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THE MECHANISM OF ACTION OF ANTICHOLINESTERASE DRUGS

BY

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During the past few years much new work on anticholinesterase substances has been published as a result of the new interest in the subject produced by the discovery of di*iso*propylfluorophosphonate (DFP) by McCombie and Saunders (1946). Many discrepancies of detail between the behaviour of individual cholinesterase inhibitors have been noted and have occasioned serious doubts as to the validity of the hypothesis that the physiological action of these substances is solely a consequence of cholinesterase inhibition. One source of error was greatly clarified by Mendel and his co-workers (Mendel, Mundell, and Rudney, 1943; Mendel and Rudney, 1943 and 1944; Hawkins and Gunter, 1946; Hawkins and Mendel, 1947), who showed that the term "cholinesterase" has been applied in the past to enzymes of two, and possibly more (Augustinsson, 1948), distinct enzymological species—termed by them "true" and "pseudo" cholinesterases, distinguishable by specific substrates. They have shown that these enzyme types differ strikingly in their distribution, their sensitivity to inhibitory agents, and their optimum substrate concentrations, and that the appearance of the pharmacological effects of anticholinesterases is related to inhibition of the "true" cholinesterase. Only when the physiological response measured is that to acetylcholine carried by the blood stream does the pseudocholinesterase in the plasma seem to be important (Heymans, Verbeke, and Votava, 1948) in determining the magnitude of acetylcholine responses. Despite this very important advance many anomalies remain to be explained, and it is the purpose of this paper to discuss the kinetics of cholinesterase inhibition by various agents and the light this sheds on our interpretation of cholinesterase inhibition under strictly physiological conditions. Some aspects of the kinetics of cholinesterase activity and inhibition have been considered by a number of workers (Straus and Goldstein, 1943; Goldstein, 1944; Mazur and Bodansky, 1946; Nachmansohn, Rothenburg, and Feld, 1947; Augustinsson, 1948; Brauer, 1948; Jansen, Nutting, and Balls, 1948; Mackworth and Webb, 1948; Nachmansohn, 1948), but there has been little attempt to interpret these results in terms of prevailing physiological circumstances, and in consequence the design of experiments on cholinesterase activity has usually been inadequate to provide relevant data. In the account that follows attention will be concentrated on the contrast between the modes of action of the two main groups of anticholinesterases, typified by eserine and tetraethyl pyrophosphate, with notes on such differences from these patterns as are found with other inhibitors.

Materials and Methods

The enzyme preparations used were: (1) fresh oxalate human plasma which contains predominantly pseudocholinesterase; (2) washed human red blood cells, lysed by the addition of 4 volumes of 0.025*M* $NaHCO_3$, containing only true cholinesterase; (3) in some experiments a highly purified bovine red cell true cholinesterase, obtained from Dr. M. L. Tainter, has been used, each mg. of which could hydrolyse 3 mg. of *d*(+) acetyl-β-methylcholine chloride per minute. The activity of the enzyme was estimated in the Warburg manometric apparatus with 0.025*M* $NaHCO_3$ as medium equilibrated with 95 per cent N_2 + 5 per cent CO_2 gas mixture at 37° C. The enzyme solution was normally placed in the main compartment of the vessel, and the substrate and inhibitor, each dissolved in 0.2 ml. of bicarbonate solution, were placed in separate side arms. The total volume of fluid used was always 3 ml. The usual final concentration of substrates were 0.025*M* *dl*-acetyl-β-methylcholine chloride, 0.007*M* benzoylcholine chloride, and 0.02*M* acetylcholine chloride. Where other concentrations of substrate have been employed they are mentioned in the text. In all experiments corrections for non-enzymic hydrolysis of the substrate were applied. Dilute solutions of the enzyme inhibitors have been made immediately before use from stock solutions, or with tetraethylpyrophosphate (TEPP) and di*iso*propylfluorophosphonate (DFP) from the pure substances.

Results

There is a tendency in pharmacology to assume an identical mechanism for two drugs that have similar actions if their dose-response relationships are similar. In Fig. 1 data for eserine and TEPP inhibition of true cholinesterase are given. Apart from the curve for TEPP being much steeper than

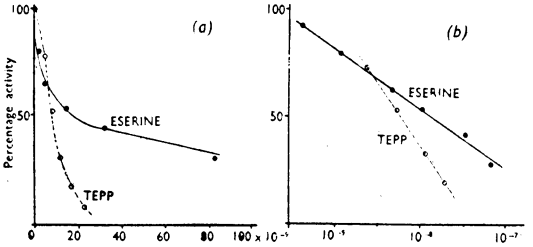


FIG. 1.—Human red cell cholinesterase. Substrate: 0.025*M* acetyl-β-methyl choline chloride. Incubation of inhibitor and enzyme for 20 min. before addition of substrate. Ordinates: velocity of hydrolysis of substrate as percentage of uninhibited control rate. Abscissae: concentration of inhibitor in g./ml. (*a*) Arithmetical scale, (*b*) log. scale.

that for eserine and consequently appearing nearly linear over the range 0–70 per cent inhibition, there is nothing in such data to suggest the striking difference in kinetics of action that will be discussed. Such differences of dose-response slope are very commonly encountered in pharmacology among apparently closely related drugs, notable examples being found in the central analgesic and local anaesthetic series, without arousing any doubts as to a unitary mechanism of action. It should be noted that this steep dose-response curve may account for a feature of TEPP action—namely, that its pharmacologically effective dose is much closer to the lethal dose than is the case with eserine or prostigmine.

Kinetics of Eserine Action

(*a*) *Rate of combination of eserine and enzyme in the absence of substrate*

At present it is not possible to measure this reaction directly; it can only be inferred from indirect data. If eserine is left in contact with the enzyme for 1, 5, or 20 min. at 37° C. before addition of substrate the subsequent rates of hydrolysis are indistinguishable, but such information is not helpful. The rate of combination in the absence of substrate—i.e., under non-competitive conditions—however, must be faster than in the presence of substrate. It will be seen in the next section that under these conditions combination occurs fairly rapidly.

(*b*) *Rate of combination of eserine and enzyme in the presence of substrate*

Fig. 2 shows some typical results illustrating the rapidity with which eserine reacts with cholinesterase when it is actively splitting acetylcholine; it will be seen that an equilibrium rate is reached in about 10 min. after adding eserine. The rate at which equilibrium is reached is dependent, however, on the concentration of inhibitor employed; with lower concentrations the rate is slower, as was predicted on theoretical grounds by Goldstein (1944), but a steady state is finally attained whose position is determined competitively by the concentrations of inhibitor and substrate.

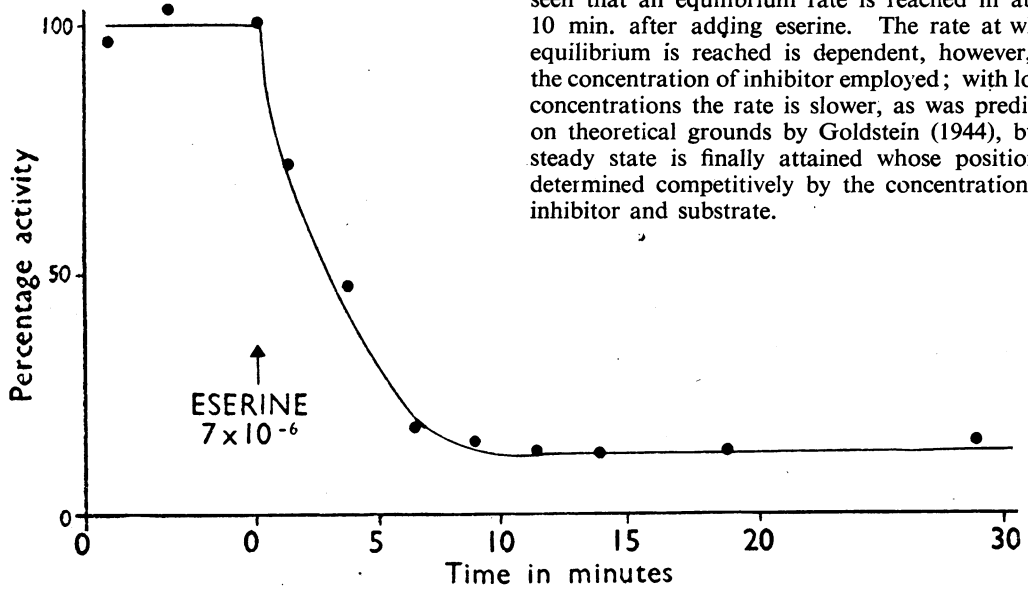


FIG. 2.—Human red cell cholinesterase. Substrate: 0.02*M* acetylcholine chloride. At zero time addition of 7×10^{-6} g. eserine sulphate/ml. Ordinates: percentage of activity before addition of inhibitor. Abscissae: time in min. after addition of inhibitor.

(*c*) *Rate of dissociation of eserine-enzyme complex in presence of substrate*

When substrate is added to a mixture of enzyme and inhibitor the enzyme-inhibitor complex is decomposed according to the Michaelis-Menten equilibrium as follows:

$$EI \rightleftharpoons E + I \quad \text{(i)}$$
$$E + S \rightleftharpoons ES \quad \text{(ii)}$$

where E = free enzyme concentration; I = free inhibitor concentration; S = free substrate concentration; EI = inhibitor-enzyme complex; ES = enzyme–substrate complex, and the velocity of acetylcholine hydrolysis is dependent on the concentration of *ES*. Fig. 3 illustrates an experiment designed to examine the velocity of this change. The initial measured rate of hydrolysis was less than 12 per cent of an uninhibited control, and the shape of the curve would suggest that under these experimental conditions the control value was probably not much less than this, but it has not been found possible to obtain reliable figures in the first 1–2 minutes owing to insufficient lapse of time for the attainment of temperature equilibrium. Over the succeeding 50 min. the rate gradually rose until it reached an equilibrium rate which was 58 per cent of the control. The rate of attainment of equilibrium is dependent on the concentration of inhibitor and substrate employed—in particular low substrate concentrations prolong the period to equilibration and high substrate concentrations speed it up. The initial value obtained is, however, virtually independent of substrate concentration and is presumably dependent purely on the position of equilibrium in equation (i) before substrate competition has appreciably shifted it. The final equilibrium level of activity reached is, however, influenced by both inhibitor and substrate competitively.

It is clear that values obtained for cholinesterase inhibition will vary according to the arbitrary conditions selected. Equilibrium values will depend both on substrate concentration and the substrate employed. The higher the substrate concentration (Fig. 4) the smaller the degree of inhibition. *d*-Acetyl-β-methylcholine, which is employed as a specific substrate for " true " cholinesterase, has a lower affinity for the enzyme than acetylcholine, and in consequence eserine shows a greater equilibrium inhibition of cholinesterase with acetyl-β-methylcholine than with acetylcholine as substrate.

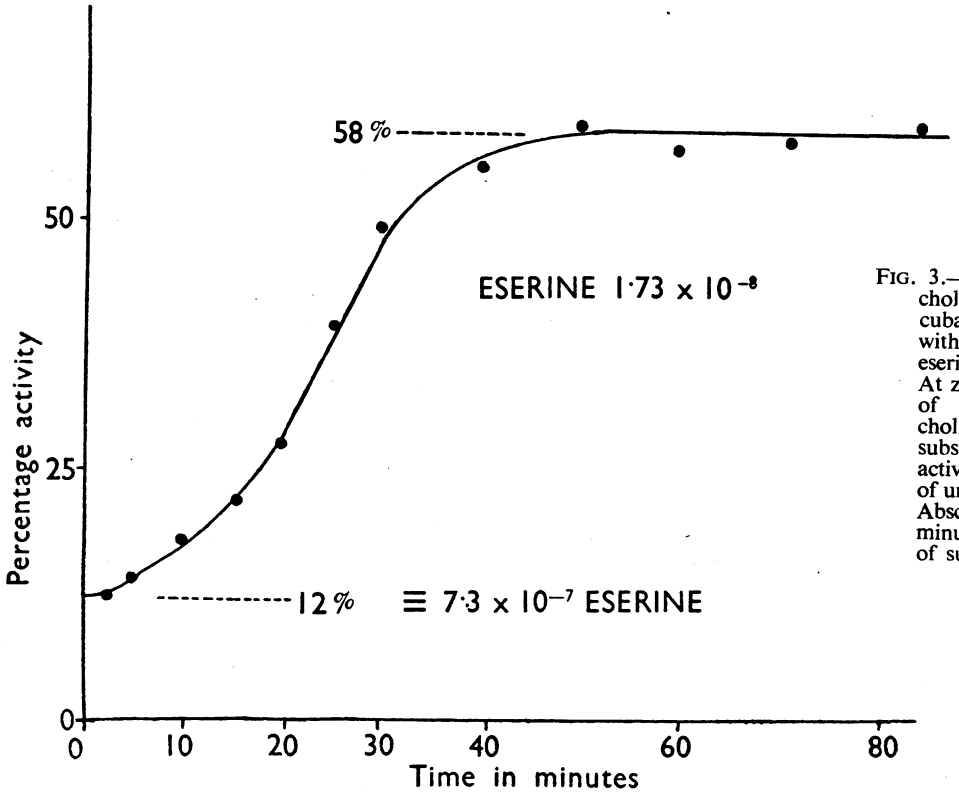


FIG. 3.—Human red cell cholinesterase. Incubated for 20 min. with 1.73 × 10^{-8} g. eserine sulphate/ml. At zero time addition of 0.005*M* acetylcholine chloride as substrate. Ordinates: activity as percentage of uninhibited control. Abscissae: time in minutes after addition of substrate.

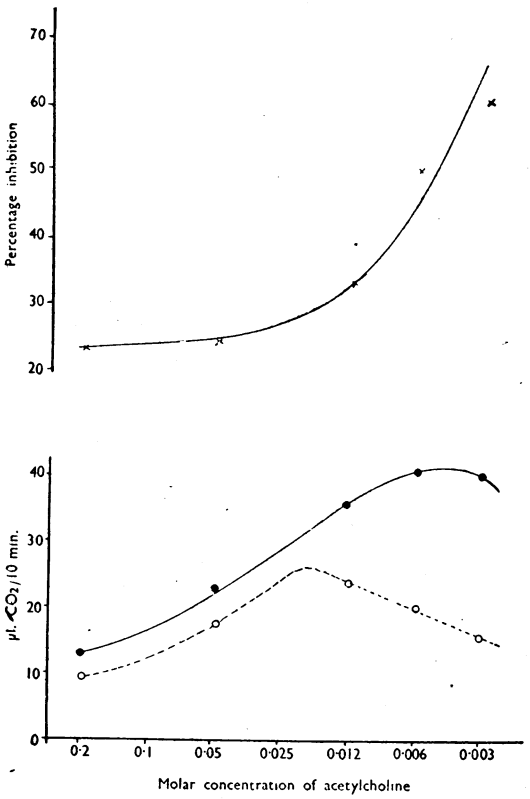


FIG. 4.—Human red cell cholinesterase. Substrate: acetylcholine chloride. *Lower curve:* Ordinates: velocity of acetylcholine hydrolysis in μl. CO_2 evolved/10 min. at equilibrium. Abscissae: molarity of acetylcholine. ●——● without addition of eserine. O- - - -O with 1.7×10^{-7} g. eserine sulphate/ml. *Upper curve:* Ordinates: percentage inhibition of cholinesterase by eserine. Abscissae: molarity of acetylcholine.

If, however, other than equilibrium conditions are considered the situation becomes chaotic. Many authors have reported results obtained in the first twenty or thirty minutes after adding substrate, but these data are influenced in such a complex way by both inhibitor and substrate concentration that their quantitative value is very doubtful. It would be of value to be able to determine the degree of non-competitive inhibition since this is unaffected by substrate concentration, but the experimental difficulties make the values only approximate, as they are necessarily obtained by extrapolation.

It is important to correlate these *in vitro* data with physiological events. The view that acetylcholine acts as a synaptic transmitter, accepted by most workers, requires that when no impulse is being transmitted at the synapse either no or an extremely small amount of acetylcholine leaks out of the cholinergic nerve terminals, whereas with the passage of an impulse a relatively large amount of acetylcholine is liberated and is present at the synaptic region for a period of milliseconds. This means that in the quiescent synapse we have essentially non-competitive conditions for cholinesterase inhibition. When acetylcholine liberation occurs its persistence is very brief compared with the long period required to establish competitive equilibrium, and is certainly insufficient to allow for more than a very small displacement of the inhibitor to occur. It would seem, therefore, that the equilibrium value discussed above grossly underestimates the effect of eserine under these conditions. The data in Fig. 3 show that the concentration of eserine employed (1.7×10^{-8}) produced only 42 per cent inhibition of the enzyme at equilibrium but produced at least 88 per cent inhibition under non-competitive conditions. To produce 88 per cent inhibition at equilibrium would require more than forty times as much eserine as was added. A further factor may come into play when the enzyme is greatly inhibited, since owing to the decreased rate of hydrolysis the acetylcholine concentration may rise sufficiently to cause some competitive decrease of the inhibition. In a prolonged nerve tetanus this displacement will increase during the course of the tetanus as acetylcholine accumulates and will reach an equilibrium value if the tetanus is of sufficient duration, but the position that this equilibrium is likely to reach cannot be estimated owing to our complete ignorance of the effective acetylcholine concentrations attained at the sites of cholinesterase activity in the synapse.

Another important aspect of these fast reactions has never been considered in connexion with cholinesterase, and that is the biphasic character of enzymic hydrolysis required by the Michaelis-Menten theory. The reaction occurs in two stages:

$$E + \text{Ac.Ch} \rightleftharpoons (E.\text{Ac.Ch}) \quad (1)$$
$$(E.\text{Ac.Ch}) \rightleftharpoons E + \text{Ac} + \text{Ch} \quad (2)$$

Stage (1) effectively removes acetylcholine without breaking it down and hence is a reaction of primary importance in disposing of acetylcholine. It is obvious that reaction (1) occurs in a shorter time than the overall reaction (1 + 2), and it is therefore of fundamental importance to know the relative rates of reactions 1 and 2. So far it has not been feasible to do this with cholinesterase, but we know from the direct measurements of Britton Chance (1943, 1948) on peroxidase and catalase that the first reaction ($E + S \rightleftharpoons ES$) may be as much as a hundred times faster than the second reaction and is mainly limited by probability considerations. If

this state of affairs can be applied to cholinesterase, it may be that the effective removal of part of the liberated acetylcholine can occur extremely rapidly. That this may be so is supported by the recent work of Eccles and MacFarlane (1949) on the effect of anticholinesterases on the frog end plate potential. They found that the upstroke of the end plate potential, which occurred in about 2 msec., rose higher and more steeply in the presence of anticholinesterases. Considering the low temperature at which they were working (16–18° C.) this time makes it probable that normally the formation of the enzyme-substrate complex is the most important factor in the initial rapid removal of acetylcholine, and that providing the enzyme capacity is adequate the actual hydrolysis of the complex is less important. Anticholinesterases will, of course, inhibit this reaction in a non-competitive manner.

Neostigmine behaves in a very similar way to eserine in all the equilibria so far considered, but other reversible inhibitors may behave differently. For instance, " 62C47 " (bis-trimethyl-aminophenyl-ethyl ketone diiodide, Glock and Mogey, 1948) reaches equilibrium more rapidly than eserine or neostigmine, so that the equilibrium under physiological conditions may be more competitive than with eserine or neostigmine. 62C47 is a less active inhibitor than eserine or neostigmine, and, as Goldstein (1944) has shown, the mass action velocities require that the less active a reversible inhibitor the more rapidly it should come into equilibrium, as is found experimentally.

Kinetics of TEPP Action

(*a*) *Rate of combination with the enzyme in the absence of substrate*

It is very easy to measure the rate of combination of TEPP with cholinesterase, because, as will be seen later, not only is the reaction almost completely irreversible *in vitro*, but the progress of the reaction is effectively blocked by addition of substrate. When cholinesterase was incubated with TEPP the enzyme slowly decreased in activity (Fig. 5) so that even after 40 min. the reaction was still proceeding, but at 2 hours little further inactivation was seen. By this time the amount of available TEPP must have fallen considerably as a result of hydrolysis, and this side reaction militates against the continued progress of the reaction. The initial rate of the reaction fits reasonably well the requirements for a bimolecular reaction. Thus if TEPP is added to the enzyme before addition of substrate it is evident that the degree of enzyme inhibition found is entirely dependent on the time of contact allowed before addition of substrate. This is in marked

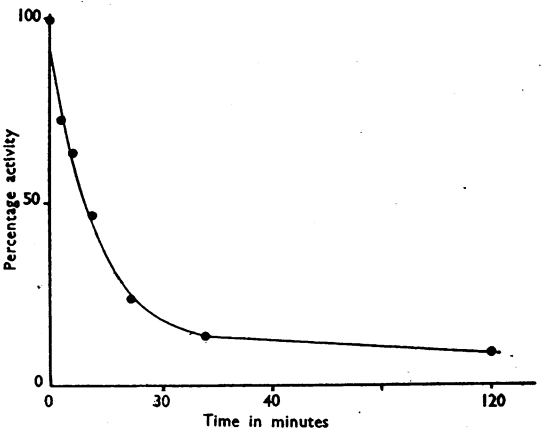


FIG. 5.—Human red cell cholinesterase. Substrate: 0.025*M* acetyl-β-methylcholine chloride. Incubation of 8×10^{-9} g. tetraethyl pyrophosphate/ml. with enzyme before addition of substrate. Ordinates: activity as percentage of control. Abscissae: period of incubation of inhibitor with enzyme before addition of substrate.

contrast to the state of affairs with eserine. In the experiment recorded in Fig. 1 20 min. contact between enzyme and TEPP was allowed. The choice of this time was arbitrary, but it is evident that for results to be comparable the same time interval must always be used. Actually, providing at least 10 min. contact is allowed, the error in estimating the potency of TEPP will be relatively small owing to the very steep concentration–inhibition relationship characteristic of this substance. It should be noted that DFP behaves very much in the same way as TEPP on the enzyme, but, as has also been found by Mackworth and Webb (1948), combines rather more slowly.

(*b*) *Combination of TEPP with enzyme in the presence of substrate*

If the experiment illustrated in Fig. 2 is repeated with TEPP as the inhibitor no detectable inhibition of cholinesterase results in the succeeding hour (Fig. 6, III), and even if TEPP and the substrate are added together very little inhibition results. This blocking by substrate is seen when either acetylcholine or acetyl-β-methylcholine is used as substrate for true cholinesterase. Even if the concentration of TEPP is increased to 10–100 times that usually required to produce inhibition, either no or only slowly developing inhibition occurs. With DFP and some other members of the group this is by no means so; for instance, with DFP some inhibition may gradually become apparent (Fig. 6, IV) even with concentrations (0.02–0.08 μg./ml.) producing considerable inhibition in

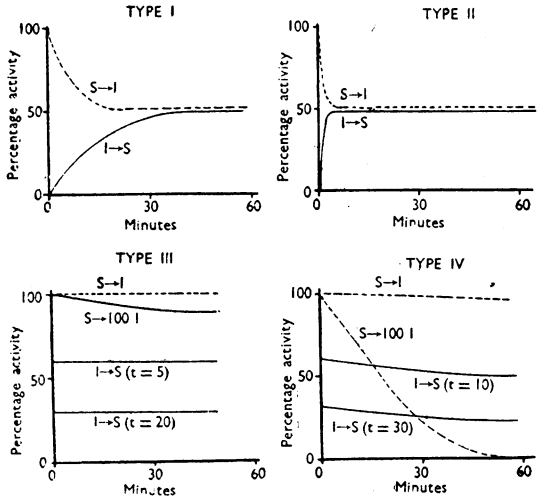


FIG. 6.—Diagrammatic representation of the four main classes of cholinesterase inhibitors studied. Ordinates: percentage activity of enzyme. Abscissae: time in minutes. S → I means substrate added before addition of inhibitor. S → 100 I substrate added before 100 times the amount of inhibitor needed to produce adequate inhibition in absence of substrate. I → S inhibitor added before substrate. The time of contact in minutes between inhibitor and enzyme, before addition of substrate, is shown in parentheses. I = typical results obtained eserine, neostigmine, and "Nu683"; II = results with "62C47." III = with TEPP. IV = with DFP.

the absence of substrate, and with higher concentrations (2–8 μg./ml.) inhibition appears quite rapidly despite the presence of substrate.

This blocking of TEPP inhibition by substrate may have some physiological significance. It may mean that at an active synapse the presence of acetylcholine may hinder the inhibitory effect of TEPP, so that the cholinesterase at that synapse is less inhibited than at a quiescent synapse. This blocking action of substrate for TEPP inhibition has been demonstrated clearly on the frog rectus abdominis preparation by Hobbiger (1949).

Blocking of TEPP inhibition by other inhibitors

The blocking action of substrate on TEPP is presumably due to competition for the same active groups on the enzyme, and it was therefore of interest to see whether cholinesterase inhibitors, which have a far greater affinity for cholinesterase than acetylcholine, would be able to block these groups as well. Fig. 7 illustrates a typical experiment of this kind. TEPP itself in a concentration of 1.33×10^{-8}, when incubated with the enzyme for 20 min., produced a 94 per cent inhibition (Fig. 7, curve *a*). Curve *b* shows the inhibition produced by increasing concentrations of eserine alone, whilst curve *c* shows the effect of incubating the enzyme with eserine for 20 min. followed by incubation for 20 min. with TEPP. It will be seen that at all concentrations of eserine some degree of protection of the enzyme results which increases with the concentration of eserine. Curve *d* is an expression of the percentage protection of the enzyme from inhibition by TEPP as a result of the prior contact of the enzyme with increasing concentrations of eserine; at a concentration of 10^{-8} eserine protects the enzyme to the extent of about 90 per cent from TEPP inhibition. It was also found that neostigmine, Nu 683, 62C47, carbachol, and choline were more or less efficient in blocking the action of TEPP, but that NaF, which by itself produces a 50 per cent inhibition of the enzyme at about 10^{-3} *M*, did not hinder TEPP inhibition and was in fact additive to it. It is easy to test rapidly whether a cholinesterase inhibitor blocks the action of TEPP by using a concentration of the reversible inhibitor that produces about 50 per cent inhibition and a concentration of TEPP that will produce 95–98 per cent inhibition in 20 min. If the substance is active as a protective agent the total inhibition will lie between 50 and 90 per cent. Koelle (1946) also showed that eserine, neostigmine, and to some extent carbachol could protect cholinesterase from irreversible inhibition by DFP. He was unable to show protection by acetylcholine or acetyl-β-methylcholine with his technique, which consisted of exposure of the enzyme to the protecting agent, followed by exposure to DFP for 30 minutes and subsequent dialysis against running water. His figures for inhibition of enzyme before dialysis, however, show clearly the effect of substrate described above. Koster (1946) has also shown that eserine has a protective action against DFP poisoning *in vivo*, and this has been confirmed by others.

The blocking of inhibitor action by substrate throws considerable light on the mechanism of the anticholinesterase action of the alkyl phosphate group of inhibitors. These agents might act in three ways: (*a*) a chemical reaction might occur between enzyme and inhibitor involving inactivation of both; (*b*) a chemical reaction might occur in which the effect of the inhibitor was catalytic and only the enzyme was changed; or (*c*) an initial reversible physical adsorption of inhibitor on the enzyme might occur followed by either (*a*) or (*b*). Nachmansohn, Rothenburg, and Feld (1947) have presented evidence that the inhibition of cholinesterase by DFP is biphasic, with an early reversible phase and a later irreversible phase. The data just presented support and extend this view. The

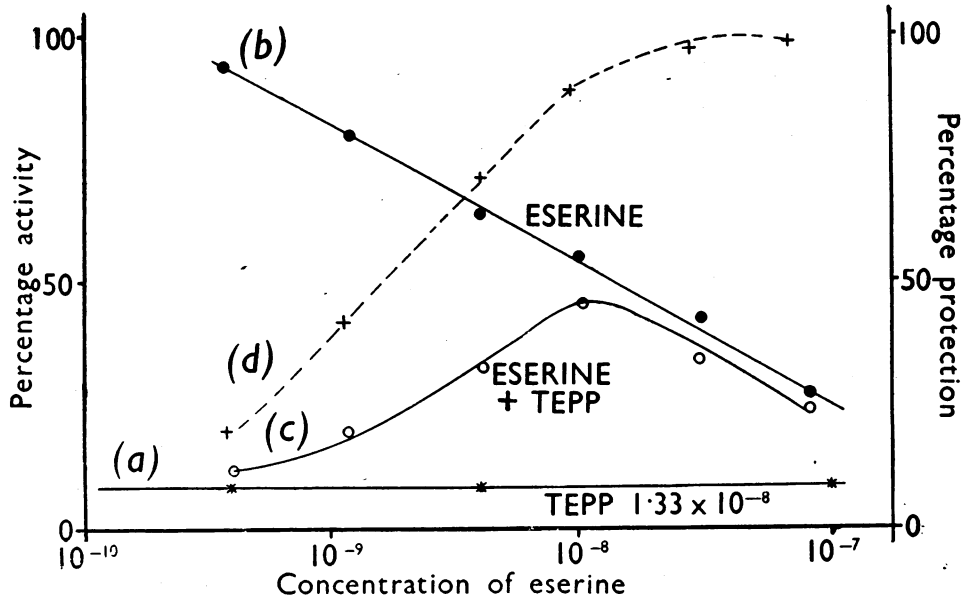


FIG. 7.—Human red cell cholinesterase. Substrate: 0.03*M* acetyl-β-methylcholine chloride. Ordinates (left): percentage activity of enzyme. Abscissae: concentration of added eserine sulphate. Curve (*a*): activity after incubation for 20 min. with 1.33×10^{-8} g. TEPP/ml. (*b*): activity after incubation for 20 min. with eserine sulphate. Equilibrium velocities. (*c*): incubation with eserine sulphate for 20 min. followed by 1.33×10^{-8} g. TEPP/ml. for 20 min. Equilibrium velocities. (*d*): percentage protection by eserine of the cholinesterase from inactivation by TEPP (right-hand ordinates).

blocking action of substrate is presumably due to the inability of TEPP to react with other than free enzyme centres. According to Michaelis and Menten (1913) the amount of free enzyme available is governed by the adsorption equilibrium:

$$E + S \rightleftharpoons ES$$

For a given substrate concentration the proportion of enzyme molecules uncombined (*E*) at any instant is fixed, and for the substrate concentrations used in our experiments is only a small proportion of the total available enzyme. If the rate of enzyme inactivation by alkyl phosphates depends entirely on the number of free enzyme centres, blocking by substrate should occur equally against DFP and TEPP, but this is not so. If, however, a reversible competitive combination is a preliminary to irreversible inactivation a second equilibrium will be involved:

$$E + I \rightleftharpoons EI$$

and the rate of inactivation of enzyme in the presence of substrate will be influenced by the dissociation constant of *EI* which will determine the amount of *EI* formed in the overall competitive reaction:

$$ES + I \rightleftharpoons EI + S$$

and just as with purely reversible inhibitors, a small dissociation constant for *EI* will favour its formation and a minimal blocking action by substrate, whereas if the dissociation constant is large—i.e., the affinity of enzyme for inhibitor is small—the blocking action by substrate will be considerable. The different ease of blocking with substrate can be explained if TEPP has a low adsorption affinity whereas that of DFP is high. The potency of an alkyl phosphate inhibitor thus depends on (*a*) the adsorption affinity, (*b*) the rapidity with which irreversible inactivation is produced in the enzyme-inhibitor adsorption complex. It remains to be established whether the second process is catalytic or involves the disappearance of inhibitor. Brauer (1948) has indicated that TEPP becomes no longer available when it is treated with cholinesterase. The quantitative nature of this change has been investigated as follows.

A constant amount of TEPP (final concentration 2.5×10^{-8}) was incubated with 0.02–0.5 mg. of purified cholinesterase per ml. at *p*H 7.2 and 37° C. for 30 min.; controls were simultaneously run containing either cholinesterase alone or 0.5, 1.0, and 2.5×10^{-8} TEPP alone. After 30 min. the *p*H was brought to 3.5 to 4.0 and the solution heated

at 96° C. for 3 min. This procedure has been found to destroy the residual cholinesterase activity completely whilst causing only very slight breakdown of TEPP. The solutions were then cooled and the *p*H adjusted to 7.2. One ml. of each solution was then incubated with 0.02 mg. of cholinesterase for 30 min. and the resultant activity of this indicator enzyme determined. The results obtained are illustrated in Fig. 8 (*a*), which shows that the more cholinesterase present initially the less the inhibition of the indicator enzyme. In order to determine whether these results could have been due merely to increasing protein concentration, the latter was held constant by adding to each tube a large excess of crystalline serum albumin; this procedure made no difference to the values obtained. Further evidence of the specificity of the reaction was obtained by the failure of either albumin alone or heat-denatured cholinesterase to decrease the available TEPP. These results fully confirm the experiments of

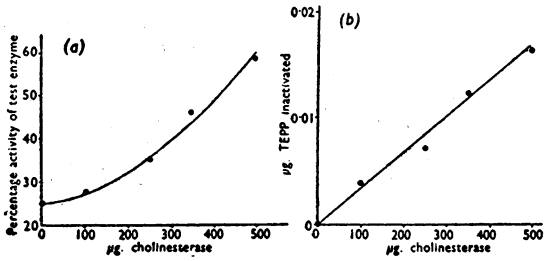
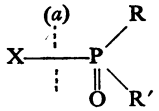


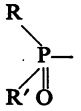
FIG. 8.—Purified bovine red cell cholinesterase. Substrate 0.025*M* acetylcholine chloride. (*a*) Ordinates: percentage activity of test enzyme. Abscissae: μg. cholinesterase added initially (experiment described in text). (*b*) Ordinates: μg. TEPP inactivated. Abscissae: μg. cholinesterase added initially.

Brauer (1948). Fig. 8 (*b*) relates the amount of cholinesterase added in the experiment of Fig. 8 (*a*) to the amount of TEPP that has become unavailable as estimated by inhibition of the indicator enzyme. It will be seen that the relationship is approximately linear, 1 mg. of cholinesterase removing about 0.034 μg. of TEPP under these conditions. A purely catalytic role of TEPP in the cholinesterase inactivation is thus excluded, and it is interesting to note that when the enzyme is heat-acid-denatured it loses its ability to combine with the alkyl phosphates as well as its enzymatic activity, and this argues further for the specificity of the chemical reaction involved.

If the biphasic interpretation of the action of the alkyl phosphates is correct, what is the nature of the secondary reaction? All the active members of the series contain the grouping



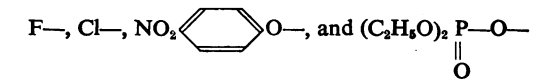
where the groups R and R′ may be a variety of structures and still retain activity, although the most active compounds are those in which R and R′ contain short alkyl chains. X is always a potentially acidic radicle so that the bond (*a*) is analogous to that in an acyl anhydride or halide and consequently rather less stable than the alkoxy or amido bonds joining R and R′ to the phosphorus. In view of this basic unit the most likely chemical change is a phosphorylation of the enzyme transferring the



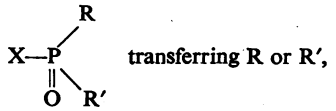
radical to some polar grouping on the enzyme.

Most of the really active inhibitors are only weak phosphorylating agents, and in general increasing reactivity of the bond (*a*) decreases anticholinesterase activity; thus di*iso*propylchlorophosphonate is a much more active phosphorylator than di*iso*-propylfluorophosphonate and yet is far less active as a cholinesterase inhibitor; in the series dimethyl-, diethyl-, and di*iso*propyl-fluorophosphonates the reactivity of bond (*a*), assessed by ease of hydrolysis, decreases in that order, yet the anticholinesterase activity increases in the same direction (Mackworth and Webb, 1948; Mazur, 1946). This is not always true; for instance, in the series of tetra-alkyl pyrophosphate derivatives in which one or both (P = O) groups are replaced by (P = S) the chemical reactivity falls as well as the anticholinesterase acitivity. It would appear that there is an optimum reactivity in the bond (*a*) which may be explained in the following way. Provided that the adsorptive affinity for the enzyme remains unchanged, if bond (*a*) is made more reactive the rate of reaction with the enzyme will be increased, but the rate of non-specific actions such as phosphorylation of random amino, hydroxyl, or phenolic groups in the protein may also increase, as will hydrolysis by water molecules. These side reactions will divert some of the active agent—an important matter with substances effective at very low concentrations. In order to explain the selectivity of the alkyl phosphates it is reasonable to assume that the adsorption complex of enzyme and alkyl phosphate introduces strain in bond (*a*) and thus facilitates phosphorylation by these weak acylating agents.

There are two alternative chemical reactions that may be considered briefly. The group X may combine with the enzyme; this is improbable because of the variety of chemical groups that are active in this part of the molecule; examples are:



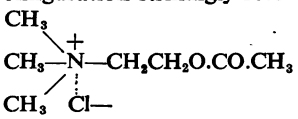
These groups differ greatly in chemical properties, and a mechanism which involves that they should all be highly active is clearly less probable than the relatively homogeneous mechanism suggested here. It is just possible that an alkylation is involved:



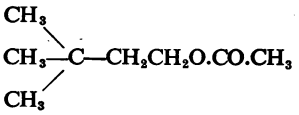
but these bonds are very stable to acid and alkaline hydrolysis, unlike bond (*a*). Further R and R′ may be alkoxy or dialkylamino without a very large change in activity and yet the groups to be transferred are vastly different in chemical potentialities; finally dialkoxy-, alkyl-, or aryl- phosphonates which have similar ester bonds but no anhydride structure are quite inert as cholinesterase inhibitors.

Brauer (1948) rejected the phosphorylation hypothesis on the basis of experiments with HETP (a mixture of alkyl phosphates obtained by heating ethyl phosphate with P_2O_5 or $POCl_3$, whose main active constituent is TEPP) labelled with P^{32}, in which he was unable to demonstrate association of the labelled phosphorus with the enzyme. His data, however, show that his preparation of labelled HETP contained only about 0.1 per cent of material active against cholinesterase; the association of this small amount of material with the enzyme would not be detectable under his experimental conditions. Michel and Krop (1949) have recently carried out a model experiment of this kind using DFP labelled with P^{32} and electrophorus cholinesterase. They found that the P^{32} of labelled DFP was precipitated with the cholinesterase by trichloracetic acid and the amount precipitated was proportional to the amount of cholinesterase added and to the degree of cholinesterase inhibition; 1 mg. of completely inactivated cholinesterase contained the phosphorus from 0.1 μg. DFP, and when allowance is made for the difference of enzyme activity and the molecular weights of DFP and TEPP this figure is very close to the one we obtained for TEPP and red cell cholinesterase by the indirect method. It can be taken therefore that members of the alkyl phosphate group inhibit cholinesterase by dialkylphosphorylation of the enzyme.

In the past it has been assumed that both acetylcholine and cholinesterase inhibitors combine with cholinesterase by virtue of the positively charged nitrogen, and it has been difficult to fit the alkyl phosphates into this pattern since they are predominantly negatively charged molecules and have no basic nitrogen. Adams (1949), however, has found that the essential feature of a substrate for true cholinesterase is the presence of an acetyl group, and that 3 : 3-dimethylbutyl acetate, which in general configuration strikingly resembles acetylcholine and yet lacks the quaternary nitrogen, is split almost as fast as acetylcholine. This work strongly suggests that combination with the enzyme occurs through the negatively charged ester linkage which is, of course, present in the alkyl phosphates. It is well known that carbamyl groups are isosteric with acetyl groups and hence the activity of the carbamyl esters may be explained without recourse to the basic nitrogen. Bloch (1939) arguing in this way prepared the acetyl and *iso*butyryl esters of *m*-hydroxyphenyltrimethylammonium (the basic half of neostigmine) and found these esters were active anticholinesterases.



Acetylcholine chloride



3 : 3-Dimethylbutyl acetate

Another aspect of the problem is the stability of the inhibited complex. Whereas both DFP and TEPP ultimately form apparently irreversible complexes with cholinesterase *in vitro*, the effects of TEPP are much shorter in duration than those of DFP *in vivo*. If the radical transferred to the enzyme were the same, the rate of recovery from the action both *in vivo* and *in vitro* would be the same, and hence the difference in duration of action between DFP and TEPP may be due to the difference between the di*iso*propylphosphoryl and the diethylphosphoryl radicals. If this theory is correct, the cholinesterase inhibition produced by diethylfluorophosphonate should be similar in duration to that produced by TEPP, and conversely the inhibition produced by tetra*iso*propylpyrophosphate should be similar in duration to that of DFP.

In support of this hypothesis may be quoted the following comment from Saunders and Stacey (1948), who compared the actions of di*iso*propyl-, diethyl-,

and dimethyl-fluorophosphonates on the eye, " We observed as early as 1941 that the dimethyl and diethyl esters produced a far less intense miosis than the di*iso*propyl ester. The effects of the dimethyl compound wore off in a matter of hours whereas that of the diethyl compound usually lasted about 2 days. . . ." The di*iso*propyl ester produced effects lasting usually for 7 days. In the cat we (Burgen, Keele, and Slome, 1949) found that the miotic effect of TEPP lasted about 1–2 days. Experiments in progress in this laboratory have shown in agreement with this theory that di*iso*propyl phosphoryl esters of different types have very similar durations of action to DFP, whereas other diethylphosphoryl esters have resembled TEPP in duration.

The breakdown of the dialkylphosphoryl-enzyme may be simply a matter of slow uncatalysed hydrolysis, and if so the *iso*propyl group would be expected to have a retarding influence on the reaction; alternatively the breakdown may occur through the operation of a phosphotriesterase. Aldridge (1949) has discovered a widely distributed enzyme in animal tissues which will hydrolyse diethyl *p*-nitrophenyl-phosphate to *p*-nitrophenol and diethyl phosphoric acid, and perhaps this or a similar enzyme could split off the dialkylphosphoryl group from the cholinesterase and so restore its activity.

Summary

1. The kinetics of inhibition of cholinesterase by both reversible and irreversible inhibitors is considered.

2. Methods of estimation in common use greatly underestimate the physiological activity of the reversible cholinesterase inhibitors because of competition with substrate and the slowness with which equilibrium is attained.

3. The action of many of the alkyl phosphate group is markedly blocked by substrate and by the presence of reversible inhibitors. Owing to this blocking by substrate the activity of this group may be overestimated *in vitro*.

4. The evidence available suggests that the alkyl phosphates form weak adsorption complexes with cholinesterase and in this activated state dialkyl phosphorylation of the cholinesterase occurs to form a stable inactive substance. This inactive enzyme may perhaps be regenerated by hydrolysis of the dialkyl phosphoryl group the rate of which is dependent on the nature of the alkyl groups. If *iso*-propyl phosphoryl groups form more stable complexes with the enzyme than diethyl phosphoryl groups, this may explain the longer duration of action of the di*iso*propyl series *in vivo*.

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