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MODIFICATION OF BRAIN MITOCHONDRIAL MONOAMINE OXIDASE ACTIVITIES BY HYDROXYETHYLHYDRAZIDE OF CYANOACETIC ACID AND SOME OTHER MONOAMINE OXIDASE INHIBITORS

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Abstract—Treatment of bovine brain mitochondrial membrane fragments with hydroxyethylhydrazide of cyanoacetic acid (HECA)—a hydrazine monoamine oxidase inhibitor in molecule of which an electronaccepting group is present—not only inhibited the monoamine oxidase activity but also induced appearance (or significant increase) of properties to catalyze deamination of various nitrogenous bases (cadaverine, histamine and others) which do not belong to the category of monoamine oxidase substrates. This phenomenon was prevented by pretreatment of the mitochondria with low concentrations of clorgyline—a selective inhibitor of monoamine oxidases of type A—but not by deprenil which is a selective inhibitor for the monoamine oxidases of type B. The data obtained suggest that HECA which inhibits the monoamine oxidase activity slowly (due to a time-dependent process) binds the active sites of monoamine oxidases of the type A and then induces qualitative modification (transformation) of their catalytic properties.

Highly purified preparations of monoamine oxidase [amine: oxygen oxidoreductase (deaminating) (flavin-containing) EC 1.4.3.4 (MAO)] from various biological sources [1, 2], including bovine brain [3], undergo qualitative modification (transformation) of their catalytic properties under conditions which favour partial oxidation of SH-groups in the enzymes. As a result of the transformation the MAO's acquire properties of deaminating (via oxidative or hydrolytic pathways) not only the monoamines but also other nitrogenous bases (for example, diamines, ω-amino acids, nucleotides) which do not belong to the category of MAO substrates. Blocking of the catalytic sites of MAO's with irreversible MAO inhibitors (iproniazid, tranylcypromine, pargyline) [4, 5] prevented the qualitative alteration (transformation) in the catalytic properties of MAO under conditions favouring partial oxidation of their SH-groups.

Treatment with reagents which modify the SH-groups induces qualitative alterations in catalytic properties of many enzymes [6, 7]. For example, treatment with *o*-iodosobenzoate caused partial oxidation of SH-groups in triosephosphate dehydrogenase (EC 1.2.1.12) with formation of sulphenic acid (–SOH) residues and induced qualitative modification (transformation) in the properties of this oxidoreductase which acquired an ability to hydrolyze acylphosphates [8, 9]. A similar effect was observed [10] after treatment of the enzyme with trinitroglycerol which may be considered as a structural analog (containing strong electronaccepting group) of naturally occurring substrates of triosephosphate dehydrogenases.

In the present paper we describe the results of experiments designed to induce qualitative modification (transformation) in catalytic properties of mitochondrial MAO's by means of a chemical compound which would attack the active sites of the enzymes and contain electronaccepting groups at the same time. As a compound which meets these requirements we have used N^2-2-hydroxyethylhydrazide of cyanoacetic acid (HECA) [11, 12]—a hydrazine MAO inhibitor possessing an electronaccepting group in the molecule: $N\equiv C\text{–}CH_2\text{–}CO\text{–}NH\text{–}NH\text{–}CH_2CH_2OH$. The hydrazine MAO inhibitors are considered as structural analogs [13] of the substrates of MAO. These inhibitors attack the active sites of MAO [14], without modification of SH-groups, and block the enzymatic activity. The inhibition by hydrazine derivatives of MAO activity is not an instantaneous but a time-depending (requiring preincubation under aerobic conditions) process [15, 16]. Thus, there is a certain time interval between the first contact of the inhibitor with the active site of the enzyme and the development of complete irreversible inhibition of the enzymatic activity. Within this time interval the electronaccepting groups of the inhibitor molecule might favour oxidation of the enzyme functional groups (for example of the SH-groups). It was shown previously [1, 2] that the hydrazine monoamine oxidase inhibitors comparatively weakly decreased activity of the modified (transformed) monoamine oxidases.

Identification of the type of MAO's, which possesses the property of undergoing transformation (qualitative alteration in enzymatic activity) in fragments of mitochondrial membranes, was also a purpose of the present work. There are, at least, two types of MAO's: A and B [17]. By definition those amine oxidases which are highly sensitive towards the inhibitory effect of clorgyline (*N*-(2,4-dichlorophen-

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oxo)-propyl-*N*-methyl-2-propynylamine hydrochloride) [18] belong to the type A of MAO's. The term MAO type B is used for designation of the amine oxidases which are highly sensitive towards the inhibitory effect of deprenil (*N*-1-phenylisopropyl-*N*-methyl-2-propynylamine hydrochloride) [19]. If the property of undergoing the transformation of catalytic activity belongs to MAO of one of these two types, one could expect prevention of the transformation of MAO by low concentrations of a suitable selective MAO inhibitor because, as it was shown previously [1, 2], one of the conditions for the transformation of MAO's is the presence of unblocked catalytic centers in these enzymes.

MATERIALS AND METHODS

Synthesis of HECA was carried out at the Chair of Pharmaceutic Chemistry (Medical School, Riga) as described previously [11, 12]. Samples of clorgyline and deprenil were kindly presented by Dr. H. J. Barber (May and Baker, Dagenham, Essex, England) and Dr. K. Magyar (Institute of Pharmacology, University Medical School, Budapest, Hungary), respectively. Tyramine.HCl, serotonin creatininesulfate, tryptamine.HCl, *L*-lysine.HCl, AMP.Na_2 (Reanal, Hungary), histamine.2HCl (G. Lawson, England), spermine.4HCl, spermidine.3HCl, cadaverine.2HCl (Serva, FRG), GABA (Calbiochem, USA), dopamine.HCl (Merck, FRG) were used without further purification. Urea and β-phenylethylamine.HCl were chemically pure compounds obtained from local producers.

Mitochondrial fractions from homogenates of fresh bovine brain were prepared by a differential centrifugation procedure developed especially for isolation of brain mitochondria [20]. Content of protein in the mitochondrial suspensions was estimated by a colorimetric procedure based on biuret reaction [21] or by Lowry's method. Deamination of nitrogenous bases was studied by measuring liberation of ammonia after incubation at 37° of one of the compounds with the mitochondria suspended in 0.1 M phosphate buffer (pH 7.4). The incubation was carried out during the time intervals (from 15 to 60 min) within which the deamination reaction followed the zero order kinetics; then the trichloroacetic acid (final concentration 7.5%) was added in order to stop the reaction. The precipitate formed was separated by centrifugation and discarded. Content of ammonia in the supernatant was estimated by isothermic diffusion in Conway units with subsequent nesslerisation.

RESULTS AND DISCUSSION

Studies on the inhibition of the MAO activity by HECA showed that after preincubation for 30 min with the bovine brain mitochondrial membranes the inhibitor caused more distinct decrease in deamination of serotonin as compared with the deamination of tyramine or β-phenylethylamine when HECA concentration was lower than 1 mM (Fig. 1). This conclusion was confirmed in studies on kinetics of inhibition of the MAO activity in the course of preincubation with 10 mM HECA (Fig. 2): under these experimental conditions the inhibition of serotonin deamination developed more rapidly than the inhibition of deamination of tyramine and, especially, of β-phenylethylamine.

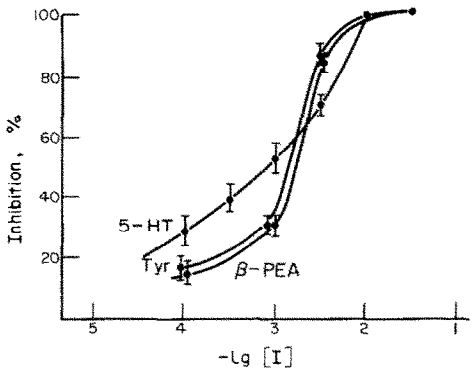

Fig. 1. Inhibition of the rates of deamination of serotonin (5-hydroxytryptamine; 5-HT), tyramine (Tyr) and β-phenylethylamine (β-PEA) in bovine brain mitochondrial fraction as a function of HECA concentration. Samples (final total vol. 1.8 ml) contained 4 mg of protein of the bovine brain mitochondrial membrane fractions, HECA (final concentrations appear at the abscissa) and 0.1 M phosphate buffer (pH 7.4). The samples were preincubated at 37° for 30 min after which one of the substrates was added to reach the following optimal ("saturating") final concentrations (mM): serotonin 5, tyramine 3.2, phenylethylamine 0.8. The samples with serotonin and phenylethylamine were then incubated at 37° for 30 min, the samples with tyramine as a substrate—45 min. After the incubation, trichloroacetic acid (final concentration 7.5%) was added in all the samples. Liberation of ammonia in the control (without HECA) samples was (in nmoles/mg protein/min): 4.8, 8.2 and 3.5 with serotonin, tyramine and β-phenylethylamine as substrates, respectively. Mean values (± S.D.) of 8 assays (2 experiments; 4 parallel samples in each of them).

On the basis of current concepts [17] on serotonin as a specific substrate for the MAO of type A, β-phenylethylamine—specific substrate for the MAO of type B, and tyramine—a substrate which is deaminated by MAO's of both types, one might assume that HECA interacts primarily with the active sites of MAO type A. After preincubation for 30 min with 1 mM HECA of bovine brain mitochondria the inhibition of serotonin deaminating activity was not

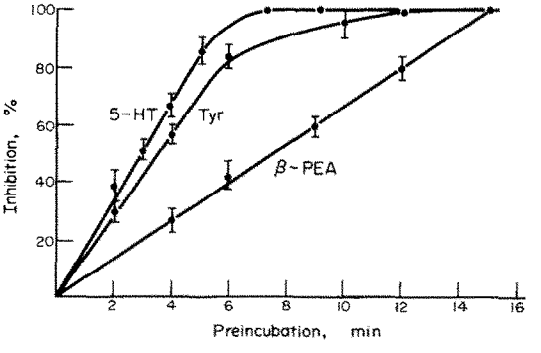

Fig. 2. Inhibition of the rate of deamination of biogenic amines in presence of 10 mM HECA as a function of duration of its preincubation with bovine brain mitochondria. For composition of the samples, experimental conditions and explanation of the designations in the Figure please see the Legend to Fig. 1. Mean values of 8–12 assays in each point.

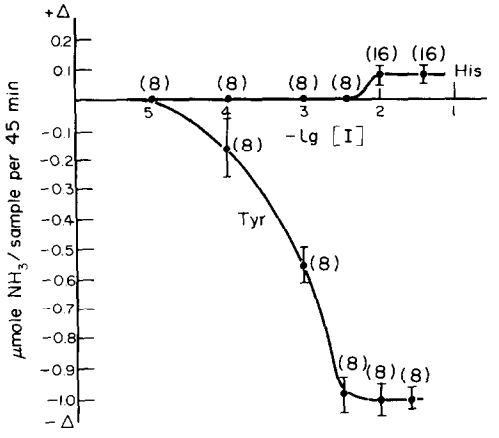

Fig. 3. Inhibition of tyramine (Tyr) deamination and appearance of the property of deaminating histamine (His) after treatment of bovine brain mitochondrial membrane fragments with 10 mM HECA during 30 min. Composition of samples and experimental conditions are described in the Legend to Fig. 1. Final concentration of histamine in the samples was 10 mM. Data are presented on the increase (+ Δ) or decrease (− Δ) in content of ammonia in the experimental minus control samples (without HECA). In parentheses-number of assays used for calculation of the mean values (± S.D.).

removed by exhaustive dialysis against 0.004 M phosphate buffer (pH 7.4). These data suggest that the time-dependent inhibition by HECA of the MAO activity was irreversible. The same conclusion was drawn from the results of similar experiments with other hydrazine MAO inhibitors [15, 16].

Treatment of the fragments of bovine brain mitochondrial membranes with high concentrations (10 mM) of HECA for 30 min caused not only complete inhibition of the tyramine deaminating activity but also induced appearance of a histamine deaminating activity which was totally absent in the mitochondria before the treatment with HECA (Fig. 3). The histamine deaminating activity reached the highest rates (approximately 20 per cent as compared with the rate of tyramine deamination by bovine brain mitochondria in control samples) if the treatment of mitochondria with 10 mM HECA was carried out not for 30 min but for 15 min (Fig. 4). Under these optimal conditions treatment of mitochondria with 10 mM HECA completely inhibited the properties of deaminating monoamines, which are usually considered as substrates for MAO. However, the deamination of dopamine after the treatment of mitochondria with 10 mM HECA was inhibited only by 50 per cent (Table 1). The residual dopamine deaminating activity was not inhibited by deprenil (0.01 mM) or clorgyline (0.01 mM) under conditions of 20 min preincubation at 37°. These experiments were carried out in 0.2 M phosphate buffer (pH 7.4) in presence of 10 mM HECA, 10 mM dopamine and 0.01 mM clorgyline or deprenil. The blanks were treated under identical conditions but did not contain dopamine. Under these experimental conditions HECA was quite stable. Lack of inhibition of the residual dopamine deaminating activity by either clorgyline or deprenil might be ascribed to the presence of a particular form of MAO, specific for dopamine [22], but a possibility that the deamination of dopamine by mitochondria pretreated with HECA might not involve an enzymic process was not followed up in these experiments. The mitochondria treated with HECA exhibited qualitatively new (or considerably increased) properties of deaminating not only histamine but also diamines (putrescine and cadaverine), polyamines (spermine and spermidine) and other nitrogenous bases—lysine, GABA or even AMP and urea which are deaminated via hydrolytic but not oxidative reactions (Table 1).

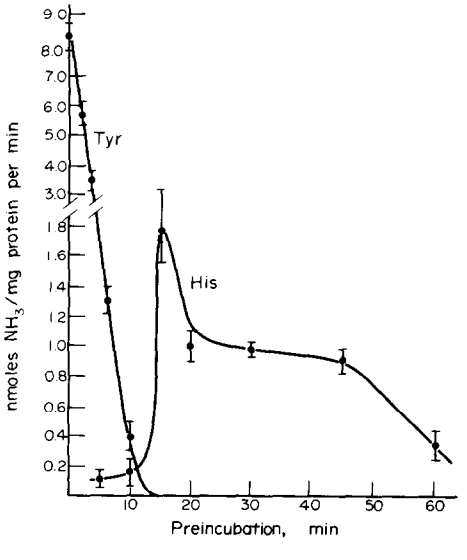

Fig. 4. Rates of deamination of tyramine and histamine by bovine brain mitochondria as a function of duration of their treatment with 10 mM HECA. Mean values of 8 assays.

We have studied in detail the conditions for inducing by treatment of bovine brain mitochondrial fragments with 10 mM HECA of a property of deaminating cadaverine as well as some characteristic features of the induced activity.

We failed in attempts to induce the cadaverine deaminating activity at pH 7.0. However, displacement of the pH value in alkaline zone from the optimum (pH 7.4) did not alter the rate of the induced cadaverine deaminating activity (Table 2). After heating for 25 min at 90° or 15 min at 100° of the suspension of mitochondria treated with 10 mM HECA for 15 min at 37°, pH 7.4, incubation (45 min, 37°, pH 7.4) of the mitochondria in presence of 10 mM cadaverine did not cause liberation of ammonia, thus suggesting that the deamination of cadaverine by the mitochondria, which were pretreated with HECA, was due to an enzymatic process. Heating in 0.2 M phosphate buffer (pH 7.4) for 25 min at 100° did not cause any decomposition of 10 mM HECA as shown by thin-layer chromatography (ethanol:water:ethyl acetate, 6:5:1) and by lack of ammonia liberation.

In samples (total vol. 1.8 ml) containing 15 mg of protein of the mitochondria pretreated with HECA in 0.1 M phosphate buffer (pH 7.4) we found after incubation for 45 min at 37° liberation of 480 ± 50 nmoles of ammonia from 1800 nmoles of added cadaverine (i.e. approximately 27% of the

Table 1. Deamination* of nitrogenous compounds by bovine brain mitochondria, treated with 10 mM HECA for 15 min

Compounds	Optimal concentrations†	Control	+HECA	Number of assays
Serotonin	5.0	4.8 ± 0.2	0	16
Tyramine	3.2	8.2 ± 0.4	0	16
Tryptamine	1.0	2.5 ± 0.3	0.4 ± 0.1	12
Dopamine	3.2	3.3 ± 0.3	1.5 ± 0.1	24
Phenylethylamine	0.8	3.5 ± 0.4	0	16
Histamine	10	0.8 ± 0.2	2.3 ± 0.3	16
Putrescine	10	0.5 ± 0.3	1.3 ± 0.1	12
Cadaverine	10	0.7 ± 0.2	2.0 ± 0.1	44
Spermine	10	0.2 ± 0.1	1.9 ± 0.5	12
Spermidine	10	0.8 ± 0.4	1.2 ± 0.1	12
GABA	20	0.5 ± 0.1	1.9 ± 0.1	12
Lysine	10	0	0.8 ± 0.2	12
AMP	10	1.0 ± 0.3	2.6 ± 0.5	12
Urea	1000	0.4 ± 0.1	1.5 ± 0.2	12

* Expressed in nmoles of ammonia liberated per min per mg of protein.
† Expressed in mM.

Table 2. Relationship between the pH values of the buffer solution*, in which preincubation (15 min) with 10 mM HECA of bovine brain mitochondria was carried out, and the rate of cadaverine (10 mM) deamination†

pH	Rate of deamination
7.0	0
7.2	1.8 ± 0.2
7.4	2.3 ± 0.2
8.0	2.3 ± 0.1
9.2	2.4 ± 0.2

* The following buffer solutions were used: 0.1 M phosphate (pH 7.0–8.0), 0.04 M borate (pH 8.0 and 9.2).
† Expressed in nmoles of ammonia liberated per min per mg of protein; mean values (± S.D.) of 12 assays at each pH studied.

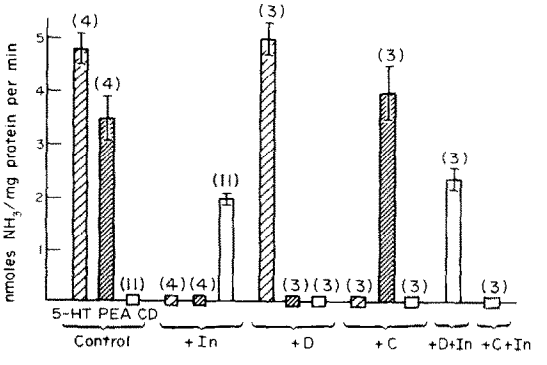

Fig. 5. Effect of deprenil (D; 0.01 mM) and clorgyline (C; 0.01 mM) on deamination of 5-hydroxytryptamine (5-HT), β-phenylethylamine (PEA) and cadaverine (CD) by bovine brain mitochondria, treated (15 min) with 10 mM HECA (In). Preincubation with the MAO inhibitors was carried out for 20 min at 37°. For concentrations of the substrates in samples please see Table 1. Mean values (± S.D.). Number of assays in parentheses.

added substrate were metabolized). We consider these data as an indication as to the catalytic nature of the process of cadaverine deamination.

Treatment with 10 mM HECA under the optimized conditions (pH 7.4, 15 min, 37°) of bovine brain mitochondria did not induce the cadaverine deaminating activity if the catalytic sites of MAO type A in these mitochondria were previously completely inactivated by pretreatment with low concentrations of clorgyline which inhibited the deamination of serotonin but did not influence the deamination of β-phenylethylamine (Fig. 5). A selective inhibitor of MAO type B deprenil under similar experimental conditions inhibited the deamination of β-phenylethylamine but did not influence the deamination of serotonin and did not prevent the inducing of the property of deaminating cadaverine (Fig. 5). These data suggest that the MAO

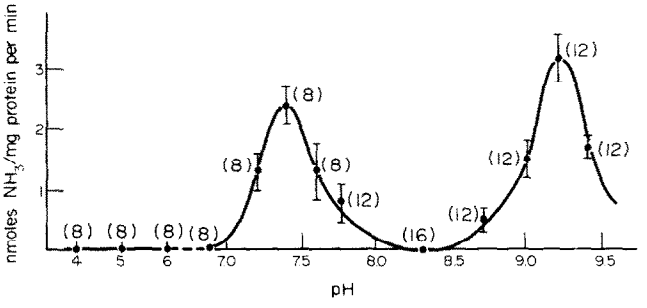

Fig. 6. Rate of cadaverine (10 mM) deamination by bovine brain mitochondrial membranes, treated (15 min, 37°, pH 7.4) with 10 mM HECA, as a function of pH. The following buffer solutions were used: 0.07 M citrate (pH 3.0–6.0), 0.1 M phosphate (pH 6.9–8.0), 0.04 M borate (pH 8.0–9.4). Number of parallel assays is shown in parentheses.

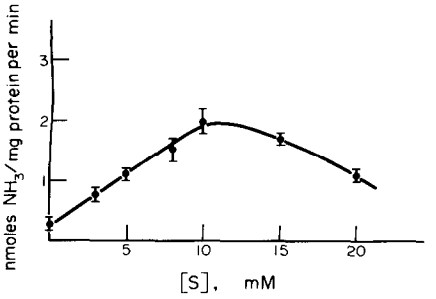

Fig. 7. Rate of cadaverine deamination by bovine brain mitochondrial membrane fragments, treated (15 min, 37°, pH 7.4) with 10 mM HECA, as a function of the substrate concentration. Mean values of 12 assays at each concentration of cadaverine.

inhibitor HECA, under the experimental conditions required for complete inhibition of MAO's of both A and B types (Fig. 1), induced the transformation (qualitative alteration in catalytic properties) only of the MAO type A.

Studies of the deamination of cadaverine as a function of pH in samples containing bovine brain mitochondria treated with 10 mM HECA (15 min, 37°, pH 7.4) showed that the rate of the induced reaction reached maximal values at pH 7.4 and 9.2 while no deamination could be detected at pH 8.2–8.4 (Fig. 6). At pH 7.4 the highest rate of the reaction of cadaverine deamination was observed in presence of 10 mM cadaverine; excess of the substrate caused a certain decrease in the rate of deamination (Fig. 7). The values, represented by the four experimental points on the ascendant branch of the curve describing the v from [S] relationship (Fig. 7), were used for replotting the data by means of the Lineweaver–Burk "double-reciprocal values" method in order to estimate the K_m; it was $2 \cdot 10^{-2}$ M. Optimal duration of incubation in presence of cadaverine of the bovine brain mitochondria treated with HECA was 45 min (Fig. 8). There was a direct relationship between the content in the samples of protein of the mitochondrial

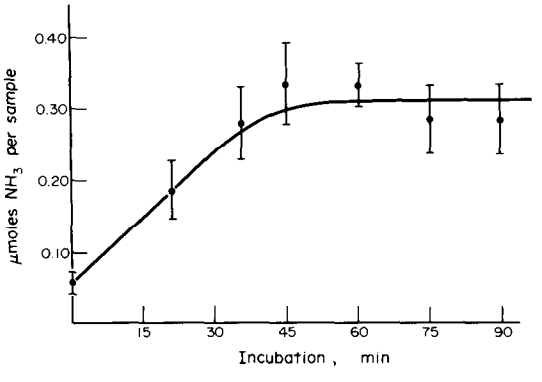

Fig. 8. The time course of cadaverine (10 mM) deamination by bovine brain mitochondrial membranes, treated (15 min, 37°, pH 7.4) with 10 mM HECA. Mean values of 12 assays at each point.

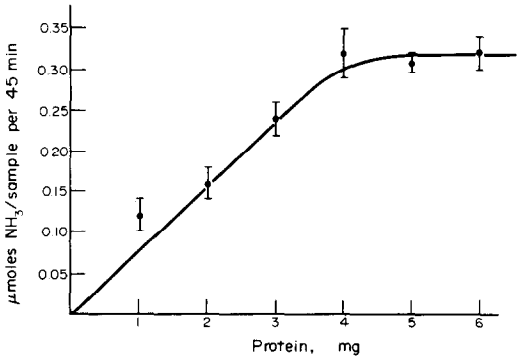

Fig. 9. Relationship between the amount of ammonia liberated after incubation (45 min, pH 7.4, 37°) of 10 mM cadaverine in presence of the bovine brain mitochondria, treated with HECA (for conditions please see the Legend to Fig. 8), and content of protein in the samples. Mean values of 8–12 assays.

membranes treated with HECA and the amount of ammonia liberated after incubation of these samples under optimal conditions in presence of cadaverine (Fig. 9).

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