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

2. DIETHYL *p*-NITROPHENYL THIONPHOSPHATE (E605) AND ANALOGUES

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(*Received* 25 *March* 1952)

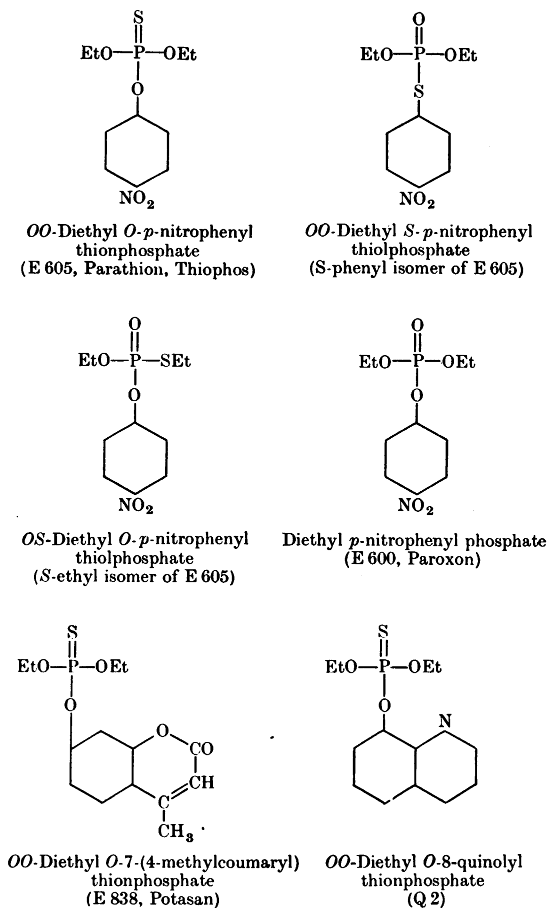
In the first paper of this series (Aldridge & Davison, 1952) it was shown that inhibition of cholinesterase by a series of substituted diethyl phenyl phosphates increased as their stability to hydrolysis decreased. During this work it was found that many of the inhibitors contained a small amount of a more active inhibitor as an impurity and evidence was produced that this was tetraethyl pyrophosphate (TEPP).

Diggle & Gage (1951*a*) have recently shown that a chromatographically purified preparation of diethyl *p*-nitrophenyl thionphosphate (E605) has an extremely low inhibitory power against cholinesterase *in vitro*. In this laboratory this observation has been confirmed using the same sample (Aldridge & Barnes, 1952). However, many workers have stated that E605 is an active inhibitor of cholinesterase *in vitro* (Aldridge, 1950; DuBois, Doull, Salerno & Coon, 1949; Engbaek & Jensen, 1951; Grob, 1950; Hecht & Wirth, 1950; Metcalf & March, 1949; Sallé, 1950; Wirth, 1949). In a report of work carried out during the war years, Schrader (1951) has stated that E605 isomerizes upon distillation and devised a series of tests to determine the diethyl *p*-nitrophenyl thionphosphate content, its purity and freedom from *S*-ethyl isomer. In Table 1 are shown the formulae of the three possible isomers of E605. The whole question of the purity and *in vitro* activity of E605 against cholinesterase has been complicated by the fact that although pure E605 has a low inhibitory power *in vitro* it is still highly toxic to animals and is converted *in vivo* to a more active inhibitor of cholinesterase (Diggle & Gage, 1951*b*; Aldridge & Barnes, 1952). In this paper we have tried to determine the true *in vitro* activity of E605 and its isomers. This has entailed investigation into the purity of our specimens of inhibitors. The specimen of E605 previously used by one of us (Aldridge, 1950) has also been re-examined and it has been found that most of its inhibitory activity is due to diethyl *p*-nitrophenyl phosphate (E600).

A 'hydrolysis technique' which takes advantage of their different rates of hydrolysis in buffers (Aldridge & Davison, 1952) was developed for the

detection of unstable active inhibitors present as impurities. In the examination of the purity of the inhibitors used in this paper (Table 1) this technique has been considerably extended and, with a method for the determination of inhibitors by their inhibitory power, it has been possible to suggest the identity of impurities. With preparations of thionphosphates whose purity has been established by

Table 1. *Inhibitors used*



OO-Diethyl *O*-*p*-nitrophenyl thionphosphate (E 605, Parathion, Thiophos)

OO-Diethyl *S*-*p*-nitrophenyl thiolphosphate (S-phenyl isomer of E 605)

OS-Diethyl *O*-*p*-nitrophenyl thiolphosphate (*S*-ethyl isomer of E 605)

Diethyl *p*-nitrophenyl phosphate (E 600, Paroxon)

OO-Diethyl *O*-7-(4-methylcoumaryl) thionphosphate (E 838, Potasan)

OO-Diethyl *O*-8-quinolyl thionphosphate (Q 2)

the methods outlined above, the relation between stability to hydrolysis and *in vitro* inhibitory power has been determined. These additional compounds confirm the relationship previously demonstrated (Aldridge & Davison, 1952).

METHODS AND MATERIALS

Cholinesterase of sheep erythrocytes has been determined by the manometric method given in detail previously (Aldridge & Davison, 1952).

In the 'hydrolysis technique' the stability of inhibitors has been determined in two buffers. The buffer used for the manometric determination of cholinesterase contains $0{\cdot}0357$ M-$NaHCO_3$, $0{\cdot}040$ M-$MgCl_2$, and $0{\cdot}164$ M-NaCl. This solution has been used for an examination of the hydrolysis of the *S*-ethyl isomer of E 605. When not gassed with 5% CO_2 in N_2 it has a pH of 8–8·5. The hydrolysis of other inhibitors has been studied in a solution at pH 10·8 containing $0{\cdot}035$ M-Na_2CO_3, $0{\cdot}164$ M-NaCl. Rates of hydrolysis of inhibitors in these buffers have been determined chemically by colorimetric estimation of the *p*-nitrophenol or *p*-nitrothiophenol liberated and also biochemically from the inhibitory power of the solution against cholinesterase. The results of such determinations have been plotted logarithmically in the usual manner to test for first-order kinetics and the velocity constants have been calculated. Throughout this paper the stability of inhibitors has been expressed by these constants (cf. Table 3). Diethyl 8-quinolyl thionphosphate (Q 2) was purified chromatographically using alumina (Peter Spence, type H) and a benzene-light petroleum (b.p. 80–100°) mixture in the proportions 1:2. The amount of esterified 8-hydroxyquinoline was determined by hydrolysis in NaOH followed by its estimation colorimetrically by coupling with diazotized sulphanilic acid. The colour was read at 510 mμ.

Rate constants for the reaction of inhibitors with cholinesterase have been calculated from the slope of the best straight line (by the method of least squares) passing through the points obtained when log (% residual activity) is plotted against concentration (M) × time (min.).

We are grateful to Dr A. H. M. Kirby for the pure E 838 and to Mr B. Topley (Albright and Wilson Ltd.) for the rest of the inhibitors. We are particularly grateful to Mr Topley and Dr Coates for the specimen of purified E 605. This sample was very laboriously purified by a combination of a chromatographic procedure and repeated fractional crystallization at low temperatures.

RESULTS

The determination of the concentration of inhibitors. The inhibition of red cell cholinesterase by E 600 and analogues shows the characteristics of a bimolecular reaction with one component in excess:

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

where K = bimolecular rate constant, t = time in min., I = molar concentration of inhibitor and b = percentage residual activity. If the time is maintained constant, a plot of I against $\log_{10} b$ will give a straight line and $\log_{10} 100/b$ may be used as a measure of inhibitor concentrations. Such a measure may be used when studying hydrolyses which have first-order kinetics and absolute concentrations are not required. In Fig. 1 the relationship is shown between the concentration of E 600 and the inhibition of sheep erythrocyte cholinesterase.

A comparison of the rate of loss of inhibitory power against cholinesterase and the rate of hydrolysis of inhibitors determined by chemical means has enabled impurities present in extremely small amounts to be detected (hydrolysis technique). By also using the quantitative method given above it

has been possible to determine the rate of hydrolysis of the impurity and thus to suggest its identity by comparison with the stability of known inhibitors. When the stability of the impurity and the compound are sufficiently different the technique may be used to determine quantitatively highly active and unstable inhibitors in more stable compounds as well as stable impurities in unstable inhibitors.

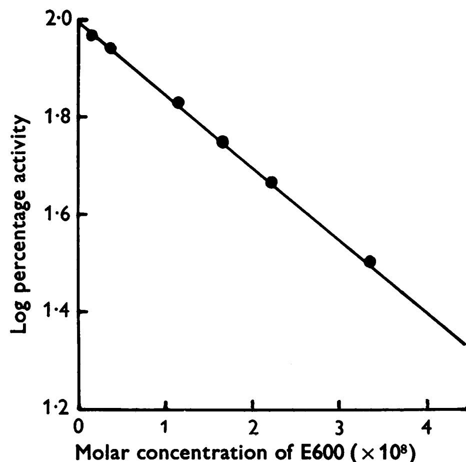


Fig. 1. The inhibition produced by incubation of sheep erythrocyte cholinesterase for 30 min. at 37° with concentrations of E600.

Inhibitory power of two inhibitors together. It has been assumed that the inhibition of cholinesterase by one phosphate ester inhibitor is not affected by the presence of another. In a previous paper (Aldridge & Davison, 1952) the concentration of TEPP in a known mixture with diethyl *p*-chlorophenyl phosphate was determined biochemically from its inhibitory power against cholinesterase under standard conditions. Even though the concentration of diethyl *p*-chlorophenyl phosphate was 150 times that of the TEPP, the determined result was in agreement with the prepared concentration. This can be interpreted as indicating that the inhibition by one inhibitor is not influenced by the other. It was thought desirable to check this point further. The relation between the inhibitory power of inhibitors separately is given by

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

and

$$K' = \frac{1}{tI'} \ln \frac{100}{b'}. \quad (2)$$

If these inhibitors do not interfere with the action of each other then from equations (1) and (2)

$$KtI + K'tI' = \ln \frac{100}{b} + \ln \frac{100}{b'}.$$

When t is maintained constant and using for convenience logarithms to the base 10 and K_1 and K_2 for K and K' respectively to account for these changes,

$$K_1 I + K_2 I' = \log_{10} \frac{100}{b} + \log_{10} \frac{100}{b'}.$$

If the concentrations of inhibitors, I and I', reduce the activity of the cholinesterase after incubation for a standard time to b and b'% of the original activity respectively then the inhibition due to both together may be derived from

$$\log_{10} \frac{100}{b} + \log_{10} \frac{100}{b'}.$$

This relation has been tested using mixtures of diethyl phenyl phosphate and E600. The phenyl compound is present in great excess over the E600, i.e. in one of the experiments 10^{-3} M as compared with 10^{-8} M E600. The results in Table 2 show that the inhibition obtained confirms the relationship derived above and with mixtures of inhibitors of this type little interference of one inhibitor with another is likely.

OO-*Diethyl* O-p-*nitrophenyl thionphosphate*, E605. Diggle & Gage (1951*a*) state that the activity of a series of samples of E605 as inhibitors of cholinesterase paralleled their content of *S*-ethyl isomer of E605. Clearly, the assessment of these results

Table 2. *The inhibition of cholinesterase by two inhibitors together*

(E600 and diethyl phenyl phosphate were incubated separately and together with washed sheep erythrocytes for 30 min. at 37°. The cholinesterase activity remaining was determined. The concentrations used were for Exp. 1, E600 2×10^{-8} M, diethyl phenyl phosphate $9 \cdot 8 \times 10^{-4}$ M; for Exp. 2, E600 2×10^{-8} M, diethyl phenyl phosphate $8 \cdot 4 \times 10^{-3}$ M; and for Exp. 3, E600 $1 \cdot 25 \times 10^{-8}$ M, diethyl phenyl phosphate $4 \cdot 0 \times 10^{-3}$ M. b = % residual activity.)

	Diethyl phenyl phosphate		E600		Diethyl phenyl phosphate + E600: Determined values		Diethyl phenyl phosphate + E600: Calculated values	
Exp. no.	Activity (%)	(1) $\log_{10} \frac{100}{b}$	Activity (%)	(2) $\log_{10} \frac{100}{b}$	Activity (%)	$\log_{10} \frac{100}{b}$	Activity (%)	(1)+(2) $\log_{10} \frac{100}{b}$
1	83·4	0·079	48·1	0·318	41·7	0·380	40·1	0·397
2	21·0	0·678	46·9	0·329	12·0	0·921	10·1	1·007
3	47·5	0·323	66·2	0·179	31·1	0·507	31·7	0·502

Table 3. *The rate of hydrolysis of inhibitors in sodium carbonate at* pH 10·8 *and* 37°

(The rate of hydrolysis of inhibitor has been determined chemically by the estimation of *p*-nitrophenol and *p*-nitrothiophenol liberated, or biochemically by the determination of the inhibitory power of the solution against sheep erythrocyte cholinesterase (see text, p. 664). All hydrolysis rates were determined in a solution containing Na_2CO_3 (0·035 M), NaCl (0·164 M), except the *S*-ethyl isomer which was hydrolysed too quickly in this medium. The expected rate at pH 10·8 was calculated from values at pH 7·6 (0·067 M-phosphate) and pH 9·64 (Michaelis veronal-acetate) using the expression $K = K_{H_2O} + K_{OH}[OH]$ and neglecting any catalysis by $(H_3O)^+$ ions.)

Inhibitor	Hydrolysis determined chemically: First-order constant (min.$^{-1}$)	Hydrolysis determined chemically: Time for 95% hydrolysis (hr.)	Hydrolysis determined biochemically: First-order constant (min.$^{-1}$)
E600	2.7×10^{-3}	18·5	2.9×10^{-3}
E605	4.1×10^{-4}	122	3×10^{-4}
S-ethyl isomer	5×10^{-1}	Approx. 0·1	—
S-phenyl isomer	9.6×10^{-2}	0·53	—
Impurity in *S*-ethyl isomer	—	—	3.1×10^{-3}
Impurity in E605	—	—	2.5×10^{-3}

depends on the specificity of the method of estimation (Gage, 1952) of this isomer in complex mixtures. It will be shown later that the specimen of purified *S*-ethyl isomer used by Diggle & Gage contained roughly 4% of E600. The chemical structure of E605 makes it unlikely that it would have no inhibitory activity against cholinesterase. It was, therefore, decided to see if pure E605 had any activity or whether the low activity of the purest sample available is also due to a trace of the *S*-ethyl isomer. Because of the extreme instability of the *S*-ethyl isomer in aqueous solution compared with E605 (cf. Tables 3 and 4) it was possible to investigate this point. A solution of the sample of purified E605 was prepared in sodium carbonate solution (pH 10·8). Under these conditions 95% of any *S*-ethyl isomer present would be hydrolysed in 6 min., the *S*-phenyl isomer in 32 min. and E600 in 18 hr. (Table 3). When the inhibitory power of the solution was determined at various times it was found that there was 75% of the activity left at 20 hr. It is clear that the inhibitory power cannot be due to any of the above three inhibitors. The activity of the solution fell exponentially and the rate constant in Table 3 shows that the activity falls at roughly the same speed as the hydrolysis of E605 determined chemically. In view of the low solubility of E605 and the low inhibitory power of the saturated solution this is as good an agreement as can be expected, and indicates that the anticholinesterase activity of the solution is probably due to E605 itself.

The first-order rate constant for the hydrolysis of E605 has been determined in phosphate buffer, pH 7·6, at 37°, one of the hydrolysis products, *p*-nitrophenol, being estimated colorimetrically. The rate constant for the reaction of E605 with erythrocyte cholinesterase has also been determined (Table 4). In Fig. 2 these values are plotted, and it is interesting that this point falls near the line established from previous work on E600 analogues (Aldridge & Davison, 1952), i.e. the activity of this purified E605 is approximately what one would expect from its stability to hydrolysis.

E605 previously studied. Previously, one of us (Aldridge, 1950) studied a specimen of E605 which must have been impure. First-order kinetics were obtained for its reaction with cholinesterase and the rate constant for this reaction was 2×10^4 (l. mole^{-1} min.$^{-1}$), whereas it has now been shown that a purified E605 gives a rate constant of 1.2×10^2 (l. mole^{-1} min.$^{-1}$). Diggle & Gage (1951*a*) have demonstrated a relation between the *S*-ethyl isomer content of various specimens of E605 and their inhibitory activity. As will be shown later, the inhibition of cholinesterase by *S*-ethyl isomer does not follow first-order kinetics. It therefore seemed improbable that *S*-ethyl isomer was responsible for the activity, and an examination of this specimen of E605 has been made. Its inhibitory power has remained unaltered since it was first examined and at a concentration of 1.3×10^{-6} M it inhibits sheep erythrocyte cholinesterase 50% after incubation for 30 min. at 37°. The rate of loss of inhibitory power of a solution of this inhibitor in sodium carbonate, pH 10·8, has been determined. The rate constant for this hydrolysis is given in Table 3, and indicates that the impurity cannot be the *S*-ethyl or the *S*-phenyl isomer and is in all probability E600. 1·6% E600 would account for the inhibitory power of this specimen of E605.

OO-*Diethyl* S-p-*nitrophenyl thiolphosphate* (S-*phenyl isomer*). This isomer is a pale yellow crystalline solid and is therefore much more readily purified than a liquid with a high boiling point. It is a highly active inhibitor producing 50% inhibition at 37° in 30 min. at a concentration of 2.8×10^{-8} M. The graph of $\log_{10}$ residual activity against the time of incubation of inhibitor with cholinesterase shows that the lines are slightly curved (Fig. 3). This can be

accounted for by the expected loss of inhibitor due to aqueous (non-enzymic) hydrolysis alone. In phosphate buffer, pH 7·6, at 37° the first-order hydrolysis constant is $5{\cdot}3 \times 10^{-4}$ (min.$^{-1}$); in 2 hr. therefore 7% of the inhibitor would have been destroyed by this means.

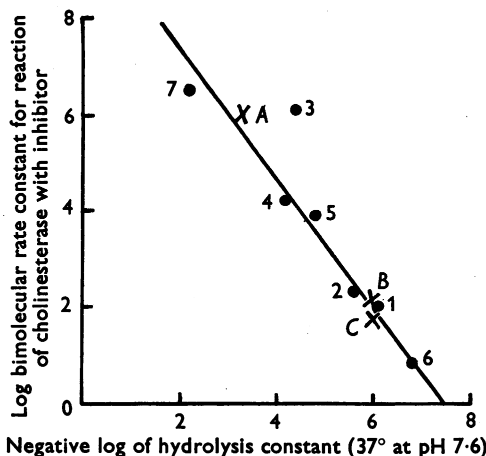


Fig. 2. The relation between stability to hydrolysis and inhibitory power. ●, points taken from Aldridge & Davison (1952). ×, compounds studied in this paper. 1–7 derivatives of diethyl phosphoric acid: 1, *p*-chlorophenyl; 2, *o*-chlorophenyl; 3, *p*-nitrophenyl (E600); 4, *o*-nitrophenyl; 5, *m*-nitrophenyl; 6, phenyl; 7, tetraethyl pyrophosphate. *A*, *S*-phenyl isomer of E605; *B*, E605; *C*, Q2.

Table 4. *Velocity constants for the reaction of inhibitors with cholinesterase and for their hydrolysis in phosphate buffer*, pH 7·6

(Rates of hydrolysis were determined colorimetrically by the estimation of *p*-nitrophenol or *p*-nitrothiophenol liberated for all inhibitors except Q2. A rough value for Q2 has been determined by calculation from the loss of inhibitory power at 37° in Na_2CO_3 solution, pH 10·8 (Na_2CO_3 (0·035 M), NaCl (0·164 M)) and in a $NaHCO_3/Na_2CO_3$ solution, pH 8·96 (Na_2CO_3 (0·0037 M), $NaHCO_3$ (0·0238 M), NaCl (0·164 M)) using the expression $K = K_{H_2O} + K_{OH}[OH]$ and neglecting any catalysis by $(H_3O)^+$ ions. The velocity constants for reaction with cholinesterase were calculated from the slope of the best straight line obtained on plotting concentration of inhibitor (M) × time (min.) against ln % activity of cholinesterase.)

Inhibitor	Rate constant (*K*) for reaction of inhibitor with cholinesterase (l.mole^{-1} min.$^{-1}$)	Rate constant (*K*) for hydrolysis of inhibitor at pH 7·6 in phosphate buffer (min.$^{-1}$)
E600	$1{\cdot}1 \times 10^6$	$3{\cdot}9 \times 10^{-5}$
E605	$1{\cdot}2 \times 10^2$	$9{\cdot}6 \times 10^{-7}$
S-Ethyl isomer of E605	—	$1{\cdot}1 \times 10^{-3}$
S-Phenyl isomer of E605	$9{\cdot}7 \times 10^5$	$5{\cdot}3 \times 10^{-4}$
Q2	$4{\cdot}6 \times 10^1$	Approx. 1×10^{-6}
E838	$1{\cdot}5 \times 10^2$	—

The constants obtained for this inhibitor fit the relationship between rate of reaction with cholinesterase and stability to hydrolysis (Fig. 2).

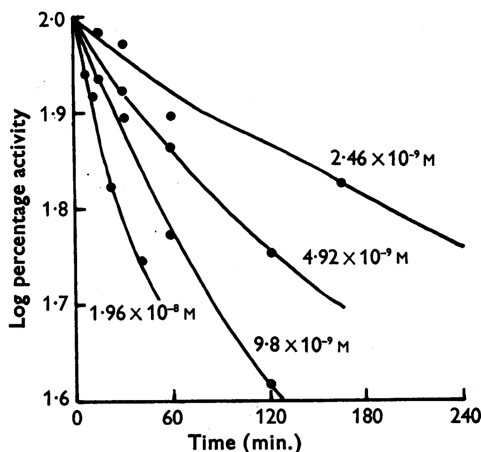


Fig. 3. The inhibition of sheep erythrocyte cholinesterase by the *S*-phenyl isomer of E605. Concentrations of *S*-phenyl isomer shown against each curve.

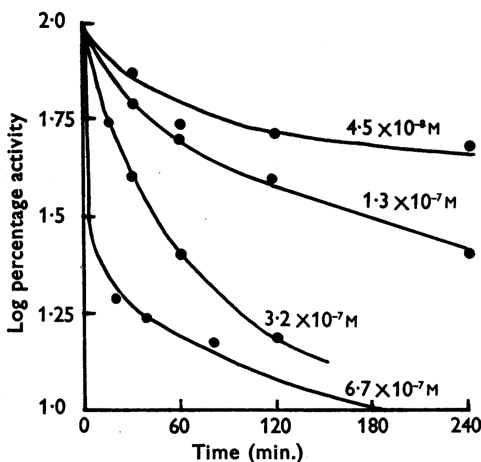


Fig. 4. Inhibition of sheep erythrocyte cholinesterase by *S*-ethyl isomer of E605 (impure). Concentration of *S*-ethyl isomer shown against each curve.

OS-*Diethyl* O-p-*nitrophenyl thiolphosphate* (S-*ethyl isomer*). The rate of reaction of this inhibitor with cholinesterase was determined at several concentrations. Fig. 4 shows that a series of curves was obtained when log (% residual activity) was plotted against time. These results resembled those obtained with the analogues of E600 which were found to be contaminated with TEPP (Aldridge & Davison, 1952). TEPP is destroyed by sheep erythrocytes. An initial rapid inhibition due to the TEPP was obtained, but after 20–30 min. this had all been destroyed and the residual rate was due to

the E600 analogue being examined. The *S*-ethyl isomer is unstable in water and the first-order rate constant at pH 7·6 for its hydrolysis is $1{\cdot}1 \times 10^{-3}$ (min.$^{-1}$) (Table 4). This means that during the 240 min. incubation of inhibitor with cholinesterase (Fig. 4) approximately 22 % of the inhibitor would have been hydrolysed. Although sheep erythrocytes do not catalyse the hydrolysis of E600, experiments were carried out to see if the *S*-ethyl isomer was hydrolysed. When *S*-ethyl isomer (approx. 1 mg./ml.) was incubated in Warburg vessels, little difference in output of CO_2 was observed with and without erythrocytes. This indicates that the hydrolysis of *S*-ethyl isomer by sheep erythrocytes must be small. When erythrocytes were incubated with a dilute solution of *S*-ethyl isomer, samples withdrawn at various times, the cells centrifuged down and the supernatant re-incubated with fresh erythrocytes, no loss of inhibitory power of the solution could be demonstrated (Table 5). This experiment should assess the total loss of *S*-ethyl isomer due to both non-enzymic and enzymic hydrolysis. It is clear that there was in the solution an inhibitor which was not destroyed under these conditions.

Table 5. *Inhibitor remaining in solution after incubation of sheep erythrocytes with* S-*ethyl isomer*

(*S*-Ethyl isomer, $5{\cdot}6 \times 10^{-7}$ M, was incubated with washed sheep erythrocytes. The erythrocytes were centrifuged down after various times of incubation and the inhibitory power of the supernatant was determined by incubation with fresh erythrocytes for 30 min.)

Time of incubation (min.)	Activity of cholinesterase of erythrocytes after incubation with supernatant (%)
0	20
10	21
20	20
40	20
90	18

The observation that the rate of inhibition decreased with time (Fig. 4) suggested that an equilibrium was being reached between enzyme and inhibitor. If this were so it would be expected that the inhibition, like that due to eserine, would be reversible. Repeated washing of erythrocytes after 4 hr. incubation with inhibitor gave no increase in enzyme activity, however.

These observations differ from our previous experience with inhibitors of this type. Pure substituted diethyl phenyl phosphates all give first-order kinetics and the rate is directly proportional to the concentration of inhibitor. However, abnormal kinetics were obtained when the original unpurified inhibitors contained as an impurity the highly active and unstable TEPP. It was therefore important to consider the purity of our specimen of *S*-ethyl isomer.

There is no specific chemical method for the determination of *S*-ethyl isomer, and evidence of purity must be deduced from other data. Topley (personal communication) has carried out detailed kinetic measurements on the hydroxyl ion-catalysed hydrolysis of this specimen of *S*-ethyl isomer. First-order kinetics were obtained with no detectable drift in the rate constant. It was concluded that the compound is at least 90 % pure. The measurements we have made from which the rate constant given in

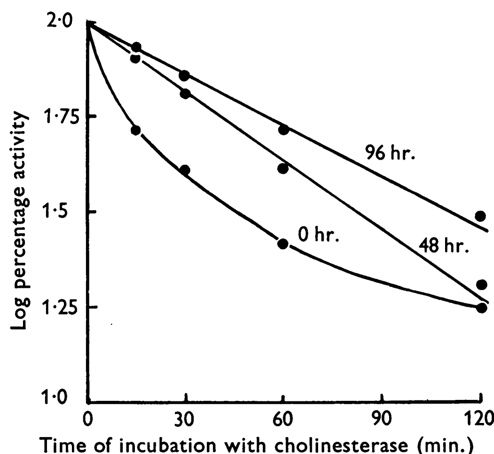


Fig. 5. Inhibitory activity of $2{\cdot}24 \times 10^{-6}$ M-*S*-ethyl isomer of E605 in bicarbonate buffer, pH 8·4, after incubation at 37°. Time of incubation shown against each curve. The solution was diluted seven times for incubation with cholinesterase to determine inhibitory power.

Table 4 was calculated confirm this view, and elementary analysis is not at variance with it. However, when hydrolysis of the isomer was followed in bicarbonate buffer, pH 8·4, at 37°, estimating the inhibitory power of the solution against cholinesterase, results were obtained which did not agree with chemical measurements. When the isomer had decomposed (as shown by the liberation of *p*-nitrophenol) the solution still possessed most of its inhibitory power (Fig. 5). In contrast to the initial results, the rate of inhibition of cholinesterase by the solution after 48 hr. incubation follows first-order kinetics. It was deduced from these observations that our *S*-ethyl isomer contained as an impurity an active inhibitor of cholinesterase which is considerably more stable than *S*-ethyl isomer. An attempt was therefore made to identify this impurity. When a solution of the isomer which has been left for 7 days in bicarbonate buffer at room temperature is extracted with chloroform, washed free from *p*-nitrophenol with bicarbonate, the chloroform evaporated and the residue taken up in buffer, the resulting solution

inhibited cholinesterase and the rate of inhibition followed first-order kinetics. This solution, which lost its activity slowly over the following weeks, became pale yellow. The yellow colour was extracted with *n*-butanol from acid solution and then transferred back to a small volume of buffer. The visible and ultraviolet absorption spectra of this solution, both acid and alkaline, were identical with that of *p*-nitrophenol.

The impurity present is more stable than the *S*-ethyl isomer and liberates *p*-nitrophenol upon hydrolysis. Diethyl *p*-nitrophenyl phosphate (E 600) is a highly active inhibitor which fulfils these conditions. This possibility has been examined. It has been shown that the inhibitory power of a solution of the isomer prepared 1 hr. previously in sodium carbonate solution, pH 10·8, falls at the same rate as that of a solution of E 600. This value agrees with the rate of hydrolysis of E 600 determined chemically (Table 3). In sodium carbonate buffer, pH 10·8, and at 37° the *S*-ethyl isomer itself will be 95% hydrolysed in 6 min. and any *S*-phenyl isomer in 32 min. It is concluded, therefore, that the impurity is E 600. An estimate of the amount of E 600 present may be obtained from the curves given in Fig. 5 on the assumption that all the activity at 48 and 96 hr. is due to E 600. The concentrations of E 600 necessary to produce this inhibition are calculated from the known bimolecular rate constant ($1 \cdot 1 \times 10^6$ l.mole^{-1} min.$^{-1}$) and this is then corrected for aqueous hydrolysis back to zero time. By this means we obtain 4·5 and 4·1% from the results after incubation for 48 and 96 hr. respectively.

OO - *Diethyl* O - 7 - (4 - *methylcoumaryl)thionphosphate* (*E*838). This inhibitor which is crystalline (m.p. 41·5°) shows little activity *in vitro* against erythrocyte cholinesterase. A saturated solution contains approximately 5 µg./ml. and at this concentration ($1 \cdot 3 \times 10^{-5}$ M) only 54% inhibition is produced in 5 hr. No loss of activity of a solution of this compound occurred after incubation at 37° for 14 days at pH 8. It is, therefore, concluded that this sample of inhibitor did not contain any unstable active inhibitor as an impurity. Because of its low solubility and activity the data on the kinetics of inhibition are limited. Sufficient has been obtained to suggest that first-order kinetics are obtained and on the assumption that it is bimolecular the rate constant has been calculated (Table 4). Due to the lack of a method of high sensitivity for the determination of 7-hydroxy-4-methylcoumarin we have been unable to determine the rate of hydrolysis of E838.

OO - *Diethyl* O - 8 - *quinolyl thionphosphate* (*Q*2). Aldridge (1950) showed that inhibition by this substance followed first-order kinetics, but the straight line obtained when $\log_{10}$% activity was plotted against time did not pass through the zero. Part of this inhibition was reversible and the amount below the zero where the lines cut the ordinate [2 − ($\log_{10}$ % activity)] was a measure of this reversible inhibition. In view of the impurities in other inhibitors derived from thiophosphoric acid this compound has been re-examined. A solution of Q2 in a 1:2 benzene-light petroleum mixture was passed through an alumina column. A preparation of Q2 was obtained which contained the theoretical amount of combined 8-hydroxyquinoline. When the inhibitory power of this purified sample was examined, first-order kinetics were obtained (Fig. 6) and the straight lines passed through the zero (100% activity).

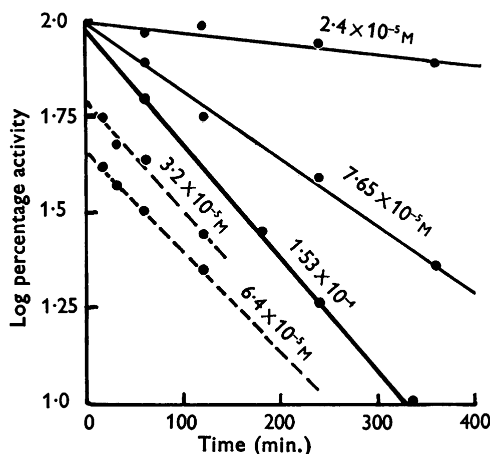


Fig. 6. Inhibition of sheep erythrocyte cholinesterase by Q2. Concentrations of inhibitor shown against each curve. ——, purified as in text; - - - - -, unpurified sample as used by Aldridge (1950).

Further determinations with the unpurified sample confirmed the original observations of Aldridge (1950), but the material(s) responsible for the reversible inhibition have not been identified. Since Q2 is only soluble to approx. 40 µg./ml. long incubation times have been used in order to obtain reasonable inhibitions. The reaction appears to be bimolecular and the rate constant has been determined from the limited data available (Table 4).

DISCUSSION

Many investigators have shown that E 605 is an *in vitro* inhibitor of cholinesterase. Estimates of the inhibitory power of different samples have varied, but until the observations of Diggle & Gage (1951*a*) it was not necessary to consider the possibility that E 605 is converted *in vivo* to a more active inhibitor of cholinesterase. Evidence has been produced that this is so (Diggle & Gage, 1951*b*; Aldridge & Barnes, 1952). Diggle & Gage (1951*a*) have concluded that the inhibitory power of the specimens of E 605 they

examined was due to their content of *S*-ethyl isomer. However, Gage (1952) states that using his methods of analysis the highly active inhibitors, *S*-phenyl isomer and E600, will both behave like the relatively inactive E605 and will be reported as such. Evidence has been presented which indicates that pure E605 has an *in vitro* inhibitory activity of its own. The *S*-ethyl isomer studied in this paper is from the same preparation that Diggle & Gage (1951*a*) used as their standard. We have shown that it contains approximately 4% E600. The high inhibitory activity of a specimen of E605 used previously by one of us (W.N.A.) has been explained by the presence of 1·6% E600. It is clear that caution must be observed before any generalizations are made about the nature of the impurities in various E605 preparations. The known possibilities are the *S*-ethyl isomer, the *S*-phenyl isomer and E600, and it is probable that the proportions of these substances present will depend on the methods of preparation and purification. It is clear from the reports of the work of Schrader (1951) that any form of distillation is inadvisable as a method of purification.

In a previous paper (Aldridge & Davison, 1952) it was demonstrated that as the stability to hydrolysis of analogues of E600 increased their *in vitro* inhibitory power against cholinesterase decreased. The results in this paper show that the *S*-phenyl isomer fits well into this relation and the limited data obtained with E605 and Q2 show that there is no reason to doubt that they also fit the same relation. The hydrolysis constant for E838 could not be determined either by chemical or biochemical methods. The *S*-ethyl isomer does not have the inhibitory activity expected from its stability to hydrolysis. Its first-order hydrolysis constant is $1{\cdot}1 \times 10^{-3}$ (min.$^{-1}$) in phosphate buffer, pH 7·6, at 37°. From the relation shown in Fig. 2, *S*-ethyl isomer should have a bimolecular rate constant for its reaction with cholinesterase of about 10^6 (l.mole^{-1} min.$^{-1}$). This value corresponds to 50% inhibition after incubation for 30 min. at 37° with a concentration of 2×10^{-8}M. It is clear from Fig. 4 that 50% inhibition is produced by approximately 3×10^{-7}M and this is uncorrected for the inhibitory power of the 4% E600 present in this specimen of isomer. This isomer does not give the usual first-order kinetics on reaction with cholinesterase, but until we have obtained a purer specimen of this inhibitor any suggestions of reasons for such behaviour would be speculative. It is interesting that Kilby & Youatt (1952) have recently shown that, acting on trypsin, the *S*-ethyl isomer is a more potent inhibitor than E600.

Many organo-phosphorus compounds are being prepared by research organisations. Traces of impurities may profoundly alter their biological activity. Without further evidence statements of inhibitory power (as concentrations to produce 50% inhibition) must be regarded as only applying to the particular specimen of inhibitor which has been examined. The 'hydrolysis technique' we have developed for testing inhibitors for small amounts of impurities is based on the determination of the concentration of an inhibitor by its inhibitory power. It is, therefore, possible to compare the rate of hydrolysis of the main constituent determined by chemical methods and the rate of loss of inhibitory power determined biochemically. When these do not agree impurities should be suspected and often these may be identified from their hydrolysis rates under standard conditions. These tests will detect unstable impurities in stable inhibitors (TEPP in analogues of E600) and stable impurities in unstable inhibitors (E600 in *S*-ethyl isomer). It is not possible to detect impurities of a similar stability. Many impurities may be eliminated by rigorous purification of the materials used for synthesis but others may arise through side reactions. Traces of impurities in liquids with high boiling points may be extremely difficult to remove by the conventional chemical techniques. The purification achieved can, at present, only be assessed by a biochemical examination.

Bimolecular rate constants are a better measure of inhibitory power than the conventional concentration for 50% inhibition. An examination of the kinetics of inhibition will often indicate the presence of impurities. These will remain undetected with a simple determination of 50% inhibition concentration. TEPP which is at least 90% hydrolysed in 25 min. by sheep erythrocytes gives an apparently normal inhibition curve on plotting $\log_{10}$M concentration of inhibitor against percentage inhibition. In all cases except one (*S*-ethyl isomer) abnormal kinetics of inhibition have been shown to be due to impurities, or to aqueous and enzymic hydrolysis of the inhibitor or a combination of both factors. However, if first-order kinetics are obtained it does not necessarily mean that the sample is pure. This is quite clear from the results on a specimen of E605 containing 1·6% E600 (Aldridge, 1950).

In conclusion, it is suggested that an examination of the kinetics of inhibition of erythrocyte cholinesterase together with the application of the 'hydrolysis technique' will help in the detection and sometimes the determination of active impurities in organo-phosphorus inhibitors. The converse is that before it is stated that the reaction of an inhibitor with erythrocyte cholinesterase does not follow first-order kinetics, it must be demonstrated that the inhibitor is not appreciably hydrolysed during the experiment either enzymically or by aqueous hydrolysis and also that it does not contain highly active impurities.

SUMMARY

1. Pure E605 has a low *in vitro* activity against cholinesterase.

2. A specimen of E605 previously used (Aldridge, 1950) owed its high activity to the presence of E600.

3. The *S*-phenyl isomer of E605 is a highly active inhibitor and the inhibition follows first-order kinetics. The specimen of *S*-ethyl isomer contains approximately 4% E600.

4. It was observed (Aldridge & Davison, 1952) that in a series of diethyl phenyl phosphates substituted in the aromatic ring, the more stable to hydrolysis the inhibitor the less effective it is as an inhibitor. This relationship has been extended to include E605, Q2 and the *S*-phenyl isomer of E605.

5. A 'hydrolysis technique' has been developed and kinetic measurements have been utilized for the detection and determination of impurities in organophosphorus inhibitors. These methods are fully discussed.

Our thanks are due to Miss J. Wheatley for valuable technical assistance.

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Amylases of *Clostridium butyricum* and a *Streptococcus* Isolated from the Rumen of the Sheep

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(*Received* 13 *March* 1952)

In the course of an investigation into the digestion of starch by the pig, Baker, Nasr, Morrice & Bruce (1951) isolated a micro-organism responsible for the breakdown of starch in the caecum and identified it as a strain of *Clostridium butyricum*. Later Masson (1951) isolated another strain of *Cl. butyricum* which was the chief agent of starch breakdown in the rumen of a sheep fed on a flaked maize and hay diet. Continuing these investigations into the digestion of starches by the sheep, MacPherson (forthcoming publication) obtained several strains of a *Streptococcus*, probably related to *Strep. bovis*, from the rumen, one being selected for use throughout this work. The extracellular amylase formed when the pig caecum strain of *Cl. butyricum* was grown on a medium containing soluble starch was examined by Whelan & Nasr (1951) and was shown to be of the α-amylase type. Both the sheep rumen *Clostridium* and *Streptococcus* formed an extracellular amylase when grown in a medium containing dissolved starch, and an investigation of these amylases is the subject of this paper. The amylases would appear to be constitutive and not adaptive enzymes since, when the *Clostridium* was grown in a medium containing maltose as the only carbohydrate, and the *Streptococcus* on a glucose-containing medium, the other constituents being the same as in the starch medium, amylase activity was present in the cell-free filtrates.

There are at present known to be two major groups of starch-hydrolysing enzymes, the α- and β-amylases. In addition, there is the amylase of *Bacillus macerans* which has a synthetic as well as a hydrolytic function, as it catalyses the synthesis of cyclic 'Schardinger dextrins' from starch and starch fractions. Recently, also, various authors have reported the isolation of a maltase from a bacterium and moulds which is also capable of hydrolysing starch entirely to glucose (French & Knapp, 1950; Kerr, Cleveland & Katzbeck, 1951; Philips & Caldwell, 1951 *a*, *b*). The only sugar produced during the hydrolysis of starch-type polysaccharides by β-amylase, which is found in ungerminated cereals, is maltose. Until recently it was thought that amylose, the 'linear' component of starch, was entirely