SUMMARY

1. Stearic, oleic, vaccenic and linoleic acids, within the range of concentrations used, produce an inhibition of respiration of washed suspensions of *Bacillus subtilis* and a concomitant inhibition of respiration and growth of growing cells.

2. The inhibition is transient, its degree and duration being dependent on the ratio acid : cells.

3. Washed suspensions and growing cells which have recovered from the inhibition are resistant to further additions of acid at concentrations higher than the initially inhibiting dose.

4. Treatment with any one acid produces resistance against any of the other acids.

5. A time lag ensues on treatment with a fatty acid before resistance develops.

6. Low concentrations of acid, which do not inhibit growth or oxygen uptake, nevertheless produce resistance.

7. The tolerance of growing cells with subsequent development of resistance is slightly greater than that of washed suspensions.

8. The resistance is lost on subculturing the cells in absence of fatty acid.

9. The evidence suggests that the resistance of acid-treated cells is due to the development of an adaptive enzyme, which is formed subsequently to the incorporation into the cell and the metabolic degradation of the fatty acid.

I am indebted to Dr I. D. Morton for supplying me with the synthetic vaccenic acid.

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The Inhibition of Erythrocyte Cholinesterase by Tri-Esters of Phosphoric Acid

1. DIETHYL *p*-NITROPHENYL PHOSPHATE (E 600) AND ANALOGUES

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The ester diethyl *p*-nitrophenyl phosphate (E 600) is a powerful and irreversible inhibitor of cholinesterase. The kinetics of its reaction with cholinesterase have been studied (Aldridge, 1950).

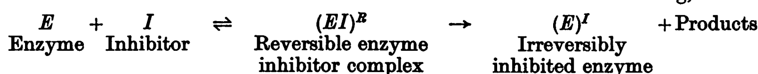
The work to be reported here began as a study of the inhibitory powers of analogues of E 600 containing different substituents in various positions on the phenyl ring. Comparisons of the activities of other groups of irreversible inhibitors of cholinesterase have been reported. Mackworth & Webb (1948) compared the activity of dialkyl fluorophosphonates prepared by McCombie & Saunders (1946) against a purified preparation of horse-serum cholinesterase. Metcalf & Marsh (1949) compared the activity of substituted diethyl phenyl phosphates and thiophosphates against bee-brain cholinesterase. In these reports a direct comparison was made between the concentrations of inhibitor that produced 50% inhibition of the cholinesterase preparation.

Aldridge (1950) showed that the inhibition of erythrocyte cholinesterase by E 600 followed first-order kinetics and was bimolecular. The bimolecular rate constants were considered to be a much better index of inhibitory power than the usual 50% inhibition concentrations. When these constants were determined for this series of compounds it was found that the kinetics of the inhibition were not like those obtained for E 600. It was then realized that these differences could be accounted for by the presence of a very active, but unstable, inhibitor of cholinesterase. When this impurity was removed the compounds reacted with cholinesterase in the same way as E 600. This aspect of the work seemed to be important, and the work carried out with one of

these compounds is given in detail. With our samples of inhibitors, therefore, the determination of the concentration necessary for 50% inhibition would have given misleading values for their activity. As will be pointed out, the possibility of the detection of this impurity rested on our choice of cholinesterase preparation and also on a detailed study of the kinetics of inhibition of the enzyme.

A knowledge of the kinetics of inhibition of erythrocyte cholinesterase rests almost entirely on work using E 600. It seemed desirable, therefore, to extend this work to other inhibitors of the same type, for Diggle & Gage (1951) have recently shown that diethyl *p*-nitrophenyl thiophosphate (E 605), purified chromatographically, has an extremely low inhibitory action against cholinesterase. The high inhibitory power of the sample used by Aldridge (1950) must have been due to the impurities it contained. These were most probably the isomers, *OO*-diethyl *S*-*p*-nitrophenyl thiolphosphate and *OS*-diethyl *O*-*p*-nitrophenyl thiolphosphate.

It was suggested (Aldridge, 1950) that the mechanism of inhibition of cholinesterase could be expressed by the equation:



and that the inhibitor was hydrolysed during the inhibitory process. There is evidence (Jansen, Nutting & Balls, 1949) that when chymotrypsin is inhibited by di-*iso*propyl fluorophosphonate (DFP), di-*iso*propyl phosphate remains attached to the inactivated enzyme and that one molecule of acid (presumably HF) is liberated during the inhibitory process. Hartley & Kilby (1950) have shown that one molecule of *p*-nitrophenol is liberated during the inhibition of one molecule of chymotrypsin by E 600. When horse-serum cholinesterase is inhibited by DFP containing ^{32}P phosphorus becomes firmly attached to the cholinesterase (Boursnell & Webb, 1949), and Jandorf & McNamara (1950) have also shown that after a dose of DFP containing ^{32}P to rabbits, the elimination of ^{32}P from the red cells paralleled the reappearance of cholinesterase activity. When mammalian cholinesterase has been prepared in a pure state, a direct experiment to test the hypothesis that the inhibitor is hydrolysed during the inhibitory process may be carried out. At the present time indirect methods must be used, and for this reason it was of interest to compare the stability of hydrolysis at physiological pH and temperature of this series of inhibitors with their inhibitory power. The results confirm the hypothesis stated above.

METHODS

Erythrocytes from defibrinated sheep blood have been used as a source of cholinesterase. Activity has been determined by the method of Nachmansohn & Rothenberg (1945), as previously described in detail (Aldridge, 1950). With long incubations (2–6 hr.) it was sometimes found that the activity of the control fell appreciably. The addition of 20 μg./ml. of KCN to the buffer for all incubations longer than 1 hr. effectively prevents such loss of activity and does not alter the inhibition by E 600 analogues.

Rates of inhibition have always been determined in double-armed flasks, the red cells in the flask and the inhibitor solution and acetylcholine in the side arms. At zero time, after gassing and temperature equilibration, the inhibitor is tipped into the centre compartment and the incubation finally terminated by tipping in the acetylcholine at the required time.

During the course of this work it was found necessary to have some quantitative measure of reversible and irreversible inhibition. This has been determined by the following washing technique: A red cell suspension of such a concentration of cholinesterase that 0·5 ml. will give an output of approximately 8 μl. CO_2/min. is incubated with the inhibitor. At the end of the incubation, 5 ml. are pipetted into 45 ml. of buffer in a centrifuge tube and 0·5 ml. directly into 3·0 ml. buffer containing the acetylcholine in a Warburg flask (to determine the total inhibition). The diluted blood is centrifuged at 2500 rev./min. for 5 min., the supernatant removed and the cells washed a further four times with buffer. After the final washing, the cells are resuspended and diluted to 5 ml. and a sample taken for determination of cholinesterase activity (irreversible inhibition). With cells of low activity larger amounts of blood (up to 3·0 ml.) may be taken, thus increasing the sensitivity six times. Buffer containing 20 μg./ml. of KCN was used for all the washing procedures. Control blood samples with no inhibitor added were always carried through the whole procedure. Stock solutions of all the inhibitors have been prepared in absolute ethanol and dilutions made from them for the experiments.

The following inhibitors, derived from diethyl phosphoric acid, were used: diethyl phenyl phosphate, diethyl *p*- and *o*-chlorophenyl phosphate, diethyl *p*-nitrophenyl phosphate (E 600), diethyl *o*- and *m*-nitrophenyl phosphate and tetraethyl pyrophosphate (TEPP). (*Editorial note.* The names 'diethyl *o*- and *p*-chlorophenyl phosphate' are used in this paper for the sake of consistency with other diethyl phenyl phosphates, although they are contrary to the alphabetical order for prefixes now customary in the *Biochemical Journal*; cf. *J. chem. Soc.* (1950), p. 3699.)

Rates of hydrolysis of the inhibitors in phosphate buffer, pH 7·6, at 37° were determined using the following standard procedure: A weighed amount of ester (approx. 500 mg.) was dissolved in 10 ml. ethanol. At zero time 5 ml. were pipetted into 495 ml. of Sorensen's phosphate buffer (pH 7·6) previously warmed to 37°. As preservatives during the hydrolysis of the *o*- and *p*-nitrophenyl esters, the buffer contained 0·02% (w/v) thymol while for the rest of the substituted phenyl esters 0·10% (w/v) merthiolate was used. Samples were withdrawn at various times and the corresponding phenols estimated. *o*- and *p*-Nitrophenol were determined directly by their yellow colour at pH 7·6. Phenol, *o*- and *p*-chlorophenol and *m*-nitrophenol were determined by the method of Gottlieb & Marsh (1946).

The rate of hydrolysis of TEPP was determined manometrically in $NaHCO_3$ buffer. The volume of CO_2 evolved after complete hydrolysis amounted to 1·94 mol. CO_2/mol. TEPP.

RESULTS

Washing technique for the determination of irreversible inhibition. As has been shown previously (Aldridge, 1950) intact red cells are a very convenient source of cholinesterase. Inhibitors may be added, allowed to react and then removed by the simple process of washing the cells with buffered saline. The results given in Table 1 show that the cholinesterase activity of normal sheep red cells is not appreciably affected by a prolonged washing equivalent to a dilution of 10^{18} times. If the cholinesterase activity of these cells is inhibited by treatment with E 600, a similar washing process reactivates the enzyme by less than 5%. When red cells are inhibited by eserine, more than 50% of the inhibition is reversed after only one washing (a dilution of 10 times). Our standard procedure for the determination of irreversible inhibition has been to wash five times which is equivalent to diluting 10^5–10^6 times.

Table 1. *Washing of sheep red cells before and after treatment with E 600*

(Washed sheep red cells incubated with $6·5 \times 10^{-7}$ M E 600 for 30 min. Each washing is equivalent to a dilution of 10 times.)

No. of washings	Activity (μl. CO_2/min.) Without E 600	With E 600
0	9·6	0·7
5	9·4	0·9
18	8·9	1·0

Enzymic hydrolysis of inhibitors. In a preliminary note (Aldridge, 1951) it has been shown that there are enzymes in mammalian tissues which can hydrolyse E 600 and it is, therefore, important to be sure that the cholinesterase preparation used (which is necessarily impure) is free from enzymes which will hydrolyse the inhibitor. This has been checked using E 600, the most unstable to hydrolysis of the substituted diethyl phenyl phosphates we have used. Sheep red cells at a concentration which possessed a cholinesterase activity of 7–10 μl. CO_2/min. produced a negligible output of CO_2 ($< 0·3$ μl./min.) when E 600 was used as a substrate at a concentration of 2 mg./ml. This means that this preparation is, from a practical point of view, free from any enzyme which will hydrolyse the substituted phenyl inhibitors used in this work. However, red cells catalyse the hydrolysis of TEPP appreciably. When red cells similar to the preparation used above are incubated with TEPP at concentration of about 4 mg./ml., 4 μl. CO_2/min. are evolved. (This figure has been corrected for the aqueous hydrolysis of TEPP.) As will be explained later inhibition of red-cell cholinesterase by TEPP does not give the kinetics of the other inhibitors and this is due to the destruction of TEPP by the enzyme preparation used. This fact has been of great value in the detection of the impurities in our inhibitors.

Inhibition of cholinesterase by diethyl p-*chlorophenyl phosphate.* The rate of reaction of the diethyl *p*-chlorophenyl phosphate has been determined using double-armed flasks so that the inhibitor may be added to the enzyme after temperature equilibration and the reaction stopped later by the addition of acetylcholine from the other side arm. In Fig. 1 are shown the results we obtained. With E 600 first-order kinetics are obtained at each concentration of inhibitor, whereas in this case there is a very rapid, followed by a slow, progressive inhibition.

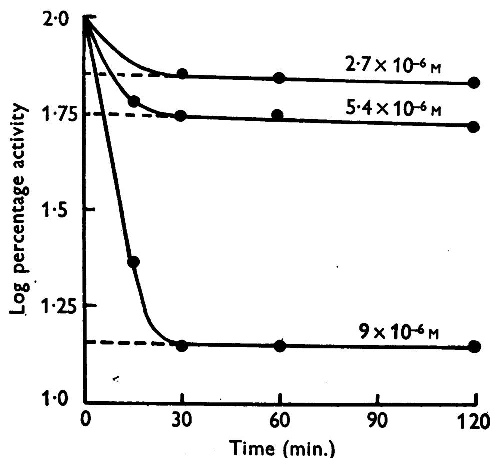


Fig. 1. Rate of inhibition of cholinesterase by unpurified diethyl *p*-chlorophenyl phosphate. Concentration of inhibitor shown against each curve.

These results were similar to those previously determined with diethyl 8-quinolyl thiophosphate (Aldridge, 1950) when it was shown that the initial rapid inhibition was reversible and could be removed by washing. A determination of irreversible inhibition was, therefore, carried out using the technique described earlier in this paper. As will be seen, in Table 2, none of this initial rapid inhibition is reversible by washing.

Since such closely related compounds as diethyl *p*-nitrophenyl and *p*-chlorophenyl phosphates produce such dissimilar results when the rate of their reaction with cholinesterase is examined it seemed probable that impurities were responsible. We should expect the type of kinetics obtained if the rapid irreversible inhibition were produced by a

Table 2. *Washing of red cells after inhibition with diethyl* p-*chlorophenyl phosphate*

(Cells inhibited for 15 min. at 37°. Each washing is equivalent to a dilution of 10 times.)

Exp. no.		Activity (μl. CO_2/min.) No washes	Five washes
1	Cells (no inhibitor)	8·5	8·7
	Cells + $1 \cdot 9 \times 10^{-4}$ M-inhibitor	0·24	0·24
2	Cells (no inhibitor)	7·6	7·8
	Cells + $2 \cdot 4 \times 10^{-5}$ M-inhibitor	0·8	0·9

highly active but unstable inhibitor. The highly active inhibitor reacts rapidly with cholinesterase, but is being destroyed so rapidly itself that after about 20–30 min., virtually all of it has been removed. The final slow inhibition would then be due to the diethyl *p*-chlorophenyl phosphate itself. Two observations convinced us that this was probably true. When a chloroform solution of diethyl *p*-chlorophenyl phosphate was shaken with weak caustic soda solution, then with water, dried and the chloroform evaporated, its inhibitory power was considerably reduced. Secondly, a solution of the diethyl *p*-chlorophenyl phosphate which had been incubated at pH 7·6 at 37° for 864 hr., but had only undergone 8% hydrolysis, had a low inhibitory power and when examined for its rate of reaction with cholinesterase gave straightforward kinetics for each concentration tried (Fig. 2).

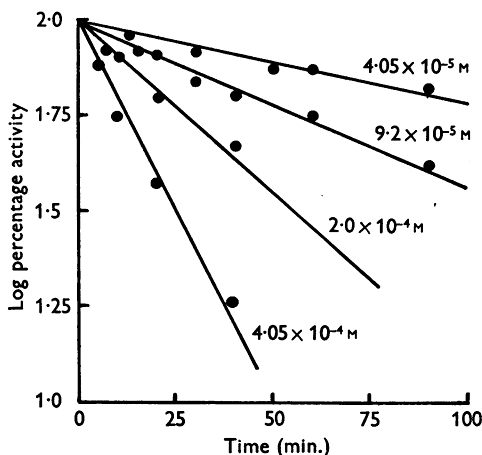


Fig. 2. Rate of inhibition of cholinesterase by purified diethyl *p*-chlorophenyl phosphate. Concentration of inhibitor shown against each curve.

It could be argued that some other change has occurred during its incubation in buffer. In order to check this point, the distribution coefficient of the parent compound between liquid paraffin and water has been compared with that of the solution of inhibitor after 864 hr. in buffer at pH 7·6 and at 37°. Chloroform, toluene, ether and *n*-amyl alcohol were of little value since the compound was too soluble in them. The results in Table 3 show that the compound in the buffer has the same distribution between liquid paraffin and water as the original compound. If an ethyl group had been removed during the incubation in buffer a di-substituted phosphoric acid would result which would be much more soluble in water. It is clear, therefore, that our sample of inhibitor contains an active inhibitor which is more readily hydrolysed in buffer than is the diethyl *p*-chlorophenyl phosphate.

Table 3. *Distribution coefficient of diethyl* p-*chlorophenyl phosphate between liquid paraffin and water*

(Concentration of diethyl *p*-chlorophenyl phosphate determined after hydrolysis in NaOH as *p*-chlorophenol using the method of Gottlieb & Marsh (1946). Free *p*-chlorophenol was determined before hydrolysis with NaOH. The solution of inhibitor in phosphate buffer was shaken with an equal volume of liquid paraffin and the concentration of free and bound *p*-chlorophenol repeated on the aqueous layer.)

Compound	Distribution coefficient (room temp.) liquid paraffin/buffer
p-Chlorophenol	1·6
Fresh diethyl *p*-chlorophenyl phosphate	6·6
Diethyl *p*-chlorophenyl phosphate after incubation for 864 hr. at pH 7·6 and 37°	6·9

An examination was made of the speed with which this active inhibitor was hydrolysed in water. A solution of the original compound was prepared in the bicarbonate buffer used for the cholinesterase estimations. Samples were withdrawn after 0, 18 and 40 hr. at room temperature and the determination of rate of reaction repeated. In Fig. 3 the results are plotted, and it will be seen that the amount of the rapid initial inhibition decreases rapidly according to the time the inhibitor has been in buffer, until after 40 hr. there is no rapid initial inhibition and the kinetics are first order over the whole experiment. During this period the hydrolysis of the diethyl *p*-chlorophenyl phosphate is negligible.

Our sample of diethyl *p*-chlorophenyl phosphate, therefore, contains an active inhibitor which is sufficiently unstable to be completely hydrolysed after 3 days in bicarbonate buffer at room temperature. However, an examination of Fig. 3 shows that this inhibitor must be removed in 20–30 min. in the presence of sheep red cells. TEPP is an inhibitor which is hydrolysed readily in water, and it has been shown earlier in this paper that the hydrolysis of TEPP is catalysed by sheep red cells and the fact

that the diethyl *p*-chlorophenyl phosphate is a derivative of diethyl phosphoric acid (half of the TEPP molecule) also suggests that TEPP may be the impurity. We have, therefore, added TEPP to a solution of the diethyl *p*-chlorophenyl phosphate which has been incubated in buffer at room temperature for several days and have repeated the determination of rate of reaction with cholinesterase. The results given graphically in Fig. 4 show that these results are similar to those in Fig. 3. Further, if the rate of inhibition of red-cell cholinesterase by TEPP alone is examined, it is found that there is no further inhibition after 30 min. (Fig. 5). It can be argued that this is due to an equilibrium reaction similar to that found with the reversible inhibitor eserine. However, if TEPP is incubated with sheep

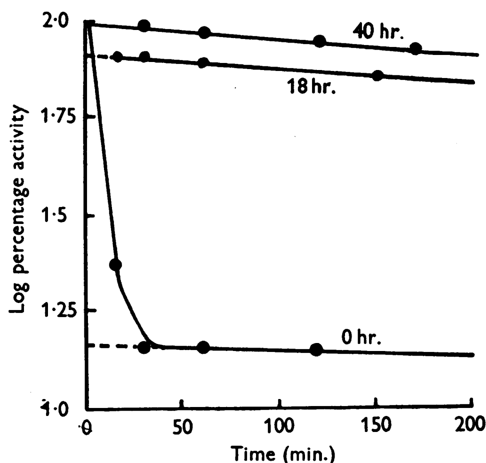


Fig. 3. Rate of inhibition of cholinesterase by 9×10^{-6} M-diethyl *p*-chlorophenyl phosphate after leaving in buffer solution at room temperature. Time in buffer shown against each curve.

red cells for 30 min., the cells centrifuged and the supernatant added to fresh red cells, the cholinesterase activity of these red cells is not inhibited; therefore, there is no TEPP remaining in solution and this is presumably due to its enzymic hydrolysis.

It is proved, therefore, that our sample of diethyl *p*-chlorophenyl phosphate contains as an impurity an active cholinesterase inhibitor which is completely inactivated within 3 days at room temperature in bicarbonate buffer and which is inactivated by sheep red cells at low concentrations in 20–30 min.; this substance is in all probability TEPP, since the addition of TEPP to diethyl *p*-chlorophenyl phosphate from which the active inhibitor has been removed by preferential hydrolysis, gives, on examination, the same kinetic picture as the original diethyl *p*-chlorophenyl phosphate. It has been shown that the inhibitor is reduced to a very low level by 40 hr. If it is assumed that the inactivation is kinetically of the first order and that it is reduced to 2 % of its original value, we obtain a first-order constant of $1{\cdot}6\times10^{-3}$ (min.$^{-1}$). A measured rate of hydrolysis of TEPP at room temperature under similar conditions gave a value of $1{\cdot}14\times10^{-3}$ (min.$^{-1}$). It is, therefore, very

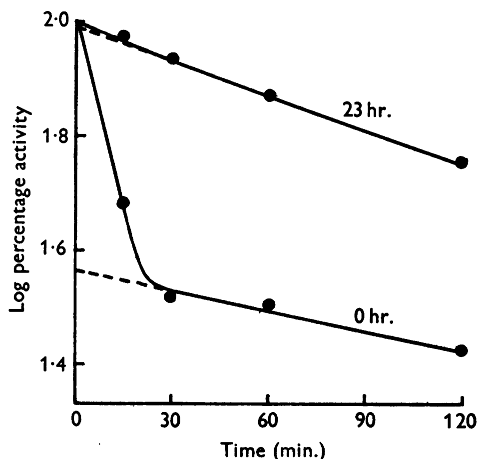


Fig. 4. Rate of inhibition of cholinesterase by $2{\cdot}7\times10^{-8}$ M-TEPP plus $3{\cdot}97\times10^{-5}$ M-purified diethyl *p*-chlorophenyl phosphate, at zero time and after 23 hr. in bicarbonate buffer at room temperature.

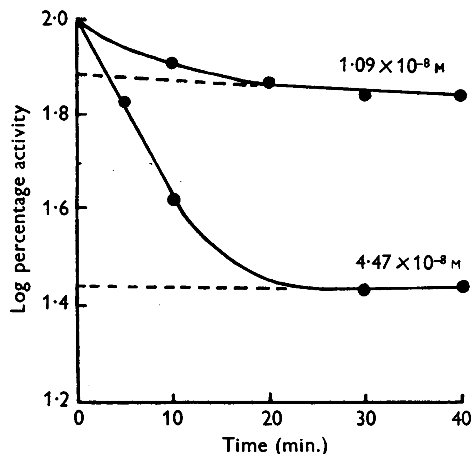


Fig. 5. Rate of inhibition of cholinesterase by TEPP. Concentration of inhibitor shown against each curve.

probable that the impurity present is TEPP and the amount of impurity will be expressed as TEPP (cf. Table 6).

The determination of concentration of TEPP in inhibitors. Aldridge (1950) has shown that the inhibition of red-cell cholinesterase by E 600 shows first-order kinetics and is bimolecular, i.e.

$$K=\frac{1}{tI}\ln\frac{100}{b}, \quad (1)$$

where K = bimolecular rate constant, t = time in min., I = molar inhibitor concentration and b = percentage residual activity. If the time is maintained constant at 30 min. a plot of log b against I, the inhibitor concentration, should give a straight line. This graphical method can be conveniently used for the determination of the concentration of an inhibitor from its inhibitory power against cholinesterase. TEPP has been shown to be destroyed during the incubation with red cells. If it is assumed that the kinetics of the inhibition of cholinesterase by TEPP are similar to those obtained for E 600 and that the kinetics of the hydrolysis of TEPP during the incubation period are also first order (cf. Van Slyke, 1942, for a discussion of enzyme kinetics at low substrate concentrations), then a plot of log % residual activity against inhibitor concentration should give a straight line. This is found to be so (Fig. 6).

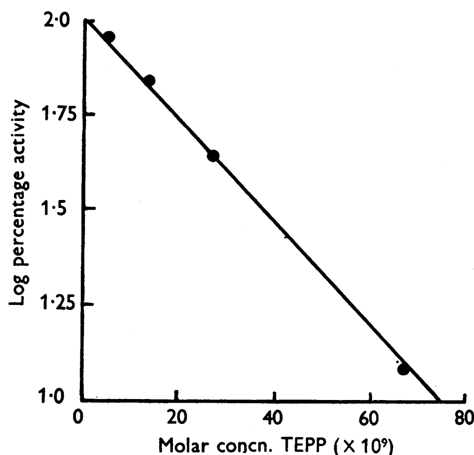


Fig. 6. Inhibition of sheep red cells by TEPP incubated for 30 min. at 37°.

The amount of inhibition due to TEPP in our diethyl p-chlorophenyl phosphate may be readily determined by extrapolating the straight line portion of the curves (after 30 min.) back to the ordinate. The amount below the origin will give a measure of the inhibition due to TEPP. This has been checked using the results obtained with our synthetic mixture of TEPP and purified diethyl p-chlorophenyl phosphate (Fig. 4). At zero time an extrapolation in this way shows that the line crosses the ordinate at 1·59 which from Fig. 6 is equivalent to $3{\cdot}0 \times 10^{-8}$M-TEPP; the amount added was $2{\cdot}7 \times 10^{-8}$M. This method has been used to obtain an estimate of the amount of TEPP present in our inhibitors. Our main purpose has been to show that the amounts present are too small to be detected by analysis of the original inhibitor and could only be determined by a biochemical method.

Calculation of bimolecular rate constants. If an inhibitor reacts bimolecularly with an enzyme, equation (1) shows that with the concentration of inhibitor (I) constant, a straight line will be obtained when log % residual activity is plotted against time, i.e. the system will show first-order kinetics. If the inhibitor concentration (I) is varied then log % residual activity as ordinates plotted against the product of time and concentration (tI) as abscissae will give a straight line crossing the ordinate at 2·0 (100 % activity). For all of the compounds (except TEPP) examined the point where the line cuts the ordinate has been determined and shown to be 2·0 within experimental error. This is illustrated by Figs. 2 and 7 using our results on diethyl p-chlorophenyl phosphate.

Examination of other inhibitors. Each inhibitor has been examined by the technique previously described for diethyl p-chlorophenyl phosphate

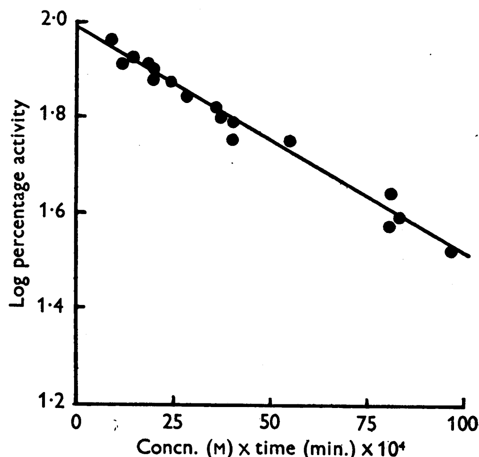


Fig. 7. Graph of all results for cholinesterase inhibition by purified diethyl p-chlorophenyl phosphate. Bimolecular rate constant derived from slope of line is $1{\cdot}1 \times 10^2$ (min.$^{-1}$ l. mol.$^{-1}$).

(Fig. 3). Except for E 600 the presence of a similar active and unstable inhibitor has been shown. It has been assumed that this is TEPP. The concentrations for 50 % inhibition (after 30 min. incubation at 37°) for the original compound, and after it has been purified (by preferential hydrolysis), are calculated as previously described and are given in Table 4.

The amount of impurity (expressed as TEPP) present in all (except phenyl (2)) of these samples of inhibitor is below 0·7 %; this concentration would be undetectable by the methods of analysis.

The inhibition of cholinesterase by all of the purified inhibitors given in Table 4 has been shown to give first-order kinetics and to be bimolecular by the methods previously illustrated using diethyl

p-chlorophenyl phosphate. Using these data, the bimolecular rate constants have been calculated and are given in Table 5. The value given for TEPP is one corrected for hydrolysis of TEPP during the experiment on the assumption that this hydrolysis will be first order and that the TEPP concentration is reduced to 95% of its original value in 30 min. Also included in Table 5 are the first-order constants for aqueous hydrolysis at pH 7·6 and 37° in phosphate buffer.

Table 4. *Concentrations for 50% inhibition by unpurified and purified inhibitors and the concentration of TEPP present as impurity*

(Samples purified by preferential hydrolysis of impurity for 3–4 days at room temperature in bicarbonate buffer. Concentrations of TEPP calculated as given in the text.)

Inhibitor	Concn. for 50% inhibition (M) Unpurified	Purified	Impurity (TEPP) (%)
p-Chloro-	5.75×10^{-6}	2.10×10^{-4}	0·68
o-Chloro-	2.24×10^{-5}	1.1×10^{-4}	0·053
p-Nitro-(E 600)	2.0×10^{-8}	2.01×10^{-8}	—
o-Nitro-	4.58×10^{-7}	1.48×10^{-6}	0·33
m-Nitro-	1.2×10^{-6}	3.0×10^{-6}	0·55
Phenyl (1)	1.35×10^{-4}	3.77×10^{-3}	0·025
Phenyl (2)	2.35×10^{-7}	3.77×10^{-3}	5·4

The results in Table 5 indicate that the more stable the inhibitor the slower is its inhibitory action upon cholinesterase. These results have been plotted in Fig. 8 where the relation between stability of an inhibitor in buffer at physiological pH and temperature and its inhibitory power is shown.

TEPP and E 600 appear to be exceptional. This might be expected with TEPP, since it has a different chemical structure to the rest of the inhibitors. However, E 600 appears to have a far higher inhibitory power than would be expected from its rate of hydrolysis. E 600 and diethyl *o*-nitrophenyl phosphate have roughly the same stability at pH 7·6 and 37°, but E 600 is 70 times more efficient as an inhibitor. There is no point in speculating why this might be so until more inhibitors of this type with rate constants higher than 10^4 (l.mol.$^{-1}$ min.$^{-1}$) have been examined. It is of interest that Metcalf & Marsh (1949), in work on bee-brain cholinesterase, found E 600 to be an outstanding inhibitor in a large series of compounds.

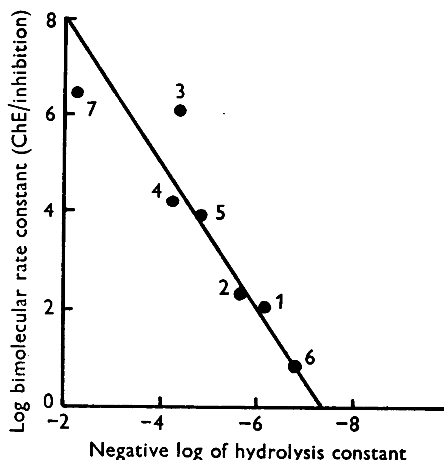


Fig. 8. Relationship between rate of hydrolysis in buffer at pH 7·6 and 37° and the bimolecular rate constant for the reaction of cholinesterase (ChE) with inhibitor. Compounds numbered as in Table 5.

DISCUSSION

The work described above began with an attempt to compare the rate of hydrolysis at pH 7·6 and 37° and the rate of reaction with cholinesterase of analogues of E 600. During the initial experiments it was found that the reaction of all these analogues with cholinesterase had peculiar kinetics quite unlike E 600. A further examination led to the idea that an unstable active inhibitor was present as an impurity. This view had been substantiated. The impurity appeared to be the same in all of our compounds (based on rate of destruction in bicarbonate buffer) and therefore it is unlikely that it could have

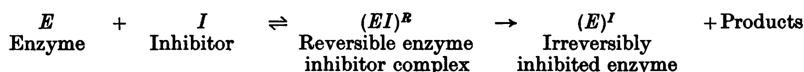
Table 5. *Constants for rate of reaction of inhibitors with cholinesterase and for their hydrolysis in buffer*

(Since most of the compounds are very stable, the long time required for their complete hydrolysis made necessary the calculation of the non-enzymic hydrolysis constants from data involving incomplete hydrolyses. The proportion of the compound hydrolysed for the period during which the determinations were made is given in brackets. ChE = cholinesterase. Numbers of compounds refer to points in Fig. 8.)

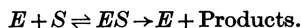
Inhibitor	No.	Rate of reaction of inhibitor with ChE K (min.$^{-1}$ l.mol.$^{-1}$)	Rate of hydrolysis of inhibitor in phosphate buffer, pH 7·6, at 37° K (min.$^{-1}$)
p-Chloro-	1	1.1×10^2	7.2×10^{-7} (4·2%)
o-Chloro-	2	2.1×10^2	2.3×10^{-6} (7·9%)
p-Nitro-(E 600)	3	1.1×10^6	3.9×10^{-5} (65%)
o-Nitro-	4	1.6×10^4	5.6×10^{-5} (78%)
m-Nitro-	5	7.7×10^3	1.4×10^{-5} (45%)
Phenyl	6	6·1	1.6×10^{-7} (2·9%)
TEPP	7	3.3×10^6	6.0×10^{-3} (100%)

been an impurity common to all the substituted phenols used in the synthesis of the compounds. All the analogues are derivatives of diethyl phosphoric acid and this suggested that tetraethyl pyrophosphate might be the impurity. The following evidence confirms this view: (1) reasonable agreement between the rate of hydrolysis of TEPP and the rate of disappearance of impurity in bicarbonate buffer; (2) sheep red cells remove TEPP in the same time (25–30 min.) as that taken to remove the impurity; and (3) a model system in which TEPP was added to the purified diethyl *p*-chlorophenyl phosphate produced the same kinetic picture as that of the unpurified inhibitor.

After purification by leaving a solution of the compound in bicarbonate buffer at room temperature for 2–3 days, the kinetics of the rate of reaction of these inhibitors with cholinesterase was re-examined. They all now followed first-order kinetics at any given inhibitor concentration and by the usual tests the reactions were bimolecular. The bimolecular rate constants for these reactions have been determined and have been shown in general to run parallel to the rate of hydrolysis of the inhibitors in water. The more unstable the compound, the more active it is as an inhibitor. This is evidence in favour of the view that hydrolysis is a part of the inhibitory process. The mechanism originally suggested (Aldridge, 1950),



is the same as that of the Michaelis-Menten equation for the enzyme-substrate system,



The two processes of inhibition and hydrolysis of substrate are probably analogous. The enzyme may form a complex with the inhibitor, activate it and the inhibitor is then hydrolysed. In the case of the inhibitor the active centre is blocked by the retention of one of the products of hydrolysis. The final proof of this hypothesis could only come with a sufficiently pure and concentrated preparation of cholinesterase, so that the products of this reaction could be produced in quantities great enough to be determined chemically.

Intact red cells have been used and therefore the cholinesterase is not in solution and the system is obviously a heterogeneous one. This reaction of inhibitor and enzyme differs from most other surface reactions in the important respect that the active surface is itself being inactivated. It is probable that the concentration of even the most powerful inhibitor (10^{-8} M E 600) is considerably in excess of the concentration of enzyme considered in terms of its active centres. On this assumption, therefore, the concentration of inhibitor will be sensibly constant throughout the reaction, and first-order kinetics can be expected. The frequency of a fruitful collision of inhibitor and active centre (i.e. a collision which leads to irreversible inhibition of the active centre) will be dependent upon the number of available active centres, and since these will be continuously depleted by the reaction itself, an exponential reaction rate will result.

Finally, a consideration of the conditions which enabled us to detect the impurity in our compounds will illustrate some of the possible sources of error to be considered when a value for the inhibitory power of these compounds is determined. The detection of the impurity depended on its instability and hydrolysis by enzymes present in our preparation of cholinesterase. In fact, if we had been able to use a pure preparation of cholinesterase we should have obtained the expected kinetics. These would have been attributed to the particular inhibitor being examined, though in fact they were really those of the active impurity. Since they have activities of the same order TEPP cannot be detected in E 600. Only high concentrations of TEPP would have altered the kinetics obtained with E 600 and this could have been detected by the normal methods of chemical analysis. A stable inhibitor present as an impurity could not be detected. For instance we could not trace by these methods E 600 in the diethyl *o*- and *p*-nitrophenyl phosphates. This possibility can be minimized by using substituted phenols of high purity in the preparation of the inhibitors. These results suggest the need for care in comparing the activity of inhibitors when concentrations of impurities undetectable by ordinary chemical means may produce large changes in a biochemical system. The value of a complete study of the kinetics of the reaction between enzymes and inhibitors is illustrated.

SUMMARY

1. Kinetics of inhibition of sheep red-cell cholinesterase by six substituted diethyl phenyl phosphate inhibitors have been examined and shown to be first order and bimolecular.

2. All the inhibitors examined contained as an impurity a small amount of an active and unstable inhibitor of cholinesterase. This is in all probability tetraethyl pyrophosphate. A simple method of purification is given and the methods used to detect and determine tetraethyl pyrophosphate are given in detail.

3. A relation between the stability of the inhibitors to aqueous hydrolysis at physiological pH and temperature and their power as inhibitors of cholinesterase has been found. The more stable the inhibitor, the lower its inhibitory power.

4. The mechanism of inhibition of cholinesterase by these compounds is discussed in relation to the above facts.

Our thanks are due to Mr B. Topley, Messrs Albright and Wilson Ltd., for providing us with the samples of all the inhibitors used in this work, and to Miss J. I. Wheatley for valuable technical assistance.

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The Metabolism of Aminophenols, *o*-Formamidophenol, Benzoxazole, 2-Methyl- and 2-Phenyl-benzoxazoles and Benzoxazolone in the Rabbit

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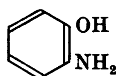
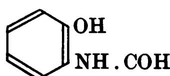
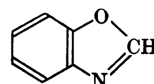
In this investigation some aspects of the metabolism of *o*-, *m*- and *p*-aminophenols, *o*-formamidophenol (II), benzoxazole (III), 2-methylbenzoxazole (IV), 2-phenylbenzoxazole (V), and benzoxazolone (VI) have been examined. The compounds (II) to (VI) can be regarded as derivatives of *o*-aminophenol (I), which might be formed from them on hydrolysis. These compounds may, therefore, be considered as having potential centres for conjugation (see Bray, Ryman & Thorpe, 1948; Thorpe, 1950). Williams (1938, 1943) and Smith & Williams (1949) found that the rabbit excretes the aminophenols mainly as *O*-conjugates, i.e. ethereal sulphates and glucuronides. The only other compound of this group which appears to have been investigated previously is benzoxazolone, which Gressly & Nencki (1890, 1891) showed to be hydroxylated in the dog.



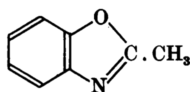
(I)



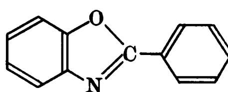
(II)



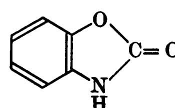
(III)



(IV)



(V)



(VI)

In this study we have attempted to draw up balance sheets for these compounds and to isolate their principal metabolites. Minor metabolites were detected by means of paper chromatography. The results obtained show the influence of substituents upon the stability of the oxazole ring.

EXPERIMENTAL

Materials. The aminophenols were purchased (British Drug Houses Ltd. and Light and Co.). *o*-Formamidophenol was prepared by the action of acetic and formic acids on *o*-aminophenol (Béhal, 1900), benzoxazole and its 2-methyl and 2-phenyl derivatives by dry distillation of *o*-amino-