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THE INHIBITION OF CHOLINESTERASES BY 3-(DIETHOXYPHOSPHINYLOXY)-*N*-METHYLQUINOLINIUM METHYLSULPHATE AND ITS TERTIARY BASE

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Neutral organic phosphates of the type

$$RO—\overset{\overset{O}{\|}}{P}—(OR')_2$$

where R′ represents an alkyl group and R an aryl group are potent inhibitors of cholinesterases. It is now generally assumed that substances of this kind are actually hydrolysed by cholinesterases in the same manner as acetylcholine; but, because of the great stability of the enzyme-dialkyl phosphate complex which is formed during hydrolysis, their turnover is negligible, and enzyme inhibition is the predominant feature. This "phosphorylation theory" is well supported by two findings; firstly, within a homologous series of organic phosphates the inhibitory potency is inversely proportional to the stability of the RO–P link (Aldridge and Davison, 1952a and b); secondly, the stability of the enzyme-inhibitor complex is a function of the dialkyl phosphate group and is always identical where inhibitors with the same dialkyl phosphate group are used (Burgen and Hobbiger, 1951; Wilson, 1952; Aldridge, 1953c). Another strong argument in favour of the phosphorylation theory is derived from the results obtained with crystalline chymotrypsin which, like cholinesterase, is also a carboxylic esterase. With chymotrypsin the inhibition by organic phosphates, such as TEPP, DFP, E600 and related compounds, consists of a dialkyl phosphorylation of the enzyme with the liberation of one mole of acid or phenol per mole of enzyme inhibited (Jansen, Fellowes-Nutting, Jang and Balls, 1950; Fleischer, Jandorf, Summerson, and Norton, 1950; Hartley and Kilby, 1950). Unfortunately, cholinesterase is not yet available in a crystalline form and so accurate quantitative studies of its reaction with anticholinesterases of the organic phosphate type cannot be carried out. All the evidence in favour of the phosphorylation theory is by necessity, therefore, only indirect and studies of the reactions of cholinesterase with new organic phosphates which exceed in potency the known compounds are of considerable value and interest.

In continuation of the work on 3-dialkylphosphato-*N*-dimethylanilines and their quaternary salts (Burgen and Hobbiger, 1951) 3 - (diethoxyphosphinyloxy)-*N*-methylquinolinium methylsulphate became available, and was found to possess an anticholinesterase potency greater than that of any other organic phosphate so far described. The following account describes *in vitro* and *in vivo* experiments with this compound and its tertiary base; the results agree closely with those previously obtained with other organic phosphates containing the same dialkyl phosphate group (Burgen and Hobbiger, 1951), and thus give additional support to the phosphorylation theory.

METHODS

Cholinesterase activity was determined manometrically at 37° C. in an atmosphere of 95% N_2 and 5% CO_2. The enzyme solutions were placed in the main compartment of the vessel; substrate and inhibitor, each dissolved in 0.2 ml., were placed in separate side arms. The total volume of fluid was always 3 ml. The final substrate concentrations were: 0.025 M-acetylcholine chloride, 0.03 M-(±)-acetyl-β-methylcholine chloride and 0.01 M-benzoylcholine chloride.

The enzymes used were:

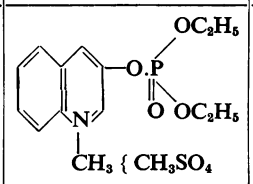
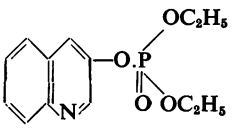
(*a*) From human red cells (twice washed with 0.9% NaCl) and from heparinized human plasma.

(*b*) Purified bovine erythrocyte cholinesterase (Winthrop-Stearns Inc.) and purified human plasma cholinesterase (Plasma fraction IV-6-3; prepared in the late Dr. Cohn's laboratory according to the method of Surgenor, Strong, Taylor, Gordon, and Gibson, 1949).

Except when otherwise stated, all experiments were carried out in a buffer 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).

Throughout the text the cholinesterase of red cells will be referred to as true cholinesterase and the cholinesterase of plasma as pseudo-cholinesterase.

The anticholinesterases used were:

Code No.	Structural Formula	Chemical Name
Ro 3–0422		3-(diethoxyphosphinyloxy)-*N*-methylquinolinium methylsulphate
Ro 3–0419		3-(diethoxyphosphinyloxy)-quinoline

Both substances are pure compounds, and fresh solutions were made each time before use. Ro 3-0422 is a crystalline, water soluble, hygroscopic salt, whereas Ro 3-0419, the tertiary base, is an oil. Both compounds,* which were synthesized in the Research Department of Roche Products Ltd. by Dr. K. J. M. Andrews, were unstable in solution. At 45° C., 0.1% solutions of Ro 3-0422 and Ro 3-0419, in distilled water, lost 50% of their activity in 67 hours, and in 260 hours respectively.

Tetraethylpyrophosphate (TEPP) was made up as a 1% (w/v) stock solution in dry propylene glycol, and dilutions were made immediately before use.

Results

In vitro *Experiments with 3–0422*

Inhibition as a Function of Inhibitor Concentration, and the Effect of Substrate on Inhibition. —Ro 3–0422 is a potent inhibitor of true and pseudo-cholinesterase. Table I gives the molar concentrations required to produce 50% inactivation of the enzymes under standard conditions. As with most other organic phosphates the slope of the inhibition curve is steep and, on the average, a fourfold difference in the concentration of the inhibitor covers the range from 20 to 80% inhibition. With the substrate concentrations used the inhibition produced by a given concentration of Ro 3–0422 was the same for true cholinesterase when either acetylcholine or acetyl-β-methylcholine was the substrate, and for pseudocholinesterase it was the same with either acetylcholine or benzoylcholine.

With the highly purified enzyme preparations in concentrations roughly equimolar to the unpurified preparations, the amount of the inhibitor required for 50% inhibition was of the same order as that necessary to produce the same degree of inhibition in the crude preparations (Table I).

TABLE I

INHIBITION OF CHOLINESTERASE *IN VITRO* AT 37° C.

The substrate (0·025 M-acetylcholine chloride) was added 20 min. after the inhibitor; the total CO_2 output, between 5 and 35 min. after addition of substrate, was the measure of enzyme activity.

Source of Enzyme and Amount per 3 ml.	Activity in μl. CO_2/10 min. in the Absence of Inhibitor	Molar Concentration of Ro 3–0422 Required for 50% Inhibition
0·06 ml. red cells ..	90·5	$3·3\times10^{-10}$
80 μg. purified true cholinesterase ..	86·6	$3·0\times10^{-10}$
0·2 ml. plasma ..	81·0	$1·2\times10^{-9}$
30 μg. purified pseudocholinesterase ..	76·0	$1·0\times10^{-9}$

If the substrate was added to a solution of true or pseudo-cholinesterase, after the enzyme had been in contact with Ro 3–0422 for 20 minutes, no further inactivation took place so long as the substrate concentration was maintained at a high level. Under the same conditions no enzyme reactivation by substrate occurred (Fig. 1), i.e., the CO_2 output remained constant over at least two consecutive periods of 10 minutes. If, on the other hand, the substrate was added together with, or before, the inhibitor a very marked protection of the enzyme was seen, and this could only be overcome by depletion of substrate or by increasing the concentration of inhibitor (Fig. 1).

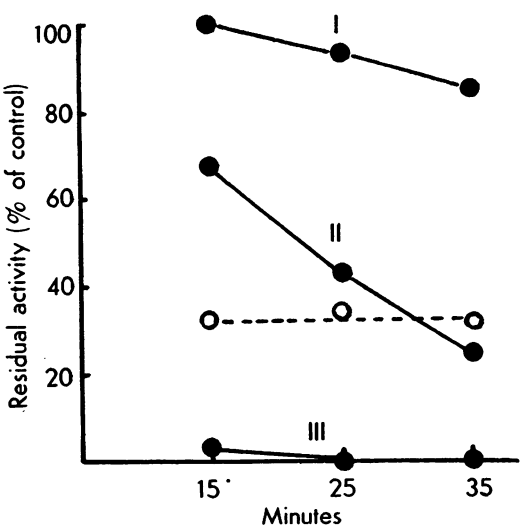


FIG. 1.—Effect of substrate (0.03 M-acetyl-β-methylcholine) on inhibition of true cholinesterase (0.06 ml. washed red cells per 3 ml.). Ordinate: Residual activity as % of control. Abscissa: Time in min. after addition of substrate (each circle gives the activity over the preceding 10 min.). ○ – – – ○: 4.8×10^{-10} Ro 3–0422 added to enzyme 20 min. before substrate. ●——●: Substrate added to enzyme 5 min. before inhibitor. I: 4.8×10^{-10} Ro 3–0422; II: 4.8×10^{-9} Ro 3–0422; III: 4.8×10^{-8} Ro 3–0422. All concentrations of Ro 3–0422 are molar and final.

* The chemistry of these compounds will be published shortly elsewhere.

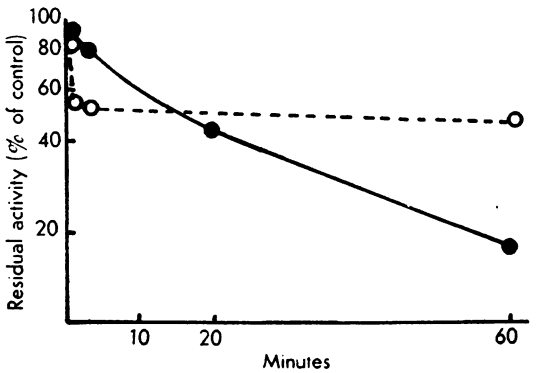


FIG 2.—Rate of combination of Ro 3-0422 with cholinesterase at 37° C. ●——●: 60 μg./3 ml. purified true cholinesterase in a 3×10^{-10} M solution of Ro 3-0422. ○ - - - ○: 30 μg./3 ml. purified pseudo-cholinesterase in a 1×10^{-9} M solution of Ro 3-0422. Ordinate: Residual activity as % of control. Abscissa: Time allowed for contact of Ro 3-0422 with enzyme before addition of substrate.

From these experiments it can be concluded that Ro 3-0422 competes with ACh for the same part of the enzyme surface; but, because of the relatively stable nature of the enzyme-inhibitor complex, the competitive nature of this reaction cannot be demonstrated when the substrate is added to the enzyme after the inhibitor.

Rate of Combination with Cholinesterases.—The inhibition of cholinesterases by organic phosphates (except those containing a dimethyl group) shows the characteristics of a bimolecular reaction with one component—the inhibitor—in excess (Aldridge and Davison, 1952a and b). With Ro 3-0422 the results were as follows:

When purified true cholinesterase was incubated with Ro 3-0422 a progressive inactivation of the enzyme took place; but, as can be seen from Fig. 2, the rate of enzyme inactivation showed a decrement with time which was greater than that which would be expected from a first order reaction. With purified pseudo-cholinesterase this was even more marked and, with a given concentration of Ro 3-0422, inhibition reached a maximum value within the first two minutes (Fig. 2). The same results were obtained with crude enzyme preparations.

One possible reason for the decrement in the rate of inhibition is that the inhibitor may undergo spontaneous hydrolysis. This, however, can be excluded because of the difference in behaviour of true and pseudo-cholinesterase and also by the fact that a 5×10^{-9} molar solution of Ro 3-0422 in buffer loses 50% of its activity at 37° C. only after 18 hours.

Another reason for the inexplicable time course of inhibition—especially of pseudo-cholinesterase—could be the enzymatic destruction of the inhibitor by a phosphatase. This point was followed up and was also excluded.

Enzymatic Hydrolysis of Ro 3-0422.—When the enzymatic hydrolysis of a 3×10^{-3} M solution of Ro 3-0422 was determined manometrically in the usual buffer solution no hydrolysis of the ester was detectable with crude or purified true cholinesterase, or purified pseudo-cholinesterase. Human plasma, on the other hand, metabolized the ester at a slow rate and 50% of the total hydrolysis was achieved in about 200 min. When $MgCl_2$ was omitted from the buffer solution, or when the experiments were carried out in a medium consisting only of 0.025 M-$NaHCO_3$, the activity of human plasma was increased about tenfold; no activity, however, could be detected under the same conditions with purified true or pseudo-cholinesterase or with crude true cholinesterase.

With any given sample of human plasma the rate of hydrolysis was characteristic of a first order reaction (Fig. 3). Using plasma taken from different persons or animals the rate of hydrolysis of Ro 3-0422 varied considerably and no correlation between cholinesterase activity and phosphatase activity could be found.

This enzymatic hydrolysis of the ester is affected most strongly by *p*-chloromercuribenzoic acid. Table II lists all the inhibitors studied.

These results closely resemble Aldridge's for the enzymatic hydrolysis of E600 and its dimethyl homologue (Aldridge, 1953a, b, and c), and it may

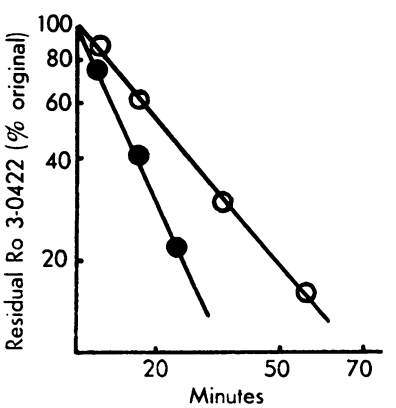


FIG. 3.—Hydrolysis of 3×10^{-3} M sol. of Ro 3-0422 by 0.2 ml. heparinized human plasma/3 ml. Ordinate: Residual Ro 3-0422 as % of original amount. Abscissa: Time in min. ●——●: Subject I; cholinesterase activity of the same sample 292 μl. CO_2/10 min. ○——○: Subject II. Cholinesterase activity of the same sample=202 μl. CO_2/10 min.

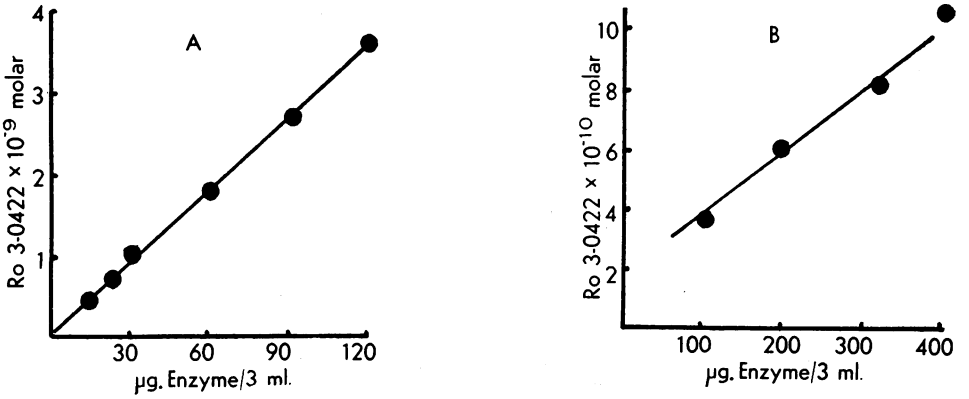


FIG. 4.—Relation between enzyme conc. and molar conc. of Ro 3–0422 required for 50% inhibition of enzyme in 20 min. at 37° C. A: purified pseudo-cholinesterase. B: purified true cholinesterase. Ordinate: molar concentration of Ro 3–0422. Abscissa: µg. of enzyme/3 ml.

TABLE II

INHIBITION OF THE ENZYMATIC HYDROLYSIS OF A 3×10^{-3} M SOLUTION OF Ro 3–0422

By 0·2 ml. of heparinized human plasma in a total volume of 3 ml. (medium: 0·025 M-$NaHCO_3$). All determinations were manometric.

	Molar Concentration	Time Required for 50% Hydrolysis in Min.	% Inhibition
Control	—	19	—
p-Chloromercuri-benzoic acid ..	10^{-4}	335	95
	10^{-5}	20	5
Nickel sulphate ..	10^{-4}	37	49
	10^{-5}	19	0
Copper sulphate ..	10^{-4}	32	41
	10^{-5}	20	5
Potassium oxalate ..	10^{-2}	136	86
	10^{-3}	19	0
Beryllium sulphate ..	10^{-2}	63	70
Sodium cyanide ..	10^{-2}	20	5
„ fluoride ..	10^{-2}	5	Activation

be that the same type of enzyme (called A-esterase by Aldridge) is responsible.

Effect of Enzyme Concentration on Inhibition.—It was shown by Nachmansohn, Rothenberg, and Field (1948) and Bain (1949) that the inhibition of cholinesterase by TEPP and DFP is not only a function of the inhibitor concentration but also of the enzyme concentration. The same applies to the inhibition of cholinesterases by Ro 3–0422. Fig. 4 shows that with both cholinesterases the amount of the inhibitor required for a 50% inhibition of the enzyme is a linear function of the enzyme concentration.

The ratio

$$\frac{[I]_{50} \text{ for enzyme concentration } 2x}{[I]_{50} \text{ for enzyme concentration } x}$$

is 1.63 for true cholinesterase and 2.0 for pseudo-cholinesterase. Assuming that only 1 inhibitor molecule combines with 1 enzyme centre, we can conclude from these figures that the molar concentration of active enzyme centres, in a preparation which, under the experimental conditions used in these experiments, has an activity of 100 µl. CO_2/3 ml./10 min. (substrate: 0.025 M-acetylcholine chloride), cannot be greater than 2.4×10^{-9} for pseudo-cholinesterase, and must be less than 6×10^{-10} for true cholinesterase.

Inhibition of Cholinesterase in Mixtures Containing True and Pseudo-Cholinesterases.—If the above figures approach the true values (which cannot be worked out at present because of the lack of a sufficiently pure enzyme preparation) the specificity of Ro 3–0422 as shown in Table I might not be a true one but might be a reflection of differences in the enzyme concentration. This point was therefore reinvestigated using mixtures of true and pseudo-cholinesterase instead of a single enzyme. Under such conditions, as Table III shows, the results are the reverse of those presented in Table I; Ro 3–0422 has thus to be con-

TABLE III

INHIBITION OF PURIFIED TRUE AND PSEUDO-CHOLINESTERASE, SINGLY AND IN A MIXTURE

The substrate (0·03 M-acetyl-β-methylcholine and 0·01 M-benzoylcholine) was added 20 min. after the inhibitor in a final concentration of 8×10^{-10} M; the total CO_2 output between 5 and 35 min. after addition of substrate was the measure of enzyme activity.

Enzyme and Amount per 3 ml.	Activity (as % of Control) Determined by Hydrolysis of	
	Acetyl-β-methylcholine	Benzoylcholine
80 µg. purified true cholinesterase	12	—
30 „ „ pseudo-cholinesterase	—	60
80 „ „ true cholinesterase plus 30 „ „ pseudo-cholinesterase	93	65

sidered an inhibitor with a higher affinity for pseudo- than for true cholinesterase.

Stability of the Enzyme-inhibitor Complex.—The stability of an enzyme-inhibitor complex which is formed between cholinesterase and organic phosphates with a diethyl phosphate or di*iso*propyl phosphate group is of an order which makes it impossible to detect enzyme recovery in short-lasting experiments or in the presence of free inhibitor. Enzyme recovery after inhibition, however, can be demonstrated either by allowing the enzyme-inhibitor complex to react with nucleophilic reagents (Wilson, 1951 and 1952) or by measuring enzyme activity at daily intervals after removal of the free inhibitor (Hobbiger, 1951; Burgen and Hobbiger, 1951; and Davison, 1953). The latter procedure was used to study the stability

TABLE IV

TIME COURSE OF REACTIVATION OF TRUE CHOLINESTERASE *IN VITRO* AFTER REMOVAL OF THE UNCOMBINED INHIBITOR AT 37° C.

Intact human red cells were suspended in a 0·9% NaCl soln. containing the inhibitor and kept at room temperature for 30 min. The excess of uncombined inhibitor was then removed by centrifugation and repeated washing of the red cells with fresh saline; 1 ml. of 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 24 and 48 hr. incubation, by taking a small sample each time from the incubated solution. The substrate was 0·03 M-acetyl-β-methylcholine.

Inhibitor	After Removal of Uncombined Inhibitor		After 24 hr.		After 48 hr.	
	μl. CO_2/ 10 min.	Activity (% of Control)	μl. CO_2/ 10 min.	Activity (% of Control)	μl. CO_2/ 10 min.	Activity (% of Control)
Exp. I:						
Control	39·5	100	36·9	93	37·5	95
TEPP (10^{-6}M)	3·3	8	11·9	32	18·0	48
Ro 3–0422 (10^{-7}M)	2·9	7	9·4	26	17·7	47
Exp. II:						
Control	47·2	100	45·5	88	47·2	81
TEPP (10^{-6}M)	0·0	0	8·5	18	17·5	37
Ro 3–0422 (10^{-8}M)	1·9	4	9·0	19	16·1	34

of the enzyme-inhibitor complex formed between true cholinesterase and Ro 3–0422. As Table IV shows, Ro 3–0422 cannot be classified as an irreversible inhibitor like DFP, but forms an enzyme-inhibitor complex which has the same stability as that of cholinesterase and TEPP. As the results were the same with crude and purified enzyme preparations we can exclude the possibility that the enzyme recovery shown in Table IV is significantly affected by an action of a second enzyme—such as, for example, a phosphatase.

These results agree well with those previously described for other organic phosphates containing the same dialkyl phosphate group; they must, therefore, be regarded as affording additional support for the phosphorylation theory.

The *in vitro* results on the stability of the enzyme-inhibitor complex are paralleled by the *in vivo* recovery of cholinesterase activity of red cells in rabbits given a single injection of Ro 3–0422. With a dose that reduced enzyme activity by 90% the activity returned to normal within four days, reaching a level of 44% after 24 hours, 62% after 48 hours and 90% after 72 hours. This is in agreement with what happens after an injection of TEPP, or other organic phosphates containing the same dialkyl phosphate group.

In vivo *Experiments with Ro 3–0422*

The parenteral administration of Ro 3–0422 produced effects in rats, rabbits, and cats which could be fully attributed to its action upon cholinesterase. The first observable changes were muscarinic—salivation, lacrimation, and bradycardia. When the dose was increased nicotinic effects—fasciculations and twitchings—were seen in addition.

The intravenous LD50 in mice was 20 μg./kg. Ro 3–0422 is, therefore, more toxic than any other organic phosphate; this was to be expected from its high anticholinesterase activity *in vivo* (Jones, Meyer and Karel, 1948).

In cats, intravenous doses of 0.1–1 μg./kg. were sufficient to potentiate and prolong the actions of injected ACh and of vagal stimulation, and to produce bradycardia. With 5 μg./kg. salivation was abundant, micturition occurred, intestinal activity was increased, fibrillary muscular twitchings were seen, and the response of skeletal muscles to indirect stimulation was markedly potentiated.

On the isolated rat diaphragm a dose as small as 0.01 μg./ml. potentiated twitch tension, and on the isolated rabbit intestine an increase in tone and spontaneous peristalsis occurred at similar dose levels.

In studies on the distribution of Ro 3–0422 *in vivo* (judged by the inhibition produced at various sites after parenteral administration) the results closely resembled those with Ro 3–0340 (Burgen and Hobbiger, 1951). Thus at low doses of the inhibitor the inhibition of blood cholinesterases was more marked than was that of tissue cholinesterases; but if the dose was increased a state of uniform inhibition was achieved. Again, as with the quaternary salts of 3-dialkylphosphato *N*-dimethylanilines, the brain was never inhibited even after a lethal dose of the inhibitor.

Action of Ro 3–0419, the Tertiary Base of Ro 3–0422

The tertiary base of Ro 3–0422 is as potent as the latter in inhibiting pseudo-cholinesterase but less active against true cholinesterase. Under standard conditions, such as those which are given in Table I, the molar concentration of Ro 3–0419 required for 50% inhibition was 3.8×10^{-9} for pseudo-cholinesterase and 7.8×10^{-8} for true cholinesterase. With both enzymes the reaction between enzyme and inhibitor followed the time course of a first order reaction, and no enzymatic destruction of the inhibitor by plasma or any other of the enzyme preparations was noticed. The inhibition of cholinesterase was competitive with substrate, and the substrate gave marked protection. Enzyme recovery *in vitro* and *in vivo* was similar to that after inhibition by TEPP and by Ro 3–0422.

The intravenous LD50 in mice was 1 mg./kg. In contrast to Ro 3–0422, brain cholinesterase was not spared from inhibition when the inhibitor was injected into an animal; the degree of inhibition of brain cholinesterase after a given dose of the inhibitor was of the same order as that of blood cholinesterases.

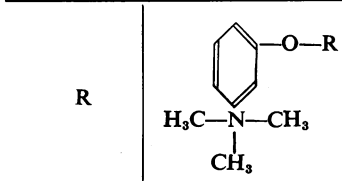
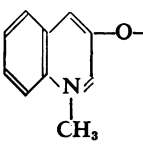
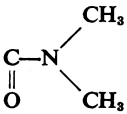
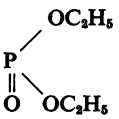
Discussion

The potency of an anticholinesterase of the organic phosphate type depends on certain structural features of the inhibitor molecule, and on the reactivity of the RO–P bond (Mackworth and Webb, 1948; Brauer, 1948; Aldridge and Davison, 1952a and b; Aldridge, 1953c). As would be expected, both these points play a significant part in determining the anticholinesterase potency of Ro 3–0422. Table V shows the concentration required for 50% inhibition of true cholinesterases for the 3-hydroxy, 3-dimethylcarbamoyloxy and 3-diethylphosphato derivatives of trimethylanilinium and methylquinolinium salts. As can be seen from this Table, the introduction of a dimethyl carbamate group leads, in both series, to a comparable gain in activity. This would be expected, since with the dimethylcarbamates two points of attachment are available (quaternary ammonium group and carbamate group) against the one point of attachment (quaternary ammonium group) in the parent substances. If the dimethyl carbamate group in both series is replaced by a diethyl phosphate group, only the phosphate in the methylquinolinium series shows a gain in activity over its corresponding dimethylcarbamate. This cannot be explained on the basis of the structural features of the two phosphates,

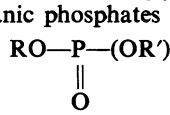
Table V

COMPARISON OF THE ANTICHOLINESTERASE POTENCY OF A SERIES OF TRIMETHYLANILINIUM AND METHYLQUINOLINIUM DERIVATIVES

The unbracketed numerals in this table are the molar concentrations of inhibitor required for 50% inhibition. Enzyme from 0·06 ml. washed human red cells. Inhibitor added to enzyme 20 min. before substrate (0·025 M-acetylcholine); the CO_2 output between 5 and 35 min. after addition of the substrate was the measure of enzyme activity

R		
H	$1{\cdot}7 \times 10^{-4}$ (Ro 2–2561)	$2{\cdot}7 \times 10^{-3}$ (Ro 3–0472)
	6×10^{-8} (Neostigmine)	6×10^{-6} (Ro 1–5384)
	8×10^{-8} (Ro 3–0340)	$3{\cdot}3 \times 10^{-10}$ (Ro 3–0422)

since the changes in both series are identical. The only difference is found in the RO–P bond, which in Ro 3–0422 is much less stable than in Ro 3–0340. If Ro 3–0422 only formed a simple addition complex with cholinesterase this liability of the ester link would reflect itself in a considerable decrease —and not an increase—in activity. The results shown in Table V are strongly in favour of the theory that organic phosphates of the type



are treated by cholinesterase in the same manner as substrates but differ from the latter only in the stability of the enzyme-dialkyl phosphate complex.

It has been pointed out in a previous paper (Burgen and Hobbiger, 1951) that if the phosphorylation theory is correct the stability of the enzyme-inhibitor complex should be the same whenever the inhibitor has the same dialkyl phosphate group. With cholinesterase inhibited by Ro 3–0422, or its tertiary homologue Ro 3–0419, the stability of the enzyme-inhibitor complex is of the same order as that formed between cholinesterase and TEPP, or Ro 3–0340. All these four inhibitors have in common a diethyl phosphate group, and so the results presented in this paper fully support the phosphorylation theory.

Although Ro 3–0422 is an inhibitor of great potency it is not at present possible to say what is the quantitative relationship of the reaction between enzyme and inhibitor. The data available from the literature on the turnover of substrate by cholinesterase provide only approximate figures, since they have either been obtained indirectly from work with inhibitors (Easson and Stedman, 1936; Goldstein, 1944; Myers, 1951; and Berry, 1951) or apply to work done with cholinesterase from a non-mammalian source (Rothenberg and Nachmansohn, 1947). Until compounds of the type described by Funke, Depierre and Krucker (1952), which inhibit cholinesterase in 10^{-14} and 10^{-16} molar concentrations, have been studied more extensively, and until a pure enzyme preparation of cholinesterase is available, it will not be possible to interpret the reaction between enzyme and Ro 3–0422 on a quantitative basis.

Its high anticholinesterase activity makes Ro 3–0422 very suitable for an *in vivo* analysis of the function of the acetylcholine-cholinesterase system. If the compound is used for such a purpose it must be remembered that the distribution on a cellular level will be affected by the physicochemical properties of the substance (Ro 3–0422 is not lipoid soluble) and could also reflect enzymatic destruction of the inhibitor by an enzyme which has all the characteristics of the A-esterase described by Aldridge (1953a and b).

Summary

1. 3-(Diethoxyphosphinyloxy)-*N*-methylquinolinium methylsulphate (Ro 3–0422) is the most potent anticholinesterase so far synthesized. It inhibits true cholinesterase in concentrations as low as 10^{-10} molar. With true and pseudocholinesterase the degree of enzyme inhibition is a function of enzyme and inhibitor concentration.

2. The enzyme-inhibitor complex formed between cholinesterase and Ro 3–0422 *in vivo* and *in vitro* is only slowly reversible and shows the same stability as the enzyme-inhibitor complex formed between cholinesterase and other diethyl phosphates such as TEPP.

3. Crude human plasma contains an enzyme (probably the A-esterase described by Aldridge) which hydrolyses Ro 3–0422 in the concentrations required to inhibit cholinesterase.

4. Ro 3–0422 is more toxic than any other organic phosphate and its LD50 in mice is 20 μg./kg. Doses below 1 μg./kg. produce in cats the typical symptoms of cholinesterase inhibition.

5. Ro 3–0419, the tertiary base of Ro 3–0422, is a weaker anticholinesterase than is Ro 3–0422.

6. The results obtained with Ro 3–0422 and Ro 3–0419 fully support the theory that inhibition of cholinesterase by organic phosphates is a dialkyl phosphorylation of the enzyme.

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