Two Selective Inhibitors of Cholinesterase

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One of the means used to characterize cholinesterases is to study their behaviour towards inhibitors. A number of substances have been used as so-called 'selective inhibitors', i.e. inhibitors which will attack preferentially either true or pseudo cholinesterase (pseudo ChE) (Adams & Thompson, 1948; Hawkins & Mendel, 1949), but in general the selectivity is not high. The most promising of the selective inhibitors appears to be di*iso*propyl phosphorofluoridate (DFP) (Hawkins & Mendel, 1947)) to which pseudo ChE appears to be about 100–200 times as sensitive as true ChE, and the *N*-*p*-chlorophenyl-*N*-methylcarbamate of *m*-hydroxyphenyltrimethylammonium bromide (Nu 1250) (Hawkins & Mendel, 1949) which selectively inhibits true ChE. However, these two, as well as several others which have been described, have the disadvantage that when applied to mixtures of the two types of ChE in concentration sufficiently high to cause 90–100 % inhibition of one enzyme they also cause measurable inhibition of the other. In spite of this, much valuable work has been carried out. The selectivity of an inhibitor varies considerably according to the species of animal studied. Hawkins & Mendel (1949) found that true ChE was about 1000 times more sensitive to Nu 1250 than pseudo ChE in human or rat blood, but only 20 times in the dog and 5 times in the horse. Aldridge (1953) has reported similar species differences. Such species variation might prove embarrassing when attempting to classify enzymes which are neither true nor pseudo, such as occur in the rat (Ord & Thompson, 1951), fowl (Earl & Thompson, 1952) or sheep (Davies, Risley & Rutland, 1952). Todrick (1952) has used the selective inhibitors *N*-(2-diethylaminoethyl)phenothiazine hydrochloride (Diparcol) and the diethylaminoethyl ester of 1-phenyl*cyclo*pentane carboxylic acid (Parpanit) which inhibit pseudo ChE, and the diethiodide of 1:5-di(8′-quinolyloxy)pentane (RP 3381) and the dimethiodide of 1:5-di(*o*-dimethylaminophenoxy)-pentane (RP 3565) which inhibit true ChE. The selectivities appear to be of the order of 200–500 as measured by the ratio I_{50} (rat brain ChE):I_{50} (rat intestinal mucosa ChE), but comparison with other selective inhibitors is difficult, partly because the ChE of rat intestinal mucosa is not quite the same as the classical pseudo ChE (Ord & Thompson, 1951), and partly because no data are given as to the inhibition of one type of ChE when the other is about 95 % inhibited. (I_{50} is the concentration giving 50 % inhibition.)

It was, therefore, with great interest that we received reports of two inhibitors which appeared to have a higher selectivity than any so far studied. A personal communication from Dr W. N. Aldridge, which has since been published (Aldridge, 1953), mentioned that the pseudo ChE of horse serum was 10 000 times more sensitive than the true ChE of horse erythrocytes to *NN′*-di*iso*propylphosphorodiamidic anhydride (DPDA);* and Fulton (1952) reported that rat-brain ChE, which is predominantly true, was 50 % inhibited by 10^{-6} M-dimethobromide of 1:5-di(*p*-*N*-allyl-*N*-methylaminophenyl)-pentan-3-one (284C51), whereas the hydrolysis of benzoylcholine by horse serum was unaffected by a concentration of 10^{-3} M. We have therefore examined these inhibitors more fully, in an endeavour to decide on their usefulness as tools for the study of cholinesterases. Some of the results have been the subject of a preliminary communication (Austin & Berry, 1953).

EXPERIMENTAL

Enzyme preparations. Oxalated blood was centrifuged and the erythrocytes washed twice with 0·9 % NaCl. Plasma and cells were diluted as required with 0·025 M-$NaHCO_3$ in order to give an output of about 200 μl. CO_2/hr. in the Warburg apparatus.

Substrates. Acetylcholine (ACh; 0·006 M and 0·06 M), benzoylcholine (BCh; 0·03 M), acetyl-β-methylcholine (β-acetoxypropyltrimethylammonium chloride; MCh; 0·03 M) and butyrylcholine (BuCh; 0·03 M), were used as stock solutions in distilled water, kept in the refrigerator, and made up freshly once a week. Some data obtained by Miss J. E. Risley are reported in which propionylcholine (PrCh; 0·06 M) was used. These concentrations were final.

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* This compound, $[(C_3H_7NH)_2PO]_2O$, was formerly called bis(di-*iso*propylamino)phosphonous anhydride and given the abbreviation *iso*-OMPA (Aldridge, 1953; Austin & Berry, 1953). This abbreviation incorrectly suggests that the compound is an isomer of OMPA ($[(Me_2N)_2PO]_2O$; DuBois, Doull & Coon, 1950); we therefore suggest that the use of *iso*-OMPA be abandoned in favour of the abbreviation DPDA.

Measurement of enzyme activity. The enzyme activity was measured in the Warburg apparatus and is given as the q_{CO_2}XCh, i.e. the vol. of CO_2 in μl./hr./ml. plasma or packed erythrocytes when using XCh as substrate.

For several experiments double-side-arm flasks were used, so that inhibitor and substrate could be added separately to the enzyme in the main vessel. In most experiments with 284C51 the enzyme was placed in the side arm and tipped into a mixture of substrate and inhibitor, in order to obtain equilibrium as rapidly as possible.

Inhibitors. DPDA was prepared in this Establishment. 284C51 was presented by Dr Copp of the Wellcome Research Laboratories. The inhibitors were dissolved in distilled water, final concentrations being arrived at by v/v serial dilution.

RESULTS

The first experiments were carried out with the ChE's of human blood. The plasma contains a typical pseudo ChE and the erythrocytes a typical true ChE (Adams, 1949; Adams & Whittaker, 1949).

Reversibility of the inhibition

DPDA. A dilution of human plasma in bicarbonate-Ringer (Krebs & Henseleit, 1932) was divided into two parts, one of which was treated overnight at room temperature with 5×10^{-6}M-DPDA. The activity of a sample of both portions was measured. 10 ml. of each portion were dialysed for 20 hr. at 0° against five changes of 500 ml. bicarbonate-Ringer, and the activity determined. It was presumed that dialysis removed surplus DPDA, but the data of Table 1 show that it did not cause dissociation of the enzyme-inhibitor complex.

Table 1. *Reversibility of inhibition of human plasma cholinesterase by DPDA*

	Activity (%)	
	Before dialysis	After dialysis
Control	100	95
Treated with 5×10^{-6}M-DPDA	3	4

284*C*51. The human erythrocyte preparation is a suspension of particles, and so lends itself to the process of washing by centrifuge, which is much less tedious than dialysis. A suspension of erythrocytes in bicarbonate-Ringer was divided into two parts, one of which contained 10^{-6}M-285C51. After 10 min. exposure to the agent, samples were taken for measurement of activity. The remainder of both portions was washed twice with 50 vol. bicarbonate-Ringer, allowing the mixture to stand 10 min. before centrifuging for 10 min. at 3500 rev./min. in an angle centrifuge. The packed cells were sampled after each washing. Table 2 shows that the inhibition was readily and completely reversed by this treatment.

Table 2. *Reversibility of inhibition of human erythrocyte cholinesterase by* 284*C*51

	Activity (%)
Control, before washing	100
Control, after washing twice	93
Inhibited by 10^{-6}M-284C51	7
Inhibited, washed once	72
Inhibited, washed twice	93

Combination of the inhibitors with the active centre

A competitive inhibitor may be defined as one which combines with the same active centre as the substrate. If the inhibition is reversible, the degree of inhibition varies with the concentration of both substrate and inhibitor, and a simple test for discovering this type of behaviour was given by Lineweaver & Burk (1934). This test cannot be used with true ChE assayed under the conditions here described, because the optimum ACh concentration is near the limit below which accurate measurements of enzyme activity are impossible. An alternative

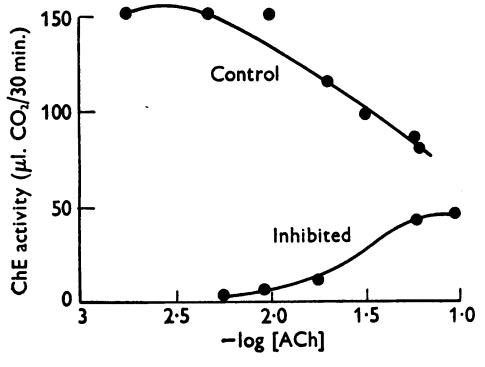

Fig. 1. Increase in the optimum ACh concentration of human erythrocyte ChE in the presence of $1{\cdot}3 \times 10^{-6}$M-284C51, showing competitive nature of the inhibition. (The increased optimum appears to be about −(log ACh) = 1.)

test is available. Augustinsson (1948) has argued that the optimum substrate concentration ought to be increased in the presence of a competitive inhibitor, and has produced some evidence in support (see also Augustinsson & Nachmansohn, 1949). It is shown in the Appendix that this assertion has a theoretical basis, and that if the inhibition is non-competitive (i.e. degree of inhibition uninfluenced by substrate concentration) the optimum is unchanged. In Fig. 1 it is shown that the optimum ACh concentration for human-erythrocyte ChE is considerably increased by 284C51, from about 0·003M to somewhere in the region of 0·1M. (The value of 0·1M is probable, although we have not

employed a sufficiently high ACh concentration to demonstrate that this is certainly a maximum.) It is shown in the Appendix that the optimum is increased by the factor $\left(\frac{K_i+[I]}{K_i}\right)^{\frac{1}{2}}$, so that the data of Fig. 1 suggest a value for K_i, the enzyme-inhibitor equilibrium constant, of about $1{\cdot}2 \times 10^{-9}$ M. This value is not exact because of the uncertainty of estimating the optima, but a fairly high affinity is indicated.

Neither the Lineweaver-Burk nor the Augustinsson tests can be applied to irreversible inhibitors. The reversal by substrate, if it occurs, is too slow to be measured under the conditions of such a test,

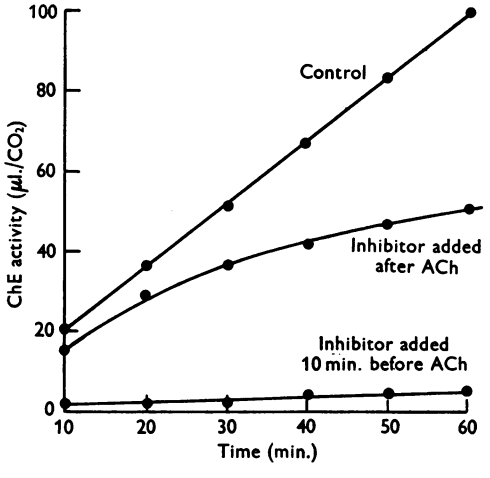

Fig. 2. Protection by ACh of human plasma ChE against inhibition by 10^{-4}M-DPDA, showing competitive nature of the inhibition.

and consequently irreversible inhibitors are apparently non-competitive (see Mackworth & Webb, 1948; Augustinsson & Nachmansohn, 1949). We have here applied the test of protection by substrate. DPDA (10^{-4}M) causes 93% inhibition of human-plasma ChE in 10 min. in the absence of ACh (Fig. 2). If the same concentration is added after ACh, the rate of inhibition is slowed down, so that the 93% level is reached only after some 60–70 min. The fact that the substrate can protect is evidence that the inhibition is competitive.

The rate of inhibition of human plasma ChE by DPDA was found to be first order for most of several concentrations of inhibitor, and was presumed so for the concentration $2{\cdot}7 \times 10^{-5}$M (Fig. 3). The time to 50% inhibition was found inversely proportional to the concentration of inhibitor (Fig. 4). Consequently the inhibition reaction is bimolecular, with a rate constant of $4{\cdot}2 \times 10^{4}$ l. mol.$^{-1}$min.$^{-1}$.

Selectivity of the inhibitors

It seemed from the foregoing results on human blood that the selectivity of DPDA for the pseudo

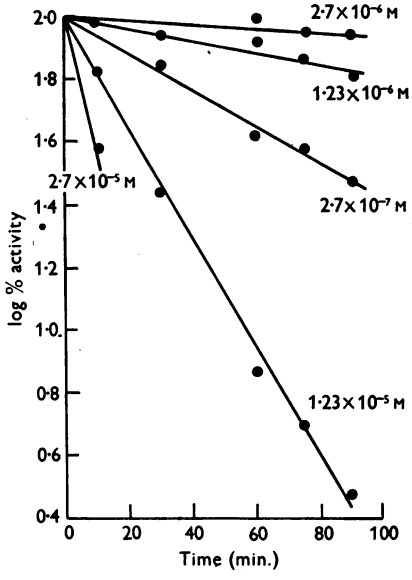

Fig. 3. Rate of inactivation of human plasma ChE by DPDA. The concentration of inhibitor is shown against each line. (The rate for $2{\cdot}7 \times 10^{-5}$M is presumed first order.)

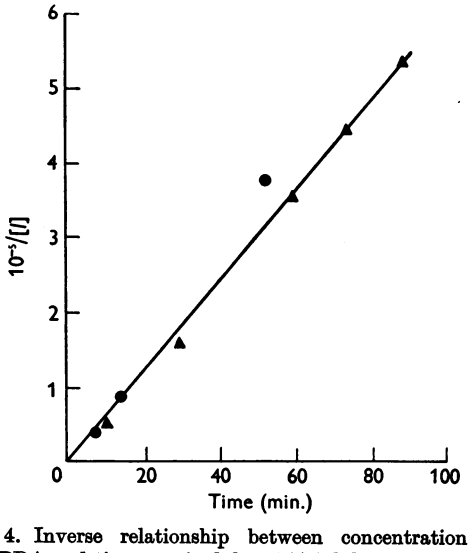

Fig. 4. Inverse relationship between concentration of DPDA and time required for 50% inhibition of human plasma ChE, showing that the inhibition reaction is bimolecular. ●, from Fig. 3; ▲, additional experiments.

ChE of the plasma was not nearly as high as had been found for horse blood. This discrepancy was the subject of correspondence between Dr Aldridge and

ourselves and was confirmed in both laboratories. We found the ratio I_{50} (cells):I_{50} (plasma) to be about 50 for human and upwards of 6500 for horse blood. Aldridge (1953) has reported 56 and 11300 respectively. According to Whittaker and his collaborators (Adams, 1949; Adams & Whittaker, 1949; Mounter & Whittaker, 1950; Sturge & Whittaker, 1950) the plasma enzymes of the two species agree closely to a substrate specificity pattern which has been designated 'butyrocholinesterase' and the erythrocyte enzymes to another 'aceto-cholinesterase' pattern. In spite of similarities in behaviour towards substrates, there seemed to be profound differences towards DPDA. Also, Fulton (1952) has claimed that 10^{-3}M-284C51 is without effect on the hydrolysis of BCh by horse serum, whereas we find an I_{50} of 3×10^{-4}M using ACh, and for human serum an I_{50} of 10^{-3}M.

We have therefore examined the species variations in the selectivity of the inhibitors, using the plasma and erythrocytes of man, rhesus monkey, horse and guinea pig, as well as plasma of the domestic fowl.

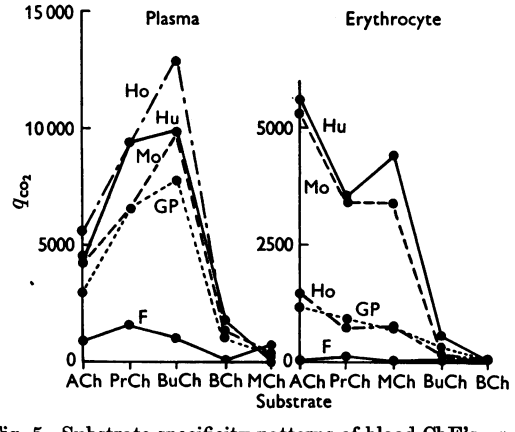

Fig. 5. Substrate specificity patterns of blood ChE's. q_{CO_2} values in μl./hr./ml. of plasma or packed erythrocytes. ACh, acetylcholine; BCh, benzoylcholine; BuCh, butyrylcholine; MCh, acetyl-β-methylcholine; PrCh, propionylcholine. Ho, horse; Hu, human; Mo, monkey; GP, guinea pig; F, fowl.

Characterization of the enzymes

The substrate specificity patterns of the enzymes in question are given in Fig. 5 (Davies, Risley & Rutland, 1952). It will be seen that the mammalian plasmas are broadly similar in the relative rates of hydrolysis of the various esters. There is a suggestion that there are two pairs of enzymes differing in the ratio $q_{PrCh}:q_{ACh}$. In human and guinea-pig plasma this is about 2, whereas in monkey and horse it is about 1·6. In all cases the $q_{BuCh}:q_{ACh}$ is about 2·3. The mammalian erythrocyte enzymes are also very similar and appear also to be divisible, on the basis of the $q_{ACh}:q_{BuCh}$ ratio, although some doubt is admissible because of the low values of q_{BuCh} and consequent inaccuracy. The enzymes of fowl blood are markedly different. Of fowl plasma more will be said later; but studies on fowl erythrocytes are profitless because of the extremely low q values.

Behaviour of the various enzymes towards the inhibitors

DPDA. Table 3 shows the concentrations of DPDA required to give 50% inhibition of the hydrolysis of various choline esters by the several plasmas. Inhibitor and enzyme were in contact for 30 min. at 38° before addition of substrate. For the mammalian plasmas the I_{50}'s for ACh, BCh and BuCh are practically identical, as might be expected if all substrates are hydrolysed by the same pseudo ChE. These I_{50}'s range between $1·3 \times 10^{-7}$ and 9×10^{-6}M: whereas for the erythrocytes the values are 3×10^{-4} to $3·6 \times 10^{-3}$M. In consequence, it might be supposed that the increased concentrations required to inhibit hydrolysis of MCh by plasma indicate that part, at least, of this hydrolysis is carried out by true ChE. However, in order to measure MCh hydrolysis with any accuracy, a very concentrated plasma was used. It is much more likely that the preparation had a relatively high binding power for inhibitor, due to substances other than ChE.

The almost equal sensitivity for all substrates in fowl plasma, and the finding that the I_{50} is much the same as with pseudo ChE, confirms the conclusion of Earl & Thompson (1952) that this tissue contains one enzyme resembling pseudo ChE and capable of hydrolysing MCh.

The selectivity of the inhibitor is fairly high, as shown by the ratios of I_{50}'s in the last column of Table 3. The shape of the percentage activity/log (inhibitor concentration) curves is such that there is a wide gap in concentration between 95% inhibition of the pseudo and 5% inhibition of true ChE. Even with human blood it appears that 3×10^{-5}M ought to give 95% inhibition of the pseudo and only 10% inhibition of true ChE. This concentration appears to be generally useful for discriminating between the two types.

284*C*51. Table 4 shows the I_{50}'s for 284C51 of the various erythrocyte preparations, using ACh and MCh. The values (ACh) range between 10^{-8} and 10^{-7}M. For pseudo ChE the values are $2·8 \times 10^{-4}$ to 10^{-2}M. The selectivity is high, at least 3000. Although the dose-response curves are flatter than with DPDA, there still exists a range of concentrations, 10^{-6} to 10^{-5}M, in which it should be possible to cause at least 95% inhibition of true but not more than 5% of pseudo ChE.

Table 3. *Inhibition by DPDA of hydrolysis of choline esters by plasma of various species. Inhibitor in contact with enzyme for* 30 *min. at* 38° *before addition of substrate*

Species	$I_{50} \times 10^7$ Acetylcholine (0·06M)	Butyrylcholine	Benzoylcholine	Acetyl-β-methylcholine	Ratio: I_{50} (cells): I_{50} (plasma) for acetylcholine
Man	60	60	60	300	50
Monkey	1·3	1·5	1·3	1300	5000
Guinea pig	30	26	26	1600	1200
Horse	4	6	5	2500	6500
Fowl	87	87	100	87	—

Table 4. *Inhibition by* 284*C*51 *of hydrolysis of choline esters by erythrocytes of various species*

Species	$I_{50} \times 10^7$ Acetylcholine (0·006M)	Acetyl-β-methylcholine	Ratio: I_{50} (plasma): I_{50} (cells) for acetylcholine
Man	1·0	50	10 000
Monkey	1·0	4·5	2 800
Guinea pig	0·12	0·64	217 000
Horse	1·0	1·6	2 800
Fowl plasma	$>10^5$	7 200	—

The differences in I_{50} between ACh and MCh can be rationalized when it is remembered that for a reversible competitive inhibitor the I_{50} depends on the enzyme-substrate affinity as well as on the enzyme-inhibitor affinity. In this connexion it must be observed that accurate measurement of the equilibrium constant K_s for the formation of the enzyme-substrate complex is virtually impossible using the method and conditions chosen here. We have attempted to verify this explanation for the considerable difference in I_{50}'s for fowl plasma, the more especially since this is a new type of enzyme. It is well known that $I_{50} = K_i(1 + [S]/K_s)$ whence the ratio of I_{50}'s can be calculated if the respective values of $[S]$ and K_s are known. We have found that for both ACh and MCh the relationship between activity and substrate concentration follows the Michaelis-Menten formula: the respective values of K_s are $7{\cdot}6 \times 10^{-4}$ and $1{\cdot}04 \times 10^{-2}$M. In determining the I_{50}'s given in Table 4 the values of $[S]$ were, respectively, 6×10^{-2} and 3×10^{-2}M. The calculated ratio of I_{50}'s is therefore 20·8; found, >14. K_i is $1{\cdot}85 \times 10^{-4}$M.

DISCUSSION

This work was undertaken to examine the value of the inhibitors DPDA and 284C51 as tools for classifying cholinesterases. Both seem to be valuable for different purposes.

If an inhibitor is to be used with a variety of substrates it should be irreversible so that variations in enzyme-substrate affinity will not affect its behaviour. DPDA appears to be very well suited to such studies, especially since our data suggest that it may be rather better than most at inhibiting pseudo ChE without affecting true ChE. This feature is not always present, to judge from results given by Hawkins & Mendel (1947, 1949) and Adams & Thompson (1948). Todrick (1952) has not yet reported on this aspect of the selective inhibitors used by him. Treatment with 3×10^{-5}M DPDA for 30 min. at 38° before addition of substrate might prove a useful means of discriminating between the two types of ChE.

If only one substrate, e.g. ACh, is to be used, 284C51 seems to be valuable, because of the wide separation of I_{50}'s between true and pseudo ChE's and because, in spite of flatter dose-response curves, there seems still to be a tenfold range of concentration, 10^{-6} to 10^{-5}M, over which it might be possible to suppress true ChE activity without there being any measurable effect on the pseudo enzyme.

It was suggested above that the plasma ChE's might be divisible into two pairs on the basis of the relative rates of hydrolysis of PrCh and ACh. These pairs were: human and guinea pig, monkey and horse. The former pair is rather less sensitive to DPDA, the I_{50}'s being $3\text{–}6 \times 10^{-6}$M as against $1\text{–}5 \times 10^{-7}$M (Table 3). The data are scanty and further work is necessary, but they support other findings (Hawkins & Mendel, 1949; Berry, 1951; Myers, 1952; Aldridge, 1953) that there are species differences in sensitivity to inhibitors among groups of ChE's which have much slighter differences in relative substrate hydrolysis rates. Since these phenomena have been studied in extremely crude preparations, it is not impossible that they are associated rather with variations in the non-cholinesterase fractions, competing for inhibitor, than with real differences in ChE's. When the substrate specificity pattern is markedly different,

as with fowl plasma, the tissues of rat (Ord & Thompson, 1951), sheep (Davies *et al.* 1952) or rabbit serum (Koelle, 1953) there can be less doubt that the cholinesterases themselves are different.

SUMMARY

1. Two new inhibitors of cholinesterase have been studied, namely, *NN'*-di*iso*propylphosphorodiamidic anhydride ($[(C_3H_7NH)_2PO]_2O$; bis(di*iso*propylamino)phosphonous anhydride; DPDA) and the dimethobromide of 1:5-di(*p*-*N*-allyl-*N*-methylaminophenyl)pentan-3-one (284C51). DPDA is a competitive irreversible inhibitor, selectively inhibiting pseudo cholinesterases, while 284C51 is a competitive reversible inhibitor, selectively inhibiting true cholinesterases.

2. The selectivity of the inhibitors, as expressed by the ratio I_{50} (true):I_{50} (pseudo) is generally high, although this ratio varies considerably among some mammalian species if blood erythrocytes and plasma are the sources of true and pseudo cholinesterases respectively. It appears that 3×10^{-5}M-DPDA (in contact 30 min. at 38° before addition of substrate) or 10^{-6} to 10^{-5}M-284C51 could be used to inhibit pseudo or true cholinesterase respectively without measurable effect on the other.

3. Some evidence is given suggesting that the apparently typical pseudo cholinesterases of mammalian plasmas may be capable of subclassification.

4. Further properties of fowl plasma cholinesterase are described, confirming that it is a pseudo cholinesterase in its behaviour towards inhibitors, although the substrate specificity pattern is unlike that of classical pseudo cholinesterase.

We wish to thank Dr A. H. Ford-Moore for supplies of DPDA and Dr Copp of the Wellcome Research Laboratories for the sample of 284C51. Thanks are due also to Dr W. N. Aldridge for certain of his then unpublished data.

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APPENDIX

A Note on the Reaction with Inhibitors of Enzymes which are Inhibited by Excess Substrate

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Equations connected with enzyme kinetics are often transformed into linear form, so that experimental data may be examined with the minimum of mathematical labour (cf. Lineweaver & Burk, 1934). Transformations are used not only to determine kinetic constants, but also to establish, among other things, mechanisms of inhibition.

The equations for enzymes which are inhibited by excess substrate cannot be transformed in this manner, but give rise to quadratic or higher order curves. Consequently for some purposes, such as are discussed in the accompanying paper (Austin & Berry, 1953), tests based on linear transformations cannot be used, unless it is possible to work below