The Selective Inhibition of Cholinesterases

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In recent years it has become apparent that cholinesterase preparations from different tissues exhibit fundamental differences in specificity and other properties (Alles & Hawes, 1940; Glick, 1941; Richter & Croft, 1942). Mendel & Rudney (1943) have suggested a classification of these enzymes into two main types: (1) the 'true' cholinesterases, active only towards certain esters of choline, and (2) the 'pseudo-cholinesterases', active towards both choline and non-choline esters.

In a further paper (Mendel, Mundell & Rudney, 1943) it is stated that the 'true' cholinesterases readily hydrolyze acetyl- β -methylcholine, but are inactive towards benzoylcholine, while the reverse is true for the 'pseudo' enzyme. This difference in substrate specificity is put forward as a practical criterion for the differentiation of the two types of enzyme. From their experiments with brain and blood of different species it appears that in general brain and red blood cells contain a preponderance of the 'true' while human serum or plasma, and that of several other species, contains chiefly the 'pseudo' enzyme. Another point of differentiation was revealed in the concentrations of acetylcholine at which maximum enzymic activity was obtained, the 'true' cholinesterase showing a maximum rate of hydrolytic activity at much lower concentrations of acetylcholine than those required by the 'pseudo' enzyme (Mendel & Rudney, 1943; Bissegger & Zeller, 1943).

Studies of the action of inhibitors on these enzymes have indicated further differences. Thus, Zeller (1942) found that the cholinesterase in human or rat brain is much less sensitive to inhibition by certain pyrazolone derivatives than the cholinesterase in human serum. Hottinger & Bloch (1943) examined the action of tri-*o*-cresyl phosphate on the cholinesterase activity of rabbit and human serum, liver and brain, but found that although the human tissues were in general more sensitive to inhibition by this compound than those of the rabbit, the three tissues of either species were inhibited to approximately the same extent. Zeller & Bissegger (1943), comparing the enzymes in human serum, brain and erythrocytes, showed that whereas morphine inhibited all three esterases to approximately the same extent, percarine inhibited the enzyme in serum more powerfully than that in brain or erythrocytes, while with caffeine the reverse was true; the

concentrations of inhibitor used by these latter workers were, however, high. In a study of the action of certain prostigmine analogues Hawkins & Gunter (1946) found that the dimethylcarbamate of (2-hydroxy-5-phenylbenzyl)-trimethylammonium bromide (Hoffmann-La Roche, Nu 683) is, both *in vitro* and *in vivo*, a potent inhibitor of pseudo-cholinesterase, a concentration of 1×10^{-8} M producing 92% inhibition of the hydrolysis of benzoylcholine by human plasma.

Diisopropyl fluorophosphonate, which, like other alkyl fluorophosphonates, possesses eserine-like properties in the animal body (McCombie, Adrian, Kilby & Kilby, 1941), was shown by Mackworth (1942) to inhibit serum cholinesterase at high dilutions. Mazur & Bodansky (1943, 1946) and Mendel & Hawkins (1947) have demonstrated that, while the esterase in human serum is highly sensitive to this compound, significantly higher concentrations are required to inhibit the cholinesterase in brain tissue.

It has been shown in this laboratory (Thompson, 1943, 1947; see also Gilman & Philips, 1946) that certain of the 'nitrogen mustards' (e.g. $\beta\beta'$ -dichlorodiethyl-*N*-methylamine) are inhibitors of brain cholinesterase. Mackworth (1943), on the other hand, obtained only slight inhibition of the enzyme in serum. From these early results it seemed therefore as if $\beta\beta'$ -dichlorodiethyl-*N*-methylamine might also be a differential inhibitor of the cholinesterases, but in the reverse sense to the fluorophosphonates, i.e. the 'true' enzyme in brain might be significantly more sensitive to inhibition by this compound than the 'pseudo' enzyme in serum. A more detailed comparison of the inhibition of certain cholinesterases by this compound has therefore been carried out with both acetylcholine and the 'specific' substrates of Mendel *et al.* (1943), and the results obtained with this inhibitor compared with those found with eserine and diisopropyl fluorophosphonate.

EXPERIMENTAL

Source of enzyme

Pigeon brain. Pigeons were killed by decapitation, and this was followed by immediate removal of the brain. The whole brain, wiped free from blood, was then dispersed by grinding in 10 times its weight of 0.024 M-NaHCO₃, and filtered through muslin.

Human brain. Fresh samples of normal human cerebellum, removed during the course of operations for the excision of cerebellar tumours or chronic encapsulated abscesses, were kindly provided by Mr J. Pennybacker, F.R.C.S. After removal from the cranium the tissue was placed in ice-cold bicarbonate buffer. Large blood vessels and any adherent membranes were first dissected away, and the tissue washed free from blood clots with glass-distilled water. The weighed sample was then dispersed by grinding with bicarbonate buffer. Since appreciable quantities of blood were still present, the dispersion was strained through muslin, centrifuged, and the blood-stained supernatant liquid discarded. The precipitate was washed once more, and finally suspended in bicarbonate so that 2 ml. of the final suspension corresponded to 1 g. of original tissue.

Human plasma and serum. Freshly drawn blood was either added to potassium oxalate (1.0 mg./ml.) or allowed to clot and the clot broken up with a glass rod; separation by centrifuging followed in either case.

Human red blood cells. The cells obtained after removal of the oxalated plasma were washed five times with 0.9% NaCl, and finally lysed by suspending in sufficient distilled water to make up to the original volume of blood.

All the enzyme preparations were stored, when necessary, at 0°, under which conditions their activity remained almost unchanged for several days. No attempt at purification was made. Plasma was used more often than serum in order to obtain more easily red cell preparations from the same blood sample. Massart & Dufait (1933*a, b*) have stated that oxalate is appreciably inhibitory towards horse-serum cholinesterase, due presumably to the removal of activating Ca^{++} ; Gautrelet & Scheiner (1939), on the other hand, who used a biological method, have claimed that oxalate has no effect on serum cholinesterase. With human blood the final concentrations of oxalate used in this work produced less than 10% inhibition of the plasma enzyme. Further, the effects of the various inhibitors on serum or on plasma were the same, within the limits of experimental error. Consequently serum and plasma have been used interchangeably throughout the work.

Substrates

(1) *Acetylcholine chloride* (ACh) (British Drug Houses Ltd.).

(2) *Acetyl- β -methylcholine chloride* (MCh) (Savory and Moore Ltd.).

(3) *Benzoylcholine bromide* (BCh), prepared and kindly given to us by Dr A. H. Ford-Moore, Experimental Station, Porton.

Each substrate was dissolved in 0.024M-NaHCO₃ immediately before use, and a sufficient volume of the solution added to the reaction mixture to bring the final concentrations to 0.006M for ACh and BCh, and 0.03M for MCh. Rough determinations of the Michaelis curves have shown that these concentrations lie upon the flat part of the curves obtained with the 'pseudo' enzyme, or are in excess of the peak concentrations for the 'true' enzyme (Mendel & Rudney, 1943).

Inhibitors

(1) $\beta\beta'$ - *Dichlorodiethyl - N - methylamine hydrochloride* (DDM). Since this compound has been shown to undergo fairly rapid change in aqueous solution, with the formation of cyclic (ethylenimonium) and hydroxy derivatives

(Hartley, Powell & Rydon, 1942; Boyland, 1942; Cameron & Rydon, 1942; Bergmann & Fruton, 1944; Gilman & Philips, 1946), the solid was dissolved in 0.024M-NaHCO₃ immediately before adding to the enzyme-bicarbonate mixture.

(2) *Diisopropyl fluorophosphate* (DFP).

(3) *Eserine sulphate* (British Drug Houses Ltd.).

(4) *Trichlorotriethylamine hydrochloride*.

The latter inhibitors were also freshly made up in bicarbonate for each experiment.

Estimation of enzymic activity

Esterase activity was determined manometrically, using Ammon's (1933) adaptation of the Warburg technique, in which the acid produced by enzymic hydrolysis of the substrate is measured by CO₂ evolution from bicarbonate buffer at pH 7.4 (gas space filled with 95% N₂ and 5% CO₂). All measurements were made in duplicate at 38°. Non-enzymic hydrolysis was controlled by including a pair of vessels containing substrate but no enzyme.

The enzyme preparation together with the inhibitor was placed in the main compartment of the Warburg vessels, and the substrate in the side bulbs, the total volume being made up to 3 ml. by the addition of 0.024M-NaHCO₃ to the main compartment. After gassing the vessels were placed in the thermostat, and 15 min. allowed for temperature equilibration; the substrate was then tipped in from the side bulbs and readings taken at 10 min. intervals. The inhibitors therefore remained in contact with the enzyme, and in the absence of substrate for 5–10 min. at room temperature, followed by a further 15 min. during the attainment of temperature equilibrium.

The first 10 min. readings have been neglected and the activity recorded over the period 10–40 min. Apart from errors introduced during the removal of the manometers from the bath in order to tip in the side-bulb contents, it was found that the non-enzymic hydrolysis was usually larger during the first few minutes. Further, in the case of reversible inhibition a measurable time must elapse to allow the new enzyme-substrate-inhibitor system to approach equilibrium (Eadie, 1942).

As far as was possible the quantity of enzyme taken was adjusted to that which gave, or would have given, 180–200 $\mu\text{l. CO}_2/30 \text{ min.}$ with acetylcholine as substrate.

RESULTS

To test the validity of the earlier work, the effects of different concentrations of DDM were first tested on the cholinesterase activity of pigeon brain and human plasma. These tissues were chosen, largely on account of their accessibility, as sources of the true and pseudo-enzymes respectively, and experiments have been carried out using both acetylcholine and the appropriate specific substrate of Mendel. In agreement with the results of Mendel *et al.* (1943) it was found that while both preparations readily hydrolyze acetylcholine, the brain enzyme is active against MCh but not against BCh, while the reverse is true for the serum enzyme. The inhibitions of enzymic activity produced are summarized in

Table 1. *Percentage inhibition by DDM of cholinesterase activity of pigeon brain and human serum*

(See p. 171 for explanation of abbreviations here and in following tables.)

Enzyme source	Substrate	Concentration DDM ($\times 10^{-4}$ M)							
		0.17	0.44	0.87	1.75	3.5	6.1	8.7	17.5
Pigeon brain	ACh	42	69	86	93	95	—	96	—
	MCh	39	62	84	93	—	—	—	—
Human plasma	ACh	—	—	8	16	31	47	56	62
	BCh	—	11	19	28	40	—	58	—

Fig. 1. Percentage inhibition of cholinesterases by DDM.

Table 1, the values for each concentration of inhibitor being the mean of several determinations, which in general agreed to within a few per cent.

The difference in the sensitivity to DDM of the cholinesterases in these two tissues is also shown in Fig. 1, from which it can be seen that, in order to produce 50% inhibition of the enzymic hydrolysis of ACh, the concentration of inhibitor required for the plasma enzyme is about 30 times that required for pigeon brain. A similar difference exists when the specific substrates MCh and BCh are used with their respective enzymes, although appreciably more inhibition is obtained at low concentrations with the BCh-plasma enzyme system.

Since in these experiments, however, the two

types of cholinesterase were derived from different animal species, the interpretation of the results is complicated by possible species differences in sensitivity to DDM. To overcome this, experiments were also carried out with preparations from human erythrocytes and human brain. The enzyme in each of these two tissues conforms to Mendel's criterion for a true cholinesterase in readily hydrolyzing ACh and MCh, while showing only a negligible activity with BCh. The results obtained with these tissues are shown in Table 2 (see also Fig. 1).

The human brain used in this work was in each experiment taken from the postero-lateral surface of the superior cerebellar lobule. Since the samples of tissue excised were not in each case identical

Table 2. *Percentage inhibition by DDM of cholinesterase in human erythrocytes and human brain*

Enzyme source	Substrate	Concentration DDM ($\times 10^{-4}$ M)					
		0.17	0.44	0.87	1.75	3.5	8.7
Human erythrocytes	ACh	18	34	55	74	86	90
	MCh	20	37	60	74	89	—
Human brain	ACh	—	34	48	69	83	85
	MCh	—	36	53	72	82	—

anatomically, considerable variations in activity were observed between them (one sample of cerebrum from the prefrontal cortex showed only negligible activity); weight for weight, the level of activity was about one eighth that of whole pigeon brain.

From Table 2 it will be seen that the activities of human erythrocytes and brain are inhibited to approximately the same extent by DDM; and although less sensitive to this substance than pigeon brain, each of these tissues is very considerably more sensitive than human plasma, 10^{-4} M DDM causing 58 and 52 % inhibition with human erythrocyte and human brain respectively, but only 9 % with human plasma.

Differences in the response of an unpurified enzyme in different tissue preparations to the presence of a given concentration of an inhibitor might be due to variations in the amount of enzymically inert substances competing with the enzyme for the inhibitor. With this possibility in mind, and in view of the selective inhibition of the true and pseudo-cholinesterases by diisopropyl fluorophosphonate (Mazur & Bodansky, 1946; Mendel & Hawkins, 1947), a series of experiments was carried out under identical conditions using DFP instead of DDM, in order directly to compare the effects of these two inhibitors on our enzyme preparations. Table 3 summarizes the results obtained with DFP on the various tissues studied.

From these results, which confirm those of Mazur & Bodansky (1943, 1946) and Mendel & Hawkins (1947), it will be seen that with the same enzyme preparations functioning under the same conditions DFP inhibits selectively, but in the opposite direction to that of DDM, i.e. the plasma enzyme is very much more sensitive than that in brain to inhibition by DFP.

This contrasting behaviour of the cholinesterases from these sources is shown more clearly in Table 4, in which is given the concentrations (I_{50}) of these inhibitors which are required to produce 50 % inhibition of enzymic activity. The selective nature of the inhibitors may be conveniently presented as the ratio of these concentrations (I_{50} ratio).

In order to bring these results into line with the classical work on eserine we have also compared the effects of eserine sulphate on these enzymes under our conditions, using both acetylcholine and the specific substrates, and have confirmed the earlier findings (Mendel *et al.* 1943) that eserine shows no marked difference in its ability to inhibit the two types of cholinesterase; under our conditions $0.05 \mu\text{g./3 ml.}$ resulted in 75, 52 and 57 % inhibition of the hydrolysis of the specific substrates by the human plasma, pigeon brain and human red cell esterases respectively.

A few experiments have also been carried out with another of the 'nitrogen mustard' class of

Table 3. *Percentage inhibition of cholinesterases by DFP*

Enzyme source	Substrate	Concentration DFP ($\times 10^{-8}$ M)									
		3.6	7.1	9.1	14.4	18.2	29	36.4	59	73	144
Pigeon brain	ACh	—	11	—	19	—	30	—	52	—	85
	MCh	—	9	—	17	—	29	—	52	—	84
Human erythrocytes	ACh	8	24	—	45	—	77	—	97	—	—
	MCh	16	32	—	53	—	80	—	96	—	—
Human brain	ACh	—	—	35	—	61	—	80	—	96	—
	MCh	—	—	40	—	68	—	85	—	95	—
Human plasma	Substrate	Concentration DFP ($\times 10^{-8}$ M)									
		0.02	0.04	0.09	0.18	0.36	0.71	1.01			
		ACh	0	7	18	35	58	87	100		
	BCh	—	—	16	34	79	86	—			

Table 4. *Concentrations (I_{50}) of inhibitors required to produce 50 % inhibition of esterase activity*

Enzyme source	Substrate	I_{50} conc. of DDM ($\times 10^{-5}$ M)	I_{50} conc. of DFP ($\times 10^{-5}$ M)	I_{50} ratio (DDM/DFP)
Pigeon brain	ACh	2.4	0.057	42
	MCh	2.7	0.059	46
Human erythrocytes	ACh	7.4	0.015	493
	MCh	6.5	0.013	500
Human brain	ACh	8.7	0.013	669
	MCh	8.0	0.012	667
Human plasma	ACh	69	0.00029	2.4×10^5
	BCh	58	0.00026	2.2×10^5

compounds, trichlorotriethylamine hydrochloride. Although this compound is less selective than DDM in its effects on true and pseudo-cholinesterases, the enzyme in pigeon brain was again found to be more sensitive than that in human plasma.

DISCUSSION

The ability of certain compounds to inhibit the pseudo-cholinesterase at concentrations far below those required to affect the true cholinesterase has been well established. In addition to those substances already mentioned, Harris & Harris (1944) demonstrated the presence of another unidentified selective inhibitor of the pseudo-enzyme in certain curare preparations. A compound capable of inhibiting the true cholinesterase at low concentrations, while leaving the pseudo-enzyme relatively unaffected, does not, however, appear yet to have been described. Zeller & Bissegger (1943), it is true, showed that high concentrations of caffeine exerted this effect; their work, however, was carried out before the introduction of specific substrates for these enzymes, and, using a concentration of acetylcholine of 6.6 mM, they required a concentration of 6 mM-caffeine to produce 40 % inhibition of enzymic activity.

From the experiments described here, DDM, in so far as its effects on esterases are concerned, would appear to fulfil the role of a selective inhibitor of the true enzyme. It is, admittedly, a much less potent inhibitor than either DFP or eserine, but it must be remembered that its action has so far only been studied on unpurified tissue extracts. Further, few compounds of the 'nitrogen mustard' group have been even superficially investigated as cholinesterase inhibitors. It is not necessarily to be expected, therefore, that DDM is either the most potent or the most selective inhibitor among these substances. It is known that DDM also inhibits certain phosphokinases present in such tissue extracts (Dixon & Needham, 1946), so that it is not unlikely that the purified cholinesterase would show a significantly greater sensitivity to this substance. Even with our crude preparations, however, 50 % inhibition of the true cholinesterase has been obtained with concentrations varying from 2.5 to 8.7×10^{-5} M, whereas amounts of from roughly 10–30 times these concentrations are required to produce an equivalent inhibition of the pseudo-enzyme.

Of the large number of substances which have been described as inhibitors of cholinesterase it is now possible to distinguish three main types, examples of which are given below:

A. Selective inhibitors for true cholinesterase:

$\beta\beta'$ -Dichlorodiethyl-*N*-methylamine.
Caffeine.*

B. Selective inhibitors for pseudo-cholinesterase:

Alkyl fluorophosphonates.
Nu 683.
Curare (unauthenticated samples).
Percaine.*
Pyrazolone derivatives.*

C. Inhibitors of both types of cholinesterase:

Eserine.
Tri-*o*-cresyl phosphate.*
Morphine.*

* Not tested against specific substrates.

It is of interest that the values for the I_{50} concentrations of either DDM or DFP for the enzyme from a given source agree closely whether the substrate be ACh or the specific ester (Table 4), since, in the case of MCh, the concentration of substrate used differed widely from that of ACh.

The I_{50} concentrations of DDM and DFP given in Table 4 suggest strongly that, while the cholinesterase in human erythrocytes and human brain are at least very similar, they are fundamentally different from the enzyme in human plasma. Further, although the results obtained with pigeon brain differ appreciably from those obtained with the true cholinesterase in human tissues, they are in support of the general classification of the cholinesterases into true and pseudo types put forward by Mendel.

Inhibition by DFP is irreversible, and we have not found it to vary with changes in substrate concentration; DDM on the other hand appears to inhibit competitively, so that the I_{50} value will depend on substrate concentration. The I_{50} ratio for these two inhibitors will therefore also depend on substrate concentration as well as on the varying effect of enzymically inert substances present in the preparations which may be competing with the enzyme for each of the inhibitors. If, however, two more closely related inhibitors are used, interference by inert substances is less likely to affect them to different degrees. It is hoped, therefore, to extend this work to the study of two competitive inhibitors, in order to determine whether the I_{50} ratio can, under these conditions, be used as an aid in the characterization of related esterases.

SUMMARY

1. The inhibitory action of $\beta\beta'$ -dichlorodiethyl-*N*-methylamine on the true and pseudo-cholinesterases of brain, erythrocytes and plasma has been studied using both acetylcholine and Mendel's specific substrates, and the effects compared with those produced by diisopropyl fluorophosphonate and eserine.

2. $\beta\beta'$ -Dichlorodiethyl-*N*-methylamine has been shown to be a selective inhibitor of the true enzyme.

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* Information available on application to Ministry of Supply, London.

The Formation of Nicotinamide from Nicotinic Acid by the Rat

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Part of the essential content of this paper has been communicated to the Biochemical Society (Ellinger, 1946a). The publication of details was postponed because the quantitative estimation of the nicotinamide formed, was carried out by an indirect biological method, which involved some uncontrollable factors. Confirmation of the results was desired using a more exact method which would permit the separate determination of nicotinamide and nicotinic acid in the same solution; but no suitable method has so far been found. No method based on the König (1904) principle is sensitive enough for the small amounts of nicotinamide and nicotinic acid to be measured in these experiments, and the errors involved in the estimation of nicotinamide by the differences found before and after alkaline hydrolysis are far too large. Scudi's (1946) method, based on the fluorimetric measurement of a pigment developed from nicotinamide with cyanogen bromide and alkali and extracted by isobutanol, permits the measurement of as little as

1 μ g. of nicotinamide in the presence of nicotinic acid; the latter does not give a fluorescent product, but aneurin gives with the reagents a product of so high fluorescent efficiency that the method cannot be used for the determination of nicotinamide in the presence of aneurin. Microbiological methods using bacteria such as *Pasteurella* strains (Koser, Berkman & Dorfman, 1941) or *Leuconostoc mesenteroides* (Johnson, 1945; Krehl, de la Hueraga, Elvehjem & Hant, 1946) including some strains of A.T.C.C. 9135 proved unsatisfactory, as all strains of both types of bacteria so far available responded to nicotinamide as well as nicotinic acid. It has been decided, therefore, to publish the results so far obtained.

Surviving rat liver, but not kidney or muscle, methylate nicotinamide to nicotinamide methochloride. None of the tissues mentioned converts nicotinic acid into either nicotinamide methochloride or trigonelline (Perlzweig, Bernheim & Bernheim, 1943). Ellinger & Coulson (1944) have