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TRIALS OF CHOLINESTERASE REACTIVATORS AS NEOSTIGMINE ANTAGONISTS

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Neostigmine is an anticholinesterase drug of the carbamate group. The highest decurarizing dose of the drug, 0.05 mg/kg [12], is more than three times greater than the dose sufficient to give rise to marked symptoms of poisoning in a normal individual [9], and for that reason, during its clinical use, signs of overdosage appear relatively often, sometimes terminating in death [7]. The use of atropine to prevent complications does not abolish disturbances associated with excitation of nicotinic acetylcholine receptors [8].

Since carbamylation leads to the formation of a less stable complex with the enzyme than phosphorylation, it can be postulated that cholinesterase reactivators used in cases of poisoning by organophosphorus insecticides will be more effective against carbamate poisoning. It has been found in practice that only single preparations are effective, and they vary, moreover, in their effectiveness against poisoning by different carbamates [13].

Reactivators (HS-3, HI-6, HGG-12, etc.) distinguished by ability to act on acetylcholine receptors have recently been described [6]. Accordingly in the investigation described below an attempt was made to chose a reactivator which, in conjunction with cholinolytics, could give a high protective effect against neostigmine poisoning. The pyridinaldoxime derivatives were provided by Yu. V. Lupandin.

EXPERIMENTAL METHOD

Lethal (LD_{50}), toxic (TD_{50}), and effective (ED_{50}) doses of the preparations were determined by the tabular method [3] on 250 male albino mice. To prevent poisoning, cholinolytics were injected subcutaneously 15 min before, and cholinesterase reactivators intraperitoneally 1 min before subcutaneous injection of neostigmine.

The reactivating action of HI-6 and TMB-4 in concentrations of 10^{-5}-10^{-4} M was estimated in experiments *in vitro* after incubation for 30 min with purified acetylcholinesterase, inhibited by neostigmine ($5 \cdot 10^{-8}$ M) in a medium of 0.08 M KCl at 37.4°C and pH 7.4. The velocity of enzymic hydrolysis of acetylcholine iodide was determined by the method of continuous potentiometric titration [1]. The affinity constant (K_a), velocity constant of carbamylation (K_c), and the pseudomonomolecular inhibition constant (K_i) were determined graphically [11].

EXPERIMENTAL RESULTS

To choose a reactivator the index of effectiveness (the ratio of LD_{50} to ED_{50}), the dose necessary to prevent death of half of the animals poisoned with LD_{99} of neostigmine, was determined. The experimental results (Table 1) showed that reactivator HI-6 had an index of effectiveness 1.5 times higher than that of TMB-4 and twice as high as the other preparations.

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TABLE 1. LD_{50} and ED_{50} of Cholinesterase Reactivators Injected Intraperitoneally into Mice 1 min before Subcutaneous Injection of LD_{99} of Neostigmine (M ± m)

Cholinesterase reactivator	LD_{50}, mg/kg	LD_{50}, mg/kg	Index of effectiveness
HI-6	515±65	28±8	18,4
TMB-4	178±20	14±5	12,7
HS-3	224±30	22±6	10,2
HGG-12	178±20	18±5	10,0
HS-6	355±45	43±11	8,2
Toxogonin	178±20	22±15	8,2
Isonitrosin	1540±140	Not effective	—

TABLE 2. Constants of Interaction of Neostigmine with Acetylcholinesterase in the Presence of HI-6

Concentration of HI-6, M	$K_a \cdot M$	K_c, min^{-1}	$M^{-1} \cdot K_i$, min^{-1}
0	$7{,}6 \cdot 10^{-7}$	1,67	$2{,}08 \cdot 10^{6}$
10^{-5}	$9{,}70 \cdot 10^{-7}$	1,49	$1{,}61 \cdot 10^{6}$
$5 \cdot 10^{-5}$	$1{,}31 \cdot 10^{-6}$	1,49	$1{,}16 \cdot 10^{6}$

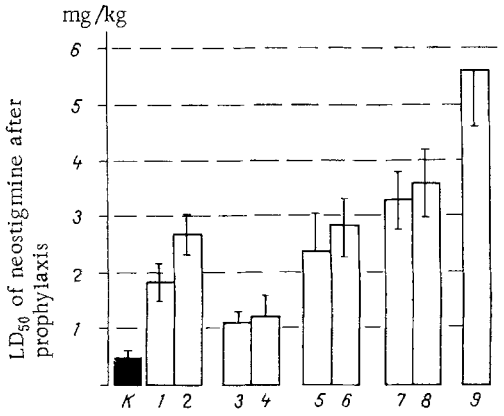

Fig. 1. Changes in LD_{50} of neostigmine for albino mice under the influence of cholinolytics, cholinesterase reactivators, and a mixture of them. Height of columns indicates LD_{50} of neostigmine (in mg/kg). Black column (control) gives LD_{50} of neostigmine for intact mice. Numbers below columns are serial numbers of experiments, corresponding to the following probabilities of errors of significance of difference from the effect of atropine (1). 2) P = 0.1 (arpenal), 3) P = 0.05 (TMB-4), 4) P = 0.1 (HI-6), 5) P = 0.5 (atropine + TMB-4), 6) P = 0.1 (arpenal + HI-6), 7) P = 0.02 (atropine + arpenal), 8) P = 0.05 (atropine + HI-6), 9) P = 0.005 (atropine + arpenal + HI-6).

Cholinolytics were tested by one of us (V.B.P.) previously [2]. Of 17 substances belonging to different subgroups, studied in experiments on mice, only arpenal gave a high protective effect against neostigmine poisoning.

In the present series of experiments all drugs given prophylactially were used in doses equal to 0.1 TD_{50} (the 50% toxic dose). LD_{50} of neostigmine, and also the values of p — the probability of error of significance of difference in effectiveness compared with atropine (5 mg/kg), are given in Fig. 1. The effectiveness of arpenal (10 mg/kg), of a combination of arpenal with HI-6 (20 mg/kg), and also a combination of atropine with TMB-4 (8 mg/kg) was not significantly higher than the effectiveness of atropine alone. However, a combination

of atropine with arpenal or with HI-6 and, more especially simultaneous administration of all three drugs gave a significantly greater increase in LD_{50} of neostigmine than atropine used alone.

In experiments *in vitro* TMB-4 and HI-6, even in such high concentrations as 10^{-4} M, did not reactivate the enzyme when carbamylated by neostigmine.

Comparison of K_a and K_c for interaction between neostigmine and acetylcholinesterase in the absence and presence of high concentrations of HI-6 (Table 2) indicates that with an increase in concentration of the reactivator only the affinity of neostigmine for the enzyme was altered in the competitive stage of formation of a reversible complex. The velocity of carbamylation, i.e., the noncompetitive stage of the reaction, remained constant. These findings show that compound HI-6 has some affinity for acetylcholinesterase, for it affects the anionic region just as neostigmine, but only in concentrations 1000 times higher. It is evident that such negligible activity in the doses used can be disregarded for practical purposes.

Ability to prevent death of the animals without reactivation of cholinesterase was described for compound HI-6 previously in poisoning by some organophosphorus compounds. This question has been examined in detail by Clement [6], who considers that a cholinolytic action is involved in the phenomenon of protection. However, according to data in the literature [5], the muscarinic cholinolytic action of HI-6 is exhibited only in a concentration of $1.9 \cdot 10^{-4}$ M. A decrease in reactivity of the ganglia has been described in cats after injection of 3 mg/kg. Meanwhile compound HGG-12 exhibits the same action in a dose ten times smaller [10], and this is not accompanied by any increase in effectiveness.

The possibility of participation of a ganglion-blocking effect in the action of the drug was tested by studying its ability to protect mice against dimethylphenylpiperazine. It was shown that LD_{50} of this gangliomimetic was increased by only 1.3 times, compared with 2.9 times after arpenal. Consequently, if the ganglion-blocking action does play a role when HI-6 is used alone, in conjunction with arpenal this role is masked. The presence of muscle-relaxing properties likewise is unimportant, for addition of tubocurarine to a mixture of atropine and arpenal did not increase the coefficient of protection in neostigmine poisoning [4].

A combination of atropine and arpenal gives a high prophylactic effect, accompanied by blockade of both muscarinic and nicotinic cholinergic systems. Meanwhile addition of HI-6 to it significantly potentiated its protective action.

The appearance of a potentiation phenomenon after the addition of HI-6 can most likely be explained by ability of this compound to modulate the properties of receptors, so increasing the effectiveness of the cholinolytics.

Consequently, the use of HI-6, a pyridinaldoxime derivative, can substantially enhance the effectiveness of prevention of side effects arising after administration of large doses of neostigmine. The mechanism of action of compound HI-6 can conjecturally be explained by a noncompetitive decrease in reactivity of the acetylcholine receptors.

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