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INTRACELLULAR LOCALIZATION AND CO-FACTOR REQUIREMENT OF AMPHETAMINE-TETRAZOLIUM REDUCTASE OF GUINEA-PIG BRAIN

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Abstract—Amphetamine dehydrogenase of guinea-pig brain is localized in the crude mitochondrial fraction and for full activity supplementation with soluble supernatant fraction or extra added NADP are necessary. The co-factor present in the supernatant fraction is heat-stable and dialysable. The pH–activity curve of the crude mitochondrial system showed two maxima, the peak in the alkaline region being more elevated than that observed at pH 7·0. Optimal concentrations of NTC and NADP were found to be 0·5 mg/ml and 200–300 μg/ml of the reaction mixture respectively and higher NTC concentration inhibited the enzyme activity slightly whereas any further increase in NADP concentration showed no further increase in diformazan production. K_m values for *d*-amphetamine and NADP under the present experimental conditions were found to be $1{\cdot}66 \times 10^{-3}$ M and $1{\cdot}33 \times 10^{-4}$ M respectively. The crude mitochondrial enzyme was found to be inhibited by KCN in the presence of supernatant or NADP, the inhibition by KCN being reversed by dialysis or increasing the amount of NADP. The enzyme activity was also inhibited by metal chelating agents like EDTA, *o*-phenanthroline, α-α′dipyridyl, 8-hydroxyquinoline, cupferron and dithizone suggesting the possible involvement of a metal ion.

THE EXISTENCE in rat and guinea-pig brain of an active dehydrogenase* catalysing the reduction of neo-tetrazolium chloride in the presence of amphetamine and related psychoactive drugs has recently been demonstrated.[1] It has been observed that the enzyme in guinea-pig brain is localized in the crude mitochondrial fraction and requires supplementation of soluble supernatant fraction for its activity. The intracellular distribution of the enzyme and its co-factor requirement were studied, the results of which are presented in this communication.

MATERIALS AND METHODS

Whole brain tissue of adult male guinea-pigs (500–600 g) was 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). The crude mitochondrial and other

* Following the recent nomenclature the enzyme is described here 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.

sub-cellular fractions were obtained by the differential centrifugation method of Brody and Bain.[2] All the particulate fractions were finally suspended in isotonic sucrose to give 20 per cent suspensions. In subsequent experiments, the crude mitochondrial fraction thus prepared was once washed by recentrifugation and finally resuspended in 0·25 M sucrose.

The standard assay system contained 0·025 M phosphate buffer pH 7·0, 100 mg of tissue homogenate or its equivalent when sub-cellular fractions were employed, 0·01 M *d*-amphetamine and 0·5 mg NTC in a final volume of 2 ml. The amount of supernatant fraction whenever added as a source of co-factor was equivalent to 100 mg of tissue homogenate while the amount of NADP used was 100 μg unless stated otherwise. 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 formed was extracted and measured at 520 nm according to the method of Lagnado and Sourkes.[3]

RESULTS

The sub-cellular distribution pattern of amphetamine dehydrogenase activity is shown in Table 1. The enzyme is chiefly localized in the crude mitochondrial fraction and full activity is restored upon addition of soluble supernatant fraction indicating the presence in the latter of a co-factor essential for enzyme activity. The data presented in Table 2 indicate that the co-factor present in the supernantat fraction is heat-stable and dialysable. Among the co-factors tested (Table 3) it was observed that only NADP can effectively replace the co-factor present in the soluble supernatant fraction while FAD had a slight stimulatory effect.

The pH–activity curve (Fig. 1) of the crude mitochondrial amphatamine dehydrogenase showed two peaks, the peak in the range of pH 8·5–9·0 being more elevated than that observed at pH 7·0. Similar peaks were also observed with brain homogenates[1] where the peak in the region of pH 9·0 was less marked. It was observed that high concentrations of NTC slightly inhibited diformazan production and optimal

TABLE 1. SUBCELLULAR DISTRIBUTION OF AMPHETAMINE DEHYDROGENASE ACTIVITY IN GUINEA-PIG BRAIN

Systems	Diformazan formed (μmoles/100 mg tissue/30 min)
Homogenate	14·2
Nucleus	0·3
Crude mitochondrial fraction	4·4
Microsomes	0·6
Soluble supernatant	nil
Nucleus + supernatant	0·6
Crude mitochondrial fraction + supernatant	12·1
Microsome + supernantant	1·3

The reaction mixture contained 0·025 M phosphate buffer pH 7·0, 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.

TABLE 2. EFFECT OF HEAT TREATMENT AND DIALYSIS OF SOLUBLE SUPERNATANT OF GUINEA-PIG BRAIN ON STIMULATION OF AMPHETAMINE DEHYDROGENASE ACTIVITY

Systems	Diformazan formed (μmoles/100 mg tissue/30 min)
Crude mitochondrial fraction	4·4
Crude mitochondrial fraction + untreated supernatant	12·1
Crude mitochondrial fraction + supernatant dialysed for 1 hr	6·2
Crude mitochondrial fraction + supernatant dialysed for 2 hr	4·6
Crude mitochondrial fraction + supernatant dialysed for 3 hr	4·4
Crude mitochondrial fraction + heat treated supernatant	12·1

Supernatant fractions were dialysed for 30 min with several changes of phosphate buffer (0·001 M) pH 7·0. Heat treatment was carried out in boiling water bath for 5 min followed by high speed centrifugation to sediment any protein coagulated. Other details are same as in Table 1.

TABLE 3. EFFECT OF VARIOUS CO-FACTORS ON AMPHETAMINE DEHYDROGENASE ACTIVITY OF CRUDE MITOCHONDRIAL FRACTION OF GUINEA-PIG BRAIN

Co-factor added (100 μg)	Diformazan formed (μmoles/100 mg tissue/30 min)
1. None	4·4
2. NAD	4·4
3. NADH	3·5
4. NADP	10·3
5. NADPH	4·2
6. Lipoic acid	4·9
7. FAD	6·3
8. FMN	3·6
9. Glutathione	4·4

Experimental details are same as in Table 1. Some interference in the estimation of the colour intensity of diformazan were observed when FAD or FMN were used in the test system.

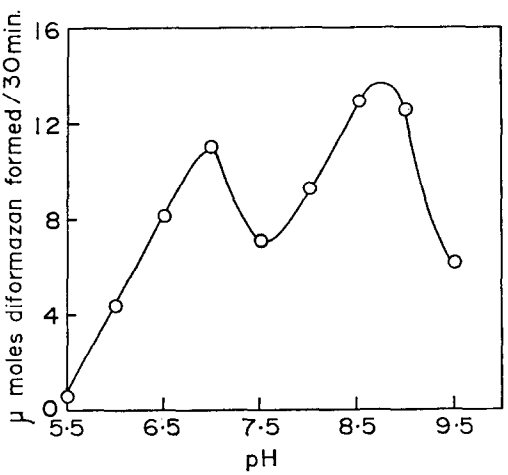

FIG. 1. Influence of pH on amphetamine dehydrogenase activity of crude mitochondrial fraction of guinea-pig brain.

Enzyme activity is expressed as μmoles diformazan produced per 100 mg tissue in 30 min presence of 100 μg NADP. Other details are given in the text.

concentration of NTC was found to be 0·5 mg/ml of the reaction mixture under the present experimental conditions (Fig. 2). The formation of diformazan, however, increased with increasing concentrations of NADP (Table 4) up to 200–300 μg/ml of the reaction mixture and any further increase in co-factor concentration failed to elevate diformazan production.

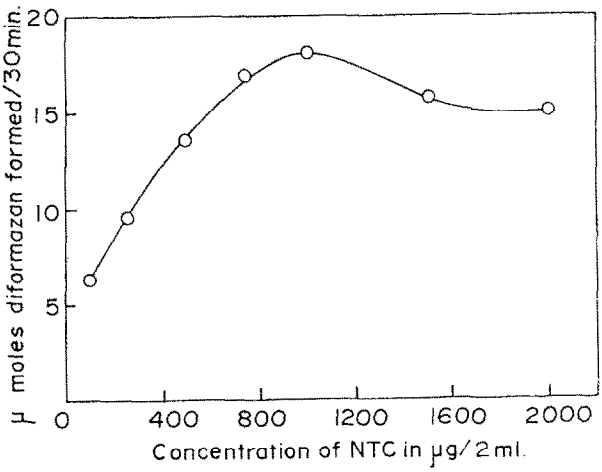

FIG. 2. Effect of varied concentrations of NTC on amphetamine dehydrogenase activity of crude mitochondrial fraction of guinea-pig brain.
The assay mixture contained 400 μg NADP and NTC was added as indicated. Other details are given in the text.

TABLE 4. EFFECT OF DIFFERENT CONCENTRATIONS OF NADP ON AMPHETAMINE DEHYDROGENASE ACTIVITY OF CRUDE MITOCHONDRIAL FRACTION OF GUINEA-PIG BRAIN

NADP added	Diformazan formed (μmoles/100 mg tissue/30 min)
1. Nil	4·4
2. 50	8·7
3. 100	10·7
4. 200	12·6
5. 400	13·8
6. 600	14·5
7. 1000	14·5

Experimental details are same as in Table 1.

Using optimal concentrations of both NADP and NTC it was observed that *d*-amphetamine at concentrations above 0·02 M slightly inhibited diformazan production (Table 5). K_m values for *d*-amphetamine and NADP under the present experimental conditions were calculated from the respective reciprocal plots of Lineweaver and Burk[4] and found to be $1{\cdot}66 \times 10^{-3}$ M and $1{\cdot}33 \times 10^{-4}$ M respectively (Figs. 3a and b).

TABLE 5. EFFECT OF HIGH CONCENTRATIONS OF *d*-AMPHETAMINE ON AMPHETAMINE DEHYDROGENASE ACTIVITY OF CRUDE MITOCHONDRIAL FRACTION OF GUINEA-PIG BRAIN

Final concentrations of *d*-amphetamine (M)	Diformazan formed (μmoles/100 mg/30 min)
0·01	17·8
0·02	21·0
0·03	18·6
0·04	18·6

Reaction mixture is same as in Table 1 with the exception that varied doses of *d*-amphetamine were used as indicated and the concentrations of NTC and NADP were 1·0 mg and 400 μg/2 ml of the reaction mixture respectively.

The inhibition of amphetamine dehydrogenase by KCN has previously been reported.[1] The crude mitochondrial enzyme with added supernatant or when fortified with NADP were inhibited by KCN (Table 6). It was further observed that when the crude mitochondrial fraction was incubated with KCN and subsequently dialysed for a short period, reactivation of enzyme activity followed. The nature of KCN inhibition was further studied by the method of Ackermann and Potter[5] and was found to be irreversible (Fig. 4). The results presented in Table 7 indicate that inhibition of amphetamine dehydrogenase by KCN can be reversed to a greater extent by increasing the amount of added NADP. However, even at a very high dosage of NADP, a residual inhibition of the enzyme always persisted.

The data presented in Table 8 indicate that metal chelating agents like EDTA, *o*-phenanthroline, α-α′-dipyridyl, cupferron and dithizone are potent inhibitors of amphatamine dehydrogenase suggesting that some metal component is essential for enzyme activity.

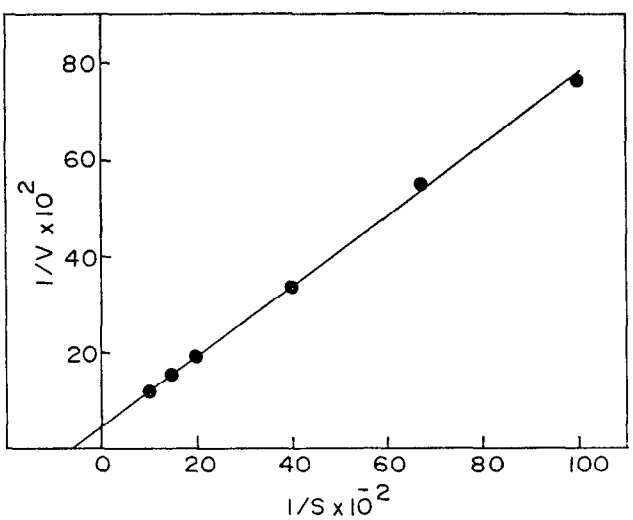

FIG. 3a. Determination of K_m for *d*-amphetamine of amphetamine dehydrogenase of crude mitochondrial fraction of guinea-pig brain.
The assay mixture contained 400 μg NADP and 1·0 mg NTC. Other details are given in the text.

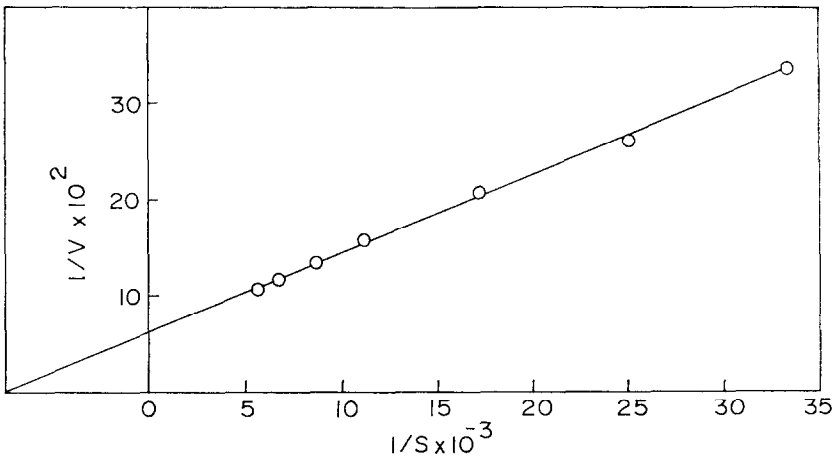

FIG. 3b. Determination of K_m for NADP of amphetamine dehydrogenase of crude mitochondrial fraction of guinea-pig brain.
The assay mixture contained 0·01 M *d*-amphetamine and 1·0 mg NTC. Other details are given in the text.

TABLE 6. INHIBITION OF AMPHETAMINE DEHYDROGENASE OF CRUDE MITOCHONDRIAL FRACTION OF GUINEA-PIG BRAIN BY KCN

Systems	KCN concentrations (M)	Per cent inhibition in systems supplemented with Supernatant fraction equivalent to 100 mg tissue	NADP (100 μg)
1. Crude mitochondrial fraction preincubated with KCN for 5 min	0·02	60	60
	0·002	75	40
2. Crude mitochondrial fraction treated with KCN and dialysed before addition of supernatant	0·002	Nil	—

Control systems similarly treated were also run, dialysis of normal and KCN treated crude mitochondrial systems were done in the cold for 30 min against 0·001 M phosphate buffer pH 7·0 with several changes of buffer. Other experimental details are given in the text.

DISCUSSION

The above findings indicate that amphetamine dehydrogenase of guinea-pig brain is mainly localized in the crude mitochondrial fraction. Since the crude mitochondrial fraction used in this study is a mixture of myelin fragments, synaptosomes and free mitochondria, more detailed study is necessary to decide whether the enzyme is truly mitochondrial or not. Washed brain mitochondria showed a residual enzyme activity which was greatly enhanced upon addition of soluble supernatant fraction or NADP. The co-factor present in the soluble fraction is dialysable and heat-stable. Among the co-factors tested only NADP could effectively replace the supernatant fraction. The

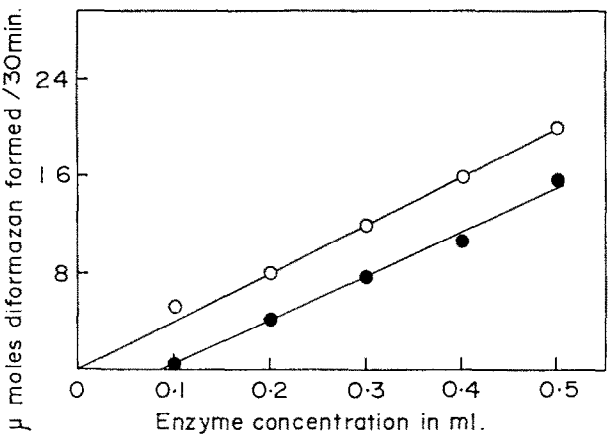

FIG. 4. Inhibition of amphetamine dehydrogenase activity of crude mitochondrial fraction of guinea-pig brain by KCN.

KCN was added to the reaction mixture simultaneously with NTC and *d*-amphetamine. Each system contained 100 mg equivalent of supernatant fraction and mitochondria were added as indicated. 0·1 ml of crude mitochondrial suspension is equivalent to 35 mg of tissue. Other details are given in the text.

○——○ without KCN
●——● with 0·002 M KCN.

TABLE 7. REVERSAL OF KCN INHIBITION OF AMPHETAMINE DEHYDROGENASE BY EXCESS ADDITION OF NADP

Dose of NADP (μg)	Per cent inhibition with 0·02 M KCN	0·002 M KCN
50	90	67
200	58	48
400	44	36
1000		25

All conditions are same as in Table 1 with the exception of NADP which was added as indicated. After an initial incubation period of 5 min, KCN was added along with NTC and *d*-amphetamine.

crude mitochondrial enzyme exhibited two pH maxima like that observed with brain homogenates,[1] but with the crude mitochondrial fraction the peak in the alkaline region was more marked. The observed differences in the pH–activity curves of homogenate and crude mitochondrial suspension perhaps reflect the influence of the environmental factors of the enzyme molecule.

It was previously shown[1] that the dehydrogenase differs from the deaminase described by Axelrod[6,7] or the hydroxylases[8,9] acting on amphetamine. The deaminase is localized in microsomes and requires NADPH and oxygen while the hydroxylase belongs to a group of enzymes resembling dopamine β-oxidase. Amphetamine dehydrogenase as shown earlier[1] is more active anaerobically with NTC as the electron acceptor and requires NADP when crude mitochondrial suspension is used instead of homogenate. Although the co-factor present in the supernatant fraction has not yet

TABLE 8. EFFECT OF METAL CHELATORS ON AMPHETAMINE DEHYDROGENASE ACTIVITY OF CRUDE MITOCHONDRIAL FRACTION OF GUINEA-PIG BRAIN

Metal chelators	Final concentrations (μM)	Per cent inhibition with 100 mg tissue equivalent of supernatant	Per cent inhibition with 100 μg NADP
1. EDTA	20,000	77	43
	10,000	60	
	5000	50	
	2500	33	
2. *O*-phenanthroline	5000		62
	1000	85	30
	500	45	
3. α-α′-Dipyridyl	5000		57
	1000	77	45
	500	50	
4. 8-Hydroxyquinoline	5000		92
	1000	80	35
	500	50	
5. Cupferron	5000	85	
	500	60	57
6. Dithizone	50		58
	10	75	
	5	35	

The enzyme was preincubated with the metal chelators for 5 min followed by addition of NTC and *d*-amphetamine. Other details are same as in Table 1.

been identified, it appears that the factor may possibly be identical with NADP. Apart from NADP, the possible involvement of a metal ion essential for the activity of the dehydrogenase is indicated by the inhibitory effect produced by various metal chelating agents. The inhibition of enzyme activity by cyanide may be due to its interaction with some metal ions necessary for enzyme activity or formation of cyanopyridine complex.[10,11] Although it is not definitely known whether the metal is present in the crude mitochondrial fraction or in the supernatant fraction, restoration of enzyme activity by dialysis of the system containing mitochondria and cyanide reveals the fact that even if cyanide acts on the metal, it is either not present in the crude mitochondrial fraction or the metal–cyanide complex is dissociated during dialysis. However the presence of the metal in the crude mitochondrial fraction is strongly suggested from the inhibitory effects produced by the metal chelators in the presence of NADP.

From the data presented in Fig. 4 it was observed that inhibition produced by cyanide is not of a reversible type. However, the crude mitochondrial fraction treated with KCN showed no inhibition of enzyme activity when dialysed for a brief period. On the other hand partial restoration of enzyme activity was achieved when NADP was added in excess to the reaction mixture containing KCN. This suggests that cyano-pyridine complex formation at least partly accounts for cyanide inhibition. However, even in the presence of high doses of NADP some amount of inhibition persisted which indicates that KCN may also act at a site other than NADP.

The exact role of the metal is at present not known and the dual possibility that the metal may serve as a component in electron transfer between NADP and tetrazolium

acceptor or between the enzyme and NADP does exist. Solubilization and subsequent purification of the enzyme may serve in the identification and understanding of the role of the metal ion involved.

The effect of high concentrations of NTC revealed that beyond a certain optimal concentration NTC inhibits the enzyme activity slightly. It was previously observed[1] that anaerobiosis increased NTC reduction. It is however not known whether the terminal acceptor of electrons is oxygen or not but the results suggest a possible competition between oxygen and NTC of which any direct evidence is lacking.

Seiler[12] described slow oxidation of mescaline in mouse brain homogenate and mitochondria. Recently the dehydrogenation of mescaline, amphetamine and ephedrine has been reported.[1] A comparison between the data indicates that mescaline oxidase and amphetamine dehydrogenase are not identical. Further confirmatory evidence is necessary to decide whether the dehydrogenation of these psychoactive agents[1] is carried out by a single or multiple enzyme systems.

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