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THE INHIBITION OF CHOLINESTERASES BY ALKYLPHOSPHATES AND ALKYLPHENOLPHOSPHATES

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The study of anticholinesterases belonging to the group of alkylphosphates has presented some new and interesting kinetic problems. The inhibition of cholinesterase by eserine, neostigmine, and related compounds is due to the formation of a reversible complex between the enzyme and the entire inhibitor molecule (Aeschlimann and Reinert, 1931 ; Bloch, 1939 ; Randall and Lehmann, 1950). The duration of action *in vivo* of anticholinesterases of this type depends on the rate of dissociation of the enzyme-inhibitor complex and the rate at which the free inhibitor is removed from the body by metabolism and excretion.

Another type of cholinesterase inhibition has been found with a group of compounds derived from dialkylphosphoric esters. Evidence has been adduced to show that these alkylphosphates inhibit by phosphorylation of the active enzyme centre of cholinesterase (Burgen, 1949b ; Wilson and Bergmann, 1950). This type of inhibition has commonly been called " irreversible " but closer study has shown marked differences in the duration of action between different members of this group (Grob, 1950 ; Hobbiger, 1951). If alkylphosphates inhibit cholinesterase by means of a phosphorylation process, the stability of the enzyme-inhibitor complex should be the same whenever the same alkylphosphate group is transferred, or in other words the nature of the alkyl group should be the determining factor in the rate of enzyme recovery. This problem can be studied by using alkylphosphates of a homologous series, where the only variable is the alkyl group. We have studied the behaviour of a series of such compounds, which combined the aminophenol feature of neostigmine and the dialkylphosphoryl group of tetraethylpyrophosphate (TEPP) and di*iso*propylfluorophosphonate (DFP). In the account that follows the kinetics of cholinesterase inhibition by these compounds are described and correlated with those of neostigmine, TEPP, and DFP. Investigations of their action *in vivo* are also presented.

METHODS AND MATERIALS

Fresh oxalated human plasma and washed human red cells, lysed by the addition of four volumes of 0.025 M-$NaHCO_3$, were used as a source of cholinesterase II (pseudo cholinesterase) and cholinesterase I (true cholinesterase) respectively. The activity of the enzyme was estimated by the Warburg technique (Ammon, 1933) at 37° C. with 0.025 M-$NaHCO_3$ as medium, equilibrated with a 95 per cent N_2 + 5 per cent CO_2 gas mixture. The enzyme solution was placed in the main compartment

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of the vessel, and substrate and inhibitor, each dissolved in 0.2 ml. 0.025 M-$NaHCO_3$, were placed in separate sidearms. The total volume of fluid was always 3 ml.; *dl*-acetyl-β-methylcholine (final concentration 0.03 M) and benzoylcholine (final concentration 0.01 M) were used as substrates for cholinesterases I and II respectively. Corrections for non-enzymic hydrolysis of the substrates were applied in all experiments.

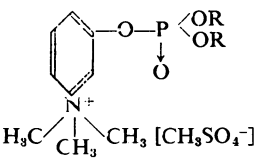
In order to study the recovery of cholinesterase activity *in vitro* human red cell esterase was used ; the details of the experimental procedure are given in the text.

The duration of action *in vivo* was studied in rats and rabbits. In rats the chromodacryorrhoea response, as described by Burgen (1949a), was used as an overall indicator of the duration of enzyme inhibition.

The duration of action of anticholinesterases on striated muscle was studied on the isolated phrenic nerve-diaphragm of the rat (Bülbring, 1946). The diaphragm was suspended in 100 ml. Tyrode solution (containing 0.2 per cent (w/v) glucose) aerated with 95 per cent $O_2 + 5$ per cent CO_2. The phrenic nerve was stimulated through submerged electrodes with slightly supramaximal square pulses of 0.25 msec. duration from an electronic stimulator. A spring-loaded lever was used for recording the contractions. After the diaphragm had been set up for 30 minutes the anticholinesterases were added to the bath for one hour, during which time a continuous stimulation at a frequency of 3 stimuli per minute was applied to the nerve. After the preparation had been in contact with the anticholinesterase for an hour 3 bursts of tetanic stimulation (25, 50, and 100 stimuli per second), each lasting 5 seconds, were given at 30-second intervals. The bath fluid was now replaced by fresh Tyrode solution (without anticholinesterase) and later again every 10 minutes. The response of the diaphragm to tetanic stimulation was tested each time before replacement of the bath fluid.

The distribution of the anticholinesterases *in vivo* was studied in male albino wistar rats of 150 to 200 g. weight, and details of the experimental procedure are given in the text.

The compounds studied had the following general chemical structure:



Code No.	R	Chemical name	Short title
Ro 3-0412 ..	$-CH_3$	*m*-(dimethylphosphato)-N-trimethylanilinium methylsulphate	Dimethylester
Ro 3-0340 ..	$-CH_2CH_3$	*m*-(diethylphosphato)-N-trimethylanilinium methylsulphate	Diethylester
Ro 3-0411 ..	$-CH(CH_3)_2$	*m*-(di*iso*propylphosphato)-N-trimethylanilinium methylsulphate	Di*iso*propylester
Ro 3-0397 ..	$-CH(CH_3)(CH_2CH_3)$	*m*-(di*sec*butylphosphato)-N-trimethylanilinium methylsulphate	Di*sec*butylester

All these compounds, which are referred to in the text by the above-mentioned short titles, or in general as " dialkylphosphostigmines," were synthesized in the Research Department of Roche Products, Ltd.* They were made available as pure crystalline

* The chemistry of these compounds will be published elsewhere.

substances readily soluble in water. Fresh aqueous solutions were always made before use.

TEPP and DFP were made up as 1 per cent (w/v) stock solutions in dry propylene glycol and dilute aqueous solutions were made from these immediately before use.

Neostigmine was used as the methylsulphate.

RESULTS

Inhibition of cholinesterase in vitro

In these experiments the inhibitor was added at room temperature to the enzyme solution, which was incubated at 37° C. for 20 minutes. The substrate was then added and the total CO_2 output between 5 and 35 minutes after addition of the substrate was taken as the measure of enzyme activity.

TABLE I

INHIBITION OF CHOLINESTERASE *in vitro* BY NEOSTIGMINE, TEPP, AND DIALKYLPHOSPHOSTIGMINES

Inhibitor	Molar concentration required for 50% inhibition of		Ratio $\frac{A}{B}$
	Red cell esterase A	Serum esterase B	
Neostigmine	1.14×10^{-7}	6.9×10^{-7}	0.17
TEPP	1.2×10^{-8}	8×10^{-10}	15
Dialkylphosphostigmines:			
Dimethyl-	7.5×10^{-8}	6.3×10^{-9}	12
Diethyl-	8×10^{-8}	1.2×10^{-9}	66
Di*iso*propyl-	2.1×10^{-7}	3×10^{-9}	70
Di*sec*butyl-	1.6×10^{-7}	9×10^{-9}	18

It will be seen from Table I that the dialkylphosphostigmines are powerful inhibitors of cholinesterase *in vitro*. Their potency compares favourably with that of neostigmine and TEPP. All four esters are more potent inhibitors of serum (Ch.E. II) than of red cell esterase (Ch.E. I), and the difference is especially marked with the diethyl- and di*iso*propyl-esters, which inhibit serum esterase by 50 per cent at dilutions respectively 66 and 70 times greater than those required to produce the same degree of inhibition of red cell esterase.

Kinetics of the cholinesterase-inhibitor complex

A closer study of the time course of inhibition and the kinetics of the dissociation of the cholinesterase-inhibitor complex showed that the inhibition of cholinesterase by dialkylphosphostigmines proceeds at a rather slow rate, and with a given concentration of inhibitor a maximal inhibition is not reached even after two hours, except with the dimethylester which produces a maximal inhibition, for a given concentration, within an hour.

It is not possible to make an accurate comparison of the time course of inhibition produced by dialkylphosphostigmines with that produced by TEPP and DFP, since the two latter compounds are much less stable in aqueous solutions (Table II).

The kinetics of inhibition differ according to the ester used, and generally we can divide the dialkylphosphostigmines into two main groups:

TABLE II

NON-ENZYMATIC HYDROLYSIS OF AQUEOUS SOLUTIONS OF TEPP, DFP, AND DIALKYLPHOSPHOSTIGMINES

Inhibitor	Initial concentration g./100 ml.	Times in hours required for 50% hydrolysis	Incubation temperature
DFP	1	7.0	37° C.
TEPP	1	3.5	37° C.
Dialkylphosphostigmines:			
Dimethyl-	1	200	45° C.
Diethyl-	1	430	45° C.
Di*iso*propyl-	1	750	45° C.
Di*sec*butyl-	0.01	180	37° C.

(a) The dimethylester.—When cholinesterase was first incubated with the dimethylester, and then the substrate added, the enzyme activity increased steadily during the first 30 minutes, by which time a state of equilibrium was reached (Fig. 1). The slope of the dose-response curve was similar to that obtained with a

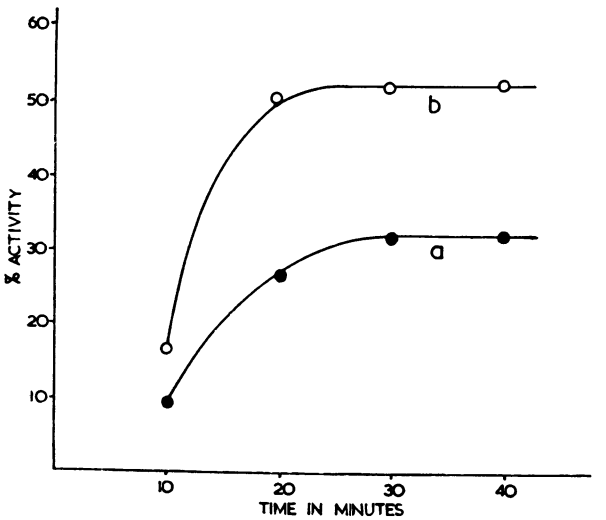


FIG. 1.—Human red cell esterase incubated for 20 min. with dimethylphosphostigmine in a molar concentration of 1.4×10^{-6} (*a*) and 1.5×10^{-7} (*b*): at zero time addition of 0.025 M-*dl*-acetyl-β-methylcholine as substrate. Ordinate : activity in per cent of uninhibited control for periods of 10 min. Abscissa: time in minutes after addition of substrate.

reversible inhibitor like neostigmine, and the concentration of the inhibitor had to be increased roughly 100 times in order to increase the inhibition from 30 to 70 per cent.

(b) The diethyl-, diisopropyl-, and disecbutyl-esters.—With these three esters addition of the substrate after the inhibitor had been in contact with the enzyme for 20 minutes merely prevented further enzyme inactivation, and no enzyme reactivation occurred. If the substrate was given before the inhibitor (the concentration of inhibitor being that which by itself caused 50 per cent reduction of cholinesterase activity in 20 minutes) no enzyme inactivation was seen during the next 40 minutes. This blocking effect by the substrate could be overcome with higher concentrations of the esters (Fig. 2). The slope of the dose-response curve was steep, and, on the

average, increasing the concentration of these esters that produced 30 per cent inhibition threefold raised the percentage inhibition of cholinesterase to 70 per cent. These results are identical with those found with TEPP and DFP (Burgen, 1949b).

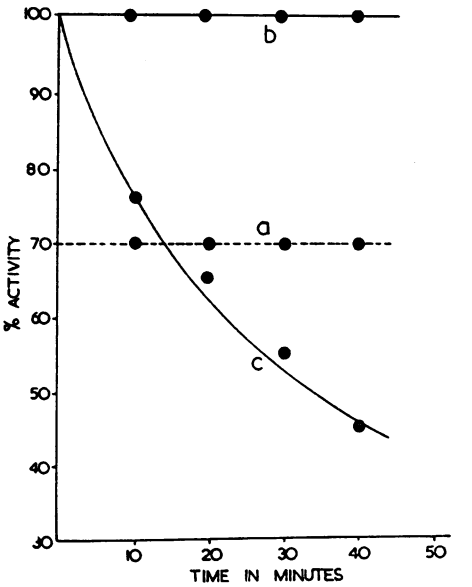


FIG. 2.—Inhibition of human red cell esterase by diethylphosphostigmine. Ordinate: activity as percentage of uninhibited control. Abscissa: time in minutes. Substrate: *dl*-acetyl-β-methylcholine. In (*a*) the enzyme was incubated with 5×10^{-8} M-diethylester for 20 min. before addition of the substrate. In (*b*) the diethylester (final conc. 5×10^{-8} M) was added 5 min. after the substrate. In (*c*) the diethylester (final concentration 5×10^{-6} M) was added 5 min. after the substrate.

It has been shown that cholinesterase previously inactivated by TEPP slowly regains its activity *in vitro* as soon as hydrolysis of the uncombined inhibitor molecules has occurred; this is not true with cholinesterase inhibited by DFP (Hobbiger, 1951). Experiments of this type were repeated with cholinesterase inhibited by dialkylphosphostigmines, the following technique being used:

Intact human red cells were suspended in a 0.9 per cent NaCl solution containing the inhibitor, and kept at room temperature for 30 minutes. The excess uncombined inhibitor was then removed by centrifugation and repeated washing of the red cells with fresh saline; 1 ml. packed red cells was then haemolysed in 49 ml. 0.025 M-$NaHCO_3$ and the haemolysate incubated at 37° C. The cholinesterase activity was measured shortly after haemolysis, and again after incubation for 24 and 48 hours.

Cholinesterase, which had been inactivated in this way by the diethylester, showed reactivation *in vitro* after removal of the uncombined inhibitor. The time course of enzyme recovery was similar to the time course of enzyme recovery after TEPP (Table III). This recovery was a true reactivation and was not due to bacterial action, since bacteriostatic agents did not influence it significantly.

When cholinesterase was inhibited by the di*iso*propyl or di*sec*butyl ester no reactivation of the enzyme was found *in vitro*. These esters behave therefore like DFP (Table III).

The duration of the actions of neostigmine, TEPP, DFP, and dialkylphosphostigmines on the isolated rat phrenic nerve diaphragm preparation

In Fig. 3 the responses to tetanic stimulation at 25, 50, and 100 stimuli/sec. are shown. The normal type response is seen in Fig. 3*a*(C). It can be seen from Fig. 3

TABLE III

TIME COURSE OF REACTIVATION OF CHOLINESTERASE I *in vitro* AFTER REMOVAL OF THE UNCOMBINED INHIBITOR AT 37° C.

Inhibitor	After removal of uncombined inhibitor		After 24 hours		After 48 hours	
	μl. CO_2 output/10 min.	Activity in % of control	μl. CO_2 output/10 min.	Activity in % of control	μl. CO_2 output/10 min.	Activity in % of control
(1) Control ..	52.5	100	46.2	88	44.7	85
+ TEPP (10^{-6}M)	1.9	4	18.1	41	23.3	52
+ Diethylphosphostigmine (10^{-5}M).. ..	10.0	18	19.7	42	23.0	51
(2) Control ..	39.5	100	36.9	93	37.5	95
+ TEPP (10^{-6}M)	3.3	8	11.9	32	18.0	48
+ Diethylphosphostigmine (10^{-5}M).. ..	6.3	15	11.2	31	17.9	47
(3) Control ..	56.6	100	51.8	92	38.8	70
+ DFP (10^{-5}M)	4.8	9	1.3	3	1.3	4
+ Di*iso*propylphosphostigmine (10^{-5}M) ..	7.1	12	4.5	9	3.2	8

that the response to a tetanus was always reduced to a single twitch after the preparation had been in contact with an anticholinesterase, whereas in a normal preparation a tetanus was always fully maintained. Fig. 3*a* illustrates that the effect produced by neostigmine is readily reversible and that 30 minutes after removal of the inhibitor a tetanus is again fully maintained with all three frequencies of stimulation. In the experiments with dimethylphosphostigmine (Fig. 3*b*) this recovery was somewhat slower, but occurred, as with neostigmine, with all three frequencies. Diethylphosphostigmine, however, had a much longer duration of action (Fig. 3*d*), and no recovery of the tetanic response to a stimulation with 100 stimuli/sec. could be seen within 90 minutes after removal of the inhibitor, by which time the response to a lower rate of stimulation (50 stimuli/sec.) was still less than it was 30 minutes after the removal of neostigmine or dimethylphosphostigmine. TEPP (Fig. 3*c*) behaved exactly like diethylphosphostigmine. With DFP and di*sec*butylphosphostigmine no significant recovery of the response to tetanic stimulation with 50 stimuli/sec. took place within 90 minutes after removal of the inhibitor. With di*iso*propylphosphostigmine the recovery for the same frequency of stimulation is somewhat more marked, but still much less than with TEPP. No recovery was seen for the three inhibitors with higher rates of stimulation.

Duration of action in vivo

The duration of action *in vivo* was studied in rats, the chromodacryorrhoea response as described by Burgen (1949a) being used. In this test the amount of acetylcholine required to produce the secretion of red tears is a measure of cholinesterase activity. After an injection of an anticholinesterase the threshold for

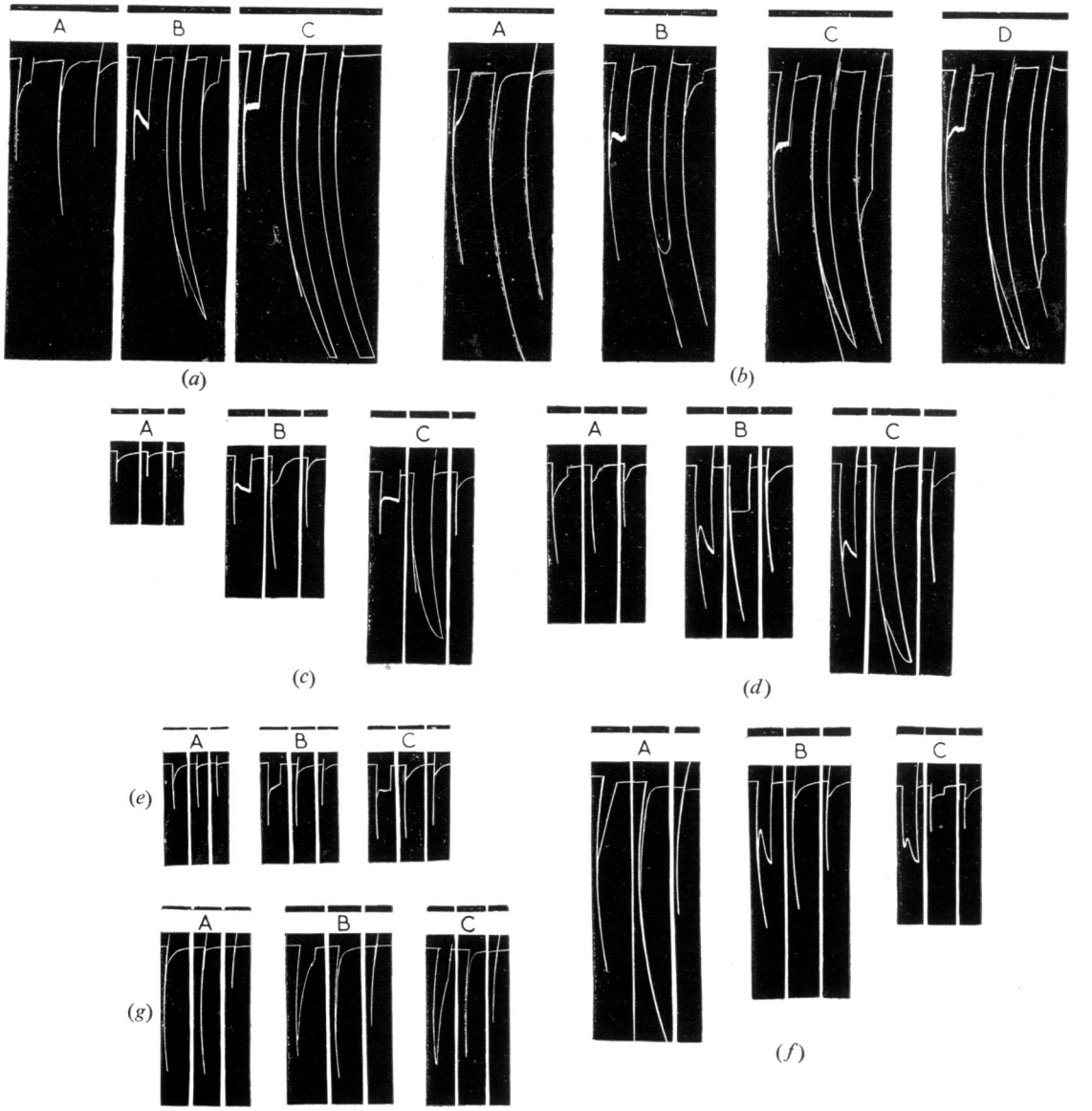


FIG. 3.—Rat phrenic nerve diaphragm (see text). Responses to a 5 sec. tetanus of 25, 50, and 100 stimuli/sec. lasting for 5 sec. (*a*) A: in presence of 0.2 mg. neostigmine methylsulphate/100 ml., left in contact with the diaphragm for 1 hr. previously. B: 10 min., and C: 30 min. after removal of the inhibitor. (*b*) A: in presence of 2 mg. dimethylphosphostigmine/100 ml. left in contact with the diaphragm for 1 hr. previously. B: 10 min., C: 20 min., and D: 40 min. after removal of the inhibitor. (*c*)–(*g*) Responses (A) 10 min., (B) 50 min., and (C) 90 min. after removal of the inhibitor, which had been in contact with the diaphragm for 1 hr. The inhibitors were: (*c*) 2 mg. TEPP/100 ml. (*d*) 5 mg. diethylphosphostigmine/100 ml. (*e*) 2 mg. DFP/100 ml. (*f*) 5 mg. di*iso*propylphosphostigmine/100 ml. (*g*) 5 mg. di*sec*butylphosphostigmine/100 ml.

acetylcholine is lowered, and in our experiments we gave a dose of the inhibitor which produced an increase in the sensitivity of about tenfold in each experiment. With neostigmine the acetylcholine threshold returned to normal on the second

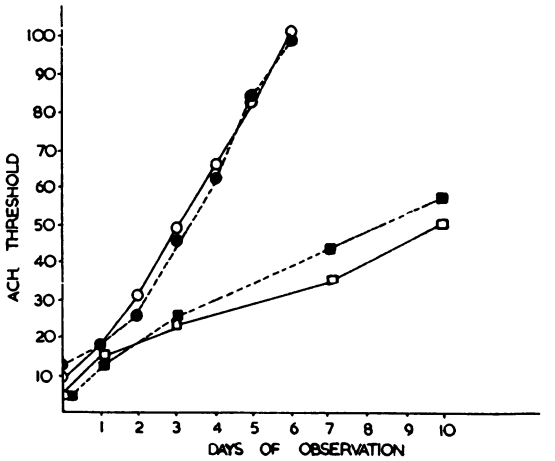


FIG. 4.—Time course of recovery of cholinesterase activity in rats, tested by the chromodacryorrhoea response. Ordinate: amount of acetylcholine (as % of 3 tests before injection of inhibitor) which had to be given to stimulate the secretion of red tears. Abscissa: days of observation. O———O: TEPP (0.4 mg./kg.). □———□ DFP (4 mg./kg.). ●- - - -●: diethylester (3 mg./kg.). ■- - - -■ di*iso*propylester (13 mg./kg.).

day; the action of the dimethylester wore off in four days; the diethylester and TEPP acted for about seven days. With DFP, the di*iso*propyl- and di*sec*butylesters, which all behaved very similarly, even after 10 days the rats were still twice as sensitive to acetylcholine as before the injection of the inhibitor (Fig. 4).

In rabbits the recovery of red cell esterase was studied after intravenous injection of the inhibitors. Here again the dimethylester had the briefest action of the dialkylphosphostigmines, the diethylester very closely resembled TEPP, and the di*iso*propylester behaved like DFP.

Inhibition of cholinesterase by dialkylphosphostigmines in blood and tissues in vivo

Parenteral administration of dialkylphosphostigmines in rats produced symptoms of intoxication which were closely correlated with the degree of inhibition of cholinesterase.

Injection of the dimethylester caused the appearance of muscarinic and nicotinic symptoms of acetylcholine poisoning, closely resembling those seen after the injection of neostigmine. The diethyl-, di*iso*propyl-, and di*sec*butyl-esters, in doses which had no effect on striated muscle, produced marked symptoms of the muscarinic type, e.g., diarrhoea, lacrimation, salivation, and bradycardia. In higher doses they also caused twitching and fibrillation, and at the same time the muscarinic symptoms became very pronounced.

The inhibition of cholinesterase in blood and tissues *in vivo* was studied in rats which received a subcutaneous injection of various doses of the diethyl- and di*iso*propyl-esters. The animals were killed 30 minutes after the injection of the inhibitor, and the cholinesterase activities of the blood, Harderian glands, intestine, and brain were estimated by the usual Warburg technique. Blood was removed from the three latter tissues by repeated washings and the tissues were then homogenized. As will be seen from Fig. 5, a concentration of the inhibitor which produced a reduction of

50 per cent in the esterase activity of red cells and serum did not significantly affect the cholinesterase activity in brain, Harderian glands, and intestine. Higher concentrations of the inhibitor, which reduced the activity of the esterases in blood by 80 per cent, caused a similar degree of enzyme inhibition in Harderian glands and intestine, but had no effect on the cholinesterase in brain. TEPP and DFP, on the other hand, given in a dose which inhibited red cell esterase by 80 per cent, reduced the activity of brain cholinesterase by 50 and 90 per cent respectively.

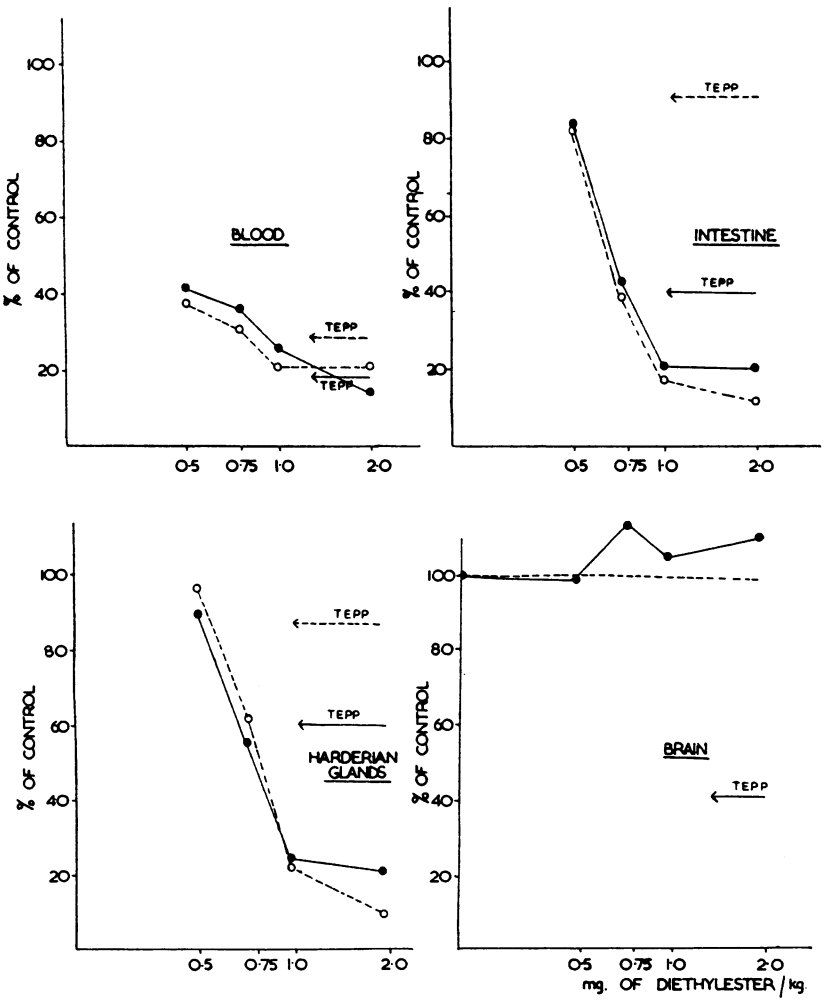


FIG. 5.—Inhibition of cholinesterase in rats *in vivo* by diethylphosphostigmine. Ordinates: cholinesterase activity expressed as a percentage of untreated controls. Abscissae: dose of the diethylester in mg./kg. The inhibitor was given by s.c. injection 30 min. after the injection of 2 mg. atropine/kg. The rats were killed 30 min. after the injection of the diethylester. The arrows on the right side give the degree of inhibition produced by 0.2 mg. TEPP/kg. ●———● cholinesterase I, O- - - -O cholinesterase II.

Toxicities of dialkylphosphostigmines, neostigmine, TEPP, and DFP

Acute toxicities were determined in white mice, weighing 15–20 grammes, by the intravenous route. As can be seen from Table IV, the dialkylphosphostigmines

TABLE IV

	Mouse LD50 by intravenous injection in mg./kg.	Mouse LD50 / 1,000 × I_{50} *in vitro* (Ch.E. I)
Neostigmine	0.30	2.6
TEPP	0.18	8.0
DFP	0.4	1.7
Dialkylphosphostigmines:		
Dimethyl-	0.57	7.6
Diethyl-	7.5	94
Di*iso*propyl	25	120
Di*sec*butyl-	12.8	80

are, with the exception of the dimethylester, much less toxic than neostigmine, TEPP and DFP. The ratio of the mouse LD50 to the amount producing 50 per cent inhibition of the enzyme *in vitro* (I_{50}) is reasonably constant for the diethyl-, di*iso*propyl-, and di*sec*butyl-esters at levels far above those for the other compounds examined. On the other hand, the ratio for the dimethylester is not markedly different from that for neostigmine or TEPP. The remarkably low toxicity of the first-named esters is surprising and cannot be adequately explained at the present time.

Discussion

The inhibition of cholinesterase by TEPP and DFP was interpreted as a phosphorylation process by Burgen (1949b). Later Wilson and Bergmann (1950), studying the inhibition produced by alkylphosphates in relation to the *p*H of the incubation medium, came to the same conclusion. However, owing to the lack of a crystalline cholinesterase preparation no study as conclusive as that of Jansen *et al.* (1949 ; 1950) on α-chymotrypsin inhibition by DFP has yet been carried out. These authors found that 1 mol DFP combined with 1 mol chymotrypsin and at the same time 1 mol free HF appeared in solution. It is very likely that a similar process occurs with cholinesterase. There is good evidence that the phosphorylation of the enzyme is preceded by an initial attachment of the inhibitor to the enzyme. Once phosphorylation has occurred reactivation of cholinesterase could be brought about by an uncatalysed or enzymic hydrolysis. The kinetics of these reactions can be presented in the following way:

$$[E] + [I_{a+b}] \underset{\rightleftharpoons}{K_1} [E][I_{a+b}] \quad \ldots\ldots\ldots\ldots\ldots\ldots (1)$$

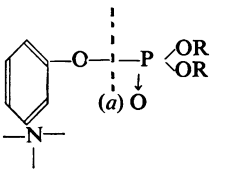
$$[E][I_{a+b}] \xrightarrow{K_2} [EI_a] + [I_b.H] \quad \ldots\ldots\ldots\ldots\ldots (2)$$

$$[EI_a] + [H_2O] \xrightarrow{K_3} [E.H] + [I^aOH] \quad \ldots\ldots\ldots (3)$$

where E = enzyme, I_{a+b} = whole inhibitor, I_a = dialkylphosphoryl moiety of the inhibitor, I_b = residue of the inhibitor molecule.

The velocities and equilibrium points of the above three reactions are determined by the different features of the structure of the inhibitor molecule. Reaction (1) depends on the general fit and bonding properties of the inhibitor molecule. With reversible inhibitors of the eserine and neostigmine type this is the only reaction involved, and it would be expected that the closer the structural resemblance of a phosphorylester to these inhibitors, or the enzyme substrates, the further equilibrium (1) will be shifted to the right.

The velocity of process (2) will depend on the initial lability of the ester linkage in the molecule, i.e., in the compounds described here most probably in bond (*a*):



It will thus be a function of both the left-hand part of the molecule and the nature of the alkyl groups.

The velocity of process (3), on the other hand, will depend on the stability of the EI_a complex. Assuming that enzymic breakdown of this complex does not occur (Hobbiger, 1951) the stability relations can be predicted in a more or less satisfactory manner from the physicochemical properties of the alkylester group. A greater stability of the EI_a complex would be expected with (i) increasing length of the alkyl chain and (ii) with a branched alkyl chain. It would seem that the only other important factor involved in this reaction in the body is the *p*H of the enzyme environment; this might cause differences in the duration of inhibition in various tissues.

To date the only relevant reference to the duration of action in a homologous series is that by Saunders and Stacey (1948), who found that the miotic action of dimethyl- and diethyl-fluorophosphonates was briefer than that of DFP.

In the experiments reported here strong evidence is given to support the theory that the dialkylphosphoryl residue of the inhibitor molecule determines the duration of action. The duration of action of diethylphosphostigmine both *in vitro* and *in vivo* resembles the duration of action of TEPP, and similarly the duration of action of di*iso*propylphosphostigmine resembles that of DFP. The di*sec*butylphosphostigmine does not offer any further prolongation of action because the di*iso*propylphosphoryl-enzyme complex is already so stable that the rate of recovery is probably dependent on the rate of enzyme resynthesis (Koelle and Gilman, 1949).

It should be made clear that these kinetics only apply if the alkylphosphates produce their inhibition by phosphorylation. The evidence suggests that the brief duration of the action of dimethylphosphostigmine is due to failure of phosphorylation rather than to the formation of an unstable phosphorylated enzyme. This can be seen on three grounds: (1) The decrease of inhibition with addition of substrate, showing that a large part of the inhibition is competitive and reversible. (2) The fact that the inhibition reaches an equilibrium point and maintains it. If an unstable phosphorylated enzyme were formed this would mean a steady shift of the dimethylester through the three reactions to its breakdown products, and no equilibrium

would be reached. Fig. 1 shows that that is not so. (3) The degree of inhibition increases only slowly with increase of the inhibitor concentration; this is characteristic of the reversible type of cholinesterase inhibition. The kinetics of the dimethylphosphostigmines therefore resemble closely those of neostigmine and other reversible inhibitors.

Actions of dialkylphosphostigmines in vivo

In comparing the actions of various anticholinesterases *in vivo* we have to consider the following points: distribution via the blood stream, passage from blood to tissues, access to cholinesterase, kinetics of the cholinesterase inhibitor complex, etc. In spite of equal potency *in vitro* anticholinesterases can therefore very likely produce a different degree of enzyme inhibition in various cholinergically innervated structures *in vivo*; thus the brain is completely spared from inhibition by the dialkylphosphostigmines *in vivo*, even with lethal doses, but *in vitro* these esters are powerful inhibitors of brain cholinesterase; the *in vivo* findings can therefore only be explained by the action of the "blood-brain barrier" and the physicochemical properties of these anticholinesterases, which are lipoid insoluble compounds.

The diethyl- and di*iso*propyl-esters act preferentially on organs where acetylcholine exerts its muscarinic type of action. These actions are more marked than with TEPP or DFP. In previous work (Hobbiger, 1951) it was shown that TEPP, which *in vitro* inhibits Ch.E. II in concentrations one-fifteenth of those required to inhibit Ch.E. I, does not show such a preferential inhibition *in vivo*, and in the intestine even the reverse is the case. The diethyl- and di*iso*propyl-esters of the dialkylphosphostigmines, which are even more specific inhibitors of cholinesterase II *in vitro*, show the same lack of differentiation between the two enzymes *in vivo*, as can be seen from Fig. 5, and the muscarinic symptoms produced by them *in vitro* can be fully explained by the inhibition of true esterase.

Summary

1. The dimethyl-, diethyl-, di*iso*propyl-, and di*sec*butyl-esters of *m*-hydroxy-N-trimethylanilinium methylsulphate are potent inhibitors of cholinesterase.

2. *In vitro* they inhibit human red cell cholinesterase 50 per cent at concentrations ranging from 7.5×10^{-8} M to 1.6×10^{-7} M. Serum cholinesterase is inhibited by lower concentrations than red cell cholinesterase, especially by the diethyl- and di*iso*propyl-esters.

3. The dimethylester is a reversible inhibitor with properties similar to neostigmine, but with a longer duration of action.

4. The inhibition of cholinesterase by the diethylester is very similar to that produced by TEPP. With both compounds reactivation of the enzyme occurs slowly *in vitro*. The time courses of recovery of enzyme activity and disappearance of physiological effects *in vivo* are identical for the two inhibitors.

5. The di*iso*propyl- and di*sec*butyl-esters produce an inhibition of cholinesterase that is not reversible *in vitro*. The duration of action *in vivo* is longer than that of the diethylester and very similar to that produced by DFP.

6. In rats none of these esters inhibit brain cholinesterase *in vivo* in doses which produce a marked decrease of enzyme activity in blood and tissues.

7. Compared with their anticholinesterase activity the diethyl-, di*iso*propyl-, and di*sec*butyl-esters are much less toxic than neostigmine, TEPP, DFP, or the dimethyl-ester.

8. The evidence presented provides strong support for the theory that inhibition of cholinesterase by TEPP and DFP is due to a dialkylphosphorylation of the enzyme and that the duration of action of the inhibition depends on the nature of the alkyl groups.

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References

Ammon, R. (1933). *Pflüg. Arch. ges. Physiol.*, **233**, 486.
Aeschlimann, J. A., and Reinert, M. (1931). *J. Pharmacol.*, **43**, 413.
Bloch, H. (1939). *Arch. exp. Path. Pharmak.*, **193**, 292.
Bülbring, E. (1946). *Brit. J. Pharmacol.*, **1**, 38.
Burgen, A. S. V. (1949a). *Brit. J. Pharmacol.*, **4**, 185.
Burgen, A. S. V. (1949b). *Brit. J. Pharmacol.*, **4**, 219.
Grob, D. (1950). *Johns Hopk. Hosp. Bull.*, **87**, 95.
Hobbiger, F. (1951). *Brit. J. Pharmacol.*, **6**, 21.
Jansen, E., Nutting, F., Fellows, M. D., and Balls, A. K. (1949). *J. biol. Chem.*, **179**, 201.
Jansen, E., Nutting, F., Fellows, M. D., Jang, R., and Balls, A. K. (1950). *J. biol. Chem.*, **185**, 209.
Koelle, G. B., and Gilman, A. (1949). *J. Pharmacol.*, Part II, **95**, 166.
Randall, L. O., and Lehmann, G. (1950). *J. Pharmacol.*, **99**, 16.
Saunders, B. C., and Stacey, G. J. (1948). *J. chem. Soc.*, 695.
Wilson, I. B., and Bergmann, F. (1950). *J. biol. Chem.*, **185**, 479.