatant.

Purification of guanine deaminase from whole brain

Supernatant enzyme. The results obtained in a typical experiment are given in Table 4 and Fig. 1.

The enzymic activity was separable into two distinct components during chromatography on DEAE-cellulose. The first fraction, enzyme A, was eluted with 100mM-phosphate buffer, pH 7.5, and the second, enzyme B, with 200mM-phosphate buffer, pH 7.5 (Fig. 1). The first was enriched 70-fold (23% yield) and the second 600-fold (20% yield). The specific activity of enzyme B was about 7-fold as high as that of enzyme A.

Enzyme from light mitochondria. (a) Activation and solubilization. Experiments involving the methods for the activation and solubilization of guanine-deaminase inhibitor described by Kumar *et al.* (1965a) are reported in Table 5. The enzyme was readily obtained in soluble form (140 000g supernatant) by treatment with Triton X-100.

Fig. 1. Elution pattern of rat-brain supernatant guanine deaminase from DEAE-cellulose column. ○, Enzymic activity; ●, protein. The concentration of orthophosphate in the eluate was 50mM, pH 6.5, in fractions 1-4, 100mM, pH 6.5, in fractions 5 and 6, 100mM, pH 7.5, in fractions 7-10, and 200mM, pH 7.5, in fractions 11-18. Other details are given in the Materials and Methods section and in Table 4.

(b) Purification. The results obtained in a typical experiment are given in Table 6.

The enzyme was eluted as a single peak from the DEAE-cellulose column and was obtained in about 30% yield and 50-fold enrichment. The specific activity of the enzyme was 13 units/mg. of protein, as assayed at pH 8.0. As shown below (Fig. 2), the particle-associated enzyme had optimum pH 7.5, at which pH it was about 30% more active than at pH 8.0. The specific activity of the enzyme at pH 7.5 would therefore be expected to be about 17 units/mg. This is about one-twentieth of the activity of the purest preparation of soluble enzyme B and about one-quarter of the activity of enzyme A.

Purification of guanine-deaminase inhibitor

The heavy-mitochondrial fraction sedimenting between 700g and 5000g from homogenates of whole brain and liver and the mixed nuclear and heavy-mitochondrial fraction of cerebellum sedimenting at 5000g were used as source of the inhibitor.

Activation and solubilization. The maximum exposure of activity and solubilization of the inhibitor was obtained on freezing and thawing, followed by grinding in a VirTis homogenizer and mixing with Triton X-100 (Kumar *et al.* 1965a). The solubilization (presence in 140 000g supernatant) was achieved with less readiness than with the enzyme from light mitochondria.

Purification. (a) Whole brain. An extract obtained from mitochondria treated as above, but centrifuged at 15 000g, was brought to 0.90 saturation with ammonium sulphate and the precipitated material dialysed against repeated changes of water. The solid that separated out during dialysis

Table 5. *Exposure and solubilization of guanine-deaminase activity from light mitochondria*

The preparation of the light mitochondria and the special treatments were as described in the text and by Kumar *et al.* (1965a). A 5.0ml. sample of the light-mitochondrial suspension was employed in the experiments.

Treatment of light mitochondria	Activity (units/g. equiv. of tissue)		
	Whole suspension	140 000g supernatant	140 000g residue
None, fresh	17.5	—	—
Triton-treated	33.8	33.8	Nil
VirTis-disrupted	19.2	20.9	Nil
VirTis-disrupted and Triton-treated	22.0	23.6	Nil
Ultrasonically disintegrated and Triton-treated	32.3	32.3	Nil

Table 6. *Purification of guanine deaminase from light mitochondria*

The starting material was the frozen-and-thawed light-mitochondrial fraction, sedimenting between 5000g and 15000g, from whole brain of ten adult male rats in 250mm-sucrose. Column chromatography was carried out in one batch. Protein estimation in all fractions was by colorimetry. Other details are as in the text.

Fraction	Vol. (ml.)	Total activity (units)	Total protein (mg.)	Recovery (%)		Sp. activity (units/mg. of protein)	Purifi- cation
				Activity	Protein		
I. Triton-treated light-mitochondrial suspension	80	181.3	691.8	(100)	(100)	0.262	(1)
II. 0.30-0.70 saturation-(NH ₄) ₂ SO ₄ precipitate	25	144.7	170.5	79.8	24.6	0.848	3.2
III. Dialysed and clarified by centrifugation (15000g, 20min.)	45	125.4	58.7	69.1	8.4	2.136	8.1
IV. Adsorbed on calcium phosphate gel (protein/gel ratio 1:5) and eluted with 200mm-phosphate buffer, pH 6.5	15	106.4	10.5	58.6	1.5	10.13	38.7
V. Negative adsorption on alumina gel (protein/gel ratio 1:6)	15	76.0	7.2	41.9	1.4	10.55	40.2
VI. Dialysed and clarified by centrifugation (15000g, 20min.)	20	64.6	6.12	35.2	0.88	10.55	40.2
VII. DEAE-cellulose chromatography: elution with 100mm-phosphate buffer, pH 6.5	5	53.2	4.12	29.3	0.59	12.91	49.2

was extracted with 1% (w/v) sodium chloride. The extract was treated with calcium phosphate gel to remove inert material (protein/gel ratio 1:6) and the inhibitory activity in the supernatant (endogenous guanine-deaminase activity was absent at this stage) adsorbed on alumina C_γ gel (protein/gel ratio 1:10). The protein was eluted from the gel with increasing concentrations of tris buffer, pH 8.0, starting with 20mm. The inhibitor was eluted with 50mm- and 100mm-tris buffer. The results are reported in Table 7.

The inhibitor was obtained in a yield of about 25% and about 35-fold purification, with a specific activity of about 100 units/mg. of protein.

(b) Cerebellum. The processing of cerebellum differed from that of whole brain in that the extract was fractionated with ammonium sulphate, the 0.30-saturation precipitate being discarded, and that positive adsorption was carried out with calcium phosphate gel.

The inhibitor was obtained in a yield of about 25% and 40-fold enrichment. Its specific activity was about one-quarter that of the purest sample from whole brain. The preparation was unstable and was completely inactivated on storage at -16° for a week, unlike the preparation from whole-brain mitochondria, which was stable for long periods when stored frozen.

The specific activity of the inhibitor in extracts of cerebellum was less than in those of the whole brain. In repeated experiments, the activity of the cere-

bellar extracts was in the range 0.6-1.3 units/mg. of protein. Since the inhibitor was localized in the cerebellum and the hemispheres were devoid of it, it was to be expected that the activity in extracts of cerebellum would be higher than in extracts of whole brain. An explanation for the apparent discrepancy may be traced to the comparative instability of the preparations from cerebellum and the fact that the nuclear fraction of cerebellum contributed more protein than the mitochondrial fraction.

(c) Liver. The method of preparation was similar to that from cerebellum, with the difference that alumina gel was used in place of calcium phosphate gel. The inhibitory material was obtained in 50% yield and 45-fold enrichment. The preparation was slightly less active than that from cerebellum. Its activity was stable to storage at -16° for a week.

Properties of guanine-deaminase inhibitor

The methods employed for enriching the inhibitor (Tables 7 and 8) were those in common use for enzyme purification. It appeared that the inhibitor was a protein, or closely associated with protein. The inhibitor (purified from whole brain) was precipitated by trichloroacetic acid and the entire inhibitory activity was recovered from the precipitate after removal of excess of trichloroacetic acid with ether (Kumar, Tewari & Krishnan, 1965b). A solution of the inhibitor at pH 8.0 was completely inactivated on heating at 50° for 5min. Since the

Table 7. *Purification of guanine-deaminase inhibitor from heavy mitochondria from whole brain*

The starting material was the frozen-and-thawed heavy mitochondrial fraction of whole brain from 12 adult male rats. The source of guanine deaminase was the 15000g supernatant of whole brain, of which 979 μ g. of protein with 0.68 unit of activity was employed in the assay system. Experimental details are as given in the text.

Fraction	Vol. (ml.)	Endogenous guanine- deaminase activity (units)	Total inhibitory activity (units)	Total protein (mg.)	Recovery (%)		Sp. activity (units/mg. of protein)	Purification (1)
					Inhibitor	Protein		
I. VirTis-homogenized Triton-treated heavy mitochondria: 15000g (60min.) supernatant	110	42.0	935	336	(100)	(100)	2.78	
II. 0.90 saturation-(NH ₄) ₂ SO ₄ precipitate	15	37.5	925	315	98.9	93.7	2.93	1.06
III. Dialysed:								
(a) Whole suspension	45	36.7	825	290.2	88.2	86.3	2.8	1.01
(b) Supernatant (15000g, 20min.)	45	36.0	Nil	110.7	—	32.9	—	—
(c) Residue	15	Nil	797	172.7	85.2	51.4	4.6	1.6
IV. Residue extracted with 1% NaCl and clarified at 15000g, 20min.	25	3.05	583	24.3	62.3	7.2	23.9	8.6
V. Negative adsorption with calcium phosphate gel (protein/gel ratio 1:6)	24	Nil	350	4.26	37.4	1.2	82.1	29.5
VI. Alumina-gel adsorption (protein/gel ratio 1:10) and elution with tris buffer, pH 8.0								
(a) 50mm	10	Nil	93.3	1.00	10.0	0.29	93.3	33.4
(b) 100mm	10	Nil	133.2	1.25	14.2	0.37	106.6	38.3

Table 8. *Purification of guanine-deaminase inhibitor from cerebellum*

The starting material was the frozen-and-thawed combined nuclei and heavy mitochondria of cerebellum sedimenting at 5000g from eight adult male rats. The source of guanine deaminase was the 15000g supernatant of cerebral hemispheres, of which 700 μ g. of protein with 0.847 unit of activity was employed in assay system. Experimental details are as given in the text.

Fraction	Vol. (ml.)	Total inhibi- tory activity (units)	Total protein (mg.)	Recovery (%)		Sp. activity (units/mg. of protein)	Purifi- cation
				Inhibitor	Protein		
I. VirTis-homogenized Triton-treated particles: 15000g (20 min.) supernatant	17	55.4	92.0	(100)	(100)	0.60	(1)
II. 0.30–0.70 saturation-(NH ₄) ₂ SO ₄ precipitate	5	54.2	25.0	97.8	27.2	2.2	3.7
III. Dialysed:							
(a) Whole suspension	18	46.9	19.1	84.7	20.8	2.5	4.2
(b) Supernatant (15000g, 20 min.)	18	Nil	3.3	—	3.6	—	—
(c) Precipitate	7	45.6	11.4	80.5	12.4	4.0	6.7
IV. Precipitate taken up in 1% NaCl and centrifuged (15000g, 20 min.)							
(a) Extract	17	27.4	2.4	49.5	2.6	11.4	19.0
(b) Residue	6	8.0	7.2	14.2	7.8	1.1	1.8
V. Calcium phosphate-gel adsorption of extract (protein/gel ratio 1:1): eluate with 100 mM-tris buffer, pH 8.0	9	13.6	0.56	24.5	0.6	24.3	40.5

inhibitor was non-diffusible, there was no easy means of its removal at the end of the reaction with the enzyme and establishing whether the inhibitor functioned reversibly or irreversibly. The conventional kinetic plots of experimental data are not applicable to irreversible inhibitors. Guanine-deaminase inhibitor bore no chemical resemblance to the substrate; the inhibition was therefore unlikely to be of the competitive type. When assays with 20 μ g. of protein of soluble enzyme B were carried out in the presence of 1 μ g. of protein of inhibitor preparation and substrate in the concentration range 0.165–1.65 μ moles/2.0 ml., the double-reciprocal plot (Lineweaver & Burk, 1934) yielded an elevated slope and intercept, but a K_m value identical with that obtained in the absence of inhibitor (1.42 mM). A characteristic of non-competitive inhibition is the absence of any effect of the inhibitor on the K_m value for substrate. Kinetic studies would therefore lead to the conclusion that guanine-deaminase inhibitor functioned non-competitively. The degree of inhibition was, however, dependent on enzyme concentration. A truly non-competitive inhibitor yields the same degree of inhibition irrespective of enzyme concentration. With most known inhibitors, the amounts needed to produce a significant inhibition are very large compared with the amount of enzyme taken. In the present case, the protein in purified enzyme preparation was considerably in excess of protein in

purified inhibitor preparation. It is obvious that guanine-deaminase inhibitor has a high affinity. Expressing the concentration of inhibitor in g. of protein/l. in the equation of Dixon & Webb (1964), the apparent K_i was calculated to be 0.5 mM.

Comparison of the effectiveness of the inhibitor from cerebellum, liver and kidney

The source of inhibitor from cerebellum was the extract of the mixed nuclear and heavy-mitochondrial fraction (5000g residue) and from liver the extract of the heavy-mitochondrial fraction. The extracts were prepared by subjecting the particles of VirTis homogenization and Triton action after freezing and thawing and clarifying by centrifugation at 15000g. The inhibitor from kidney was in the form of a frozen-and-thawed suspension of the heavy mitochondria in sucrose medium. The source of guanine deaminase was the 15000g supernatant of homogenates of cerebral hemispheres (equivalent to 25%), liver (equivalent to 15%) and kidney (equivalent to 20 or 10%).

On addition of the same amount of liver inhibitor to the three enzyme sources, it was observed (Table 9) that they were almost equally inhibited, the degree of inhibition being calculated in terms of the exogenously added enzyme. The inhibitor from cerebellum showed some specificity; it was maximally active against brain supernatant; liver

Table 9. *Comparison of the inhibitor from cerebellum, liver and kidney of rat*

The portion employed of the 15000g supernatant fraction contained 0.65 unit of guanine-deaminase activity and 0.6mg. of protein for the cerebral hemispheres, 0.65 unit of enzyme and 1.39 mg. of protein for the liver and 0.39 unit of enzyme and 3.1 mg. of protein for the kidney. The results reported, (a) and (b), are from two different rats.

Source of inhibitor	Protein in portion used of inhibitor (mg.)	Endogenous guanine-deaminase activity (unit)	Inhibition of guanine-deaminase activity of 15000g supernatant (%)		
			Cerebral hemispheres	Liver	Kidney
Cerebellum					
(a)	1.1	Nil	50	30	16
(b)	1.1	Nil	46	37	Nil
Liver					
(a)	0.5	0.33	60	60	50
(b)	0.7	0.33	63	63	43
Kidney*					
(a)	0.5	Nil	16	16	66
(b)	0.8	Nil	Nil	Nil	40

* For testing kidney inhibitor, the 15000g supernatant of cerebral hemispheres and liver was diluted 1:1; the kidney supernatant used in (a) had 0.39 unit of enzyme and 3.1 mg. of protein and in (b) 0.33 unit of enzyme and 3.2 mg. of protein.

supernatant was less inhibited; the kidney enzyme was either not inhibited at all or inhibited to a slight extent. The kidney inhibitor was highly specific; the liver and cerebral-hemisphere enzyme was either not inhibited at all, or inhibited to a low extent, contrasting with the marked inhibition of the kidney enzyme.

Distinctive nature of guanine deaminase from light mitochondria

For comparison of properties, use was made of the purified preparations of the enzyme from light mitochondria and the supernatant. Of the two preparations of supernatant enzyme, greater emphasis was placed on enzyme B, because of its higher specific activity. Different batches of the enzymes were used in the various experiments; also, some of the preparations had been stored with occasional thawing and freezing. The specific activities of the enzymes were therefore not the same in the various experiments.

pH-activity relationship. With 10 and 5 μ g. of protein respectively of supernatant enzyme A and B and 25 μ g. of protein of the enzyme from light mitochondria and veronal buffer in the pH range 3.5–9.5, enzyme B was found to have a broad range of maximum activity in the range pH 7.5–9.0, with a maximum at 8. The pH-activity relationship of enzyme A was practically the same as that of enzyme B. The profile of the enzyme isolated from the light mitochondria was quite different. The enzyme had a fairly sharp peak of activity at

Fig. 2. pH-activity relationship of the forms of guanine deaminase from rat brain. Details are as given in the text. O, Supernatant enzyme A; ●, supernatant enzyme B; Δ, light-mitochondrial enzyme.

pH 7.5; at pH 8.0, the activity was about 30% less and at pH 9.0 about 45% less than at pH 7.5 (Fig. 2).

The activity of the enzyme preparations was unaffected when 100mm-tris buffer, pH 8.0 or 7.5, was substituted for veronal buffer.

Substrate affinity. Whereas the K_m value for guanine of soluble enzyme A, as determined from a double-reciprocal plot (Lineweaver & Burk, 1934), was in the range 0.20–0.38mm and that of soluble enzyme B 1.2–1.66mm, the enzyme from light mitochondria had K_m in the range 0.88–1.22mm. The particle-associated enzyme thus had more or less the same apparent substrate affinity as soluble enzyme B, but enzyme A had a distinctly lower K_m .

The enzyme preparations of the present authors

Table 11. *Effect of heavy-mitochondrial inhibitor on forms of brain guanine deaminase*

The assay system was as described in the text. Owing to storage and repeated thawing and freezing, the enzymes had decreased specific activity. Purified whole-brain mitochondrial inhibitor (2 μ g.) was added in the assay systems, inclusive of system (a) with the enzyme from light mitochondria. In system (b) of this enzyme, the inhibitor concentration was raised to 25 μ g. of protein.

Enzyme sample	Amount of protein in enzyme sample (μ g.)	Total added activity (units)	Residual activity (units)	Inhibition (%)
Supernatant A	12	0.55	0.32	42
Supernatant B	15	0.50	0.20	60
From light mitochondria				
(a)	25	0.61	0.61	Nil
(b)	25	0.61	0.61	Nil

Table 12. *Immunochemical distinction between the enzymes from the supernatant fraction and the light mitochondria by the precipitin reaction*

The antiserum prepared against guanine deaminase from light mitochondria was diluted 1:3 and 0.30 ml. mixed with the different enzyme preparations, in total 0.60 ml. volume. After 60 hr. incubation at 0–4°, the tubes were centrifuged; any precipitate formed was washed with 0.30 ml. of 0.9% NaCl and the wash liquid mixed with the main supernatant. The precipitate was suspended in 100 mm-tris buffer, pH 7.5, and the volume made to 0.60 ml. In control tubes, a sample each of the enzyme was stored for the same period in the cold, to serve as control. Only one dilution of each enzyme was stored; the residual activities of the other dilutions (recorded in parentheses) were calculated assuming proportionate change.

Amount of protein in enzyme sample (μ g.)	Enzyme activity without reaction		Enzymic activity after precipitin reaction with antiserum			
			Supernatant		Precipitate	
	Initial (units)	Stored (units)	(units)	Inhibition (%)	(units)	Recovery (%)
Supernatant						
Enzyme A						
(a) 16	0.604	0.431	0.431	Nil	No precipitate	
(b) 24	0.906	(0.646)	0.653	Nil	No precipitate	
Enzyme B						
(a) 7	0.638	0.638	0.638	Nil	No precipitate	
(b) 14	1.276	(1.276)	1.264	Nil	No precipitate	
From light mitochondria						
(a) 8	0.95	0.855	Nil	100	0.191	22.3
(b) 16	1.90	(1.710)	0.638	62.7	0.255	14.9
(c) 24	2.85	(2.565)	1.064	58.5	0.383	14.9
(d) 32	3.80	(3.42)	1.702	50.2	Nil	Nil

24 μ g. of protein of enzyme A, with 0.604 and 0.906 unit of activity; (c) 7 and 14 μ g. of protein of enzyme B, with 0.64 and 1.3 units of activity. The volume was brought to 0.60 ml., and the tubes were stored in the cold for about 60 hr. and centrifuged in the cold. When a precipitate was sedimented, it was washed once with 0.9% sodium chloride and the wash liquid mixed with the main supernatant. The precipitate was suspended in 100 mm-tris buffer, pH 7.5, and the volume made to 0.60 ml. The solutions and the suspensions of the precipitates were separately assayed for guanine-deaminase

activity. In control tubes, a sample each of the enzymes was stored for the same period in the cold. The results of an experiment are reported in Table 12.

When the antiserum prepared with mitochondrial enzymes was mixed with the light-mitochondrial enzyme, precipitation occurred in all the tubes. The supernatant on centrifugation was devoid of any enzymic activity at the lowest enzymic concentration tested, i.e. 8 μ g. of protein; the precipitate contained about 20% of the total activity. In the higher concentrations of enzyme, as much as

50–60% activity was left in the supernatant; the residue had about 15% of the total activity, or none at all.

At comparable levels of enzymic activity where the enzyme isolated from the light mitochondria was precipitated totally or in part, the supernatant guanine-deaminase preparations were not precipitated by the mitochondrial form of antiserum. All the initial activity of the enzymes could be recovered in solution at the end of incubation with the antiserum.

Electrophoretic characterization

The two forms of supernatant enzyme travelled an equal distance towards the anode as a single compact spot each at pH 7.0 on starch gel. The protein of the enzyme preparation isolated from the light mitochondria migrated more slowly, and the spot was elongated with considerable tailing. About 70–90% of the activity of enzymes A and B could be recovered after electrophoresis in the position of the protein spot. Over 80% of the activity of the enzyme from the light mitochondria was recoverable from the gel immediately behind the well-stained region, at the beginning of the tail. When the enzyme from the light mitochondria (7.5 μ g. of protein) and supernatant enzyme B (7.5 μ g. of protein) were mixed and subjected to electrophoresis (4–5 v/cm.; 20 hr. at 2–6°), there was separation into two distinct regions of enzymic activity, one (presumably the enzyme from light mitochondria) located between 0.5 and 1.5 cm. from the point of application and the other (presumably the supernatant enzyme) between 4.2 and 5.5 cm.

Chromatography of a mixture of supernatant and the light-mitochondrial enzyme

Six units of supernatant enzyme B (75 μ g. of protein) in 50 mM-phosphate buffer, pH 6.5, were mixed with 6.9 units of enzyme from light mitochondria (985 μ g. of protein) in 50 mM-phosphate buffer, pH 6.5 and the enzyme activity adsorbed on DEAE-cellulose. Elution with 100 mM-phosphate buffer, pH 6.5, gave 6.6 units of activity in a total volume of 2.5 ml. The subsequent use of 100 mM-phosphate buffer, pH 7.5, did not elute any enzymic activity. When this was followed with 200 mM-phosphate buffer, pH 7.5, 5.6 units of activity were eluted in 2.5 ml. The identity of the first fraction with the light-mitochondrial enzyme (Table 6) and the second with the supernatant enzyme (Table 4) was established by testing the action of the inhibitor isolated from whole-brain heavy mitochondria. On mixing 5 μ g. of protein of inhibitor with a portion of the first eluate fraction with 0.28

unit of activity and a portion of the second with 0.35 unit of activity, there was no inhibition of the former, but 43% inhibition of the latter occurred.

DISCUSSION

The general concept of the exclusive location of guanine deaminase in the 'supernatant' fraction of tissue homogenates needs revision. The present results obtained with brain, liver, kidney and spleen prove that guanine deaminase has a complex, but variable, pattern of subcellular distribution in the rat.

The existence of a naturally occurring inhibition of guanine deaminase raises the question whether the apparent absence of the enzyme from some tissues and from the blood of some species may be due to inhibition, especially when the analyses are performed on whole homogenates (Hirschberg, Kream & Gellhorn, 1952). The present authors find that, although rat kidney is a fair source of guanine deaminase, goat kidney is devoid of activity. Whole homogenates of the separated medulla and cortex of goat kidney and subcellular fractions derived from either were all inactive. The particulate fractions from medulla and cortex did not inhibit the 15000g supernatant of rat liver. In confirmation of the results with human and monkey cerebellum (Levine, Hall & Harris, 1963; Mansoor *et al.* 1963), rat brain cerebellum does not have guanine-deaminase activity. Whole homogenates of the cerebellum may be expected to be inactive because of the presence of inhibitor associated with the particles, but the 15000g supernatant was also inactive. Human erythrocytes are devoid of guanine-deaminase activity, in contrast with rat erythrocytes. The separated 'ghost' cells of human erythrocytes did not inhibit rat-liver guanine deaminase. The physiological role of the inhibitor is not clear. In rat brain it is concentrated in the cerebellum, but its location in rat kidney has not yet been studied. There is reason to believe that the enzymes and the inhibitors differ in brain, liver and kidney. The insensitive nature of the enzyme purified from the light mitochondria towards the inhibitor explained the guanine-deaminase activity of particulate fractions in spite of the presence of inhibitor in concentrations sufficiently high to inhibit totally an equivalent amount of supernatant activity. The same may hold good for liver.

The enzymic activity obtained with 8-azaguanine as substrate has been assumed by some authors to be a measure of guanine deaminase (Hirschberg *et al.* 1952; Levine *et al.* 1963). The findings that there exists a form of guanine deaminase that is inactive towards 8-azaguanine necessitates caution in the interpretation of data with the analogue.

Artifact formation during isolation of the forms of

guanine deaminase from whole brain appeared to be unlikely. Not only was the original pattern of chromatography on DEAE-cellulose reproducible when supernatant enzymes A and B were tested singly, but the reconstituted mixture of the two yielded the same chromatographic pattern as the original composite supernatant enzyme preparation (fraction V; Table 4). The enzyme from light mitochondria also retained its elution pattern unchanged on rechromatography. Even more significant was the result of chromatography of a mixture of supernatant enzyme and the enzyme purified from the light mitochondria.

In the present experiments, Triton X-100 was employed to expose and solubilize the enzyme from light mitochondria. The possible effect of the detergent on the properties of the enzyme had to be taken into account. The 15000g supernatant fraction of brain homogenate was mixed with Triton and fractionated for enzyme isolation. The enzyme activity was eluted from DEAE-cellulose column in two distinct fractions (Table 4). Their response to molybdate and to the action of the mitochondrial inhibitor and the antiserum prepared against the light-mitochondrial enzyme was similar to that of the enzyme fractions isolated from fresh supernatant. Since prior Triton treatment did not affect the properties of the enzyme forms isolated from the supernatant fraction, the conclusion seems justified that the distinctiveness of guanine deaminase from light mitochondria was not an artifact of Triton action.

Guanine deaminase therefore constitutes another member of the small group of enzymes that exist in isoenzymic forms at different subcellular location, the best-known example of which is malate dehydrogenase (Thorne, 1960). The techniques employed in the present investigations were not sufficiently sensitive to establish whether a micro-heterogeneity existed in light-mitochondrial guanine deaminase as in mitochondrial malate dehydrogenase (Kitto, Wassarman, Michjeda & Kaplan, 1966). Evidence for a distinction between supernatant enzyme fractions A and B was not as strong as between enzyme B and the enzyme isolated from light mitochondria. Nevertheless, it

was significant that the supernatant enzyme did not reveal contamination with the light-mitochondrial form of the enzyme in the immunochemical tests. This may be contrasted with the finding of Grimm & Doherty (1961) that, of the two malate dehydrogenase components separating on zone electrophoresis of the supernatant of ox-heart sucrose homogenate, only one was characteristic of the soluble fraction, the second having been derived from the mitochondria.

S. K. is grateful to the Council of Scientific and Industrial Research, Delhi, and V. J. to the University Grants Commission, Delhi, for the award of Research Fellowships. This Department is indebted to the Rockefeller Foundation for generous grants.

REFERENCES

- Cohen, B. & Smith, A. H. (1919). *J. biol. Chem.* **39**, 489.
- Conway, E. J. (1933). *Biochem. J.* **27**, 430.
- Davies, M. T. (1959). *Analyst*, **84**, 248.
- Dixon, M. & Webb, E. C. (1964). *Enzymes*, 2nd ed., p. 327. London: Longmans, Green and Co., Ltd.
- Grimm, F. G. & Doherty, M. D. (1961). *J. biol. Chem.* **236**, 1980.
- Hirschberg, E., Kream, J. & Gellhorn, A. (1952). *Cancer Res.* **12**, 524.
- Kabat, E. A. & Mayer, M. M. (1964). *Experimental Immunochimistry*, 2nd ed., p. 873. Springfield: Charles C. Thomas.
- Kalckar, H. M. (1947). *J. biol. Chem.* **167**, 461.
- Kitto, G. B., Wassarman, P. M., Michjeda, J. & Kaplan, N. O. (1966). *Biochem. biophys. Res. Commun.* **22**, 75.
- Kumar, S., Tewari, K. K. & Krishnan, P. S. (1965a). *Biochem. J.* **95**, 797.
- Kumar, S., Tewari, K. K. & Krishnan, P. S. (1965b). *J. Neurochem.* **12**, 1003.
- Levine, R., Hall, T. C. & Harris, C. A. (1963). *Cancer*, **16**, 269.
- Lineweaver, H. & Burk, D. (1934). *J. Amer. chem. Soc.* **56**, 658.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, A. J. (1951). *J. biol. Chem.* **193**, 265.
- Mansoor, M., Kalyankar, G. D. & Talwar, G. P. (1963). *Biochim. biophys. Acta*, **77**, 307.
- Roush, A. & Norris, F. R. (1950). *Arch. Biochem.* **29**, 124.
- Thorne, C. J. R. (1960). *Biochim. biophys. Acta*, **42**, 175.
- Warburg, O. & Christian, W. (1941). *Biochem. Z.* **310**, 384.