Biochemical Pharmacology, Vol. 22, pp. 651–657. Pergamon Press, 1973. Printed in Great Britain.

AMPHETAMINE OXIDATION IN RAT BRAIN

CHHANDA MITRA and S. R. GUHA

Indian Institute of Experimental Medicine, Calcutta, India

(*Received* 4 *May* 1972; *accepted* 6 *July* 1972)

Abstract—Amphetamine dehydrogenase activity of rat brain is localized in the crude mitochondrial fraction and is enhanced upon addition of soluble supernatant fraction whereas different co-factors tested were without any effect. The pH-activity curve indicated two peaks, the one at pH 7·5 being more elevated than that observed at pH 9·0. Optimal concentration of NTC was found to be 0·5 mg/ml and higher NTC concentration was not inhibitory. K_m for *d*-amphetamine under the present experimental conditions was found to be $2{\cdot}5 \times 10^{-3}$ M. The enzyme activity was found to increase when homogenates were stored at a temperature of 0–1° for 1 or 2 hr. The enzyme was not inhibited by NaCN, semicarbazide, various metal chelating agents and inhibitors of monoamine oxidase. It was observed that tranylcypromine is actively dehydrogenated by rat brain homogenate.

THE EXISTENCE of dehydrogenase systems in rat and guinea-pig brain tissues catalysing the oxidation of amphetamine, mescaline and ephedrine has previously been demonstrated.[1] It was further observed[2] that in guinea-pig brain amphetamine–tetrazolium reductase activity* is chiefly localized in the crude mitochondrial fraction and needs supplementation with either the supernatant fraction or NADP for full activity. The function of a metal ion in the catalytic activity of the enzyme protein was also envisaged in the case of guinea-pig brain system.[2] The dehydrogenase of rat brain appears to be different from that of guinea-pig brain, especially with regard to co-factor requirements, an account of which is presented in this communication.

MATERIALS AND METHODS

Whole brain tissue of adult male albino rats (150–200 g) were homogenized in 0·25 M sucrose to give 10% (w/v) suspensions in an homogenizer fitted with a Teflon pestle (clearance 0·005–0·007 in, 1000 rev/min). Since it was observed that enzyme activity increased on storage of the homogenate, even at ice-cold temperatures, the homogenate was usually added to the test medium within 5 min after its preparation unless otherwise stated. The crude mitochondrial and other subcellular fractions were obtained by the differential centrifugation method of Brody and Bain.[3] All the particulate fractions were then suspended in isotonic sucrose to give 20 per cent suspensions. In experiments with sub-cellular fractions, the activity of the original homogenate was assayed along with those of the different sub-cellular fractions.

The standard assay system for rat brain amphetamine dehydrogenase contained 0·025 M phosphate buffer pH 7·5, 100 mg tissue homogenate or its equivalent when

* Following the recent nomenclature the enzyme is described as amphetamine–tetrazolium reductase or amphetamine dehydrogenase. The following abbreviations are used: NTC, neo-tetrazolium chloride; NAD, nicotinamide adenine dinucleotide; NADH, reduced nicotinamide adenine dinucleotide; NADP, triphosphopyridine nucleotide; NADPH, reduced triphosphopyridine nucleotide; FMN, flavin mononucleotide; FAD, flavin adenine dinucleotide; EDTA, ethylenediamine tetraacetic acid; INH, isonicotinic acid hydrazide; *p*-CMB, *p*-chloromercuribenzoate.

subcellular fractions were employed, 0·01 M *d*-amphetamine and 0·5 mg NTC in a final volume of 2 ml. Although the optimal concentration of NTC was found to be 0·5 mg/ml of the reaction mixture, usually a sub-optimal concentration of NTC (0·25 mg/ml) was employed to reduce the endogenous diformazan values. Amphetamine dehydrogenase activity of guinea-pig brain was assayed under conditions as described previously.[2] All incubations were carried out at 37° for 30 min with air as the gas phase. When inhibitors were used, they were incubated with the enzyme for 5 min prior to addition of NTC and *d*-amphetamine. The diformazan was extracted and measured at 520 nm according to the method of Lagnado and Sourkes.[4]

The distinct areas of brain were dissected under ice-cold conditions and usually five animals were sacrificed for each experiment. After dissection the corresponding brain areas were pooled, homogenized and assayed for enzyme activity.

RESULTS

The subcellular distribution pattern of amphetamine dehydrogenase activity of rat brain is shown in Table 1. The enzyme is chiefly localized in the crude mitochondrial fraction and the activity of the crude mitochondrial fraction increased upon addition of soluble supernatant fraction indicating the presence in the latter of a co-factor

TABLE 1. SUBCELLULAR DISTRIBUTION OF AMPHETAMINE DEHYDROGENASE ACTIVITY IN RAT BRAIN

Systems	μMoles diformazan formed/ 100 mg tissue/30 min
Homogenate	16·8
Nucleus	0·7
Crude mitochondrial fraction	10·0
Microsome	nil
Supernatant	nil
Nucleus + supernatant	2·2
Crude mitochondrial fraction + supernatant	13·8
Microsome + supernatant	nil

The reaction mixture contained 0·025 M phosphate buffer pH 7·5, 0·5 mg NTC, 0·01 M *d*-amphetamine and 100 mg of tissue homogenate or its equivalent in a final volume of 2 ml.

necessary for enzyme activity. Various co-factors like NAD, NADH, NADP, NADPH, FAD, FMN, lipoic acid or glutathione were used, but none could produce the stimulating effect of the supernatant fraction. However, it was observed that the co-factor present in the supernatant fraction is heat-stable and dialysable.

The pH-activity curve (Fig. 1) of amphetamine dehydrogenase of rat brain homogenate showed two peaks, the peak at pH 7·5 being more elevated than that observed at pH 9·0. Similar peaks were also observed when rat brain homogenate was incubated with l-amphetamine. It was observed that diformazan production reached an optimum level when NTC concentration was 0·5 mg/ml of the reaction mixture and any further increase in NTC concentration failed to increase diformazan production (Table 2).

Using optimal concentration of NTC, it was noted that high concentrations of

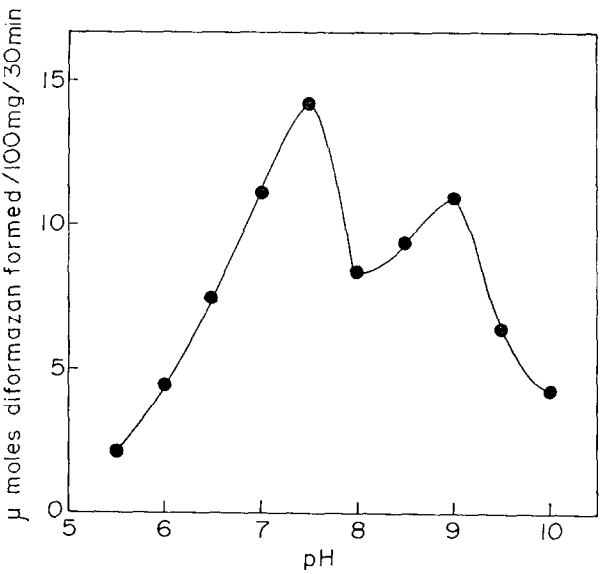


FIG. 1. Influence of pH on amphetamine dehydrogenase activity of rat brain homogenate. Enzyme activity is expressed as μmoles diformazan formed per 100 mg tissue in 30 min in presence of 0·01 M *d*-amphetamine. Other details are given in the text.

d-amphetamine failed to inhibit the formation of diformazan (Table 3). K_m value for *d*-amphetamine under the present experimental conditions was calculated from the reciprocal plot of Lineweaver and Burk[5] and found to be $2{\cdot}5 \times 10^{-3}$ M (Fig. 2).

Both in guinea-pig and rat brains, the dehydrogenase was very active in cortex and hippocampus while cerebellum and mid-brain regions exhibited significantly low enzyme activities (Table 4). The effect of storage of rat and guinea-pig brain homogenates in ice-cold temperatures (0–1°) as shown in Table 5 indicated that amphetamine dehydrogenase activity is greatly enhanced if rat brain homogenate is stored for 1 or 2 hr while a gradual decrease in enzyme activity occurred in the case of guinea-pig brain homogenate.

TABLE 2. EFFECT OF VARIED CONCENTRATIONS OF NTC ON AMPHETAMINE DEHYDROGENASE ACTIVITY OF RAT BRAIN HOMOGENATE

Concns of NTC (μg/ml)	μMoles diformazan formed/ 100 mg tissue/30 min
125	11·7
250	14·6
375	16·6
500	18·6
750	18·6

Reaction mixture is same as in Table 1 with the exception that varied doses of NTC were used as indicated.

TABLE 3. EFFECT OF HIGH SUBSTRATE CONCENTRATIONS ON AMPHETAMINE DEHYDROGENASE ACTIVITY OF RAT BRAIN HOMOGENATE

Final concn *d*-amphetamine (M)	μMoles diformazan formed/100 mg tissue/30 min
0·01	18·3
0·02	20·8
0·03	22·1
0·04	22·1

Reaction mixture is same as in Table 1 with the exception that varied doses of *d*-amphetamine were used as indicated and concentration of NTC was 0·5 mg/ml of the reaction mixture.

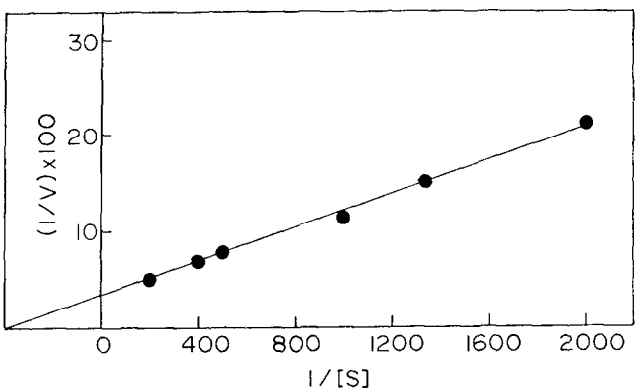


FIG. 2. Determination of K_m for *d*-amphetamine of amphetamine dehydrogenase of rat brain homogenate.
The reaction mixture contained 0·5 mg/ml NTC. Other details are given in the text.

TABLE 4. DISTRIBUTION OF AMPHETAMINE DEHYDROGENASE ACTIVITY IN DIFFERENT AREAS OF BRAIN

Brain areas	μMoles diformazan formed/100 mg tissue/30 min	
	Guinea-pig	Rat
Cortex	13·8	20·6
Cerebellum	6·9	7·9
Midbrain	5·1	8·9
Hippocampus	12·1	17·2

Experimental conditions for rat tissues are same as in Table 1 and for guinea-pig tissues as described previously.[2]

TABLE 5. EFFECT OF STORAGE ON AMPHETAMINE DEHYDROGENASE ACTIVITIES OF RAT AND GUINEA-PIG BRAIN HOMOGENATES

Storage (hr)	μMoles diformazan formed/100 mg tissue/30 min	
	Rat brain	Guinea-pig brain
0	14·6	14·2
1	17·6	9·8
2	21·5	8·7

Experimental details are same as in Table 4.

TABLE 6. EFFECT OF DIFFERENT INHIBITORS ON AMPHETAMINE DEHYDROGENASE ACTIVITY OF RAT BRAIN HOMOGENATE

Inhibitors	Final concn (mmoles)	Inhibition (%)
Tranylcypromine	0·1	nil
Pargyline	1	nil
Catron	1	nil
Iproniazid	1	nil
INH	1	nil
Semicarbazide	20	nil
Sodium cyanide	20	nil
N-Ethylmaleimide	0·1	30
p-CMB	0·1	36
α-α′-Dipyridyl	4	nil
O-Phenanthroline	5	nil
8-Hydroxyquinoline	1	nil
Cupferron	1	nil

Experimental details are given in the text.

TABLE 7. EFFECT OF TRANYLCYPROMINE ON AMPHETAMINE DEHYDROGENASE ACTIVITY OF RAT BRAIN HOMOGENATE

Additions	μMoles diformazan formed/ 100 mg tissue/30 min
d-Amphetamine (0·01 M)	14·6
d-Amphetamine (0·02 M)	18·6
Tranylcypromine (0·01 M)	13·4
Tranylcypromine (0·02 M)	17·1
d-Amphetamine (0·01 M) plus tranylcypromine (0·01 M)	18·6

Experimental details are same as in Table 1.

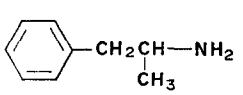
The enzyme was found to be insensitive towards carbonyl reagents like NaCN and semicarbazide (Table 6). INH and monoamine oxidase inhibitors, like tranylcypromine, pargyline, catron and iproniazid failed to inhibit diformazan production. Metal chelating agents like α- α′-dipyridyl, *O*-phenanthroline, 8-hydroxyquinoline and cupferron also failed to inhibit the dehydrogenase activity whereas *p*-CMB and *N*-ethylmaleimide produced slight inhibitory effect. It is interesting to note that tranylcypromine at 0·01 or 0·02 M concentrations was actively dehydrogenated by rat brain homogenate (Table 7). When equimolar concentrations of *d*-amphetamine and tranylcypromine were used, the amount of diformazan formed indicated that there was neither any additive nor inhibitory effect. The amount of diformazan formed in presence of both amphetamine and tranylcypromine, each at 0·01 M concentration was the same as that of the system containing only *d*-amphetamine at 0·02 M concentration.

DISCUSSION

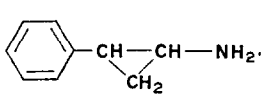
The above findings indicate that, as for guinea-pig brain, amphetamine dehydrogenase of rat brain is also localized in the crude mitochondrial fraction. It is apparent that the mitochondrial fraction employed in this study is grossly contaminated with myelin fragments, synaptosomes and free mitochondria which necessitates a more detailed study to decide whether the enzyme is truly mitochondrial or not. It was observed that the crude mitochondrial fraction itself exhibited a high activity which was further augmented when supplemented with the soluble supernatant fraction. However, in contrast to guinea-pig brain,[2] the activity of crude mitochondrial fraction of rat brain exhibited a high level of enzyme activity even without supplementation with supernatant fraction. The co-factor present in the soluble supernatant fraction of rat brain was found to be heat-stable and dialysable as was noted previously with guinea-pig brain.[2] But in contrast to guinea-pig brain system,[2] the enzyme activity did not enhance upon addition of NADP and other co-factors tested were also ineffective in enhancing the enzyme activity of crude mitochondrial fraction of rat brain.

The dehydrogenase exhibited two pH maxima which is similar to those observed when rat brain homogenate was incubated with l-amphetamine. However, on the basis of data available at present, it is difficult to decide whether a single enzyme catalyses the dehydrogenation of both the isomers of amphetamine.

It was further observed that high concentrations of NTC and *d*-amphetamine were not inhibitory to rat brain amphetamine dehydrogenase in contrast to observations with guinea-pig brain.[2] The enzyme activity in freshly prepared homogenates was found to increase on storage, the exact significance of which is not yet clear. It was also observed that tranylcypromine is not inhibitory to amphetamine dehydrogenase even at 10^{-4} M concentration. On the other hand, at 10^{-2} M concentration, tranylcypromine was actively dehydrogenated which is expected on the basis of structural similarity between amphetamine and tranylcypromine as shown below:



Amphetamine



Tranylcypromine

The absence of any additive effect when equimolar solutions of amphetamine and tranylcypromine are present indicates that both of these compounds are perhaps oxidized by the same enzyme. It is evident that for dehydrogenation a cyclysed alkyl side chain is as good as an open chain structure.

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