Table 4. *Ratio of activities by different assay methods, when activity of preparation C is taken as 1*

	Biological assay Viscosimetric assay		Biological assay Turbidimetric assay	
Enzyme source	Commercial substrate	Pure substrate	Commercial substrate	Pure substrate
A, seminal fluid	1·26	1·26	0·5	0·45
B, testis	1·38	1·22	1·17	1·0
C, testis	1	1	1	1
D, testis	1·1	1·2	0·87	1·1
E, testis	0·75	1·2	0·58	0·69
F, Staphylococcus	0·91	0·58	0·72	0·54
G, Streptococcus	2·1	0·9	0·8	0·7

markedly different upon different enzyme preparations. These observations imply some degree of heterogeneity of the enzyme preparations compared. It is of interest, from a practical point of view, that the closest agreement between *in vitro* and *in vivo* activity was obtained with the purest substrate.

SUMMARY

1. Five preparations of testicular hyaluronidase, one of streptococcal and one of staphylococcal origin were compared by an accurate skin-diffusion assay and by viscosimetric and turbidimetric methods.

2. With testicular preparations the results of the three methods agree well, provided that the potency is measured in terms of a reference preparation of enzyme, and that the pH and ionic strength of the solvents are approximately physiological. It is desirable to use highly purified substrate, and to stabilize the enzymes with gelatin rather than gum acacia.

3. The activities of the two bacterial enzymes showed a close but not complete correlation between the three tests.

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The Differentiation of True and Pseudo Cholinesterase by Organo-phosphorus Compounds

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True cholinesterase is the enzyme present in the brain and erythrocytes of many species which hydrolyses acetylcholine at a higher rate than butyrylcholine and is inhibited by excess substrate. Pseudo cholinesterase is present in the serum of many species and hydrolyses butyrylcholine at a higher rate than acetylcholine and is not inhibited by excess substrate. Mendel, Mundell & Rudney (1943) introduced the substrates acetyl β-methylcholine and benzoylcholine for true and pseudo cholin-

esterases respectively. Differentiation of true and pseudo cholinesterases has also been achieved by the use of selective inhibitors. Di*iso*propyl fluorophosphonate (DFP) inhibits pseudo cholinesterase at a lower concentration than the true (Mazur & Bodansky, 1946; Mendel & Hawkins, 1947; Adams & Thompson, 1948). However, in some species the sensitivities of the two enzymes are not very different (Ord & Thompson, 1950). A series of organo-phosphorus compounds have been ex-

amined for their differential inhibitory power. Some of these compounds are superior, in some respects, to DFP for this purpose.

Evidence has been produced which suggests that organo-phosphorus inhibitors are substrates for cholinesterase and are hydrolysed (Aldridge & Davison, 1952 a, b), but that one of the products, the substituted phosphoric acid, remains attached to the enzyme (Boursnell & Webb, 1949; Michel & Krop, 1951). The most outstanding difference between true and pseudo cholinesterases is their substrate specificity for acetates and butyrates respectively (Adams, 1949; Adams & Whittaker, 1949; Sturge & Whittaker, 1950). If the mechanism of inhibition of the true and pseudo cholinesterases by organo-phosphorus inhibitors is the same and they are attached to the ester-attracting site by the electrophilic phosphorus atom (Nachmansohn & Wilson, 1951) 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. A comparison has been made between the substrate and inhibitor specificity of true and pseudo cholinesterases of horse blood. The mechanism suggested above would explain the results we have obtained on the comparative inhibition of true and pseudo cholinesterases of horse blood by various organo-phosphorus inhibitors.

METHODS

Cholinesterase has been determined manometrically as described in detail previously (Aldridge & Davison, 1952 a). For inhibitor experiments whole horse blood has been used as the source of true and pseudo cholinesterases with acetyl β-methylcholine as substrate for cholinesterase in the erythrocytes and benzoylcholine for pseudo cholinesterase of the serum. A measurable output of CO_2 is produced with both substrates from 0·5 ml. defibrinated whole blood. For the determination of the substrate specificity horse serum was used for pseudo cholinesterase and a partially purified preparation of horse erythrocyte stroma for true cholinesterase. The purified stroma was prepared as follows: defibrinated horse blood (1200 ml.) was washed twice with 2 % w/v NaCl (saline) solution. It was centrifuged and resuspended to 1 l. in saline and poured rapidly into 1 l. 0·2 % (w/v) BeSO$_4$.4H$_2$O in saline. This causes the cells to agglutinate. It was then diluted to 10 l. with saline and the supernatant removed when the cells had settled. The washing procedure was repeated and tap water then run in up to 10 l. It was left 45 min. for the cells to haemolyse. 1 l. 20 % (w/v) NaCl solution was added. The supernatant was removed when the cells had settled and the washing with saline repeated until the top layer was only pale red (five times). After centrifuging at +1°, the pale red stroma (approx. 100 ml.) was collected. Sodium citrate (20 g./100 ml. stroma) was added and left overnight at −5°. Four times its volume of 27 % (v/v) mixture of ethanol and water was run in slowly at −5° stirring all the time. After standing 1 hr. it was centrifuged at −5°. The resulting buff-coloured precipitate has an activity against acetylcholine of 1·32 μl. CO_2/mg. dry wt./min., a 16-fold purification over the original packed cells.

The choline esters used for the determination of substrate specificity were prepared by heating 1·2 mol. of the corresponding acyl chloride with 1 mol. choline perchlorate for 1 hr. at 140–150°. The resulting choline ester perchlorates were recrystallized from dry isopropanol. All gave a positive reaction for esters by Hestrin's (1949) reaction and have the following melting points: acetyl 114°, propionyl 103–105°, n-butyryl 72°, isobutyryl 117°, and isovaleryl 35°. The n-valerylcholine iodide was kindly supplied by Mr D. R. Davies (Chemical Defence Experimental Establishment, Porton), the benzoylcholine chloride was from Roche Products and acetyl β-methylcholine bromide from L. Light and Co., Ltd. All substrates were used at a final concentration of 0·015 M. The perchlorate ion has no effect on the activity of the enzyme since true cholinesterase (horse erythrocytes) has the same activity against acetylcholine chloride and perchlorate and pseudo cholinesterase (horse serum) against n-butyrylcholine chloride and perchlorate.

In all inhibitor experiments the enzyme has been incubated with the inhibitor for 30 min. at 37° prior to the addition of substrate. Inhibitor solutions in buffer (NaHCO$_3$ 0·0357 M; MgCl$_2$, 0·040 M; NaCl, 0·164 M) have been prepared fresh for every experiment. The concentration of inhibitor necessary to produce 50 % inhibition of enzyme activity under these conditions has been determined by plotting log$_{10}$ residual activity against M concentration of inhibitor. This inhibitory power has usually been expressed as pI$_{50}$ = − log M concentration of inhibitor to produce 50 % inhibition. The rate of hydrolysis of choline esters by pseudo cholinesterase divided by that for true cholinesterase is called the substrate ratio. The concentration of inhibitor necessary to produce 50 % inhibition of true cholinesterases divided by that for pseudo cholinesterase is called the inhibitor ratio.

RESULTS

Comparison of the sensitivities of true and pseudo cholinesterase to organo-phosphorus compounds. It has been demonstrated that diisopropyl fluorophosphonate (Mazur, 1946) tetraethyl pyrophosphate (Aldridge & Davison, 1952 a), and diethyl p-nitrophenyl phosphate (Aldridge, 1953 a) are hydrolysed by the tissues of various mammalian species. A comparison of the sensitivity of true and pseudo cholinesterases, in an unpurified condition, to such organo-phosphorus inhibitors is difficult to assess. The results in Table 1 show that with diethyl p-nitrophenyl phosphate (E 600) as inhibitor less inhibitor is required to inhibit erythrocyte cholinesterase in the absence than in the presence of serum. This is due to the presence of an enzyme hydrolysing E 600 in horse serum (Aldridge, 1953 a). Two other compounds examined in this way, bismonoisopropylamino fluorophosphine oxide (Isopestox) and diisopropyl fluorophosphonate (DFP), show little difference in behaviour with or without horse serum.

Both the cholinesterases of whole horse blood may be treated with an inhibitor under the same

conditions and the effect on true and pseudo enzymes separately determined using the specific substrates, acetyl β-methylcholine and benzoylcholine (Mendel *et al.* 1943). By this means more comparable estimates of the sensitivities of the two enzymes to different inhibitors may be obtained. With some inhibitors the concentration necessary for 50% inhibition of true cholinesterase is much higher than that for pseudo cholinesterase. In this case, although the same amount of enzyme which may destroy the inhibitor is present in the two cases, it is presented with different concentrations of inhibitor. Experimental evidence of the kinetics of hydrolysis of substrates at concentrations as low as 10^{-8}M is extremely limited, but it is probable that the hydrolysis will follow first-order kinetics (Van Slyke, 1942); with a constant enzyme concentration and time of incubation (for the experiment) a constant proportion of the inhibitor will be removed. It is, therefore, unlikely that an appreciable error will be produced from this source.

The results obtained for various inhibitors using the above technique are given in Table 2. Two in-

hibitors show a high inhibitor ratio. Isopestox and tetra*iso*propyl pyrophosphoramide (ISO-OMPA). Both of these compounds are crystalline, practically non-volatile, soluble in water and are more

Table 1. *The effect of horse serum on the inhibition of horse-erythrocyte cholinesterase by various inhibitors*

(Defibrinated horse blood or washed horse erythrocytes (0·5 ml.) incubated for 30 min. at 37° with various concentrations of inhibitor. After 30 min. the acetyl β-methylcholine was tipped in and the enzyme activity remaining determined. $pI_{50} = -\log_{10}$M concentration producing 50% inhibition after incubation for 30 min. at 37°.)

	pI_{50}	
Inhibitor	Whole horse blood	Washed horse erythrocytes
Diethyl *p*-nitrophenyl phosphate (E 600)	6·31	7·33
Bismono*iso*propylamino fluorophosphine oxide (Isopestox)	3·77	3·77
Di*iso*propyl fluorophosphonate (DFP)	5·70	5·68

Table 2. *The inhibition of true and pseudo cholinesterases of horse blood by various inhibitors*

(Defibrinated horse blood (0·5 ml.) incubated for 30 min. at 37° with various concentrations of inhibitor. After 30 min. the substrates were tipped in and the remaining enzyme activity determined. Acetyl β-methylcholine was used to determine erythrocyte cholinesterase and benzoylcholine to determine the serum enzyme. $pI_{50} = -\log_{10}$M concentration of inhibitor to produce 50% inhibition. ChE = cholinesterase.)

	pI_{50}		Inhibitor ratio (True ChE/pseudo ChE)
Inhibitor	Pseudo ChE	True ChE	
Dimethyl *p*-nitrophenyl phosphate	6·10	6·27	0·67
Diethyl *p*-nitrophenyl phosphate (E 600)	6·85	6·38	2·9
Di*iso*propyl *p*-nitrophenyl phosphate	6·50	5·50	10
Tetraethyl pyrophosphate (TEPP)	8·39	6·52	73
Tetra*iso*propyl pyrophosphate	8·68	5·96	520
Di*iso*propyl fluorophosphonate (DFP)	8·18	5·75	270
Diethyl fluorophosphonate	7·80	6·09	52
Bisdimethylamino fluorophosphine oxide	3·31	1·96	22
Mono*iso*propylamino dimethylamino fluorophosphine oxide	5·10	3·42	48
Bismono*iso*propylamino fluorophosphine oxide (Isopestox)	7·42	3·82	3950
Tetra*iso*propyl pyrophosphoramide (ISO-OMPA)	6·48	2·51	9400

Table 3. *The sensitivity of the true and pseudo cholinesterases of different species to tetraisopropyl pyrophosphoramide and bismonoisopropylamino fluorophosphine oxide*

(Erythrocytes washed three times with saline were used as a source of true ChE and plasma or serum for pseudo ChE except in the case of the rat where rat-brain and rat-heart homogenates were used respectively. The enzyme was incubated with inhibitor for 30 min. at 37° prior to the addition of substrate. Acetylcholine was the substrate except for rat heart when benzoylcholine was used. $pI_{50} = -\log_{10}$M concentration of inhibitor which will produce 50% inhibition. ChE = cholinesterase.)

	ISO-OMPA pI_{50}			Isopestox pI_{50}		
Species	True ChE	Pseudo ChE	Inhibitor ratio	True ChE	Pseudo ChE	Inhibitor ratio
Horse	2·47	6·52	11 300	3·80	7·42	4 200
Human	3·52	5·27	56	4·66*	6·41*	56
Guinea pig	2·34	5·89	3 600	4·21	6·51	200
Dog	2·42	6·54	13 200	4·06	7·29	1 700
Rat	3·60	6·32	530	4·22	6·75	340
Sheep	3·20	—	—	4·41	—	—

* These results are in confirmation of an observation by Austin & Berry, 1952.

stable to hydrolysis than DFP. They are, therefore, more convenient to handle. The inhibitor ratio for DFP for some species is particularly low (Ord & Thompson, 1950). The results in Table 3 show that Isopestox and tetra*iso*propyl pyrophosphoramide also show species differences and care must also be used to differentiate true and pseudo cholinesterase with these inhibitors.

Substrate specificity of true and pseudo cholinesterase of horse blood. It is clear from the work of Whittaker (1951) that the optimum acyl group of substrates for true cholinesterase is acetyl and, for pseudo cholinesterase, butyryl. To examine the possibility that length of the alkyl chain from the phosphorus atom governed the specificity of the inhibitors it was necessary to determine the substrate specificity for a series of choline esters. For instance, calculation from covalent single-bond radii of carbon, oxygen and phosphorus atoms (Remick, 1949) shows that the length of C—C—C chain of propionylcholine is 3·08 A. while that for P—O—C chain in dimethyl *p*-nitrophenyl phosphate is 3·19 A. The lengths of the alkyl chains in these two compounds are approximately the same. On this basis the length of the alkyl chain in di*iso*propyl fluorophosphonate will be equivalent to that for *iso*valerylcholine. A series of choline esters have therefore been synthesized and their hydrolysis by erythrocyte and serum cholinesterase of horse blood has been determined. The results are given in Table 4. Up to *n*-butyrylcholine our results agree with those of Glick (1941) for serum cholinesterase and of Mounter & Whittaker (1950) for erythrocyte cholinesterase. Chain length as well as side-branching of the acyl group have a profound effect on the

rates of hydrolysis of choline esters. A comparison of the rates of hydrolysis of a choline ester by the two enzymes is given by the substrate ratio.

Mechanism of the differentiation of true and pseudo cholinesterase by organo-phosphorus compounds. When chymotrypsin is inhibited by DFP there is evidence that the inhibitor is hydrolysed and the dialkyl phosphate group remains attached to the enzyme (Jansen, Nutting & Balls, 1949; Jansen, Nutting, Jang & Balls, 1950). This has been extended

Table 4. *Hydrolysis of choline esters by horse-erythrocyte and serum cholinesterases*

(Horse serum was used directly. The erythrocyte cholinesterase was a purified stroma preparation as described under Methods. All substrate concentrations were 0·015 M. Results are corrected for non-enzymic hydrolysis. No output of CO_2 was obtained without the addition of substrate. Readings were taken every 5 min. except for *iso*valerylcholine with true ChE (30 min.). Activities are all based on acetylcholine as 100. ChE = cholinesterase.)

Choline ester	Pseudo ChE	True ChE	Substrate ratio (pseudo ChE/ true ChE)
Acetyl	100	100	1·0
Propionyl	145	78	1·9
n-Butyryl	216	2·3	94
*iso*Butyryl	91	46	2·0
n-Valeryl	145	3·3	44
*iso*Valeryl	50	0·19	263

to the inhibitor E 600 (Hartley & Kilby, 1952). If cholinesterase is inhibited by a similar mechanism, and there is indirect evidence that this is so (Aldridge & Davison, 1952a; Boursnell & Webb, 1949), then the inhibitor is acting as a substrate and being

Table 5. *A comparison of the substrate ratio and the inhibitor ratio for true and pseudo cholinesterase of the horse*

(Substrate ratios and inhibitor ratios are as given in Tables 4 and 2 respectively.)

Substrates			Inhibitors			
			Inhibitor ratios			
Acyl group of choline ester	Substrate ratio	Alkyl group of inhibitor	*p*-Nitrophenyl phosphates	Fluoro-phosphonates	Pyro-phosphates	Fluorophos-phoramide
—CO.CH₃	1	>PO.CH₃	—	—	—	—
—CO.CH₂.CH₃	1·9	>PO.OCH₃	0·68*	—	—	—
—CO.CH₂.CH₂.CH₃	94	>PO.OCH₂CH₃	2·9	51	75	—
—CO.CH(CH₃)CH₃	2·0	>PO.N(CH₃)CH₃	—	—	—	22
—CO.CH₂.CH₂.CH₂.CH₃	44	>PO.OCH₂.CH₂.CH₃	—	—	—	—
—CO.CH₂.CH(CH₃)CH₃	263	>PO.OCH(CH₃)CH₃	10	270	500	3950

* We have evidence that the inhibition by dimethyl *p*-nitrophenyl phosphate reverses rapidly. The inhibitor ratio will be influenced by the relative rates of reversal of true and pseudo cholinesterase.

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hydrolysed by the enzyme. If the mechanism of hydrolysis of acetylcholine by cholinesterase put forward by Bergmann, Wilson & Nachmansohn (1950) is accepted and the ester is attached to the ester-attracting site by the electrophilic carbon of the acyl group, then it is highly probable that the organo-phosphorus compounds are attached by the electrophilic phosphorus (Nachmansohn & Wilson, 1951). If this is so, the length of the alkoxyl chains attached to the phosphorus should play an important part in the 'fit' of these compounds on the enzyme surface in a similar way to the effect of various acyl groups on the rate of hydrolysis of choline esters. In Table 5 the substrate ratios for various choline esters are compared with the inhibitor ratio for inhibitors with similar lengths of groups from the electrophilic group. It should be pointed out that the substrate ratio is the rate of hydrolysis (based on acetylcholine = 100) for pseudo over that for true cholinesterase while the inhibitor ratio is the concentration of inhibitor for 50 % inhibition for true cholinesterase over that for pseudo cholinesterase. 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 inhibitor necessary for inhibition. It appears from a limited number of inhibitors that there is a rough correlation. For instance, on passing from n-butyrylcholine to isovalerylcholine the substrate ratio changes from 94 to 263. A change of the inhibitor ratio in the same direction is shown for three groups of inhibitors: tetraethyl and tetra-isopropyl pyrophosphate, diethyl and diisopropyl p-nitrophenyl phosphates and diethyl and diiso-propyl fluorophosphonates. There is a marked change in substrate ratio from isobutyrylcholine to isovalerylcholine. A similar change in inhibitor ratio is found from bisdimethylamino to bismono-isopropylamino fluorophosphine oxides.

DISCUSSION

Evidence has been presented that in the differential inhibition of true and pseudo cholinesterase by a series of organo-phosphorus compounds an important factor is the alkyl group attached to the phosphorus. Changes in the acyl group of choline esters produce marked changes in the relative rates of hydrolysis of these esters by true and pseudo cholinesterases. Organo-phosphorus inhibitors have been compared with choline esters with regard to length of the alkoxyl and acyl chains attached to the electrophilic phosphorus and carbon respectively. 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 (similar as regards length) on the inhibitor a compound is produced which is relatively more efficient against pseudo than true cholinesterase. It has previously been shown, by using a series of inhibitors of widely varying stabilities to hydrolysis but with the alkyl groups maintained constant (Aldridge & Davison, 1952a, b), that the more stable an inhibitor is to hydrolysis the less effective it is as an inhibitor. 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 groups attached to the phosphorus.

These observations are consistent with the view that organo-phosphorus inhibitors are substrates for cholinesterase, are hydrolysed and the phosphorus remains attached to the enzyme. Changes in the alkyl groups of an inhibitor should alter the 'fit' of the enzyme surface in a similar way that changes in the acyl groups of choline esters affect their hydrolysis by the enzyme. On the Fischer 'lock and key' principle the better the 'fit' of a substrate on the enzyme surface the more readily it is hydrolysed. In a similar way the better the 'fit' of an organo-phosphorus inhibitor the more readily it should be hydrolysed and the more efficient it should be as an inhibitor. Stability to hydrolysis of the inhibitor is also an important factor which should alter the ease with which it is hydrolysed at the enzyme surface. Such a view of the mechanism of inhibition places most of the responsibility for inhibition on the enzyme itself. This would be consistent with the fact that some stable organo-phosphorus compounds do inhibit cholinesterase. Diethyl phenyl phosphate with a half life of 8 years in phosphate buffer, pH 7·6, and at 37° does inhibit cholinesterase at 10^{-3}M concentrations (Aldridge & Davison, 1952a,b). This mechanism of inhibition is similar to that elucidated by Jansen et al. (1949, 1950) for the inhibition of chymotrypsin by DFP.

Although isopestox and ISO-OMPA are good inhibitors for the in vitro differentiation of true and pseudo cholinesterase in some species, Table 3 shows that caution must be used in the use of inhibitors for this purpose. Substrates have also been used to differentiate true and pseudo cholinesterase, but evidence is accumulating that this method is far from certain (Ellis, 1947; Metcalf & March, 1950; Levine & Suran, 1950; Earl & Thompson, 1952). It has been suggested that enzymes of the same type from different species will show a different spectrum of activities against different substrates (Augustins-son, 1951; Aldridge, 1953b). If this is accepted, the view that organo-phosphorus inhibitors are substrates for cholinesterase would be consistent with the finding that variations in the response of true and pseudo cholinesterases of different species to these inhibitors have been obtained.

SUMMARY

1. The inhibition of true and pseudo cholinesterase by a series of organo-phosphorus inhibitors has been determined.

2. Based on the effect of changes of acyl groups in choline esters on their hydrolysis by true and pseudo cholinesterase an explanation of the different sensitivities of the two enzymes for organo-phosphorus inhibitors has been suggested.

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Studies on Fumarase

2. THE EFFECTS OF INORGANIC ANIONS ON FUMARASE ACTIVITY

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It was shown quite early in the history of this enzyme that the activity of fumarase is affected by the addition of inorganic salts. Mann & Woolf (1930), working with whole washed cells of *Escherichia coli*, found that sulphates inhibited slightly the hydration of fumaric acid to L-malic acid; while phosphates and citrates strongly activated this reaction. Similarly, the activating effect of phosphates on the extracted enzyme from animal tissues has been shown by various workers (Quastel, 1931; Clutterbuck, 1928; Davydova, 1947). In this paper is reported the effect of various anions on the activity of crystalline fumarase.

METHODS

The enzyme used was obtained from pigs' hearts by the method already described (Massey, 1951, 1952) and was recrystallized twice before use. The enzyme activity was determined by the spectrophotometric method of Racker (1950), which depends on the formation or disappearance of the double bond of fumaric acid. The fumarate or L-malate concentrations used in these experiments were generally 0·0167 M-fumarate or 0·0835 M-L-malate. These concentrations are over five times the highest Michaelis constant observed with either substrate. The rate of the reaction is linear at first and then falls off as the substrate concentration decreases and the product is formed (Fig. 1). The values