RESULTS AND DISCUSSION

The maximum copper retaining power of the humic acid tested, 1600 μequiv. copper/g. humic acid, agrees reasonably well with the figure of 1400 μequiv./g. obtained indirectly on whole soils (Lees, 1948). Humic acid contains about 56% carbon (Page & duToit, 1932); therefore some 60 carbon atoms are associated with one copper atom in the fully saturated complex of copper and humic acid.

SUMMARY

1. The copper-retaining power of a humic acid from a Trinidad peat soil was 1600 μequiv. copper/g. humic acid. This is in agreement with previous results.

2. The atomic ratio of carbon:copper in the complex so formed is about 60:1.

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Some Properties of Specific Cholinesterase with Particular Reference to the Mechanism of Inhibition by Diethyl *p*-Nitrophenyl Thiophosphate (E 605) and Analogues

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A full account of the properties of substances inhibiting cholinesterase has been given in a recent review by Koelle & Gilman (1949). In the following work the properties of certain organic esters of phosphoric acid which are powerful inhibitors of cholinesterase are described. In particular, the kinetics of the inhibitor-enzyme system have been worked out and certain conclusions have been drawn as to the mode of action of these inhibitors. The properties of a convenient preparation of cholinesterase which has been used for this work are given below.

METHODS

For the cholinesterase preparation blood was taken from a goat into a flask containing 2 ml. of 30% (w/v) Na citrate per 60 ml. of blood, and was stored in the refrigerator at 4°. Washed cells lose their activity on storage. Prior to use in an experiment, the blood was centrifuged, the plasma removed and the cells washed three times with isotonic saline. They were then resuspended in buffer and diluted roughly to the original volume of blood taken.

Determination of cholinesterase activity was carried out using the method of Nachmansohn & Rothenberg (1945). The final concentrations in the Warburg flask, after tipping in the contents of the side arm, were NaCl, 0·15M; $MgCl_2$, 0·035M; $NaHCO_3$, 0·0312M and acetylcholine chloride 0·0138M. The pH of this buffer mixture, gassed with 5% CO_2 in N_2, is 7·72 (calculated from Henderson-Hasselbach equation). Stock salt solutions of 20 times the strength used in the preparation of the buffer were stored in the refrigerator in paraffin-waxed flasks. The buffer was likewise stored after dilution from these stock solutions. The total volume of fluid in each flask was 4·0 ml. except where otherwise stated. All measurements of red-cell suspensions were made with a Krogh-Keys syringe pipette. The contents of the flasks were gassed in the bath at 37° with a slow stream of 5% CO_2 + 95% N_2 for 10 min., and allowed to equilibrate for 10 min. After the addition of the acetylcholine from the side-arm, readings were taken every 10 min., for 1 hr. After a visual inspection of the readings to see that a straight line relationship between time and output of CO_2 was sensibly satisfied, the slope of the line (output in μl. of CO_2/min.) was determined by the method of least squares (Aldridge, Berry & Davies, 1949). The flasks were cleaned after each experiment by boiling in soap solution to remove all lanoline grease and protein, and once a day were further treated with ethanol and HNO_3, rinsed in distilled water and dried in a warm oven.

E 605 and its analogues, the formulae of which are given below, were dissolved initially in absolute ethanol (1 mg./ml.) and were diluted with buffer from that solution prior to the experiment. All the inhibitors were stored at 4° in the dark in bottles with stoppers sealed with paraffin wax.

Throughout this paper their code numbers (given in brackets) are used. They are all pale yellow liquids when pure, but both E 605 and Q 2 darken on standing, particularly when exposed to light. They all hydrolyse slowly in water to give a substituted phosphoric acid and *p*-nitrophenol or

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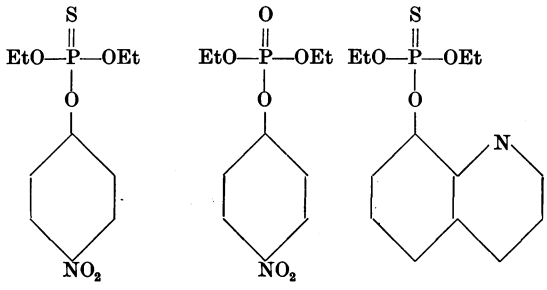
8-hydroxyquinoline. Their stability increases in the order E600, E605 and Q2. E600 is the most soluble in water (approx. 2 mg./ml.), while E605 and Q2 dissolve to the extent of approx. 20 and 40 μg./ml. respectively at 37°. Other inhibitors used were di-*iso*propyl fluorophosphonate (DFP), bis(β-chloroethyl)-*N*-methylamine hydrochloride (N mustard) and eserine sulphate.



p-Nitrophenyl diethyl thiophosphate (E605) | *p*-Nitrophenyl diethyl phosphate (E600) | 8-Quinolyl diethyl thiophosphate (Q2)

RESULTS

Red cells as a specific cholinesterase preparation. It has been shown by Paléus (1947), Brauer & Root (1945) and Augustinsson (1948) that the specific cholinesterase of red cells is attached to the red-cell membrane. It was therefore thought that intact red cells would provide a suitable preparation of cholinesterase for work with inhibitors; intact red cells afford a cleaner preparation than haemolysed cells, and it is also possible to wash them by centrifugation after inhibition of their cholinesterase and so demonstrate reversibility under appropriate conditions. Goat red cells have been used for two reasons: (1) goat serum and red cells contain little or no pseudo-cholinesterase as estimated by ability to split benzoylcholine (Mendel, Mundell & Rudney, 1943); and (2) it is possible to use the same animal repeatedly as a source of red cells. By this means it has been possible to use the activity of the fresh samples of blood as a rough check on the method; since the cholinesterase level of goat red cells from the same animal seems to remain constant for some months. The need for constant rechecking of the method and care in cleaning glass ware need no emphasis when working with such active inhibitors.

Table 1. *Properties of intact and haemolysed red cells*

Concentration of inhibitor (M)	Activity (μl. CO_2/min.)	
	Intact red cells	Haemolysed red cells
Nil	4·25	4·29
$1·6 \times 10^{-8}$ E600	2·34	2·42
$6·9 \times 10^{-7}$ eserine sulphate	2·74	2·59

The results in Table 1 not only confirm the finding of Augustinsson (1948) that the activity of haemolysed cells is the same as that of the intact cells, but also show that both are inhibited to an equal degree by the same concentration of inhibitor.

Activity of inhibitors. For the purposes of comparison with other work 50% inhibition values are given. Because the inhibition is progressive it is essential that the times of incubation are clearly stated when inhibition values are expressed. For this reason double-armed Warburg flasks have been used, one arm for the inhibitor, the other for the substrate, and the centre well containing the red-cell suspension in buffer. In this way the time and temperature of incubation are accurately known.

Table 2. *Concentrations for 50% inhibition*

(Incubation time, 30 min., 37°)

Inhibitor	Concentration for 50% inhibition (M)
E605	$1·26 \times 10^{-6}$
E600	$2·14 \times 10^{-8}$
Q2	$3·16 \times 10^{-5}$
N mustard	$9·44 \times 10^{-5}$
Eserine	$1·4 \times 10^{-6}$
DFP	$4·6 \times 10^{-7}$

The values given in Table 2 have been determined by the conventional method of plotting percentage inhibition against the negative log of the M-concentration of inhibitor. Sigmoid curves are obtained. As will be explained later, a better way for irreversible inhibitors is to plot concentration of inhibitor against log percentage residual activity when straight lines are obtained.

Irreversibility of DFP, E600 and E605. Many investigators have shown eserine and prostigmine to be reversible inhibitors by the simple procedure of dialysing the cholinesterase preparation after inhibition, whereby the activity is rapidly regained. It can also be shown that if cholinesterase is first incubated with eserine and acetylcholine is then added to determine the residual activity a curve convex upward is obtained on plotting output in μl. of CO_2 against time. This can only be due to replacement by acetylcholine of physostigmine molecules on the active groups of the cholinesterase. This phenomenon may be considered as an indication of reversibility though not necessarily so. Benzoylcholine has been shown to be a competitive inhibitor of true cholinesterase (p. 457). Table 10 gives the inhibition data for benzoylcholine at the usual concentration of acetylcholine (0·0138M). If cholinesterase is incubated with benzoylcholine and the acetylcholine then tipped in, the output of carbon dioxide is linear with respect to time over the whole range, even though readings are taken every 3 min. This indicates that although benzoylcholine is a

reversible inhibitor of true cholinesterase, an equilibrium is reached between acetylcholine and benzoylcholine so quickly that no changes in rate of output of carbon dioxide may be determined. The difference between eserine and benzoylcholine is presumably one of rate of combination.

Washing intact red cells after inhibition has also been examined as a possible demonstration of reversibility. Table 3 gives the results obtained when red cells inhibited with eserine are centrifuged and washed a varying number of times. The activity is readily recovered, as is also shown for benzoylcholine in Table 12.

Table 3. *Use of intact red cells to demonstrate reversibility*

(Red cells first incubated in buffer containing $8{\cdot}8 \times 10^{-6}$ M-eserine)

Further treatment	Original activity (%)
None	15
Centrifuged, resuspended in buffer	56
Centrifuged, washed twice	95

Studies on the reversibility of DFP by dialysis of the inhibitor-enzyme mixtures have given negative results (Mackworth & Webb, 1948). DuBois, Doull, Salerno & Coon (1949) claim that the inhibition of rat-brain cholinesterase by E 605 is readily reversible *in vivo*. Using the washing technique described above, we have failed to demonstrate any reversibility of E 605 (Table 4).

Table 4. *Irreversibility of E 605 by washing technique*

($1{\cdot}96 \times 10^{-6}$ M-E 605 incubated with red cells (ice cold) for 15 min.)

Treatment	Final activity as percentage of original activity
Uncentrifuged and determined immediately	80
Centrifuged, washed once	84
Centrifuged, washed twice	82
Centrifuged, washed three times	80
Centrifuged, washed four times	84

Nachmansohn, Rothenberg & Feld (1947) have shown a considerable degree of reversibility of DFP inhibition of cholinesterase from both electric tissue of the eel and from caudate nucleus of the ox. The principle of their 'dilution technique' was to incubate cholinesterase with a certain concentration of DFP and at various times to take identical samples, dilute one with the same concentration of DFP in buffer (U = undiluted with respect to DFP) and the other with buffer alone (D = diluted with respect to DFP). If the inhibition is reversible there should be some reactivation of the enzyme in the one diluted with buffer (D). For such a technique to give reliable results it is imperative that the reaction between cholinesterase and DFP is stopped at the time of dilution, otherwise, since this inhibition of cholinesterase is progressive (Nachmansohn, Rothenberg & Feld, 1948), the inhibition will continue at its original rate, while that in D will be very much slowed down by the dilution. Therefore, unless this progressive inhibition is stopped, a difference between the activities of U and D will always be obtained which will indicate 'reversibility'. Table 9 shows that, if acetylcholine is present with the enzyme before the inhibitor is added, little or no inhibition is obtained, and this compound has therefore been used to stop the reaction. The ratio of acetylcholine to inhibitor must be high enough to stop the reaction completely, and this can be checked for each experiment as will be described later. Nachmansohn *et al.* (1947) give no precise information when the acetylcholine was added to the cholinesterase preparation; however, for their experiments on electric tissue they state that 'the time required for taking the solution out of the thermostat, diluting it, putting it into the Warburg vessel, gassing and putting back into the thermostat was kept at the minimum necessary and did not exceed 6–8 min.' In their experiments on cholinesterase from ox caudate nucleus a further dilution was made so that it must be assumed that the acetylcholine was not added until at least 8 min. after the dilution; the inhibition in U was therefore progressing at a rapid rate, while that in D would be much slowed down. That the rate was high in U in the experiments of Nachmansohn *et al.* (1947) with caudate nucleus is shown by the fact that at zero time on their graph U shows approximately 75 % inhibition.

A method has been devised which eliminates these sources of error. In tube A are placed 5 ml. of red-cell suspension in buffer, and in tube B 5 ml. of buffer containing inhibitor (concentration $2x$ μg./ml.). Both tubes are stored in ice. Into the main compartment of one single-armed Warburg flask (U) are pipetted 4·5 ml. of buffer containing x μg. of inhibitor/ml. together with the correct concentration of acetylcholine, and into another Warburg flask (D) 4·5 ml. of buffer containing acetylcholine alone. At zero time tubes A and B are thoroughly mixed and 0·5 ml. of the mixture is placed in the side arm of the Warburg vessels using a Krogh-Keys syringe pipette. The contents of the side arm of the first pair (D and U) are tipped into the centre compartment as soon as possible (1 min. cold). The others are placed in the 37° bath and at various times the contents of the side arms are tipped into the (acetylcholine + inhibitor) or acetylcholine alone. Great care should be taken to ensure that the two flasks are treated identically in the time of placing in the bath and in the time of the mixing with acetylcholine. If necessary, due to the mixing times, the flasks were

Table 5. *Reversibility experiments by dilution technique*

(Technique as described on p. 453. D and U = diluted and undiluted with respect to inhibitor respectively. Error of normal cholinesterase estimation = $0{\cdot}11 \pm 0{\cdot}056$ (11 pairs of estimations).)

Inhibitor			DFP		E 600		E 605		Q 2		Eserine		Nitrogen mustard	
Conc. before dilution (M)			$6{\cdot}8 \times 10^{-7}$		$4{\cdot}5 \times 10^{-8}$		$1{\cdot}7 \times 10^{-6}$		$3{\cdot}4 \times 10^{-5}$		$7{\cdot}7 \times 10^{-6}$		$1{\cdot}6 \times 10^{-4}$	
Time cold (min.)	Time at 37° (min.)	Flask	CO_2 output (μl./min.)	$D-U$	CO_2 output (μl./min.)	$D-U$	CO_2 output (μl./min.)	$D-U$	CO_2 output (μl./min.)	$D-U$	CO_2 output (μl./min.)	$D-U$	CO_2 output (μl./min.)	$D-U$
1	0	D	4·64		4·86		5·36		4·96		3·21		4·86	
		U	4·57	0·07	4·76	0·10	5·08	0·28	3·03	1·93	0·85	2·36	4·12	0·74
3	5	D	4·33		4·23		4·87		4·26		3·24		4·58	
		U	4·07	0·26	3·98	0·25	4·62	0·25	3·01	1·25	0·82	2·42	4·03	0·55
3	10	D	3·43		3·33		4·42		4·25		3·05		3·91	
		U	3·11	0·32	2·98	0·35	4·12	0·30	2·81	1·44	0·72	2·33	3·53	0·38
3	20	D	2·60		2·20		3·73		3·89		3·19		3·21	
		U	2·40	0·20	2·12	0·08	3·63	0·10	2·60	1·29	0·77	2·42	2·76	0·45
3	40	D	1·67		1·09		2·60		3·53		3·25		2·19	
		U	1·33	0·34	0·89	0·20	2·35	0·25	2·35	1·18	0·72	2·43	1·94	0·25
Activity of red cells before inhibition			4·75 4·92		4·93 5·03		5·60 5·75		4·81 4·86		5·31 5·39		4·65 4·75	
Mean				**0·24**		**0·20**		**0·24**		**1·42**		**2·39**		**0·47**
Standard deviation				±0·09		±0·10		±0·07		±0·27		±0·04		±0·16
t				3·02*		1·87		3·61*		10·7*		91·3*		4·90*
Recalculation using a correction for 2% inhibition in U (see below):														
Mean				**0·18**				**0·16**						**0·41**
Standard deviation				±0·11				±0·07						±0·15
t				1·35				1·39						4·33

* Significant $P < 0{\cdot}05$.

gassed with 5% CO_2 + 95% N_2 after mixing with acetylcholine. In this technique a dilution of ten times was obtained. Before each reversibility experiment a test was carried out to determine the highest concentration of inhibitor which would allow the acetylcholine to stop the reaction at the time of mixing. This was done by adding fresh red-cell suspensions to a mixture of acetylcholine and inhibitor as in the U flask described above. In order to be on the safe side, half the highest concentration to give an output of carbon dioxide linear with respect to time under these conditions has been used. In general, it appears that a concentration of inhibitor which does not produce more than 80% inhibition in 40 min. is satisfactory for this purpose. DFP, E 605, E 600 and Q 2 have been examined in this way. An experiment with eserine is included for comparative purposes as a known completely reversible inhibitor and also one with nitrogen mustard which Thompson (1947) has stated is partially reversible (determined by a washing technique on the insoluble portion of brain homogenate).

Examination of the reversibility data given in Table 5 for E 600, E 605 and DFP indicates that a t test of the difference between D and U shows significance when tested against the value of $0{\cdot}11 \pm 0{\cdot}056$ for the ordinary determination of cholinesterase. The table also includes a recalculation of the results assuming that the inhibition in the U flask was not quite stopped by the acetylcholine and that 2% inhibition resulted. It can be seen that the results then cease to be significant. It is thought that in a test of this nature the results do not indicate any measurable reversibility. Nitrogen mustard shows some reversibility though it is interesting that the inhibition due to this compound is also progressive. Inhibition by Q 2 is also partly reversible and this observation has been checked by the direct test of inhibiting red cells and then washing with saline. The results in Table 6 confirm this reversibility.

Table 6. *Reversibility of Q 2*

(Cells incubated with $4{\cdot}8 \times 10^{-5}$ M-Q 2 for 30 min. at 37°)

No. of washings	Inhibition (%)
0	57
1	29
2	27
4	29

Rate of reaction of inhibitors with cholinesterase. It has been shown that the inhibition due to DFP is

a progressive one both with serum cholinesterase (Mackworth & Webb, 1948) and with the cholinesterase of the electric eel (Nachmansohn *et al.* 1948).

The rates of reaction of E 605, E 600 and Q 2 have been determined with cholinesterase of intact red cells using double-armed flasks so that the inhibitor

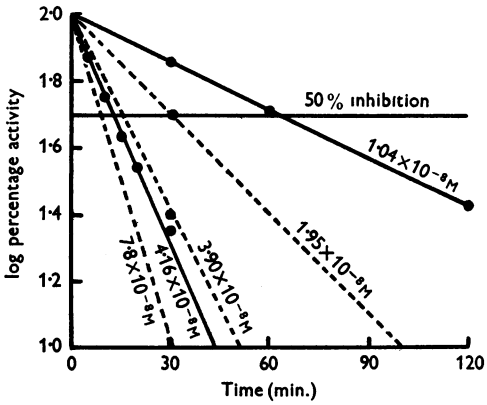


Fig. 1. Rate of inhibition of cholinesterase by E 600. Concentration of E 600 shown against each curve.

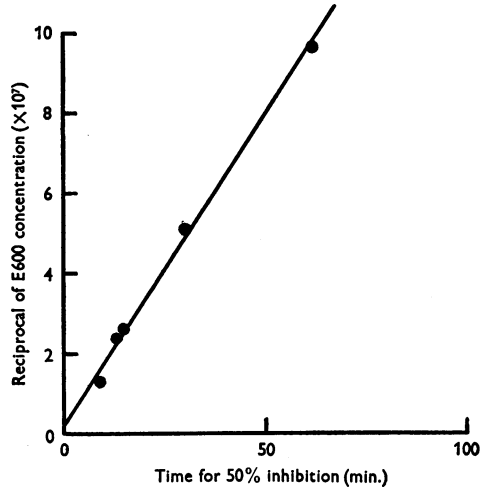


Fig. 2. Demonstration that time for 50% inhibition is inversely proportional to concentration of E 600. Data taken from Fig. 1.

may be added at 37° and the reaction stopped at the desired time by the addition of substrate from the other side arm. The results of these experiments are shown in Figs. 1 and 3 where log percentage activity is plotted against time in minutes. With E 605 and E 600 straight lines are obtained indicating that the reaction shows the characteristics of a unimolecular reaction at a particular concentration of inhibitor.

$$k_1 t = \ln \frac{a}{a-x},$$

where a = original activity, x = decrease in activity due to inhibitor, t = time in min. and k_1 = velocity constant, or

$$k_1 t = \ln \frac{100}{b}, \qquad (1)$$

where b = percentage residual activity.

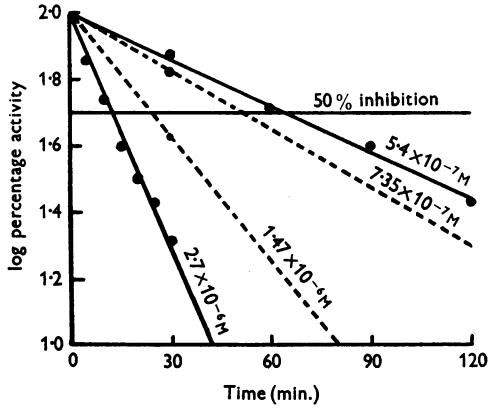


Fig. 3. Rate of inhibition of cholinesterase by E 605. Concentration of E 605 shown against each curve.

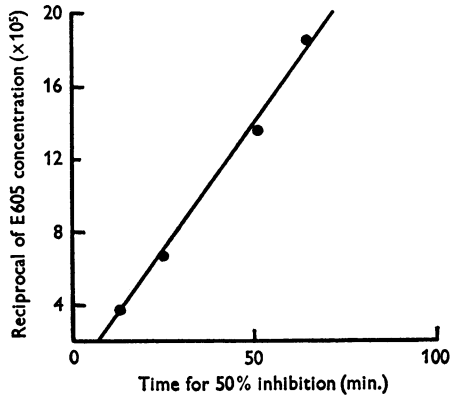


Fig. 4. Demonstration that time for 50% inhibition is inversely proportional to concentration of E 605. Data taken from Fig. 3.

Equation (1) is readily derived from the conventional formula for a bimolecular reaction when one of the reactants is in excess, i.e. in this case

$$K = \frac{1}{tI} \ln \frac{100}{b}, \qquad (2)$$

where I = molar inhibitor concentration. When I is a constant $kI = k_1$ and we have equation (1). Further when $b = 50$ and $t = t_{0.5}$ then

$$t_{0.5} = \frac{1}{kI} \ln 2 = \frac{1}{k_2 I}. \qquad (3)$$

On plotting the times for 50 % inhibition ($t_{0.5}$) against the reciprocal of inhibitor concentration straight lines are obtained, indicating that the reaction, or one of the slowest of a series of reactions, is a bimolecular reaction (cf. Figs. 2 and 4). Using these data, the value of k, the velocity constant, has been calculated, using equation (2).

Table 7. *Velocity constants for bimolecular reaction*

Inhibitor	k (l.mol.$^{-1}$ min.$^{-1}$)
E 600	$1{\cdot}1 \times 10^{6}$
E 605	$2{\cdot}0 \times 10^{4}$

The values for k given in Table 7 are much more satisfactory constants for such irreversible inhibitors than the conventional 50 % inhibition value, e.g. the inhibition at any concentration and time may be readily calculated from k.

When the rate of reaction of Q 2 with cholinesterase is examined on the same basis it is seen that straight lines are again obtained (Fig. 5) on plotting log percentage activity against time, but they differ in two important respects from those of E 600 and E 605. First, the lines produced back do not pass through the origin (2 = log 100 % activity), and secondly the lines for different concentrations are roughly parallel.

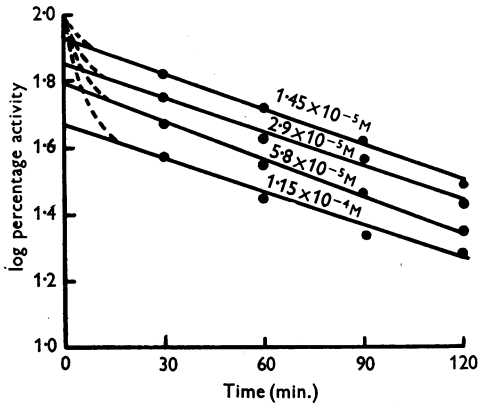


Fig. 5. Rate of inhibition of cholinesterase by Q 2. Concentration of Q 2 shown against each curve.

It has previously been shown that the inhibition of cholinesterase by Q 2 is partly reversible. Possibly in this case the straight lines give a measure of rate of 'becoming irreversibly inhibited', and the amount below the origin where the line produced back-crosses the ordinate is a measure of the reversible inhibition. Reading from the graph on this basis the following figures for reversible inhibition are obtained (Table 8).

Examination of data from the dilution technique, which will give a low result, gives approximately 25 % reversibility for $3{\cdot}4 \times 10^{-5}$ M, while the direct-washing test gave 29 % for $4{\cdot}5 \times 10^{-5}$ M, both in fair agreement with the above.

Table 8. *Percentage inhibition at zero time by Q 2 extrapolated from Fig. 4*

Concentration of Q 2 (M)	Inhibition at zero time (%) (Reversible)
$1{\cdot}45 \times 10^{-5}$	16
$2{\cdot}9 \times 10^{-5}$	29
$5{\cdot}8 \times 10^{-5}$	38
$1{\cdot}15 \times 10^{-4}$	54

The second point, that the straight lines for different concentration of inhibitor are parallel, indicates that under these conditions the rate of the reaction leading to irreversibility (the slowest in the system) is independent of concentration, i.e. as in a unimolecular reaction where

$$t_{0.5} = \frac{\ln 2}{k}.$$

Affinity of inhibitors for cholinesterase. Several workers have shown that the inhibition produced by some agents is prevented if the substrate, acetylcholine, is added first, for example, Thompson (1947) and Barron, Bartlett & Miller (1948) with the nitrogen mustards and Burgen (1949) with TEPP. It is shown here that the inhibition due to E 605, E 600 and DFP is also prevented if the substrate is added first (Table 9).

Table 9. *Protection of cholinesterase from inhibition by substrate*

Inhibitor	Concentration (M)	Inhibition: Inhibitor added before substrate (%)	Inhibition: Inhibitor added after substrate (%)
E 605	$1{\cdot}72 \times 10^{-6}$	60	3
DFP	$6{\cdot}8 \times 10^{-7}$	70	3
E 600	$4{\cdot}5 \times 10^{-8}$	73	4

These results suggest that the inhibitor attaches itself to the group or groups involved in the hydrolysis of acetylcholine.

Mendel *et al.* (1943) have shown that benzoylcholine and acetyl-β-methylcholine may be used as specific substrates for pseudo and true cholinesterase respectively. Table 10 shows that benzoylcholine acts as an inhibitor for true cholinesterase, indicating that although it is not hydrolysed by the 'true' enzyme it is attached to the active centres.

Butyrylcholine has also been shown to be a substrate for pseudo-cholinesterase (Easson & Stedman, 1936; Nachmansohn & Rothenberg, 1945). Cohen, Kalsbeck & Warringa (1949), using a continuous titration method, have reported that butyryl-

choline can also act as a competitive inhibitor for the true cholinesterase. Using the Warburg technique we have obtained the following few results. It seems that butyrylcholine is roughly three times as effective as an inhibitor as benzoylcholine. However,

Table 10. *Inhibition of specific cholinesterase by benzoylcholine*

(Conc. of acetylcholine = $1{\cdot}35 \times 10^{-2}$ M)

Conc. of benzoylcholine (M)	Inhibition (%)
$1{\cdot}24 \times 10^{-1}$	77
$6{\cdot}12 \times 10^{-2}$	71
$2{\cdot}04 \times 10^{-2}$	44
$9{\cdot}62 \times 10^{-3}$	25
$8{\cdot}3 \times 10^{-3}$	24
$5{\cdot}64 \times 10^{-3}$	15
$1{\cdot}92 \times 10^{-4}$	2

From above data 50% inhibition would be obtained with $2{\cdot}52 \times 10^{-2}$ M-benzoylcholine.

Therefore for 50% inhibition $\frac{[\text{Benzoylcholine}]}{[\text{Acetylcholine}]} = \frac{1{\cdot}86}{1}$.

Table 11. *Inhibition of specific cholinesterase by butyrylcholine*

(Conc. of acetylcholine = $1{\cdot}35 \times 10^{-2}$ M)

Conc. of butyrylcholine (M)	Inhibition (%)
$5{\cdot}97 \times 10^{-3}$	41
$5{\cdot}97 \times 10^{-4}$	9
$5{\cdot}97 \times 10^{-5}$	0

50% inhibition at approx. $9{\cdot}1 \times 10^{-3}$ M-butyrylcholine.

At 50% inhibition $\frac{[\text{Butyrylcholine}]}{[\text{Acetylcholine}]} = \frac{0{\cdot}67}{1}$.

since we did not learn of the butyrylcholine inhibition until most of this work had been completed, benzoylcholine has been used for all of the work to be described in this paper. That the inhibition due to benzoylcholine is readily reversible can be shown by the washing technique described earlier with eserine (Table 3).

Table 12. *Reversibility of benzoylcholine inhibition*

Conc. of benzoylcholine (M)	Inhibition: Before centrifuging (%)	Inhibition: After centrifuging and resuspending in fresh buffer (%)
$1{\cdot}21 \times 10^{-2}$	31	1
$4{\cdot}03 \times 10^{-3}$	10	0

Since, as has been shown above, true cholinesterase has an affinity for benzoylcholine, but does not hydrolyse it (at least, not at a measurable rate), it was thought that this property might be utilized for the determination of the affinity of inhibitors for cholinesterase. This cannot be readily done with acetylcholine because the substrate concentration is being continuously depleted. Koelle (1946) has done similar experiments using neostigmine as the reversible and DFP as the irreversible inhibitor. He dialysed to remove neostigmine and DFP after a definite time and measured the inhibition due to DFP. The principle of the method described here has been to incubate a constant concentration of inhibitor and cholinesterase (intact red cells) in the presence of various concentrations of benzoylcholine. After a definite time interval the cells are centrifuged down and resuspended in buffer, thus removing benzoylcholine from the enzyme (Table 12) and also removing excess inhibitor.

In detail the technique is as follows: in a series of centrifuge tubes are placed 5 ml. of a red-cell suspension in buffer (washed with saline three times and diluted to seven times the original volume of whole blood) and 1 ml. of varying concentrations of benzoylcholine in buffer. The solutions are now warmed to 37° and then 1 ml. of inhibitor solution in buffer, also warmed at 37°, is added in sufficient concentration to give 50–75% inhibition. These mixtures are now incubated for 30 min., when they are centrifuged and resuspended in buffer and diluted to their original volume (7 ml.). 3·5 ml. of the contents of each tube are now taken for the determination of cholinesterase activity in the usual way. The value of k, the velocity constant (see above), has been calculated for inhibitor alone and for each concentration of benzoylcholine, and by plotting log k against log M-concentration of benzoylcholine the concentration necessary to reduce k by one-half has been roughly extrapolated.

Table 13. *Affinity of E 600 and E 605 for cholinesterase*

Conc. of benzoylcholine (M)	Activity (%)	k
Inhibitor, E 600 ($4{\cdot}16 \times 10^{-8}$ M)		
$1{\cdot}2 \times 10^{-2}$	84·4	$0{\cdot}14 \times 10^{6}$
$7{\cdot}98 \times 10^{-3}$	77·2	$0{\cdot}21 \times 10^{6}$
$5{\cdot}32 \times 10^{-3}$	70·1	$0{\cdot}29 \times 10^{6}$
$3{\cdot}55 \times 10^{-3}$	60·6	$0{\cdot}39 \times 10^{6}$
0	26·9	$1{\cdot}05 \times 10^{6}$

Concentration to reduce k by one-half = $2{\cdot}1 \times 10^{-3}$ M-benzoylcholine (by extrapolation).

Inhibitor, E 605 ($1{\cdot}17 \times 10^{-6}$ M)		
$1{\cdot}2 \times 10^{-2}$	94·1	$0{\cdot}17 \times 10^{4}$
$4{\cdot}03 \times 10^{-3}$	77·7	$0{\cdot}74 \times 10^{4}$
$1{\cdot}34 \times 10^{-3}$	66·3	$1{\cdot}20 \times 10^{4}$
$4{\cdot}46 \times 10^{-4}$	55·4	$1{\cdot}73 \times 10^{4}$
0	48·3	$2{\cdot}13 \times 10^{4}$

Concentration to reduce k by one-half = $1{\cdot}9 \times 10^{-3}$ M (by interpolation).

It has been seen that the slowest reaction taking place between E 600 (or E 605) and true cholinesterase is a bimolecular one. The mechanism for the slowing down of the reaction by benzoylcholine must be by blocking some of the enzyme centres so that they are no longer available for the inhibitor. Since the concentration of benzoylcholine necessary to

reduce the velocity constant by one-half is the same for both E 600 and E 605 the bimolecular reaction is probably the primary combination of inhibitor with enzyme.

DISCUSSION

No reversibility of E 605, E 600 and DFP can be demonstrated. This has been examined by two methods, one by washing inhibited intact red cells and the other by a modification of the dilution method of Nachmansohn *et al.* (1947). The latter method gives unreliable results since the progressive inhibition characteristic of these inhibitors is not stopped at the time of dilution; during the time between dilution and the addition of acetylcholine, the inhibition of the sample undiluted with respect to inhibitor concentration will be increasing at a far greater rate than the one diluted with buffer alone. By modifying the procedure so that acetylcholine is added at the time of dilution it has been possible to conclude that neither DFP, E 600 nor E 605 exhibit any measurable reversibility *in vitro*.

Examination, on the basis of a homogeneous system, of the data obtained on the rate of inhibition of cholinesterase by E 600 and E 605 indicates that they follow the kinetics of a bimolecular reaction, i.e. the time for 50 % inhibition is inversely proportional to the concentration of the inhibitor. For each particular concentration of inhibitor the reaction shows unimolecular properties, presumably because the concentration of inhibitor is in large excess compared with the concentration of enzyme. The slowest reaction taking place in the system is therefore bimolecular. Velocity constants have been calculated for the reaction with E 600 and E 605; such constants are more satisfactory than the conventional 50 % inhibition value though they are only applicable directly to irreversible inhibitors. Fig. 6 shows a 15 min. inhibition curve for E 605 (percentage inhibition plotted against the negative logarithm of the M-concentration of inhibitor) calculated from the velocity constant

$$(k = 2{\cdot}0 \times 10^4 \text{ l.mol.}^{-1} \text{ min.}^{-1}).$$

This curve shows the usual sigmoid characteristics. Included on the graph are experimentally determined values of inhibition for 15 min. incubation at 37°. Good agreement with theory is shown.

It has been shown that benzoylcholine is a competitive inhibitor of true cholinesterase; utilizing this fact a technique to determine the affinity of these inhibitors for cholinesterase has been developed. This affinity has been expressed as the concentration of benzoylcholine necessary to reduce k, the velocity constant, by one-half. For E 600 and E 605, this concentration is approximately the same; this indicates that the slowest reaction (the bimolecular reaction) is the primary reaction of inhibitor with enzyme active centre.

A study of Q 2 has shown that its inhibition of cholinesterase is partly reversible and the rate of increase of irreversible inhibition is extremely slow. The reversible inhibition shows up when a plot of log percentage activity is made against time. Straight lines are obtained which produced back do not pass through the origin. Reversibility may be demonstrated by this method, which has been used to confirm that E 600 and E 605 are not reversible. An examination of the rate of inhibition with Q 2 shows that it is independent of inhibitor concentration, i.e. unimolecular.

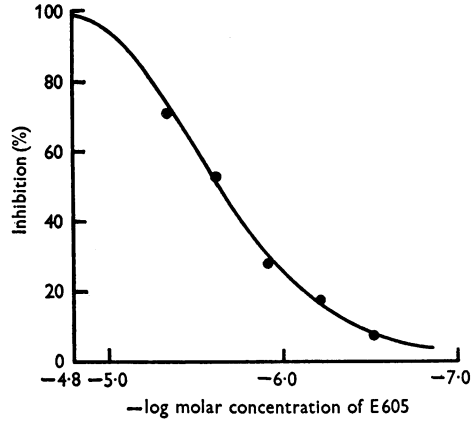


Fig. 6. Theoretical inhibition curve for E 605 calculated from $k = 2{\cdot}0 \times 10^4$ min.$^{-1}$ for incubation time of 15 min. and temperature 37°. Experimentally determined points have been added to show agreement with theory.

It has been shown for several inhibitors that inhibition is prevented by the presence of substrate with the enzyme before the inhibitor (Thompson, 1947; Barron *et al.* 1948; Burgen, 1949). The inhibition by E 600 and E 605 is similarly prevented; the inhibitor and acetylcholine must therefore have some common point of attachment to the enzyme. Since E 600, E 605 and Q 2 are structurally similar molecules, there is no reason to suppose that they will attach themselves to different sites on the enzyme, and any progressive irreversible inhibition will be produced by a similar mechanism for all three compounds. The order of their reaction rates leading to irreversible inhibition is the same as their lability to hydrolysis, i.e. the most stable one, Q 2, is the slowest inhibitor and E 600, the least stable, the most rapid. Hydrolysis of the inhibitors renders them inactive; the substituted phosphoric acid and *p*-nitrophenol and 8-hydroxyquinoline do not inhibit cholinesterase.

A possible mechanism of inhibition by these compounds can now be suggested (see below).

In the case of E 600 and E 605, the bimolecular reaction (1) is the limiting factor. With Q 2 the unimolecular reaction (2) is the slowest. Partial reversibility has been demonstrated, indicating the existence of the reversible inhibitor complex (reaction 1). Since the more stable these compounds are to hydrolysis the slower do they irreversibly inhibit cholinesterase, it is probable that reaction (2) is in fact hydrolysis of the inhibitor at the phosphate bond to liberate from E 600 and E 605, *p*-nitrophenol and from Q 2, 8-hydroxyquinoline. In this case, of course, water would be involved in the reaction making it pseudo-unimolecular. We have attempted to detect *p*-nitrophenol after the inhibition of rat-brain homogenate, the most potent preparation we had at the time, but were unable to get any positive

$$\underset{\text{Enzyme}}{E} + \underset{\text{Inhibitor}}{I} \overset{(1)}{\rightleftharpoons} \underset{\substack{\text{Reversible enzyme}\\ \text{inhibitor complex}}}{(EI)^R} \overset{(2)}{\rightarrow} \underset{\substack{\text{Irreversibly}\\ \text{inhibited enzyme}}}{(E)^I} + \overset{\text{other reaction products}}{\text{---------}}$$

results. Little information is available about the substituted phosphoric acid portion of the molecule. Brauer (1948) claims with HETP containing ^{32}P that no radioactive P remained on the cholinesterase.

From the above mechanism of inhibition it can be deduced that any inhibitor of this type (those producing progressive irreversible inhibition) showing bimolecular characteristics on reaction with cholinesterase, cannot show any reversible inhibition. On the other hand, unimolecular properties should indicate some reversible inhibition.

The concept of a bimolecular reaction may be used to give information about the concentration of enzyme. From Fig. 1 it can be seen that at a concentration of $1{\cdot}0 \times 10^{-8}$ M-E 600 a straight line is obtained. Since straight lines in a graph of this nature are obtained for bimolecular reactions only when one of the reactants is in excess, it may be possible to put an upper limit on the molar concentration of enzyme (active centres). One can easily calculate that when the inhibitor is not more than four times the enzyme concentration the line will be curved more than can be accounted for by experimental error. Therefore it can be said that, since the reaction is bimolecular (1 mol. enzyme active centre to 1 mol. inhibitor), the enzyme concentration which produces 5–6 μl. of CO_2/min. under the conditions described cannot be more than $2{\cdot}5 \times 10^{-9}$ M, and may be much lower. Nachmansohn *et al.* (1948), in their studies on the kinetics of DFP inhibition using electric tissue cholinesterase, state that the usual concentration of cholinesterase in manometric work is 10^{-11} M and that at this concentration 100,000 mol. DFP are required per mol. of cholinesterase to cause 100 % inhibition. This concentration of enzyme was based on a molecular weight of 3,000,000 determined from ultracentrifuge data. The correct result is probably between these values 10^{-11}M and $2{\cdot}5 \times 10^{-9}$ M.

SUMMARY

1. The properties of a convenient preparation of true cholinesterase are described.

2. The available methods for the demonstration of reversibility have been examined. The 'dilution technique' of Nachmansohn has been modified and new methods have been devised. *p*-Nitrophenyl diethyl thiophosphate, *p*-nitrophenyl diethyl phosphate and di-*iso*propyl fluorophosphonate do not show any significant reversibility. 8-Quinolyl diethyl thiophosphate and nitrogen mustard are partly reversible.

3. The rate of progressive inhibition of cholinesterase by *p*-nitrophenyl diethyl phosphate and *p*-nitrophenyl diethyl thiophosphate follows the characteristics of a bimolecular reaction.

4. The rate of progressive inhibition of cholinesterase by 8-quinolyl diethyl thiophosphate shows unimolecular reaction characteristics.

5. Benzoylcholine has been demonstrated to be an inhibitor of specific cholinesterase. A technique has been devised, using this property, to determine the affinity of inhibitors for cholinesterase.

6. A possible mechanism of inhibition based on the above data has been proposed.

7. A maximum figure for the M-concentration of enzyme active centres has been deduced.

Since the completion of the work we have learnt from Mr B. Topley that the sample of E 605 contained some of the isomers *OO*-diethyl-*S*-*p*-nitrophenyl thiolphosphate and *OS*-diethyl-*O*-*p*-nitrophenyl thiolphosphate.

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Studies in Detoxication

32. THE INFLUENCE OF BROMOBENZENE AND CYSTINE ON THE BROMINE CONTENT OF THE HAIR OF RATS

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The ultimate objective of this investigation was to find out how orally administered compounds possessing therapeutic activity could be made to appear in the skin and hair. It was thought that some evidence concerning this could be obtained by feeding compounds which form mercapturic acids *in vivo*, because cystine is much involved in the elaboration of hair and skin and in the formation of mercapturic acids. For this study, bromobenzene was selected as the mercapturic acid former, because it could be traced by its bromine content. It was possible that *p*-bromophenylmercapturic acid might find its way to the skin and hair in quantities sufficient to warrant studying, in a similar manner, mercapturic acid formers carrying therapeutic groups.

It has long been known that arsenic, when administered to animals, appears in the hair, skin and nails, and this is explained by the fact that arsenic compounds combine with sulphydryl groups *in vivo*. Haddow, Elson, Roe, Rudall & Timmis (1945) have shown that when the flavine, 9-phenyl-5:6-benzo-*iso*alloxazine, is injected into albino rats, an orange-yellow pigmentation of the growing parts of the hair appears. The pigment of the hair was shown to be either the original flavine or a simple derivative spectroscopically indistinguishable from it. The results of analyses of the sulphur compounds (inorganic and ethereal sulphates and neutral sulphur) of the urine of the injected animals were consistent with mercapturic acid formation, and it was suggested that the flavine might have been transported to the hair in combination with the cystine used for the elaboration of keratin. Furthermore, Crabtree (1944, 1945, 1946) has shown that a number of mercapturic acid formers, including bromobenzene, when applied locally to the skin, are probably detoxicated by mercapturic acid formation in the skin. At the same time there is a temporary decrease in the glutathione content of the skin and a delay in chemical carcinogenesis caused by 3:4-benzpyrene applied simultaneously. There is therefore ample evidence of a close connexion between the metabolism of mercapturic acid formers and keratin containing tissues.

In the present work, the plan was to feed varying amounts of bromobenzene and L-cystine to different groups of rats which had been clipped free of hair at the beginning of the experiment, and then, after an interval sufficient to allow the new hair to grow to normal length, to analyse the hair and certain organs for bromine and the hair for cystine. It will be shown that in animals showing signs of cystine deficiency as a result of feeding bromobenzene, the bromine content of the hair is much higher than that of other tissues examined.