Brit. J. Pharmacol. (1955), **10**, 356.

EFFECT OF NICOTINHYDROXAMIC ACID METHIODIDE ON HUMAN PLASMA CHOLINESTERASE INHIBITED BY ORGANOPHOSPHATES CONTAINING A DIALKYLPHOSPHATO GROUP

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(RECEIVED MAY 10, 1955)

Organophosphates which have a dialkylphosphato group $\left(\begin{array}{l} O \\ \| \\ -P \\ \end{array}\begin{array}{l} OR \\ \diagdown \\ \diagup \\ OR \end{array}\right)$ are potent inhibitors of cholinesterase. Studies of the inhibited enzyme indicate that the reaction between cholinesterase and organophosphates of this type yields a phosphorylated enzyme which then reacts with water to produce the corresponding acid and the regenerated enzyme. Reactivation in aqueous solution is fastest if R is a methyl group, but proceeds only very slowly if R is an ethyl group; no measurable enzyme recovery takes place if R is an *iso*propyl group (Burgen and Hobbiger, 1951; Aldridge and Davison, 1953; and Hobbiger, 1954). If the " phosphorylation theory " is correct, then potent nucleophilic reagents should dephosphorylate the enzyme at a greater rate than water. This is indeed so, as was shown by Wilson and Meislich (1953). They found that, after inhibition by an organophosphate with a diethyl- or di*iso*propylphosphato group, the activity of true cholinesterase of electric eel tissue could be completely restored by nicotinhydroxamic acid methiodide (NHA). The rate of enzyme reactivation by 0.1 M-NHA greatly exceeded that produced by water, and at 24° C. 91% of the enzyme activity was restored in 15 min. Wilson and Meislich's results cannot be generally applied because cholinesterases from different sources are not identical in their behaviour towards inhibitors (Austin and Berry, 1953; Davison, 1953). For this reason, and bearing in mind any possible importance in man, the effect of NHA on human plasma cholinesterase, which had been inhibited by organophosphates with a diethyl- or di*iso*propylphosphato group, was studied. The results are presented here.

METHODS

All experiments were carried out in the Warburg apparatus at 37° C. with an atmosphere of 95% N_2 + 5% CO_2. In some experiments 95% O_2 + 5% CO_2 was used and identical results were obtained. A solution containing 0.025 M-$NaHCO_3$, 0.075 M-NaCl, 0.075 M-KCl, 0.04 M-$MgCl_2$ and 0.1% crystalline bovine plasma albumin (Armour Laboratories) was used for all dilutions, and as the medium for inhibition, reactivation, and determination of enzyme activity.

The enzyme preparations were either heparinized human plasma or its highly purified cholinesterase fraction (Plasma fraction IV-6-3 which was a gift from the late Dr. Cohn and had been prepared by the method of Surgenor, Strong, Taylor, Gordon, and Gibson, 1949). Cholinesterase activity was determined by the manometric technique using 0.03 M-butyrylcholine iodide as the substrate. The CO_2 output from 5 to 45 min. after onset of substrate hydrolysis was chosen as a measure of enzyme activity and all reactivations are expressed as a percentage of the activity of controls which had been treated in the same way but without the addition of inhibitor. Dilutions and addition of the reactivating agent were carried out at room temperature, and the moment when the manometric vessels were placed in the bath was chosen as the most accurate, but necessarily arbitrary, zero point of reactivation. Thus the figures obtained for 10 min. contact with the reactivator also include the reactivation which took place at room temperature, and true reaction rates are only represented by the differences between various periods of reactivation.

Solutions of NHA were freshly made immediately before use and adjusted with NaOH to *p*H 7.4 at which all the experiments were carried out.

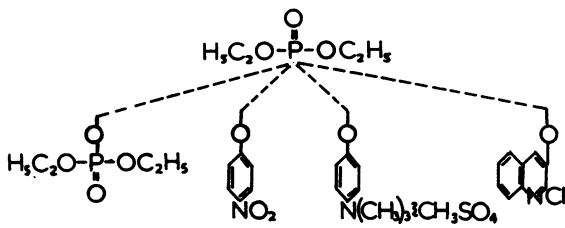
The organophosphates used had either a diethyl- or di*iso*propylphosphato group in common; their structural formulae are shown in Table I. Throughout the text the inhibitors are referred to by the abbreviations given in parentheses in Table I.

TABLE I

ORGANOPHOSPHATES USED AS INHIBITORS

(*a*) Organophosphates containing a diethylphosphato group:
1. Tetraethyl pyrophosphate (TEPP).
2. Diethyl *p*-nitrophenyl phosphate (E 600).
3. 3-(Diethoxyphosphinyloxy)-*N*-trimethylanilinium methylsulphate (Ro 3–0340).
4. 3-(Diethoxyphosphinyloxy)-*N*-methylquinolinium methylsulphate (Ro 3–0422).

(*b*) Organophosphates containing a di*iso*propylphosphato group:
5. Di*iso*propyl phosphorofluoridate (DFP).
6. Di*iso*propyl *p*-nitrophenyl phosphate (D 600).
7. 3-(Di*iso*propoxyphosphinyloxy)-*N*-trimethylanilinium methylsulphate (Ro 3–0411).
8. 3-(Di*iso*propoxyphosphinyloxy)-quinoline (Ro 3–0433).



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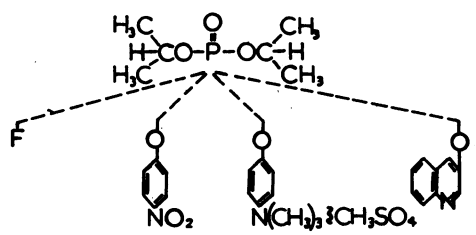

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RESULTS

Effect of NHA on Human Plasma Cholinesterase

NHA is a competitive inhibitor of human plasma cholinesterase with an I 50 of 3×10^{-2} M in the presence of 0.03 M-butyrylcholine iodide. The enzyme-inhibitor complex dissociates readily after dilution, and at 37° C. an equilibrium between enzyme, inhibitor and substrate is established within 5 min., regardless of the order in which the 3 components are added. As with other reversible anticholinesterases, NHA partially protects the enzyme against inhibition by organophosphates; but the degree of protection varies according to the type of inhibitor used.

Effect of NHA on Human Plasma Cholinesterase after Inhibition by an Organophosphate with a Diethylphosphato Group

The inhibition of human plasma cholinesterase by TEPP, Ro 3–0340, Ro 3–0422 or E 600 cannot be reversed by addition of substrate, nor after a dilution which is sufficient to reduce the inhibitor concentration to an ineffective level is there any significant enzyme recovery within 1 hr.

The addition of NHA in concentrations above 10^{-3} M to human plasma cholinesterase which has been completely inhibited by one of the four organophosphates markedly increases enzyme activity within 1 hr. The speed and degree of enzyme reactivation at 37° C. and *p*H 7.4 depend on the concentration of free inhibitor, the concentration of NHA, and the time of contact between enzyme and inhibitor.

NHA reacts with each of the four organophosphates studied, and this reaction yields products which are without anticholinesterase activity. The chemical inactivation is rapid with TEPP and Ro 3–0422 but slow with Ro 3–0340 and E 600 (Table II). Aqueous solutions of the organophosphates show the same differences with respect to their stability.

In the presence of free inhibitor the rate of reactivation of inhibited human plasma cholinesterase depends on the nature and the amount of inhibitor used. Fig. 1 shows that NHA reactivates the enzyme in the presence of large amounts of TEPP, whereas a similar excess of Ro 3–0340 prevents reactivation. Ro 3–0422 behaves like TEPP, and E 600 like Ro 3–0340.

To study the true rate of reactivation of human plasma cholinesterase by NHA it is essential to minimize, or if possible exclude, any interference by the free inhibitor with the reactivation process. Such conditions are obtained by incubating a concentrated solution of highly purified human plasma cholinesterase (plasma fraction IV-6-3) with amounts of the inhibitor just sufficient to produce nearly complete inhibition, and then diluting the enzyme-inhibitor mixture a hundredfold before addition of NHA. Using such a procedure it is found that the rate of enzyme reactivation by NHA increases exponentially if enzyme and inhibitor are in contact for not more than 10 min. before reactivation is started. If 20 or 30 min. are allowed for contact between enzyme and inhibitor before the addition of NHA the time-course of

TABLE II

INTERACTION OF NHA AND ORGANOPHOSPHATES AT 37° C.

10^{-3} M and 10^{-7} M solutions of the organophosphates in a phosphate buffer of *p*H 7.4 were incubated with 0.05 M-NHA for 1 hr. The change in anticholinesterase potency was then determined manometrically

Inhibitor	% Reduction in Activity	
	10^{-3} M	10^{-7} M
TEPP	Complete loss of activity	Complete loss of activity
Ro 3–0422		
E 600	0	24
Ro 3–0340	0	19

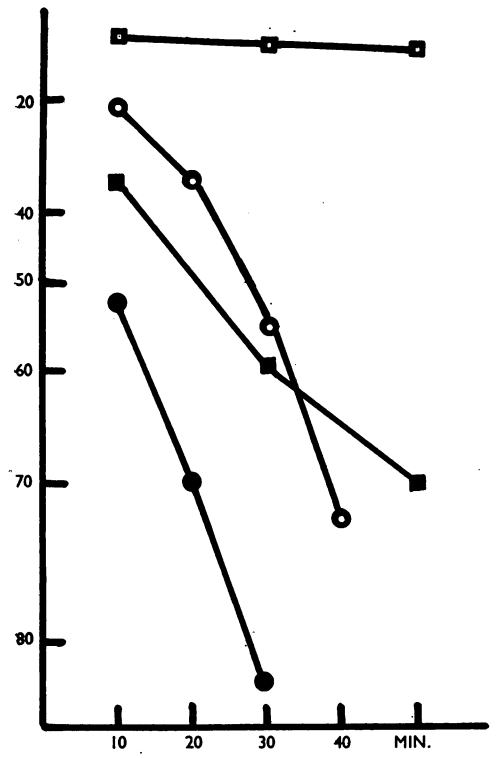

FIG. 1.—Enzyme reactivation by 0.02 M-NHA in presence of excess of free inhibitor. Heparinized human plasma was incubated with the inhibitor for 20 min. before addition of NHA (using 1 vol. NHA for 2 vol. of enzyme-inhibitor mixture). The inhibitors used were: 10^{-8} M-TEPP (●), 10^{-6} M-TEPP (○), 2.5×10^{-8} M-Ro 3-0340 (■) and 2.5×10^{-7} M-Ro 3-0340 (□). Enzyme activity was determined after a 10-fold dilution. Ordinate: enzyme activity in % of controls. Abscissa: duration of exposure of inhibited enzyme to NHA before addition of substrate.

reactivation approaches that of a first order reaction with one component in excess (Fig. 2). In either of these two cases 0.01 M-NHA restores more than 90% of the activity of a completely inhibited enzyme and an equal degree of reactivation is obtained by a given concentration of NHA regardless of the type of inhibitor used, the variations in different experiments amounting to less than 10%. The time required for maximum reactivation is inversely proportional to the concentration of NHA (Fig. 2).

These findings indicate that under the experimental conditions NHA acts on a phosphorylated enzyme. This interpretation is supported by the effect of trimethylamine on enzyme reactivation by NHA. Wilson and Meislich (1953) have shown that NHA is better than hydroxylamine as a reactivating agent for cholinesterase of electric eel tissue; the most plausible explanation for this is that the quaternary nitrogen group of NHA becomes attached to the anionic site of the enzyme. The presence of an anionic site in human plasma cholinesterase has been demonstrated by Bergmann and Wurzei (1954). If organophosphates do phosphorylate human plasma cholinesterase a block of the anionic site by trimethylamine should slow down enzyme reactivation by NHA. This is indeed so no matter which of the four organophosphates is used as the inhibitor and in spite of the fact that Ro 3-0340 and Ro 3-0422 also possess a quaternary nitrogen group which occupies the anionic site of the enzyme as long as the inhibitor molecule remains intact (Table III). Butyrylcholine—the substrate used throughout the experiments—has the same effect as trimethylamine.

If organophosphates with a diethylphosphato group are used for *in vivo* work complete enzyme recovery requires several days. Experiments were, therefore, carried out to study the effect of NHA on human plasma cholinesterase which had been incubated with the inhibitor for long periods. These experiments showed that the reactivating power of NHA decreases gradually as the time of contact between enzyme and inhibitor is increased (Fig. 3). The findings are identical with all four inhibitors and are not due to a gradual deterioration

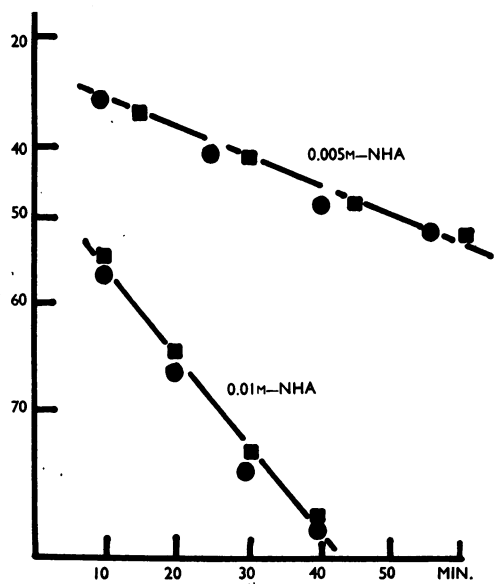

FIG. 2.—Enzyme reactivation by NHA without interference by free inhibitor. A solution of purified human plasma cholinesterase was incubated with 5×10^{-8} M-TEPP (●) or 10^{-7} M-E 600 (■) for 30 min. and then diluted 100-fold with NHA. Ordinate: enzyme activity in % of controls. Abscissa: duration of exposure of inhibited enzyme to NHA before addition of substrate.

TABLE III

EFFECT OF TRIMETHYLAMINE ON ENZYME REACTIVATION BY NHA

NHA or NHA and trimethylamine were added to purified plasma cholinesterase which had previously been incubated with 5×10^{-8} M Ro 3-0340 for 20 min. and was then diluted 10-fold. The activity of individual samples was determined after a 10-fold dilution with substrate

	Activity in % of Control after Reactivation for		
	10 min.	30 min.	50 min.
(*a*) Inhibited enzyme alone ..	3	2	2
(*b*) (*a*)+0·05 M-NHA	74	95	98
(*c*) (*a*)+0·05 M-NHA +0·7 M trimethylamine	25	49	63

of the enzyme during the prolonged incubation at 37° C. Enzyme solutions which were kept for 24 hr. at 37° C. and then incubated with the inhibitor for 30 min. behaved like freshly made enzyme solutions which had been inhibited for the same time.

Effect of NHA on Human Plasma Cholinesterase after Inhibition by an Organophosphate with a Diisopropylphosphato Group

The inhibition of human plasma cholinesterase by DFP, D 600, Ro 3-0411 or Ro 3-0433—like that produced by organophosphates with a diethylphosphato group—can neither be reversed by substrate nor is there any measurable enzyme recovery within a few hours after a dilution which is sufficient to reduce the inhibitor concentration to ineffective levels.

The addition of NHA to dilute solutions of any one of the organophosphates leads to a loss of the anticholinesterase potency of the inhibitor. The rate of interaction of the inhibitor with NHA is of the order DFP>D 600>Ro 3-0433>Ro 3-0411 and is thus a reflection of the stability of the inhibitor in aqueous solution (Table IV).

TABLE IV

STABILITY OF ORGANOPHOSPHATES WITH A DIISOPROPYLPHOSPHATO GROUP AND THEIR RATE OF INTERACTION WITH NHA

A: Loss of activity of 10^{-5} M solutions which were kept for 2 hr. at 80° C. B: Loss of activity of 10^{-5} M solutions which were kept with 0.01 M-NHA for 2 hr. at 37° C. All incubations were carried out in a phosphate buffer of *p*H 7.4

Inhibitor	% Reduction in Activity	
	A	B
DFP	99·9	72
D 600	54	13
Ro 3-0411	33	5
Ro 3-0433	39	8

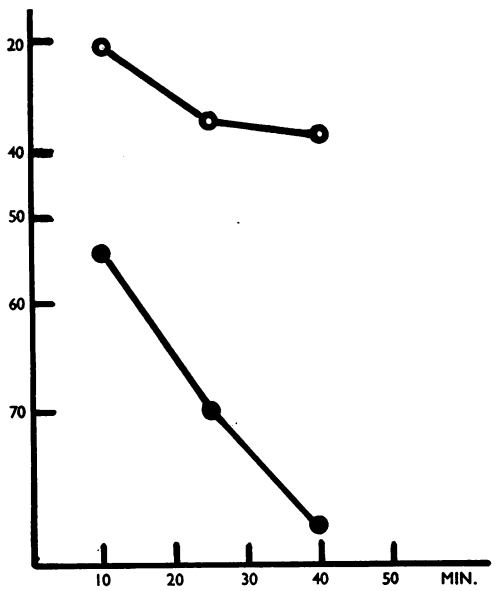

FIG. 3.—Enzyme reactivation by 0.01 M-NHA after various times of contact between enzyme and inhibitor. Purified human plasma cholinesterase was incubated with 5×10^{-8} M-E 600 for 30 min. (●) and 24 hours (○). The enzyme-inhibitor mixture was then diluted 100-fold with NHA. Ordinate: enzyme activity in % of controls. Abscissa: duration of exposure of inhibited enzyme to NHA before addition of substrate.

If human plasma cholinesterase is incubated with DFP, D 600, Ro 3-0411 or Ro 3-0433, NHA can restore some of the enzyme activity. The degree of enzyme reactivation at 37° C. and *p*H 7.4 depends on the time of contact between enzyme and inhibitor, the concentration of NHA, and the type of inhibitor used.

The presence of free inhibitor during the incubation of inhibited enzyme with NHA interferes with the reactivation process in the same way as was shown previously for the reactivation of enzyme inhibited by an organophosphate with a diethylphosphato group.

The results obtained with NHA fall into two distinct groups according to which organophosphate is used as the inhibitor:

(1) *Inhibition by DFP or D* 600.—If human plasma cholinesterase is inhibited by DFP or D 600 NHA never completely restores enzyme activity. The degree of enzyme reactivation by NHA is inversely proportional to the time of contact between enzyme and inhibitor and approaches a maximum within 10 min. (Figs. 4 and 5). If the enzyme is incubated with the inhibitor for more than 1 hr. no enzyme reactivation is obtained with 0.1 M-NHA. Trimethylamine interferes with the action of NHA, but its effect is less than after inhibition by an organophosphate with a diethylphosphato group.

(2) *Inhibition by Ro 3-0411 or Ro 3-0433.*—If NHA is added to human plasma cholinesterase which has been inhibited by Ro 3-0411 or Ro 3-0433 enzyme reactivation is always more marked than if the enzyme had been incubated with DFP or D 600 for the same time (Fig. 4). The degree of enzyme reactivation by NHA is inversely related to the duration of inhibition, but a considerable amount of enzyme activity can still be restored by 0.1 M-NHA even after 1 hr. of contact between enzyme and inhibitor (Fig. 5). Reactivation by NHA proceeds progressively, but its rate falls considerably short of that of a first order reaction.

The differences in the effect of NHA on enzyme inhibited by organophosphates of groups (1) and (2) are not due to interference by hydrolysis products which arise during phosphorylation of the enzyme.

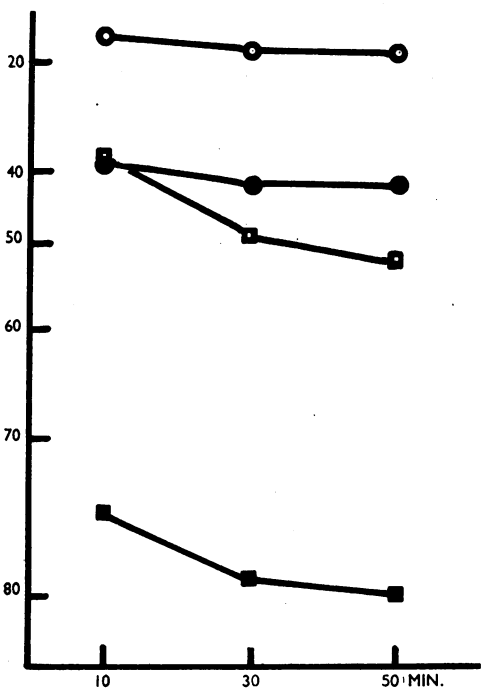

FIG. 4.—Effect of NHA on enzyme activity after inhibition by DFP or Ro 3-0433. Purified plasma cholinesterase was incubated for 10 min. with 5×10^{-8} M-DFP or 2×10^{-7} M-Ro 3-0433. Part of the enzyme-inhibitor mixture was then diluted 10-fold with NHA (final concn.: 0.1 M) and reactivation allowed to proceed for 10, 30, and 50 min. Enzyme activity was determined after a further 10-fold dilution. (●: enzyme inhibited by DFP; ■: enzyme inhibited by Ro 3-0433). The remainder of the enzyme-inhibitor mixture was diluted 100-fold with NHA (final concn.: 0.01 M) and reactivation determined after 10, 30, and 50 min. (○: enzyme inhibited by DFP; □: enzyme inhibited by Ro 3-0433). Ordinate: enzyme activity in % of controls. Abscissa: duration of exposure of inhibited enzyme to NHA before addition of substrate.

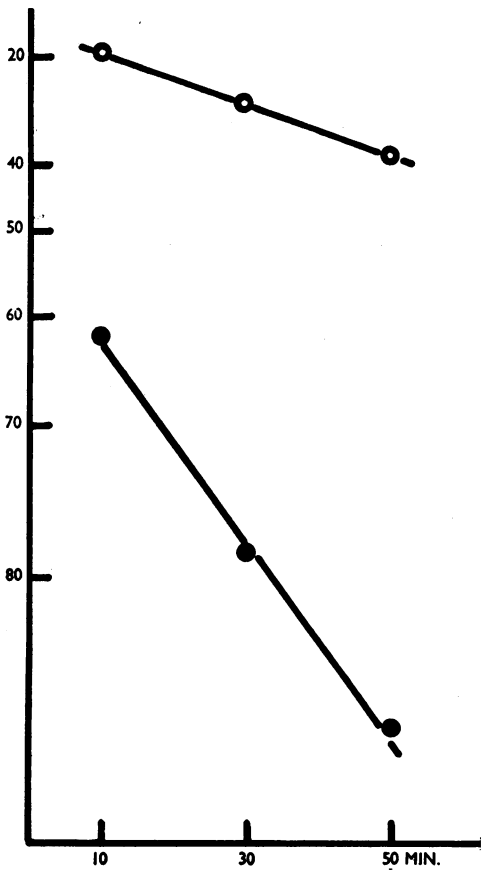

FIG. 5.—Amount of enzyme activity which cannot be restored by 0.1 M-NHA after incubation of plasma cholinesterase with 5×10^{-8} M-DFP (●) or 2×10^{-7} M-Ro 3-0433 (○). Purified plasma cholinesterase was incubated with the inhibitor for 10, 30, and 50 min. The enzyme-inhibitor mixtures were then diluted 10-fold with NHA (final concn.: 0.1 M). 40 min. were allowed for reactivation and enzyme activity was then determined after a further 10-fold dilution. Abscissa: time of contact between inhibitor and enzyme before addition of NHA. Ordinate: % of enzyme activity which cannot be restored by 0.1 M-NHA.

TABLE V

REACTIVATION OF HUMAN PLASMA CHOLINESTERASE INHIBITED BY TWO DIFFERENT ORGANOPHOSPHATES

Purified human plasma cholinesterase was incubated for 10 min. with either 10^{-7} M-DFP (*a*) or 2×10^{-7} M-Ro 3-0433 (*b*). Equal parts of *a* and *b* were then mixed (*c*) and all three samples incubated for another 50 min.; *a*, *b*, and *c* were then diluted 10-fold with NHA (final concentration 0.1 M) and 40 min. allowed for reactivation which was determined on equal volumes of *a*, *b*, and *c* after a further 10-fold dilution

	Observed Reactivation in % of Control	Calculated Reactivation in % of Control
a	2	—
b	53	—
c	28	27·5

Table V shows that the effect of NHA on human plasma cholinesterase which, after inhibition by DFP, was further incubated with another sample of the same enzyme previously inhibited by Ro 3–0433 is exactly the same as would be expected from the effect of NHA on two control samples of the enzyme which had been independently inhibited.

Discussion

Human plasma cholinesterase which has been inhibited by an organophosphate with a diethyl- or di*iso*propylphosphato group can be reactivated by NHA. The degree of enzyme reactivation which is obtained with NHA at 37° C. and *p*H 7.4 depends on the time of contact between enzyme and inhibitor, the concentration of NHA, and the type of inhibitor.

With organophosphates which contain a diethylphosphato group (TEPP, E 600, Ro 3–0340 and Ro 3–0422) the findings indicate that the inhibitory process involves the following stages:

Enzyme + inhibitor⟶addition complex⟶phosphorylated enzyme I⟶phosphorylated enzyme II.

All four organophosphates react with NHA, and this reaction yields products which are without anticholinesterase activity. The chemical inactivation of TEPP and Ro 3–0422 is much faster than that of Ro 3–0340 and E 600, but reactivation of the inhibited enzyme by NHA (in the absence of free inhibitor) is the same regardless of which inhibitor is used. Such results are only possible if NHA is acting on a phosphorylated enzyme. This interpretation is fully supported by the finding that trimethylamine, by blocking the anionic site of the enzyme, slows the rate of enzyme reactivation.

The degree of enzyme reactivation obtained with NHA is inversely related to the duration of inhibition. If the enzyme has been in contact with the inhibitor for up to 30 min., more than 90% of its activity is restored. After 24 hr. contact with the inhibitor, NHA restores less than 50% of the activity. This must mean that the diethylphosphato group of the inhibitor after its initial attachment to one reactive group of the esteratic site (phosphorylated enzyme I) is slowly transferred to another reactive group with which it forms a bond of higher stability and unable to react with NHA (phosphorylated enzyme II).

The inhibitory reaction of human plasma cholinesterase with organophosphates containing a di*iso*propylphosphato group is also a phosphorylation process which leads to the formation of two different types of phosphorylated enzyme. After equal periods of contact between enzyme and inhibitor, NHA always produced a greater reversal of the inhibition by Ro 3–0411 or Ro 3–0433 than it did of the inhibition by DFP or D 600. The rate of interaction between NHA and the four organophosphates is of the order DFP>D 600>Ro 3–0433>Ro 3–0411, so that if the reactivation obtained by adding NHA to the inhibited enzyme were due to an interaction between NHA and the intact inhibitor molecule—as it would be with an addition complex—the inhibition produced by DFP should be more easily reversed by NHA than that produced by the other inhibitors. The experimental findings show the reverse. Since there is no interference by the products which arise during hydrolysis of the organophosphates it must be assumed that Ro 3–0411 and Ro 3–0433 transfer their di*iso*propylphosphato groups to a reactive group of the esteratic site which is different from that to which the di*iso*propylphosphato group of DFP and D 600 is initially attached. If this is so it is not surprising that the rate of formation of a phosphorylated enzyme which cannot be reactivated by NHA (phosphorylated enzyme II) is different with different inhibitors. With DFP and D 600 all the inhibited enzyme is present in form of phosphorylated enzyme II if the time of contact between enzyme and inhibitor exceeds 1 hr. With Ro 3–0411 and Ro 3–0433 the formation of a stable bond between the enzyme and the di*iso*propylphosphato group is slower, but it is still considerably faster than with inhibition by organophosphates containing a diethylphosphato group.

It is possible that the findings reported here are not entirely applicable to *in vivo* conditions and that the rate of formation *in vivo* of a phosphorylated enzyme which cannot be reactivated by NHA (phosphorylated enzyme II) differs greatly. That two types of phosphorylated enzyme are formed *in vivo* is, however, indicated by the results of Davison (1953), who found that the true cholinesterase of rats with E 600 poisoning recovers 50 to 60% of its activity within 4 days, whereas the later stage of recovery proceeds at a considerably slower rate.

Wilson and Meislich (1953), who first described the use of NHA as a reactivator of phosphorylated cholinesterase, found that at 24° C. reactivation of an enzyme inhibited by DFP increased progressively during a period of 24 hr. The differences in the enzymes—human plasma cholinesterase instead of electric eel cholinesterase—and in the temperature —37° C. instead of 24° C.—are most probably the reasons for the lack of agreement between Wilson and Meislich's findings and the results reported here.

In recent years attempts have been made to study the active surface of carboxylic esterases using ^{32}P

labelled DFP as an inhibitor of chymotrypsin, bovine true cholinesterase, human plasma cholinesterase and the aliphatic esterase of red cell stromata. If the inhibited enzymes are subjected to proteolytic digestion followed by treatment with concentrated HCl, serine phosphate containing the labelled P atom can be isolated (Schaffer, May and Summerson, 1953; Cohen, Osterbaan and Warringa, 1954; and Osterbaan, Kunst and Cohen, 1955). Should the findings which are reported here also apply to other carboxylic esterases it would appear that other amino-acids, in addition to serine, participate in the inhibitory reaction, and methods of analysis will have to be found for detecting those parts of the enzyme which play an important role in those initial stages of the inhibition which lead to the formation of phosphorylated enzyme I.

Preliminary experiments have shown that the results with human plasma cholinesterase also apply to the true cholinesterase of human red cells. With TEPP as inhibitor, NHA is about 20 times less potent as a reactivating agent of true cholinesterase than of plasma cholinesterase; thus no great beneficial result could be expected from its use in human beings with organophosphate poisoning.

Summary

1. The effects of nicotinhydroxamic acid methiodide (NHA) on human plasma cholinesterase inhibited by organophosphates containing either a diethyl- or a di*iso*propylphosphato group were studied.

2. The inhibition produced by four different organophosphates with a diethylphosphato group (TEPP, E 600, Ro 3–0340 and Ro 3–0422) is reversed by NHA. The degree of reactivation depends on the concentration of free inhibitor, the time of contact between enzyme and inhibitor, and the concentration of NHA. The inhibitory reaction is a phosphorylation process producing 2 types of phosphorylated enzyme. Phosphorylated enzyme I, which is formed initially, can be fully reactivated by NHA. Phosphorylated enzyme II is formed during prolonged contact between enzyme and organophosphate and cannot be reactivated by NHA.

3. The inhibition produced by organophosphates with a di*iso*propylphosphato group also leads to the formation of two types of phosphorylated enzyme. With DFP and D 600 as inhibitors reactivation of the enzyme is obtained only if the time of contact between enzyme and inhibitor does not exceed 1 hr., whereas the inhibition produced by Ro 3–0411 and Ro 3–0433 can be partially reversed by NHA even after considerably longer inhibition.

4. The results are discussed in relation to the inhibitory mechanism and the study of the active surface of carboxylic esterases.

I wish to thank Dr. A. L. Morrison, Director of the Research Department of Roche Products, Ltd., for helpful discussions and a generous supply of all the Ro compounds used in this work. I also wish to express my thanks to Dr. R. F. Long, of Roche Products, Ltd., for the synthesis of nicotinhydroxamic acid methiodide.

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