REVIEW ARTICLE

CHOLINESTERASES AND THE MODE OF ACTION OF SOME ANTICHOLINESTERASES

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Although several reviews upon the role of cholinesterase, acetylcholine and anticholinesterase drugs have been written since 1945 (Feldberg[1], Bodansky[2], Augustinsonn[3], Koelle and Gilman[4], Whittaker[5], Holmstead[6] and Nachmansohn and Wilson[7]), progress has been so rapid during the last 2 years that yet another review is justified. The reasons for the great interest in this field are two-fold; first the results which are emerging are helping to explain the process of neuro-muscular transmission; secondly, during the last few years, several new organo-phosphorus compounds have been synthesised, which have proved to be powerful physiological and biochemical tools, and in addition, many of them have been shown to be valuable insecticides which are being extensively used in agriculture. Since many of these compounds are highly toxic to man, their safe use has given rise to a number of problems which have had to be solved in order that the full advantages of these materials could be exploited.

The following account is therefore a summary of progress mainly during the last 2 or 3 years.

Cholinesterase is the enzyme which hydrolyses acetylcholine. Inasmuch as cholinesterase is recognised by its action on acetylcholine *in vitro*, at least two separate enzymes have been described previously, which satisfy this condition. The "true" cholinesterase, known also as the "specific" enzyme, is generally found in nervous tissues and the red cells of most mammalian species. This enzyme, it is generally agreed, destroys acetylcholine at nerve endings. The pseudo enzyme, also known as the non-specific cholinesterase, is found in human serum (usually associated with the IV globulin fraction of Cohn), white matter of nervous tissue and dog's pancreas. No specific function has been found for this enzyme, neither has any naturally occurring substrate been demonstrated. Indeed, many workers believe that acetylcholine, *in vivo*, is not normally hydrolysed by the pseudo esterase.

These enzymes exhibit different kinetic properties (Alles and Hawes[8], Mendel, Mundel and Rudney[9], Augustinsonn[3]). They are differently distributed in tissues (Augustinsonn[3], Orde and Thompson[10,11]) and may be differentially inhibited by various inhibitors (Mazur and Bodansky[12], Hawkins and Mendel[13], Adams and Thompson[14], Todrick[15], Austin and Berry[16] and Aldridge[17]). They also exhibit different specificity patterns towards choline esters (Nachmansohn and Rothenberg[18], Mendel *et al.*[9], Adams[19], Adams and Whittaker[20], Mounter and Whittaker[21], Sturge and Whittaker[22]). Thus, true cholinesterase, i.e., the enzyme present in the grey matter and erythrocytes of many species, hydrolyses acetylcholine

faster than all the commonly known choline esters. The pseudo enzyme of human plasma, however, tends to exhibit a maximum activity with butyrylcholine. Mendel, Mundel and Rudney[9] showed that the true enzyme hydrolyses acetyl-β-methylcholine, but does not split benzoylcholine. On the other hand, the pseudo enzyme was shown to hydrolyse benzoylcholine but not acetyl-β-methylcholine. These authors suggested the use of this criterion to differentiate the enzymes which might be present in any given tissue.

The inadequacy of such a test was fairly quickly demonstrated for, in 1945, an enzyme was found in guinea-pig liver and kidney which would hydrolyse benzoylcholine but which did not break down acetylcholine. Sawyer[23], Ellis[24] and Ellis, Sanders and Bodansky[25] found that the plasma of certain rabbits contained a benzoylcholinesterase in addition to the true or specific cholinesterase, whereas, in the plasma of others, only the latter cholinesterase is present in significant amounts. This was confirmed by Levene, Hoyt and Suran[26] and Davies[27]. Levy[28] has also reported the presence of several other esterases in rabbit plasma. Very recently Koelle[29] has confirmed these results with rabbit serum and tissues. He partially purified the enzymes in these tissues, by means of fractional precipitation with sodium sulphate and has examined their substrate activity patterns and behaviour towards selective inhibitors. He concluded that the substrate activity pattern of acetocholinesterase, i.e., the true enzyme was similar to that of other species. The serum butyro-cholinesterase and serum benzoyl-cholinesterase exhibited virtually identical substrate activity patterns but they contrasted with that of the horse in their relatively low rates of acetylcholine hydrolysis. Inhibition studies indicated that rabbit serum benzoylcholinesterase and rabbit intestinal butyrocholinesterase are two distinct enzymes. The liver, too, appears to contain at least one additional enzyme capable of hydrolysing choline esters at a significant rate.

Davies, Risley and Rutland[30] examined the tissues of the ruminant and showed that a number of sheep tissues were active against butyrylcholine and concluded that there was a butyrocholinesterase in sheep tissue which did not hydrolyse benzoylcholine and if this were a pseudo type cholinesterase, it was certainly different from that found in rat tissue.

Levine and Suran[31] have described another variant of cholinesterase in swine serum, for they showed that, although it hydrolysed acetylcholine it did not hydrolyse either benzoylcholine or acetyl-β-methylcholine.

Earl and Thompson[32] have reported yet further modifications in the cholinesterase found in chicken serum. This enzyme hydrolyses acetylcholine, benzoylcholine and acetyl-β-methylcholine but its response to di*iso*propyl phosphorofluoridate (DFP), an inhibitor which has been extensively used to define the pseudo enzyme, suggests that it is, in fact, wholly pseudo.

Todrick[15] studied a large series of inhibitors and found 4 (see Table I) which, on the basis of their high specificity towards the cholinesterases of rat brain (true) and rat intestinal mucosa (pseudo), he used to characterise the enzymes present in rat, dog and goat heart tissue. He also used them

to investigate the type of enzymes present in the ganglia of the horse. He concluded that the rat heart enzyme is identical with the pseudo enzyme of the rat intestinal mucosa; although the enzyme of dog heart is similar to, it is not identical with that of the rat. Goat heart contains a true cholinesterase. The sympathetic chain of the horse and trigeminal

TABLE I

THE I50 CONCENTRATION OF INHIBITORS TOWARDS RAT BRAIN AND INTESTINAL MUCOSA CHOLINESTERASE

(After Todrick[15])

Inhibitor	Formula	Molar I 50 concentration × 10^{-6}		
		Rat brain	Rat mucosa	
Diethazine (Diparcol)	S, N; CH_2—CH_2—N(C_2H_5)(C_2H_5) HCl	2800	3·7	760
Caramephenium chloride (Parpanit)	C—CO·OCH$_2$CH$_2$N(C_2H_5)(C_2H_5) HCl	2500	5·1	490
Mepyramine (Neoantergan)	N, N; CH_2—OCH$_3$; CH_2—CH_2—N(CH_3)(CH_3)	56	85	0·66
R.P. 3565	—O—(CH$_2$)$_5$—O—; +N (CH$_3$)$_3$ I—; +N(CH$_3$)$_3$ I—	8·5	1500	0·0057

ganglion contain, in Todrick's view, the true enzyme almost exclusively; whereas, the ciliary ganglion and post ganglionic ciliary nerve appear to contain both enzymes.

Austin and Berry[16] have also examined two inhibitors which appeared to exhibit a high degree of specificity towards these two enzymes. *NN′*-di-*iso*propyl phosphorodiamidic anhydride (*iso*-OMPA) exhibits a high degree of selectivity toward the pseudo enzyme which it inhibits irreversibly and competitively, whilst the dimethobromide of 1:5-di(*p*-*N*-allyl-*N*-methylaminophenyl) pentan-3-one (284C51) inhibits the true enzyme reversibly and competitively. The degree of specificity of these inhibitors as measured by I 50 (true) : I 50 (pseudo) ratio is in general very high although the ratio varies considerably from species to species if erythrocytes and plasma are the source of true and pseudo enzymes respectively. The use of these inhibitors has also served to illustrate the fact that quite significant

differences may exist between enzymes which had previously been thought to be identical. According to Whittaker and his colleagues (Adams[19], Adams and Whittaker[20], Mounter and Whittaker[21], Sturge and Whittaker[22]) the esterases of human and horse blood are identical. Thus, the plasma enzymes of the two species conform to a substrate specificity pattern which they have designated "butyro-cholinersterase" and the erythrocyte enzyme to another, "aceto-cholinesterase". The I 50 (cells)/I 50 (plasma) was found to be about 50 for human and upwards of 6500 for horse using *NN'*-di*iso*propyl-phosphorodiamidic anhydride as inhibitor. A difference was also observed with 284C51. The I 50 (plasma)/I 50 (cells) being 10,000 for man and 2800 for horse. Acetylcholine was the substrate in each case.

There is little doubt from the foregoing that the term "cholinesterase" must include a family of esterases all of which hydrolyse acetylcholine *in vitro*. The specificity of these esterases towards other choline esters is quite wide, as Whittaker[5] has shown. The physiological significance of these various esterases is much less obvious, although most investigators will agree that the aceto-cholinesterase found at nerve endings is almost certainly the enzyme which is associated with neuro-humoral transmission. The substrate patterns of the "pseudo cholinesterases" however, raise a number of problems of great interest which Koelle[29] has discussed. Thus Augustinsson and Nachmansohn[33] do not believe that butyro-cholinesterase exists primarily to hydrolyse acetylcholine, an opinion with which few would disagree. Koelle[34,35], Koelle, Koelle and Fredenwald[36], have, however, presented evidence in the case of the non-specific cholinesterase of the cat's ileum to indicate that it plays an important role in the regulation of intestinal mobility, a conclusion supported by Burn, Kordek and Mole.[37]

The interesting and indeed, urgent problem is the recognition of the natural substrates for this family of enzymes. In this connection, the identification of propionylcholine in ox spleen by Bannister, Whittaker and Wijesundera[38] and also the description of a cholinesterase which hydrolyses this ester with maximal velocity (Orde and Thompson[11]) in rat heart is pertinent. Nachmansohn, Hestrin and Voripaieff[39] have presented evidence that an acetylating system of rabbit brain forms a compound possessing acetylcholine-like biological activity which is distinct from acetylcholine.

These findings indicate that the cholinergic mediator may consist not of acetylcholine alone but of acetylcholine plus one or more related compounds, a possibility which Whittaker has also suggested.[5]

THE NATURE OF THE INHIBITION OF CHOLINESTERASE BY ORGANO-PHOSPHORUS INHIBITORS

Aldridge[40] and Aldridge and Davison[41] have examined the nature of the inhibition of cholinesterase by organo-phosphorus compounds. In 1950, Aldridge had suggested that the mechanism of inhibition of cholinesterase could be expressed by the equation:

$$\underset{\text{(Enzyme)}}{E} + \underset{\text{(Inhibitor)}}{I} \rightleftharpoons \underset{\text{Reversible Enzyme Inhibitor Complex}}{(EI)^R} \longrightarrow \underset{\text{Irreversibly inhibited enzyme}}{(E)^I} + \text{Products}$$

and that the inhibitor was hydrolysed during the inhibitory process. There is a lot of evidence to support this suggestion; thus Jansen, Fellowes-Nutting and Balls[42] demonstrated that when chymotrypsin is inhibited by di*iso*propyl phosphorofluoridate, di*iso*propyl phosphate remains attached to the inactivated enzyme and one molecule of acid—presumably hydrogen fluoride is liberated during the process of inhibition. Hartley and Kilby[43] have shown that one molecule of *p*-nitrophenol is liberated during the inhibition of one molecule of chymotrypsin by diethyl *p*-nitrophenyl phosphate (E600). When horse serum cholinesterase is inhibited by di*iso*propyl phosphorofluoridate containing ^{32}P, phosphorus becomes firmly attached to the enzyme (Boursnell and Webb[44]), whilst Jandorf and McNamara[45]) have also shown that, after a dose of di*iso*propyl phosphorofluoridate containing ^{32}P to rabbits, the elimination of ^{32}P from the red cells paralleled the reappearance of cholinesterase activity. Aldridge and Davison[41] point out that the direct test of their hypothesis that the inhibitor is hydrolysed cannot be made until mammalian cholinesterase has been obtained in a pure state. They have, therefore, approached the problem indirectly and have compared the stability of hydrolysis of a series of inhibitors at physiological *p*H and temperature with their inhibitory power. They found that the more stable the inhibitor, the slower is its inhibitory action on cholinesterase. These results are shown in Table II.

TABLE II

CONSTANTS FOR THE RATE OF REACTION OF INHIBITORS DERIVED FROM TRIETHYLPHOSPHATE WITH CHOLINESTERASE AND FOR THEIR HYDROLYSIS IN BUFFER SOLUTION
(After Aldridge and Davison[41])

Inhibitor	No.	Rate of reaction of inhibitor with cholinesterase K(M^{-1}/Mol^{-1})	Ratio of hydrolysis of inhibitor in phosphate buffer *p*H 7·6 at 37° C. (K. $min.^{-1}$)	
p-Chloro-	1	$1·1 \times 10^{2}$	$7·2 \times 10^{-7}$	(4·2 per cent.)
o-Chloro-	2	$2·1 \times 10^{2}$	$2·3 \times 10^{-6}$	(7·9 per cent.)
p-Nitro-	3	$1·1 \times 10^{6}$	$3·9 \times 10^{-6}$	(65 per cent.)
o-Nitro-	4	$1·6 \times 10^{4}$	$5·6 \times 10^{-5}$	(78 per cent.)
m-Nitro-	5	$7·7 \times 10^{3}$	$1·4 \times 10^{-5}$	(45 per cent.)
Tri-phenyl-	6	6·1	$1·6 \times 10^{-7}$	(2·9 per cent.)
Tetraethyl pyrophosphate ..	7	$3·3 \times 10^{6}$	$6·0 \times 10^{-3}$	(100 per cent.)

(Since most of the compounds are very stable, the long time required for their complete hydrolysis made necessary the calculation of the non-enzymic hydrolysis constants from data involving incomplete hydrolyses. The proportion of the compound hydrolysed in the period during which the determinations were made is shown in brackets. The numbers refer to the points in Figure 1.)

In Figure 1 these results have been shown diagrammatically, where the logarithm of the bimolecular rate constant of the inhibitory reaction has been plotted against the negative logarithm of the hydrolysis constant.

These data are undoubtedly consistent with the mechanism postulated above, which as Aldridge and Davison point out, is the same as that of the Michælis–Menten equation for an enzyme substrate equation.

$$E + S \rightleftharpoons ES \rightleftharpoons E + \text{Products}$$

Later[46] these same authors extended these observations to include the S-phenyl isomer of *OO*-diethyl-*S*-*p*-nitrophenyl phosphorothionate, *OS*-diethyl *O*-*p*-nitrophenyl phosphorothionate and *OO*-diethyl-*O*-8-quinoyl phosphorothionate.

These ideas have been employed to offer an explanation of the differential inhibition of true and pseudo cholinesterase by a series of organophosphorus compounds (Aldridge[17]). The most outstanding difference between true and pseudo cholinesterase is their substrate specificity for acetates and butyrates respectively. If the mechanism of inhibition of both enzymes is the same and they are attached to the esteratic site by the electrophilic phosphorus atom (Nachmansohn and Wilson[7]), then the length of the alkoxy group attached to the phosphorus may well be important in governing the "fit" of the inhibitor on the enzyme surface and thus its ease of hydrolysis and inhibitory power. Aldridge compared the substrate ratio of a series of choline esters in which the length of the acyl group was varied, with the inhibitor ratio of a number of organo-phosphorus inhibitors with varying acyl groups. The substrate ratio is the rate of hydrolysis (based on acetylcholine = 100) for pseudo over that for true cholinesterase, whilst the inhibitor ratio is the concentration of inhibitor for 50 per cent. inhibition for true cholinesterase over that for pseudo. The ratios have been calculated in this way because if the inhibitor is a substrate for the enzyme, then the more efficiently it is hydrolysed by the enzyme, the lower will be the concentration of the inhibitor necessary for inhibition.

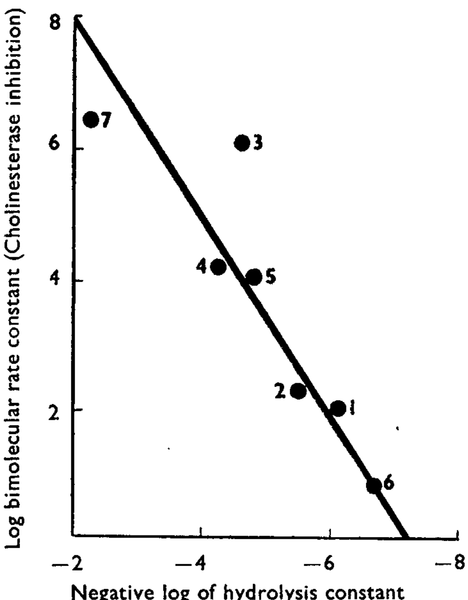

FIG. 1. Relationship between rate of hydrolysis in buffer at pH 7·6 and at 37° C. and the bimolecular rate constant for the reaction of cholinesterase with inhibitor. Compounds are numbered as in Table II. (After Aldridge and Davison[41]).

It appears from a limited number of inhibitors that there is a rough correlation. There is a marked change from *n*-butyryl to *iso*valeryl choline in the substrate ratio. In three groups of inhibitors, tetraethyl and tetra*iso*propyl pyrophosphate, diethyl and di*iso*propyl *p*-nitrophenyl phosphates and diethyl and di*iso*propyl phosphorofluoridates, there is a change in the inhibitor ratio in the same direction. There is a marked change in substrate ratio from *iso*butyrylcholine to *iso*valerylcholine and a similar change is found in the inhibition ratio from *NNN′N′*-tetramethyl phosphorodiamidic fluoride (dimifox) to *NNN′N′*-tetramethyl phosphoramidic anhydride.

Thus, considering a choline ester and an inhibitor with groups of a

similar length, if the choline ester after a change of acyl group is hydrolysed relatively more readily by pseudo than true cholinesterase, then with a similar change of groups (as regards length) on the inhibitor a compound is produced which is relatively more efficient against pseudo than against true. Thus it appears that at least two factors are involved in the efficiency of an organo-phosphorus compound as an inhibitor: its stability to hydrolysis and the particular group attached to the phosphorus.

Results such as those just quoted, together with the evidence obtained from studies on purified chymotrypsin led Aldridge[47] to postulate that the inhibitory process is a phosphorylation of the enzyme by the organo-phosphorus inhibitor. Thus Brauer[48] pointed out that those organo-phosphorus compounds which are inhibitors contain an anhydride-like structure. Furthermore, it has been shown that, with many of these inhibitors, the more unstable they are to hydrolysis, the more effective they are as inhibitors. With such a series, their efficiency in phosphorylating water parallels their ability to inhibit cholinesterase (Topley[49]). On the basis of this evidence, Aldridge has suggested the following reaction mechanism for the inhibition and reversal of inhibition of cholinesterase by symmetrical dialkyl aryl phosphates.

$$E + \underset{OR'}{\overset{(OR)_2}{P}}=O \xrightarrow{(1)} \left[E \cdots \underset{OR'}{\overset{(OR)_2}{P}}=O \right]$$

$$\xrightarrow{(2)} R'OH + \left[E - \overset{(OR)_2}{P} = O \right] \xrightarrow[H_2O]{(3)} E + \underset{OH}{\overset{(OR)_2}{P}} = O$$

(E = cholinesterase, R = alkyl, R′ = aryl.)

This reaction is very similar to that postulated by Wilson, Bergman and Nachmansohn[50] for the reaction mechanism for the hydrolysis of acetylcholine by cholinesterase.

$$E + \underset{Ch}{\overset{Ac}{|}} \xrightarrow{(1)} \left[E \cdots \underset{Ch}{\overset{AcO}{|}} \right] \xrightarrow{(2)} ChOH + [E - Ac]$$

$$\xrightarrow[+ H_2O]{(3)} E + AcOH.$$

(AcO − Ch = acetylcholine, ChOH = choline, AcOH = acetic acid.)

In this view, the two reactions are strictly analogous. Thus, just as marked changes are produced in the rates of hydrolysis of choline esters by changes in the length and shape of the aryl groups, so alterations in the length and shape of the alkoxy groups of organo-phosphorus compounds produce corresponding changes in their inhibitory powers against the true and pseudo cholinesterases. It has been pointed out by Wilson *et al.*[50] that the rate-determining step is the formation of the acylated enzyme while reaction 3 must be comparatively fast. In a similar scheme

shown for the inhibitory process, the rate-limiting step is reaction 3, thus allowing the accumulation of the inhibited enzyme by reactions 1 and 2. If reaction 3 were fast compared with reactions 1 and 2, then the enzyme would not be inhibited and the organo-phosphorus compound would be rapidly hydrolysed. Aldridge sums up his views as follows:—

"Briefly, the experimental results obtained by many workers are consistent with the view that, since organo-phosphorus inhibitors are esters, they can attach themselves to the active centre of cholinesterase in the same way as carboxylic esters; the inhibitor is then hydrolysed, but the enzyme phosphate so formed has its own stability to hydrolysis and this is dependent upon the groups attached to the phosphorus atom. On such a hypothesis, the potency of these inhibitors is a reflection not of an especially high affinity for the enzyme but of the fact that one active centre is inactivated after reaction with an inhibitor molecule".

TABLE III

A COMPARISON OF THE SUBSTRATE AND INHIBITOR RATIOS FOR THE TRUE AND PSEUDO CHOLINESTERASE OF THE HORSE

(After Aldridge[17])

Substrates			Inhibitors Inhibitor ratio			
Acyl group of Choline ester	Substrate ratio	Acyl group of inhibitor	*p*-Nitrophenyl phosphates	Phosphoro fluoridates	Pyrophosphates	Phosphoramidic fluorides
$—CO·CH_3$	1	$>PO·CH_3$				
$—CO·CH_2CH_3$	1·9	$>PO·OCH_3$	0·68			
$—CO·CH_2CH_2CH_3$	94	$>PO·OCH_2CH_3$	2·9	51	75	
$—CO·CH(CH_3)_2$	2·0	$>PO·N(CH_3)_2$				22
$—CO·CH_2CH_2CH_2CH_3$	44	$>PO·OCH_2CH_2CH_3$	10	270	500	3950
$—CO·CH_2CH(CH_3)_2$	263	$>PO·OCH(CH_3)_2$				

The Organic Phosphorus Insecticides

The organo-phosphorus compounds represent a new group of toxic chemicals which are becoming increasingly important as agricultural insecticides because of their effectiveness against a variety of harmful insects including many which are resistant toward dichlorodiphenyltrichloroethane (chlorophenothane, DDT). DuBois and Coon[51] have listed and classified many of the substances which have been seriously considered as insecticides. They are all anticholinesterases *in vivo* and produce symptoms similar to those resulting from excessive stimulation of the parasympathetic nervous system. At the present time, there are three principal groups of organic insecticides containing phosphorus, namely

the tetra-alkyl pyrophosphates, the alkyl phosphorothionates and the phosphoramidic derivatives. The individual members of each group exhibit similarities in their pharmacological behaviour, whilst the three classes of compounds vary with respect to such factors as duration of action and distribution in the tissues.

The Tetra Alkyl Pyrophosphates. These possess the general formula—

$$(RO)_2P(=O)\text{—O—}P(=O)(OR')_2$$

where R may be identical with R′.

In Table IV, the intraperitoneal LD50 for mice and the molar concentration for 50 per cent. inhibition of cholinesterase *in vitro* are listed.

TABLE IV

TOXICITY AND ANTICHOLINESTERASE ACTION OF ALKYL PYROPHOSPHATES

(After DuBois and Coon[51])

Compound		LD50 mg./kg.	Molar concentration for 50 per cent. inhibition of cholinesterase
Tetramethyl pyrophosphate	$(CH_3O)_2P(=O)\text{—O—}P(=O)(OCH_3)_2$	1·7	$1{\cdot}8 \times 10^{-8}$
Tetraethyl pyrophosphate . .	$(C_2H_5O)_2P(=O)\text{—O—}P(=O)(OC_2H_5)_2$	0·85	$4{\cdot}0 \times 10^{-9}$
Dimethyl diethyl pyrophosphate	$(CH_3O)_2P(=O)\text{—O—}P(=O)(OC_2H_5)_2$	1·1	$8{\cdot}0 \times 10^{-9}$
Dimethyl di*iso*propyl pyrophosphate	$(CH_3O)_2P(=O)\text{—O—}P(=O)(OCH(CH_3)_2)_2$	2·5	$2{\cdot}0 \times 10^{-7}$
Tetra*iso*propyl pyrophosphate	$((CH_3)_2CHO)_2P(=O)\text{—O—}P(=O)(OCH(CH_3)_2)_2$	16·0	$1{\cdot}4 \times 10^{-6}$

The most important member of this series is tetraethyl pyrophosphate (TEPP), a compound which is highly toxic to mammals, produces typical cholinergic effects and exerts a potent inhibitory action on cholinesterase *in vitro* and *in vivo*.

The Alkyl Phosphorothionates. These have the general formula—

$$RO\text{—}P(=S)(\text{—OR})\text{—O—}X$$

S and O may be interchanged to provide isomeric forms whilst R is in general an alkyl group and X is an aryl group. The more commonly occurring ones, that is those compounds which have been examined more fully for insecticidal properties are shown in Table V.

TABLE V

THE TOXICITY AND ANTICHOLINESTERASE ACTIVITY OF ALKYL PHOSPHOROTHIONATES (After DuBois and Coon[51])

Compound	Synonym	Intraperitoneal toxicity for rats mg./kg.	Molar concentration for 50 per cent. inhibition of cholinesterase *in vivo*
OO-Diethyl *Op*-nitrophenyl phosphorothionate	Parathion	5·5	$1{\cdot}2 \times 10^{-6}$
Dimethyl *p*-nitrophenyl phosphorothionate	Metacide	3·5	1×10^{-4}
Diethoxy ester of 7-hydroxycoumarin phosphorothionate	Potassan E.838	15·0	5×10^{-9}
S-(1 : 2-dicarbethoxyethyl) *OO*-dimethyl dithiophosphate (90 per cent. technical)	Malathan 4049	750·0	1×10^{-4}
Diethoxy thiophosphoric acid ester of 2-ethyl mercaptoethanol (technical)	Systox	3·0	5×10^{-7}

The best known of these substances is *OO*-diethyl *O-p*-nitrophenyl phosphorothionate (parathion) which is extensively used throughout the world. The proven value of this substance has resulted in a thorough examination of its pharmacological and biochemical properties. Various estimates of its molar concentration for the inhibition of 50 per cent. cholinesterase activity *in vivo* have been made. These estimates have varied very considerably but Aldridge and Davison[46] have shown that nearly all of the *in vitro* activity of this compound is due to the presence of impurities which are extremely potent inhibitors of cholinesterase *in vitro* and are present in too small an amount to be determined or even recognised by ordinary methods of chemical analysis. The acknowledged technical quality of at least 2 of the 5 substances quoted in Table V would suggest that most of the *in vitro* activity quoted for these substances may well be due to impurities, consequently, the inhibition values quoted should be accepted with considerable caution. This problem will be discussed more fully later in this article.

The Phosphoramide Derivatives. These constitute the most recent addition to the ever growing list of organic phosphorus insecticides. Octamethyl pyrophosphoramide (OMPA) is the only one which has thus far been released for use. It is, however, not used on food crops because of the danger of food contamination. DuBois, Doull and Coon[52] investigated the pharmacology and biochemistry of this compound extensively. It exhibits no appreciable anticholinesterase activity *in vitro* but is converted by the mammalian liver and by plants into a strong cholinesterase inhibitor. A further differentiating feature of this substance is its inability to gain access to the brain *in vivo*; its cholinergic action being therefore limited to peripheral tissues. In addition, octamethyl pyrophosphoramide is stable in aqueous solutions in contrast to the alkyl pyrophosphates and alkyl thiophosphates which undergo relatively rapid hydrolysis in the presence of moisture. These unusual properties appear

to be due to a nitrogen-phosphorus linkage in the molecule. The general formula of these compounds is—

$$(R{=}N)_2P(=O){-}R$$

Table VI gives a comparison of the toxicity and *in vitro* anticholinesterase activity of several phosphoramidates. They are all similar in their properties with respect to their stability in aqueous solution, selective action on peripheral tissues and relatively long duration of action. The active phosphoramidates which contain alkoxy phosphate linkages do not require hepatic conversion to become anticholinesterase agents.

TABLE VI

COMPARISON OF THE TOXICITY AND ANTICHOLINESTERASE ACTION OF VARIOUS PHOSPHORAMIDATES (After DuBois and Coon[51])

Compound	Structural formula	Intraperitoneal LD50 for rats, mg./kg.	Molar concentration for 50 per cent. inhibition of cholinesterase *in vitro*
Ethyl di(dimethylamido) phosphate	$[(CH_3)_2N]_2P(=O){-}OC_2H_5$	>1500	$>1 \times 10^{-2}$
Bis (dimethylamido) phosphorofluoridate	$[(CH_3)_2N]_2P(=O){-}F$	5	4×10^{-5}
Octamethyl pyrophosphoramide	$[(CH_3)_2N]_2P(=O){-}O{-}P(=O)[N(CH_3)_2]_2$	8	$>1 \times 10^{-2}$
Diethyl di(dimethylamido) pyrophosphate (symmetrical)	$(CH_3)_2N(C_2H_5O)P(=O){-}O{-}P(=O)[N(CH_3)_2](OC_2H_5)$	11·5	$4{\cdot}7 \times 10^{-7}$
Diethyl di(dimethylamido) pyrophosphate (unsymmetrical)	$(C_2H_5O)_2P(=O){-}O{-}P(=O)[N(CH_3)_2]_2$	2·7	$2{\cdot}8 \times 10^{-7}$

FACTORS INFLUENCING THE TOXICITY OF THE ORGANO PHOSPHORUS INSECTICIDES TOWARDS MAMMALS

Aldridge and Barnes[53] have drawn attention to many discrepancies in the published literature of the biological properties of a number of these compounds both *in vivo* and *in vitro*. Thus they quote the experiments of Aldridge and Davison[46,47] who studied a group of "purified" substituted diethyl phosphates. These authors showed that a good correlation exists between toxicity to mammals and their ability to inhibit cholinesterase *in vitro*. A study of the kinetics of the reaction between these compounds and cholinesterase, however, revealed that they all contained an impurity. Aldridge[47] had previously shown that the inhibition of red

cell cholinesterase by diethyl *p*-nitrophenyl phosphate was of first order kinetics and is bimolecular, i.e.—

$$K = \frac{1}{t \times I} \log_n \frac{100}{b}$$

where K = bimolecular rate constant, t = time in minutes, I = molecular concentration and b = percentage residual activity. If the time is maintained constant at 30 minutes, a plot of log b against I, the inhibitor concentration should give a straight line. This was, in fact, found to be the case not only with diethyl *p*-nitrophenyl phosphate but also with tetraethyl pyrophosphate and a specially purified specimen of diethyl

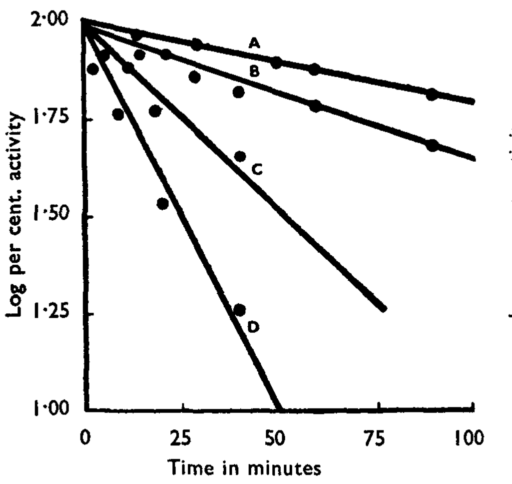

FIG. 2. Rate of inhibition of cholinesterase by purified diethyl *p*-chlorophenyl phosphate. Concentration of inhibitor shown against each curve. (After Aldridge and Davison).

A = $4{\cdot}05 \times 10^{-5}$M
B = $9{\cdot}2 \times 10^{-5}$M
C = $2{\cdot}0 \times 10^{-4}$M
D = $4{\cdot}05 \times 10^{-4}$M

FIG. 3. Rate of inhibition of cholinesterase by unpurified diethyl *p*-chlorophenyl phosphate concentrations of inhibitor shown against each curve. (After Aldridge and Davison).

A = $2{\cdot}7 \times 10^{-6}$M
B = $5{\cdot}4 \times 10^{-6}$
C = $9{\cdot}0 \times 10^{-5}$M

p-chlorophenyl phosphate (Aldridge and Davison[46,47]). Figure 2 shows the inhibition produced by incubation of purified diethyl *p*-chlorophenyl phosphate with washed sheep red cells.

Figure 3 shows the rate of inhibition by unpurified diethyl *p*-chlorophenyl phosphate. The authors came to the conclusion that the differences in the kinetic picture of the two preparations were due to the presence of small quantities of an impurity.

They studied a series of other inhibitors both unpurified and purified and determined the concentration required for 50 per cent. inhibition of the enzyme preparation (see Table VII). Then by applying a method of calculation described in their paper Aldridge and Davison[41] determined the percentage impurity which they concluded from other data was tetraethyl pyrophosphate and which was present in the unpurified preparations.

The amount of impurity is extremely small and is, in fact, too small to be detected by ordinary chemical analysis. Table VII also shows that the presence of very small quantities of impurities which are themselves potent inhibitors of cholinesterase may well influence in a most profound manner the concentration of inhibitor needed to produce 50 per cent. inhibition of the enzyme.

TABLE VII

CONCENTRATION FOR 50 PER CENT. INHIBITION BY UNPURIFIED ANALOGUES OF DIETHYL *p*-NITROPHENYL PHOSPHATE AND THE CONCENTRATION OF TETRAETHYL PYROPHOSPHATE AS IMPURITY

(After Aldridge and Davison[41])

Inhibitor	Molar concentration for 50 per cent. inhibition		Impurity Tetraethyl pyrophosphate per cent.
	Unpurified	Purified	
p-Chloro	$5·75 \times 10^{-6}$	$2·1 \times 10^{-4}$	0·68
O-chloro	$2·24 \times 10^{-5}$	$1·1 \times 10^{-4}$	0·053
p-Nitro (E600) ..	$2·0 \times 10^{-8}$	$2·01 \times 10^{-8}$	—
O-nitro	$4·58 \times 10^{-7}$	$1·48 \times 10^{-6}$	0·33
m-Nitro	$1·2 \times 10^{-6}$	$3·0 \times 10^{-6}$	0·55
Phenyl 1	$1·35 \times 10^{-4}$	$3·77 \times 10^{-3}$	0·025
Phenyl 2	$2·35 \times 10^{-7}$	$3·77 \times 10^{-3}$	5·4

As mentioned above, DuBois *et al.*[52] showed that octamethyl pyrophosphoramide was metabolised by the liver into a potent inhibitor of cholinesterase but it has only recently been appreciated that this is true also of diethyl *p*-nitrophenyl phosphorothionate. Aldridge and Barnes[53] demonstrated this both simply and effectively. Two rabbits were given 10 mg./kg., one intravenously and the other intraperitoneally. Blood cholinesterase determinations were performed before injection and at various times afterwards. Samples of blood which were taken before administration of the chemical were incubated with a concentration of the insecticide which was equal to that which would occur if all the administered substances were assumed to be circulating in the blood stream. This was, of course, substantially greater than that reached *in vivo*. In each case, the inhibition was very significantly more in the blood samples which had been treated *in vivo* than in those specimens which had been incubated with the inhibitor *in vitro*. The diethyl *p*-nitrophenyl phosphorothionate had been converted in the body, into a much more potent inhibitor. Diggle and Gage[54] had earlier suggested that this might, in fact, be the case after considering the relationships which they uncovered between toxicity and proportion of isomer present as a contaminant. Later, their colleague, Goldblatt[55] showed that hepatectomy considerably reduced the toxicity of diethyl *p*-nitrophenyl phosphorothionate. Gage and Payton[56] showed that it was converted in the animal body to diethyl *p*-nitrophenyl phosphate.

Aldridge and Barnes have repeated the experiment quoted above with 4 other organic phosphorus inhibitors (see Table VIII) and have shown that they too are metabolised to active anticholinesterases *in vivo*.

With 4 of the 5 compounds in Table VIII, the results are quite conclusive, indicating the conversion to a much more potent anticholinesterase

in the body. The di*iso*propyl compound seems to be discrepant but the authors consider that by comparison with diethyl *p*-nitrophenyl phosphate, the results indicate a similar type of conversion.

Aldridge and Barnes have also drawn attention to some other biological properties which may well lead to discrepant results if the experimenter is not aware of them. Thus there are sex species differences in their susceptibility to the toxic action of these substances. DuBois *et al.*[57] had shown

TABLE VIII

A COMPARISON OF THE INHIBITION OF RED CELL CHOLINESTERASE BY COMPOUNDS 1, ADMINISTERED TO LIVING ANIMALS AND 2, INCUBATED *in vitro* WITH BLOOD FROM THE SAME ANIMAL

(After Aldridge and Barnes[53])

For the calculation of the *in vivo* concentration in the blood, the blood volume of the rabbit has been taken to be 70 ml./kg. It was assumed that all the drug would be in the circulating blood. All injections were solutions of the compound in absolute ethanol.

Dose mg./kg.	Route of administration	Time after injection or incubation (minutes)	Percentage inhibition *in vitro*	Percentage inhibition *in vivo*
	Diethyl *p*-nitrophenyl phosphorothionate			
	Intravenous	10	8	83
10	Intraperitoneal	11	9	66
		40	20	81
	4-Methyl-7-hydroxycoumarin diethyl phosphorothionate (E838):			
15	Intravenous	20	26	94
		30		97
	Dimethyl *p*-nitrophenyl phosphorothionate:			
10	Intravenous	10	—	67
		40	8	64
15	,, ...	10	6	80
		40	5	65
	Di*iso*propyl *p*-nitrophenyl phosphorothionate:			
15	Intravenous	10	21	34
	Bis-dimethylaminofluorophosphine oxide			
20	Intravenous	7	31	88
		20	—	86
		42	—	89

that *OO*-diethyl *O*-*p*-nitrophenyl phosphorothionate is more toxic to female than to male rats. Other species do not show this differential. Male and female rats are equally susceptible to tetraethyl pyrophosphate, diethyl *p*-nitrophenyl phosphate and also to two isomers of diethyl *p*- nitrophenyl phosphorothionate. On the other hand, diethyl-4-methyl-7-hydroxycoumaryl phosphorothionate (E838) also exhibits sex differences as does *p*-nitrophenylbenzene phosphorothionate (EPN300).

Male and female rats differ in their sensitivity to both octamethyl pyrophosphoramide and *NN'*-di*iso*propylphosphorodiamidic fluoride (mipafox), but it is the male rat which is the more susceptible. With the exception of tetraethyl pyrophosphate which does not exhibit a sex differential, all the compounds mentioned have to be metabolised before exhibiting anticholinesterase properties. It may well be, therefore, that the sex

differential represents the ease and efficiency with which the two sexes affect this conversion.

Marked differences have been found in the size of the lethal dose, depending upon both the route of administration and the solvent used. Thus the lethal doses of tetraethyl pyrophosphate and diethyl *p*-nitrophenyl phosphate are only slightly reduced if the subcutaneous is substituted for the intravenous route of administration. Death occurs within 10 to 30 minutes by either route. The latter has always been injected as a solution in ethanol; the former may be given in water or ethanol without affecting the size of the dose. When diethyl *p*-nitrophenyl phosphorothionate and its methyl and *iso*propyl analogues are given in ethanolic solution, either intravenously or intraperitoneally, death normally occurs within one hour. When injected subcutaneously in small volumes (0·01 to 0·1 ml.) in strong ethanol solution, it is necessary to administer about 10 times the average lethal dose by intravenous injection and death does not occur for at least 24 to 48 hours in the case of diethyl *p*-nitrophenyl phosphorothionate and its dimethyl analogue. In the case of the *iso*propyl compound, death may be delayed for 10 to 14 days. It is possible that the compounds remain dissolved in the lipoids at the site of injection and are only slowly released.

The Turnover Number of Cholinesterase

Berry[58] has pointed out that many problems in cholinesterase kinetics would be greatly simplified by a knowledge of the concentration of the enzyme in the system being studied. This could be calculated if the "turnover number" (T) of the enzyme were known. He attempted to determine T by titrating the active centres of the true and pseudo cholinesterase with what he initially thought was a highly specific and irreversible cholinesterase inhibitor, di*cyclo*hexyl phosphorofluoridate (DCFP). Di*cyclo*hexyl phosphorofluoridate appeared to be suitable for this purpose since, in addition to the above properties it is also highly resistant to spontaneous hydrolysis.

The value of T for erythrocyte cholinesterase as determined in this way varied not only amongst species but also amongst individuals of the same species. From this, Berry concluded that these inhibitors were not as highly specific as previously believed and that the observed variations in the apparent turnover number were probably due to the presence in tissue extracts of varying amounts of substances competing with cholinesterase for the inhibitor.

Cohen, Oosterbahn and Warringa[59] have approached the determination of T for erythrocyte cholinesterase very ingeniously. Although they used a partially purified preparation of ox red cell cholinesterase, they appreciated at the outset that their preparation contained substances other than cholinesterase which were likely to combine with the inhibitor. They, therefore used a combination of irreversible inhibitor, di*iso*propyl phosphorofluoridate labelled with radioactive phosphorus, and a reversible one, butyrylcholine. The essential principle of their method was to block the active centre first with the reversible inhibitor and to then cause the

remaining molecules which will combine to react with non-labelled di*iso*propyl phosphorofluoridate.

The butyrylcholine and the excess of di*iso*propyl phosphorofluoridate were then dialysed off and the active centre now free from inhibitor treated to the action of radioactive di*iso*propyl phosphorofluoridate. Two estimates of *T* for ox red cell cholinesterase in which modifications of the above method were used were 300,000 molecules/minute and 280,000 molecules/minute.

Organo-phosphorus Compounds and Demyelination

The long term effects of the organo-phosphorus compounds have not been as fully investigated as the acute manifestations. Recently, however, 3 factory workers were accidentally poisoned whilst preparing, on a pilot plant scale, *NN'*-di*iso*propylphosphorodiamidic fluoride. Two of these developed paralysis of the legs and hands which persisted for several months (Bidstrup and Hunter[60]). Clinically, these effects were very similar to those which occur after poisoning with tri-*o*-cresyl phosphate (TOCP) and which have been described by Smith, Elvove and Frazier[61]. They have also been demonstrated as being due to demyelination of the nerve sheath (Smith, Engle and Stohlman[62]).

The toxic effects of tri-*o*-cresyl phosphate have been known since the last century. Thus Loret[63] described 6 cases of multiple neuritis in tubercular patients who had been treated with preparations of phospho creosote which contained tri-*o*-cresyl phosphate. Extensive outbreaks of paralysis occurred owing to the use of tri-*o*-cresyl phosphate as an adulterant in drinks prepared from extracts of jamaica ginger (Smith and Elvove[64]) in apiol (Ter Braak[65]) and in edible oils (Sampson[66], Hotston[67]). This substance is also used in the plastics industry and cases of poisoning in this country have been described by Hunter, Perry and Evans[68]).

Smith and Lillie[69] have described the histopathology of lesions produced by tri-*o*-cresyl phosphate in the peripheral nerves and central nervous system of animals. They found evidence of demyelination of the peripheral nerves, degenerative changes in the anterior horn cells and fatty degeneration in the white substance of the spinal cord. The syndrome of tri-*o*-cresyl phosphate poisoning is usually described as a peripheral neuritis although it is clear that cord changes are also present. Motor function is severely effected and the paralysis is more marked in the feet and legs, than in the arms. The distal muscles of the limbs are more effected than those nearer the trunk. Sensation seems to be largely unaffected. Complete recovery may ensue but in some cases, late signs of damage to the pyramidal tracts may appear and the picture has been described as being very like that seen in amyotropic lateral sclerosis (Ahring[70]).

Koelle and Gilman[4] summarised the chronic effects which have been found in rats, dogs, monkeys and cats following prolonged dosage with di*iso*propyl phosphorofluoridate. When dogs were given doses of this substance to elicit nicotinic and muscarinic responses and when such doses were repeated twice weekly for several months, functional disturbances of smooth and striate muscle occurred, which persisted after the drug was

discontinued. The first effects were fasciculations of the tongue. These spread to other muscles. Muscular weakness of the hind legs developed which eventually led to paralysis within a few weeks. Similar effects have also been observed in cats which have received either single large injections or 2 to 6 successive doses. Fasciculations followed by ataxia and extreme muscular weakness were observed. A recurrent weakness of the kind limbs was evident for as long as 147 days.

Chronic effects have also been observed in man following poisoning by the organo-phosphorus insecticides. Thus Petry[71] described chronic effects similar to the above in a man poisoned with diethyl *p*-nitrophenyl phosphorothionate, Bidstrup and Hunter[60] as mentioned above, gave an account of 3 workers poisoned with *NN'*-di*iso*propyl phosphorodiamidic fluoride, two of whom developed chronic manifestations similar to those described with di*iso*propyl phosphorofluoridate and tri-*o*-cresyl phosphate.

These long term clinical effects are, as a consequence, being more intensively investigated. Earl and Thompson[72] have studied the effect of tri-*o*-cresyl phosphate upon the changes in the enzymes of the brain, spinal cord and serum. From their experiments, it appears that the only biochemical reaction of tri-*o*-cresyl phosphate is with pseudo cholinesterase, which it inhibits selectively. Bloch[73] showed that the cholinesterase of horse serum was inhibited by this substance and later Hottinger and Bloch[74] extended these observations and showed that tri-*o*-cresyl phosphate inhibited acetylcholine hydrolysis by human and rabbit serum and rabbit liver and brain. Mendel and Rudney[75], reported that oral administration of tri-*o*-cresyl phosphate to rats caused a lowering of the serum pseudo-cholinesterase, whereas the true esterase appeared to be unaffected. Earl and Thompson[72] examined this problem in greater detail with human tissues. These authors showed that 50 μg./3 ml. tri-*o*-cresyl phosphate caused over 50 per cent. inhibition of the pseudo enzyme of the serum, of the white matter of the cerebrum and also of the grey matter from the same area. The spinal cord pseudo enzyme was equally inhibited. The sciatic nerve appeared to be even more sensitive since half this quantity of inhibitor was sufficient to cause a similar degree of inhibition. The true cholinesterase in erythrocytes, white and grey cerebral matter, spinal cord and striated muscle on the other hand was not significantly inhibited by even 200 μg./3 ml. and quantities of 300 to 1000 μg./3 ml. had little or no effect upon the true enzyme of the spinal cord or striated muscle. Similar results were obtained with rabbit and chicken tissues but the pseudo cholinesterase of albino rat appears to be insensitive to poisoning by tri-*o*-cresyl phosphate and is only partially inhibited by even high concentrations.

These observations were extended to *in vivo* studies on the chicken (Earl and Thompson[72]). 24 birds were poisoned with tri-*o*-cresyl phosphate and the brain, spinal cord and plasma esterases were examined at intervals after poisoning. For 10 days, the birds remained apparently quite normal. Between the tenth and fourteenth day, they showed the first definite evidence of paralysis. They appeared quite normal in the cage but, when let out, they walked unsteadily. On the following day,

they could be seen sitting on their heels in the cage but when released, as before, they walked unsteadily but after a few minutes were unable to lift their heels from the ground. A few days later, the legs became completely useless and the birds sat with their legs outstretched, unable to move. Apart from the paralysis, however, the birds seemed to be healthy; they held their heads high and their combs were bright. Furthermore, except in the advanced stages of paralysis, they ate well.

The pseudo cholinesterases of the plasma, brain and spinal cord are markedly diminished within 24 hours after poisoning and, in the case of the brain and spinal cord, remain at a low level for 10 days at least. The true cholinesterase in these tissues is relatively unaffected.

In discussing their results, Earl and Thompson[72] remind their readers that, from earlier work on both man and mammals, it seems generally agreed that the primary change in tri-*o*-cresyl phosphate poisoning is demyelination. Smith and Lillie[69] have even described this compound as a specific "myelin poison". Since the rapid onset of the lowering of pseudo cholinesterase activity precedes the development of clinical symptoms by about a fortnight, it is possible that the biochemical effect precedes any structural damage to the nervous system. Indeed, Earl and Thompson suggest that the inhibition of the pseudo cholinesterase may be responsible for the demyelination. In support of this suggestion, they draw attention to the fact that the two other substances which appear to produce demyelination easily are also selective inhibitors of the pseudo enzyme. They also point out that there is no evidence to connect this esterase with the inactivation of acetylcholine *in vivo* and that it is conceivable that its function may be concerned with some unknown aspect of metabolism which is of particular importance in the turnover of myelin. A transient inhibition of this enzyme process might cause no serious upset in the slow but continuous breaking down and formation of myelin, whereas, the prolonged inhibition such as occurs with tri-*o*-cresyl phosphate might, after some time, cause defects in myelination to become apparent.

Barnes and Denz[76] examined the effects of 9 organo-phosphorus compounds upon chickens, rats and rabbits. Each of these compounds were selective inhibitors of pseudo cholinesterase. 3 of them only were effective in producing paralysis in any of these species. They were, di*iso*propyl phosphorofluoridate, *NN'*-di*iso*propylphosphorodiamidic fluoride and tri-*o*-cresyl phosphate. In each case 1 mg./kg. was administered either intravenously or orally, atropine being given where necessary to control the acute symptoms. With *NN'*-di*iso*propyl phosphorodiamidic fluoride and di*iso*propyl phosphorofluoridate, these lasted 1 to 2 days but the chronic picture, as described earlier, did not develop for 10 to 14 days. No acute symptoms were produced with tri-*o*-cresyl phosphate.

In the case of the remaining 6 compounds, 3 successive doses of each were given and the birds were observed for 3 weeks after the last dose. In no case did paralysis occur.

Attempts were made to produce permanent lesions in rats and rabbits by feeding them a diet containing 300 p.p.m. of *NN'*-di*iso*propyl phosphorodiamidic fluoride. Adult rats of both sexes consumed this diet

for 3 months and showed nothing more than occasional fine muscular twitchings and some weakness when trying to climb a sloping surface. Estimations of the cholinesterase levels in the brain and heart showed this to be reduced to 5 to 10 per cent. of normal. Rabbits fed on this diet showed general weakness and head drop in 2 to 4 weeks, although the time of onset of symptoms was irregular. These signs disappeared on returning the animal to a normal diet.

Barnes and Denz describe the histological lesions in detail and the reader is advised to refer to their original paper for a full account of them. The lesions are the same with each of the 3 compounds, *NN'*-di*iso*-propyl phosphorodiamidic fluoride, di*iso*propyl phosphorofluoridate and tri-*o*-cresyl phosphate. They are also identical with those found in chickens fed on a vitamin B_1 (thiamine) deficient diet. The lesions in the spinal cord are more consistent and severe than those found in the peripheral nerve. It is thought that paralysis is due chiefly to the lesions in the cord but no explanation is offered. These authors conclude their article in the following words:—"This outline of some of the anomalies that we have found gives an idea of the difficulties met in accounting for demyelination produced with these compounds. For the present, we are concerned in showing that some organo-phosphorus compounds cause demyelination and that this is not directly attributable to their other outstanding property of anticholinesterase activity."

The views of Barnes and Denz have more recently received further confirmation from a number of enzyme studies carried out by Davison[77], Austin and Davies[78] and Myers and Mendel[79]. Davison injected a number of selective inhibitors of pseudo cholinesterase into chickens. Three of them tri-*o*-cresyl phosphate, di*iso*propyl phosphorofluoridate and *NN'*-di*iso*propyl phosphorodiamidic fluoride cause demyelination and three did not. (*iso*. E600, tetra *iso*propyl pyrophosphate (TIPP) and di*iso*propyl phosphorodiamidic anhydride. The findings of Earl and Thompson with reference to the prolonged inhibition of pseudo cholinesterase after the administration of tri-*o*-cresyl phosphate were confirmed. Initially all the other compounds inhibited the pseudo esterase powerfully, but recovery of the enzyme level was rapid (about 50 per cent. in 2 days and 100 per cent. in 12 days). Furthermore the recovery pattern was similar for those compounds which caused demyelination and also for those that did not. Austin and Davies examined another group of 4 potent phosphorus anticholinesterases, di*iso*propyl phosphorofluoridate, *iso*propyl methyl phosphonofluoridate (Sarin), 3:3-dimethylbutyryl-2-methyl phosphonofluoridate (DL33, Soman) and ethyl *NN*-dimethyl phosphoroamidocyanidate (Tabun). Di*iso*propyl phosphorofluoridate was the only substance to produce paralysis in chickens.

There was no clinical signs of paralysis nor histological evidence of demyelination with any of the other three. A carefully controlled experiment in which the cholinesterases of the central nervous system were studied in di*iso*propyl phosphorofluoridate- and *iso*propyl methyl phosphonofluoridate-treated birds failed to produce any characteristic differences in

the two groups. These authors also came to the conclusion that demyelination did not arise as a direct consequence of either true or pseudo cholinesterase inhibition.

Myers and Mendel have also found no evidence of paralysis in rats even after prolonged and complete inhibition of rat brain and spinal cord cholinesterase with tri-*o*-cresyl phosphate. The rat, however, is not a good species for the demonstration of these phenomena and other factors may well be operative.

The Significance of Cholinesterase Levels of the Blood

Cholinesterase levels of the cells and plasma or serum have been found to be valuable in poisoning by organo-phosphorus insecticides. Serum or plasma levels have also proved significant in liver disease, malnutrition and in response to succinylcholine, a drug which is being extensively used in anæsthetic practice as a skeletal muscle relaxant.

The necessary prelude to using cholinesterase levels of the blood for diagnostic purposes is a knowledge of the variations which are likely to occur under physiological conditions. Callaway, Davies and Rutland[80] have studied not only the population variation (i.e., the variation from person to person) but also the personal variation (i.e., variations in the same individual). They examined approximately 250 adults in order to determine the population variation. They consisted of two groups, one was composed of 100 service men between the ages of 18 and 30 and drawn from the Royal Navy, the Army and the Royal Air Force. Each of these had been designated "fit for combat duty anywhere". The second group was composed of 81 males between 18 and 69 years old and 66 females between 35 and 74 years. These were all blood donors drawn from the industrial and agricultural areas of S.W. England. None of them were suffering from minor infections nor had they suffered from any major serious illness for at least two years.

Distribution activity curves indicated that the distribution was normal and that the variation of normal levels could be defined by the fiducial limits of the population. It was also shown that the "coefficient of variation" could be used as a more general parameter or measure of variation and that it would provide an index which could be applied to data obtained by quite different analytical techniques.

The coefficient of variation of the plasma enzyme was 21·7 per cent. and the 95 per cent. fiducial limits of variation 57 and 143 per cent. of the mean value. Differences were observed in the variation of the red cell enzyme in service and civilian groups. Thus, the mean value for the service group was 118 (SD 12·7) and for the civilian group 108 (SD 16·7). (There was no obvious explanation of this difference.) In therefore defining the population limits, Callaway *et al.* gave separate values for the two groups. Their results are summarised in Table IX.

Wolfsie and Winter[81] examined 255 employees of the American Cyanamid Company. The coefficient of variation for the plasma enzyme in their series was significantly less than that given by Callaway *et al.* There is no obvious explanation of the discrepancy. It should, however,

be pointed out that Wolfsie and Winters assessment of normal limits is substantially less than that of most authors (Callaway *et al.*[80], Wilson *et al.*[82], Davies and Rutland[83], Vorhaus *et al.*[84], Sealey [85], McArdle[86] and McCance *et al.*[87]). The limits given in Table IX will, however, include the narrower ones of Wolfsie and Winters. No great discrepancy exists between their value for red cell enzyme limits and those of other workers.

TABLE IX

THE COEFFICIENT OF VARIATION OF PLASMA AND RED CELL CHOLINESTERASES

(After Callaway *et al.*[80])

Mean value = 100

Enzyme	Group	Number of cases	Coefficient of variation	Limits
Plasma	Total	228	21·7	57–143
Red cell	{Service ..	98	10·8	78–122
	{Civilian ..	143	15·4	69–131

Personal variations are much more difficult to assess so Callaway *et al.*[80] approached this problem from a purely practical point of view and defined it in the following terms. If a number of estimates of the normal or physiological level of the enzyme are available, what must be the difference between these and a subsequent value, in order that this latter value shall differ significantly from the previous values? They examined blood specimens on 8 occasions over a period of 4 weeks from 10 soldiers aged 18 to 30. As a result, they constructed a table showing the percentage minimum differences needed for the recognition of abnormal deviations.

TABLE X

PERCENTAGE MINIMUM DIFFERENCES FOR THE RECOGNITITION OF ABNORMAL RED CELL AND PLASMA CHOLINESTERASE VALUES IN THE SAME INDIVIDUAL

(After Callaway *et al.*[80])

No. of Initial Values	Percentage differences	
	Plasma	Cells
1	19·9	15·3
2	17·3	13·3
3	16·3	12·5
4	15·7	12·5
5	15·5	11·9
10	14·7	11·3
∞	14·1	10·9

The differences are the percentage differences calculated as the difference betwen the mean value of the early estimates and the one under discussion as percentages of the mean value.

McCance and Jones[88] examined the serum cholinesterase of umbilical cord blood and found that, at birth, the level is only about 65 per cent. of the adult level. It increases to about 90 per cent. of the normal adult level during the next 3 weeks. Hutchinson and Widdowson[89] followed up these observations in children from 6 to 15 years. At 6 years of age, the serum enzyme is 25 per cent. above the normal adult level. During the next 9 years there is a steady fall to the adult level which is reached at 15 years of age. These changes are shown graphically in Figure 4.

The Significance of Cholinesterase Levels of the Blood in Insecticide Poisoning

The authors of several recent examinations have discussed the value of blood cholinesterase levels in organo-phosphorus insecticide poisoning (Barnes and Davies[90], Bidstrup[91], Davies[92] and Marchand[93]). It is however, difficult to assess the precise significance of esterase levels of the blood in this connection for there is no close relationship between variations in the red cell or plasma enzyme levels and the onset of symptoms. Thus, both the red cell and plasma esterases of animals have been reduced to levels below 5 per cent. of the normal without apparently inconveniencing the animal. Equally so, animals have been killed with measurable quantities of enzyme in the blood. Thus, the exact significance of esterase levels is not obvious.

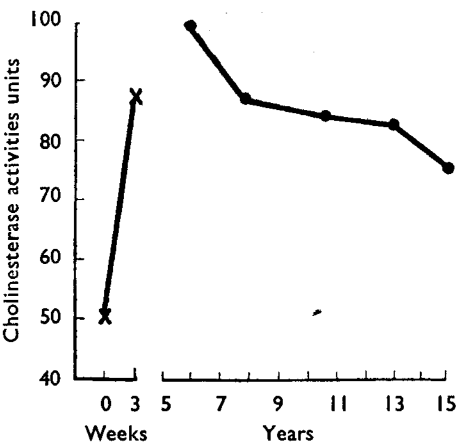

Fig. 4. Cholinesterase activities of the serum at different ages. (After Hutchinson and Widdowson[89]).

Marchand[93] gives a sound assessment of the value of these levels as far as insecticide poisoning is concerned. Variations in the levels of the red cells and plasma esterase appear to be a specific and reliable method of establishing or excluding significant intoxication by one or more anticholinesterase insecticides. Separate cholinesterase determinations of red cells and plasma when compared with pre-exposure values permit the recognition of cumulative effects of repeated exposures and the differentiation of chronic from severe acute exposures. Low plasma values indicate mild acute poisoning. Low red cell values, with normal plasma values indicate recovery from a previous acute or repeated intoxication. Low values for both indicate severe acute intoxication.

Cholinesterase estimations have also been of diagnostic value in individual cases in which the symptoms have been ambiguous and when there was need of a timely warning for accidentally exposed persons who have not yet developed symptoms. When groups are screened the extent to which individual values or the average for the group as a whole, fall below the pre-exposure controls, serve as an objective index of the need for correction of handling methods or protective routines.

Fawley *et al.*[94] stress the limitations of cholinesterase inhibition in evaluating the degree of exposure and severity for reasons which have already been indicated in the opening paragraph of this section. In chronic exposures they believe that cholinesterase measurements would be valuable in detecting slight multiple exposures. They say, however, that

both plasma and erythrocyte levels have been of value as evidence of parasympathomimetic stimulation which may be a delayed phenomenon in subacute exposure. They observed plasma and erythrocyte cholinesterase levels in animals as low as 5 per cent. of normal, several weeks before the onset of convulsions and death.

One aspect of this problem has not been adequately investigated. The organo-phosphorus insecticides are irreversible inhibitors of cholinesterase. The rate of return of both the cell and plasma enzyme is usually slow (Grob *et al.*[95]) and a question of considerable importance which has not yet been adequately answered is whether the low level of the enzyme under these conditions is indicative of a state of hypersensitivity to subsequent doses of anticholinesterases. Whatever the final answer to this problem, it is the reviewer's opinion that, in our present state of knowledge, workers whose estimated red cell cholinesterase is below 50 per cent. of normal should avoid contact with these substances.

TABLE XI

THE VARIATION OF PSEUDOCHOLINESTERASE LEVEL IN THE SERUM WITH STATE OF NUTRITION (After Hutchinson, McCance and Widdowson[98])

Subjects	Clinical assessment	Activity of pseudoenzyme Units*	
		Average and range	Standard deviation
37 British Men	Normal	79 (59 to 122)	15·1
82 I.G. Farben employes	Slightly undernourished	57 (20 to 117)	14·5
45 Civilians. Applicants for rations for œdema	More undernourished than above group	49 (26 to 99)	16·2
17 Prisoners at Siegburg	Undernourished	49 (28 to 88)	16·0
12 Civilian internees	Undernourished with œdema	61 (39 to 105)	20·3
12 Civilian internees	Undernourished without œdema	42 (25 to 56)	8·1
6 Prisoners at Kiel	Very undernourished without œdema	46 (29 to 58)	10·1
6 Prisoners at Kiel	Very undernourished with œdema	29 (15 to 40)	7·7

* C.mm. of carbon dioxide liberated/ml. of serum/minute (after McArdle[86]).

THE VARIATION OF SERUM CHOLINESTERASE IN PATHOLOGICAL CONDITIONS

Serum cholinesterase is low in liver disease (McCardle[86], Antopol *et al.*[96] and Kunkel and Ward[97]). Recently Vorhaus, Scudamore and Kark[84] have stated that serum cholinesterase is the most reliable index of liver damage. They investigated the changes in serum cholinesterase levels in 69 cases of established hepatic or biliary disease and compared their results with the results of a number of other liver function tests.

Hutchinson, McCance and Widdowson[98] studied the variation of serum cholinesterase in malnutrition. They examined groups of prisoners of war and German civilians suffering from varying degrees of malnutrition. Their results are shown in Table XI.

As can be seen from Table XI, there is a strong tendency for the mean activity of each group to reflect the clinical assessment of the nutritional status of that group. Improvements in the diet of these groups resulted

in an increase in the level of the serum enzyme. There is little doubt in the minds of these workers that an insufficiency of calories lowers the pseudo enzyme of the serum of man and that there are good grounds for using the activity of this enzyme as a sensitive index of the nutritional status of man. The normal range is too wide to make single determinations of value in individual cases but group averages have proved themselves of considerable value and the response of individuals to treatment has been equally instructive.

Berry, Cowin and Davies[99] have recently established a correlation between cholinesterase and surface fat as measured by caliper measurements of skin folds in 5 parts of the body. They found statistically significant correlations between enzyme level and skin fold measurements in 146 industrial workers at Slough and 114 youths mainly employed in industry at Salford. In 86 youths of widely varying occupations at Kingston, a positive relationship was also found but it was not statistically significant.

Succinylcholine has been used extensively during the last 2 or 3 years as a relaxant in anæsthetic practice. Bourne *et al.*[100] describe it as a safe and effective substance particularly valuable in short procedures such as intubation, electroconvulsive therapy and orthopædic manipulation Evans *et al.*[101,102] confirm this assessment of the value of the drug but also draw attention to the fact that instances of abnormal duration of apnœa have been observed. Bourne *et al.* also noted this. Both Bourne *et al.* and Evans *et al.* observed that in patients who responded abnormally to a standard dose of succinylcholine, the plasma cholinesterase was significantly depressed. Evans *et al.* found succinylcholine to be a competitive inhibitor of both "true" and pseudo cholinesterase and they suggested that the response to this drug is dependent upon the level of pseudo cholinesterase in the plasma. Thus, it seems that plasma esterase levels may well be helpful in prophesying the response of individuals to such a drug. Further, the use of this substance may well be contra-indicated in conditions such as liver disease and insecticide poisoning where the plasma esterase is likely to be significantly depressed.

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