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THE PROPERTIES OF ACETYLCHOLINESTERASE MODIFIED BY INTERACTION WITH THE ALKYLATING AGENT *N,N*-DIMETHYL-2-PHENYLAZIRIDINIUM ION*

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SUMMARY

The interaction of *N,N*-dimethyl-2-phenylaziridinium ion with bovine erythrocyte acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7) and the product of this interaction have been investigated in some detail.

Initially DPA inhibits acetylcholinesterase towards the hydrolysis of acetylcholine and isoamyl acetate. With time, an irreversible reaction develops, resulting in a product with an activity greater or less than that of acetylcholinesterase, depending on the substrate.

The product of the irreversible reaction (DPA-enzyme) has little or no affinity for compounds containing a quaternary nitrogen function but has esteratic activity towards uncharged esters. Compared with acetylcholinesterase it demonstrates considerably less substrate specificity as shown by its behaviour towards a series of aliphatic acetates (ranging from ethyl to 3,3-dimethylbutyl) and towards a series of *para*-substituted phenyl acetates. It is also characterized by uniformly low k_{cat} values. This is tentatively interpreted as resulting from difficulty in deacetylation.

INTRODUCTION

Acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7) differs from other esterases in its strong interaction with compounds containing a quaternary nitrogen. The "natural" substrate, acetylcholine, is hydrolysed considerably faster than uncharged esters by acetylcholinesterase, and phosphylation rates by organophosphorus compounds and carbamylation rates by carbamates are enhanced by the incorporation of a quaternary nitrogen function into the inhibitor molecule. This has led to the idea of a specific anionic site to accommodate the quaternary head[1]. Some re-

Abbreviations: DPA, *N,N*-dimethyl-2-phenylaziridinium ion; DPA-enzyme, acetylcholinesterase irreversibly inhibited by DPA towards acetylcholine hydrolysis.

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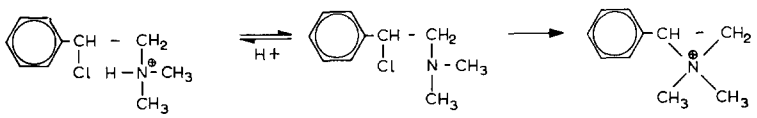
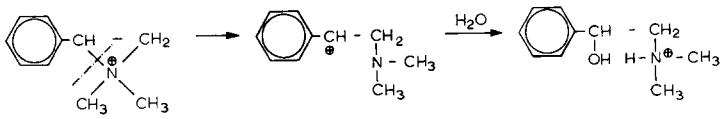
versible inhibitors showing mainly competitive behaviour have no features which would be expected to lead to high specificity, *e.g.* the simple tetraalkyl ammonium salts, while others, *e.g.* 3-hydroxyphenyltrimethylammonium iodide[2], have group(s) which could also interact with residues at the esteratic site. There is quite a variation in the quaternary heads of the inhibitors ranging from those in the heterocyclic pyridinium compounds to those in spiran quaternary ammonium salts[3] and the tri- and tetraalkyl ammonium salts. There is no apparent reason why all these should be entirely specific for one anionic site, especially as acetylcholinesterase is an acidic protein with several carboxylate side chains[4].

BELLEAU AND TANI[5] found that DPA also inhibited acetylcholinesterase, but whereas the inhibition was competitive for its *m*-hydroxy, *m*-methoxy and *m*-bromo analogues, the unsubstituted DPA showed purely non-competitive kinetics and furthermore proceeded to inhibit the enzyme irreversibly towards acetylcholine hydrolysis.

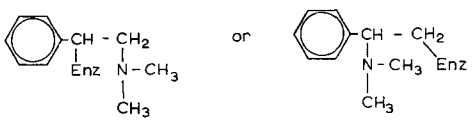
DPA is formed from the free base of the β-chloroamine as shown in the following equation:



In aqueous solution, DPA undergoes hydrolysis or reacts with any other suitable nucleophile. CHAPMAN AND TRIGGLE[6] have shown that hydrolysis proceeds by an SN_1 mechanism with the formation of the carbonium ion being the rate-determining step:



The reaction with enzyme may take place in an analogous fashion or may proceed by an SN_2 mechanism in which case reaction could occur at the α-carbon atom. Thus the end-product could be of the form



or a mixture of the two. We found that DPA-enzyme is completely inhibited only towards the hydrolysis of quaternary ammonium compounds, *e.g.* acetylcholine and acetylthiocholine, while its activity towards uncharged esters was modified showing that reaction had not occurred with any of the essential catalytic groups[7]. Besides being inactive towards acetylcholine, DPA-enzyme was little affected by the protonated form of the potent organophosphorus inhibitor Tetram (diethyl *S*-2-diethylaminoethyl phosphorothioate) although it was completely inhibited by the uncharged

reagents Sarin and Soman (isopropyl methylphosphonofluoridate and pinacolyl-methylphosphonofluoridate, respectively). We have since found DPA-enzyme to be relatively immune to tetramethylammonium bromide, 3-hydroxyphenyltrimethyl-ammonium iodide and neostigmine.

The further investigation of some of the properties of DPA-enzyme is reported in this paper.

MATERIALS

The enzyme used was a purified bovine erythrocyte acetylcholinesterase preparation from Winthrop laboratories. The contents of the vials (nominally 600–800 International Units) were dissolved in physiological saline (5 ml) containing bovine serum albumin (40 mg/ml). This stock solution was stored frozen and aliquots were diluted daily as required using an aqueous solution of 0.04 M $MgCl_2$ and 0.05 M NaCl.

Phenyl acetate and its derivatives were synthesized from the parent phenols except for *p*-nitrophenyl acetate and indophenyl acetate which were procured from Mann Research Labs. 3,3-Dimethylbutyl acetate was prepared from 3,3-dimethyl-butyric acid (Chemical Procurement Labs.) as described by SAREL AND NEWMAN[8]. Other aliphatic acetates were purchased from Fisher Chemical Co. and were purified by fractional distillation until homogeneous as shown by vapour phase chromatography. Acetylcholine bromide, acetylthiocholine iodide were from Eastman Kodak, tetramethylammonium iodide from Fisher and neostigmine methyl sulphate from Mann Research laboratories. *N,N*-Dimethyl-2-chloro-2-phenylethylamine hydrochloride was synthesised by the method of CHAPMAN AND TRIGGLE[6]. 3-Hydroxyphenyltrimethylammonium iodide was made from the tertiary amine[9].

METHODS

Titrimetric data were obtained under N_2 using a Radiometer pH stat (type TTTIC/SBR2/SBUI/TTA2). All solutions used for enzyme assays were made from water doubly distilled in glass under N_2 and stored under N_2. The titrant was 0.01 M NaOH and its normality was checked periodically with 0.1 M HCl. The reaction medium was an aqueous solution of 0.04 M $MgCl_2$–0.05 M NaCl. For all substrates not containing a quaternary nitrogen, ethanol was added to the assay medium to a concentration of 1% (v/v). During assays with volatile substrates N_2 could not be passed directly over the surface of the reaction mixture. So, to minimize CO_2 absorption, the vessel was either well flushed with N_2 beforehand and sealed during titration or the N_2 was pre-equilibrated with respect to substrate by prior passage over the surface of a solution of the substrate at the same temperature and concentration and in an identical vessel. Volumes of the reaction mixture varied from 15 to 50 ml depending on the sensitivity required. All assays were carried out at 25 $\pm$ 0.1° and pH 7.40 except where stated otherwise. Blank titrations were always carried out on the substrate alone, and for poor substrates bovine serum albumin in physiological saline was added. Poor substrates required the use of relatively high enzyme concentrations and the albumin blank was then not always negligible. The volume of the syringe delivering the titrant was 0.5 ml and this corresponded to full-scale travel of the recorder pen. In the case of *p*-nitrophenyl acetate, it was necessary to correct

for the ionization of the product. For this purpose, the pK_A of *p*-nitrophenol was taken as 7.04 (ref. 10).

For spectrophotometric assays, a Beckman DU instrument, fitted with a thermostatted cell compartment was used. The hydrolyses of indophenyl acetate and its 2',6'-dichloro-derivative were followed at pH 8.0 by the method of KRAMER AND GAMSON[11]. Using the extinction coefficients at 400 mμ given by KEZDY AND BENDER[10], the hydrolysis of *p*-nitrophenyl acetate was followed at pH 8.0. For stock solutions of these substrates ethanol was used as solvent. For phenyl acetate the release of phenol was followed by measuring the increase in absorption at 270 mμ. Mg^{2+} was not present in these assay media due to precipitate formation in the slightly alkaline phosphate buffers.

To check that transesterification was not a major effect an experiment was carried out in which the hydrolysis of $2.0 \cdot 10^{-3}$ M phenylacetate catalysed by acetylcholinesterase was followed simultaneously by spectrophotometry and titrimetry. The actual hydrolysis was carried out in the pH stat in the $MgCl_2$–NaCl medium and the rate of phenol production was measured by pipetting aliquots at intervals into 1.0 M acetic acid to stop the reaction. The phenol was then measured as before. Both ethanol and isopropanol were used at a concentration of 1% (v/v) in the reaction medium.

The DPA was generated from the parent chloroamine hydrochloride by dissolving the latter in the $MgCl_2$–NaCl medium and adding an approximately equimolar amount of NaOH. The solution was then titrated to neutrality. H^+ release, indicating cyclisation, was complete in less than 2 min. The solution was used immediately.

The conditions under which DPA-enzyme was prepared were reproduced from batch to batch as nearly as possible. The stock solution of acetylcholinesterase was diluted 20-fold with the $MgCl_2$–NaCl solution and the pH adjusted to 7.40 at 25.0°. An aliquot of DPA solution was added to give a final concentration of $2.2 \cdot 10^{-3}$ M, the pH readjusted if necessary, and the mixture incubated at 25.0°. A control without DPA was also run. Aliquots were assayed with $2.0 \cdot 10^{-3}$ M acetylcholine solution or $3.0 \cdot 10^{-4}$ M indophenyl acetate solution to check on the progress of the reaction. When activity towards acetylcholine had dropped to below 2% of the original value (approx. 6 h), the control and DPA-enzyme solutions were dialysed against several changes of $MgCl_2$–NaCl solution for 24–48 h at 4° using dialysis tubing which had been pretreated with several rinses in doubly distilled water, 5% acetic acid, water and finally with the dialysis medium. No loss in activity of the control or DPA-enzyme occurred during this treatment. The enzyme solution was stored at 4° and centrifuged before use if necessary. (The formation of a slight gelatinous precipitate did not affect the activity.)

The reversible stage of the interaction of DPA with acetylcholinesterase had already been found to be mainly non-competitive as assayed with acetylcholine[5]. The effect of DPA on the acetylcholinesterase-catalysed hydrolysis of isoamylacetate and indophenyl acetate was tested. Aliquots of a freshly made-up solution of DPA were added to the substrate solution and the acetylcholinesterase added last. The rate of alkali uptake was measured after it settled down to a steady reading. Preincubation of DPA and acetylcholinesterase was not practical due to the speed of alkylation which was lower in the presence of substrate. 5–6 concentrations of DPA were examined with freshly made solutions being used for each determination.

TABLE I

VARIATION IN KINETIC CONSTANTS WITH DIFFERENT PREPARATIONS OF BOVINE ERYTHROCYTE ACETYLCHOLINESTERASE

Acetyl-cholin) esterase batch	*Date of assay*	*Substrate*	$K_{m(app)}$ *(mM)*	v_{max} *(μM/min)*
A	Dec. 1965	Acetylcholine	0.28	48.8
C	Nov. 1967	Acetylcholine	0.32	63.6
C	Apr. 1968	Acetylcholine	0.32	64.0
D	Apr. 1968	Acetylcholine	0.19	48.2
C	Nov. 1967	Phenyl acetate	3.33	84.8
C	Apr. 1968	Phenyl acetate	3.30	84.0
D	Apr. 1968	Phenyl acetate	1.63	44.1

RESULTS

Over the period during which this study extended, it became apparent that different batches of acetylcholinesterase did not have the same kinetic constants. Some values of $K_{m(app)}$ and v_{max} for these preparations are given in Table I.

Effect of alcohol

As most of the substrates studied were not sufficiently soluble in water, especially the salt solutions used in titrimetric work it was necessary to add an organic

TABLE II

A COMPARISON OF THE EFFECTS OF ALCOHOLS ON THE RATES OF HYDROLYSES CATALYSED BY DPA-ENZYME AND ACETYLCHOLINESTERASE

The rate of indophenyl-acetate ($3.0 \cdot 10^{-4}$ M) hydrolysis was followed spectrophotometrically in 0.05 M phosphate buffer (pH 8.00) by the appearance of indophenol. For isoamyl acetate ($2.0 \cdot 10^{-3}$ M) and phenyl acetate ($1.0 \cdot 10^{-3}$ M) the rates refer to production of acetic acid measured titrimetrically in 0.04 M $MgCl_2$, 0.05 M NaCl (pH 7.40) at 25°. For each substrate the relative rate refers to unity for its acetylcholinesterase-catalysed hydrolysis in 1% (v/v) ethanol. Enzyme batch B was used.

Substrate	*Alcohol*	*(%, v/v)*	*Acetyl-cholin-esterase Relative rate*	*DPA-enzyme Relative rate*
Indophenyl acetate	Ethanol	1	1	2.57
	Ethanol	2	0.92	2.47
	Isopropanol	1	0.75	2.37
	Isopropanol	2	0.62	2.23
Isoamyl acetate	Ethanol	1	1	0.60
	Isopropanol	1	0.64	0.78
Phenyl acetate	Ethanol	1	1	0.1
	Isopropanol	1	0.68	0.1
	Tert.-butanol	1	0.79	0.1

TABLE III

THE EFFECT OF ALCOHOLS ON $K_{m(app)}$ AND v_{max} FOR THE ACETYLCHOLINESTERASE-CATALYSED HYDROLYSIS OF ACETYLCHOLINE AND PHENYL ACETATE

$K_{m(app)}$ and v_{max} were calculated from Lineweaver–Burk or Eadie plots using initial rates determined titrimetrically in the presence of 0.04 M $MgCl_2$–0.05 M NaCl (pH 7.4) at 25.0°. Enzyme batch C was used.

Medium	*Substrate*	$K_{m(app)}$ *(mM)*	v_{max} *(μM/min)*
Aqueous	Acetylcholine	0.32 ± 0.003	63.6 ± 0.4
Methanol (1%, v/v)	Acetylcholine	0.274	56.7
Ethanol (1%, v/v)	Acetylcholine	0.388	56.5
Methanol (1%, v/v)	Phenyl acetate	1.92	68.6
Ethanol (1%, v/v)	Phenyl acetate	3.33	84.8
Isopropanol (1%, v/v)	Phenyl acetate	5.04	78.4

solvent. Dioxane is unsatisfactory because of the possibility of peroxide formation, so short-chain aliphatic alcohols were considered. These were found to have different effects on the activities of acetylcholinesterase and DPA-enzyme (Table II). Table III shows that for the acetylcholinesterase-catalysed hydrolyses of acetylcholine and phenyl acetate, both $K_{m(app)}$ and k_{cat} are affected by the presence of alcohol.

The agreement in rates of phenol and acetic acid production from the acetylcholinesterase-catalysed hydrolysis of phenyl acetate in 1% (v/v) ethanol and 1% (v/v) isopropanol is shown in Table IV.

Reversible inhibition of acetylcholinesterase by DPA

As mentioned previously DPA interacts with acetylcholinesterase reversibly before irreversible inhibition is apparent. To observe the reversible phase it is necessary to have substrate present and this, to some extent, protects against the irreversible phase (see below). As with acetylcholine, the hydrolyses of isoamyl acetate and indophenyl acetate were also inhibited. With isoamyl acetate and acetylcholinesterase batch C, a plot of reciprocal of velocity *versus* DPA concentration was linear,

TABLE IV

THE RATES OF PHENOL AND ACETIC ACID PRODUCTION FROM THE ACETYLCHOLINESTERASE-CATALYSED HYDROLYSIS OF PHENYL ACETATE IN THE PRESENCE OF ALCOHOLS

The hydrolysis was carried out at pH 7.4 in the presence of 1% (v/v) alcohol and 0.04 M $MgCl_2$–0.05 M NaCl. Acetic acid and phenol were assayed titrimetrically and spectrophotometrically respectively as described in METHODS. Enzyme batch B was used.

Alcohol	*Rate of acetic acid formation (μM/min)*	*Rate of phenol formation (μM/min)*
Ethanol	8.33	8.05
	8.40	8.55
Isopropanol	6.18	5.8
	6.27	6.3

indicating either purely competitive or non-competitive inhibition. The derived K_i of $6.0 \cdot 10^{-5}$ M compared numerically with the non-competitive K_i of $5.9 \cdot 10^{-5}$ M for acetylcholine[5].

Protection against irreversible inhibition was observed in the presence of acetylcholine or isoamyl acetate. For example, no irreversible inhibition was observed after 16 min with $2.45 \cdot 10^{-5}$ M DPA when acetylcholine ($2 \cdot 10^{-3}$ M) or isoamyl acetate ($5.3 \cdot 10^{-3}$ M) was present. However, with indophenyl acetate at $3 \cdot 10^{-4}$ M (estimated $K_{m(app)}$ of $8 \cdot 10^{-4}$ M) and acetylcholinesterase batch B little if any protection was observed. The initial inhibition produced by DPA soon moved into an increase in rate as the irreversible stage set in. This gave rise to the upward swinging curves shown in Fig. 1 and made it difficult to determine the true initial velocity. It is possible that the difference between assay media is significant in that Mg^{2+} was absent for the assay of indophenylacetate hydrolysis.

Properties of DPA-enzyme

Some properties of DPA-enzyme have already been described[7]. These include (a) a very low (if any) activity towards inhibitors and substrates containing a quaternary nitrogen and (b) a modified esteratic activity towards uncharged aliphatic and aromatic acetates.

(a) Interaction with compounds containing a quaternary nitrogen. Tetramethylammonium bromide (10^{-2} M) reduced the rate of hydrolysis of indophenyl acetate by acetylcholinesterase by 48.8% but the activity of DPA-enzyme was unaffected. Higher concentrations of the inhibitor were not examined in case these could cause changes other than simple competitive inhibition.

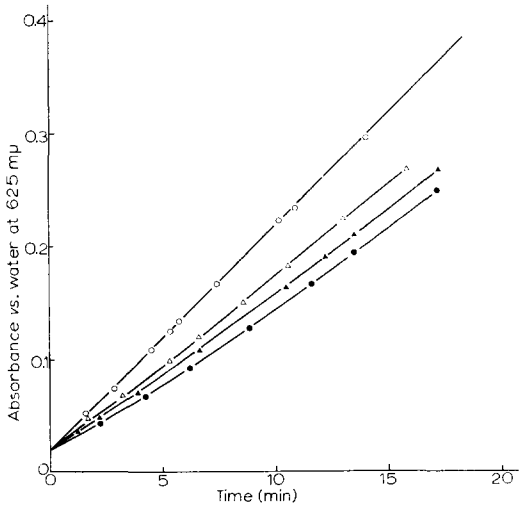


Fig. 1. The effect of DPA on the acetylcholinesterase-catalysed hydrolysis of indophenyl acetate. Details of the assay are given in METHODS. ○—○, acetylcholinesterase and indophenyl acetate; △—△, hydrolysis in the presence of $1.03 \cdot 10^{-5}$ M DPA; ▲—▲, $2.58 \cdot 10^{-5}$ M DPA; ●—●, $5.15 \cdot 10^{-5}$ M DPA. These runs were all done in duplicate. The curves do not extrapolate back to the origin because the absorbance was measured against water, for convenience, and the rates were corrected later for spontaneous hydrolysis of the substrate. This was not noticeably affected by the presence of DPA.

3-Hydroxyphenyltrimethylammonium iodide[2], a very potent inhibitor of acetylcholinesterase (K_i of about $3 \cdot 10^{-7}$ M) at $1.0 \cdot 10^{-4}$ M completely blocked the acetylcholinesterase-catalysed hydrolysis of isoamyl acetate ($5.5 \cdot 10^{-3}$ M), while the activity of DPA-enzyme was reduced by only 9.6%. Similarly, DPA-enzyme was much less sensitive to neostigmine, the dimethyl carbamate of this inhibitor. In the presence of isoamyl acetate ($2.0 \cdot 10^{-3}$ M), neostigmine ($1 \cdot 10^{-6}$ M), as methyl sulphate, very quickly inhibited acetylcholinesterase (batch C) to the extent of 97.5%, and with neostigmine ($1 \cdot 10^{-4}$ M) inhibition was complete almost immediately. However with DPA-enzyme 11% inhibition was obtained at both concentrations. Preincubation of DPA-enzyme for 16 min with $5.3 \cdot 10^{-6}$ M, $5.3 \cdot 10^{-5}$ M and $5.3 \cdot 10^{-4}$ M neostigmine followed by assay, undiluted, with $9.1 \cdot 10^{-3}$ M isoamyl acetate led to inhibitions of 5.9%, 7.9% and 16.0% respectively.

At the relatively high enzyme concentrations used in the study of uncharged substrates, the DPA-enzyme preparations always had a small amount of activity towards acetylcholine. For example, 20 μl of the control acetylcholinesterase solution catalysed the hydrolysis of $2.0 \cdot 10^{-3}$ M acetylcholine at a rate of 9.2 μM/min and 500 μl of the DPA-enzyme gave a rate of about 1.6 μM/min. This residual activity occurred with all preparations of DPA-enzyme and was of the same order of magnitude. If this is due to residual acetylcholinesterase the percentage of unreacted enzyme still present is about 0.7%. Both neostigmine ($5.3 \cdot 10^{-5}$ M) and its parent phenol reduced this activity to zero. The presence of $2 \cdot 10^{-3}$ M acetylcholine very slightly increased the rate of isoamyl acetate ($5.5 \cdot 10^{-3}$ M) hydrolysis indicating little if any interaction of the DPA-enzyme with the quaternary substrate at that concentration. The activity of DPA-enzyme towards acetylcholine is not increased by raising the pH of the assay gradually to 9.0.

(b) Interaction with uncharged substrates. Preliminary investigations showed that, on the whole, aliphatic acetates derived from alcohols containing from 2 to 6 C atoms were substrates of comparable effectiveness for DPA-enzyme and acetylcholinesterase. The series ethyl, *n*-propyl, *n*-butyl, isoamyl and 3,3-dimethylbutyl acetates were studied and $K_{m(app)}$ and v_{max} values obtained for each using Lineweaver–Burk

TABLE V

COMPARISON OF $K_{m(app)}$ AND k_{cat} VALUES FOR THE HYDROLYSIS OF A SERIES OF ALIPHATIC ACETATES CATALYSED BY ACETYLCHOLINESTERASE AND DPA-ENZYME

Acetylcholinesterase	*Substrate*	$K_{m(app)}$ *(mM)*		*Relative* k_{cat}	
		Acetylcholinesterase	*DPA-enzyme*	*Acetylcholinesterase*	*DPA-enzyme*
D	Ethyl acetate	500	156 ± 25	*	0.10
D	*n*-Propyl acetate	200 ± 20	18.5 ± 1.9	0.10	0.10
C	*n*-Butyl acetate	38.0 ± 4	11.5 ± 1.2	0.09	0.09
C	Isoamyl acetate	8.8 ± 0.8	5.4 ± 0.7	0.22	0.085
C	3,3-Dimethylbutyl acetate	4.0 ± 0.4	4.4 ± 0.3	0.55	0.07
D	Acetylcholine	0.19 ± 0.002	—	1.00	—

* Activity too low to permit measurement of v_{max}.

TABLE VI

$K_{m(app)}$ AND RELATIVE k_{cat} VALUES FOR THE HYDROLYSIS OF PHENYL ACETATE AND ITS *p*-SUBSTITUTED DERIVATIVES AS CATALYSED BY ACETYLCHOLINESTERASE AND DPA-ENZYME

No constants are given for DPA-enzyme and *p*-chlorophenyl acetate but the hydrolysis rate of this ester at $2 \cdot 10^{-3}$ M was identical with those of the other analogues. All results were obtained with acetylcholinesterase batch D.

Substrate	$K_{m(app)}$ *(mM)*		*Relative* k_{cat}	
	Acetylcholinesterase	*DPA-enzyme*	*Acetylcholinesterase*	*DPA-enzyme*
Phenyl acetate	1.6 ± 0.2	3.3 ± 0.3	0.92	0.12
p-Nitrophenyl acetate	4.7 ± 0.3	3.3 ± 0.3	0.12	0.12
p-Methoxyphenyl acetate	10.5 ± 0.5	3.3 ± 0.3	0.97	0.12
p-Chlorophenyl acetate	5.1 ± 0.2	—	0.79	—
Acetylcholine	0.19 ± 0.002	—	1.00	—

plots. Two different batches of acetylcholinesterase were used which were found to have somewhat different kinetic behaviour (see Table V) so that a quantitative comparison was difficult. However, a trend was observed, as can be seen from the results in Table V.

A study of the *para*-substituted phenyl acetates also produced some interesting results. Phenyl acetate itself is a very good substrate for acetylcholinesterase and has a high k_{cat}. All the derivatives tried were inferior and *p*-acetoxybenzoic acid was not a substrate for either enzyme. The remainder of the results are shown in Table VI.

Neither enzyme showed any activity towards 2',6'-dichloroindophenyl butyrate although the acetate was hydrolysed at rates comparable with those of indophenyl acetate.

DISCUSSION

The differences in kinetic behaviour between batches of acetylcholinesterase (as shown in Table I) appeared rather surprising. The data on the phenylacetate series were obtained with the same batch (D) and the more interesting members of the aliphatic series *i.e.* butyl, isoamyl, 3,3-dimethylbutyl, were all studied with a single batch (C). In this work comparison within a series was more informative than comparison between series, so the variation in kinetic constants of the enzyme lots did not interfere with the interpretation of our results. Similar variations in enzyme lots have been observed by other workers[12] during a study of the effect of NaCl, $MgCl_2$ on the v_{max} and $K_{m(app)}$ for phenyl acetate and acetylcholinesterase.

The use of ethanol introduced the possibility of transesterification which would cause an apparent lowering in reaction rate where the titrimetric method was used, due to formation of ethyl acetate in place of acetic acid. However, the turnover rate should at least equal the rate in a purely aqueous medium or exceed it if the alcohol competes successfully with water, and thus measurement of the product alcohol should indicate no change, or a faster rate of hydrolysis. Referring to Table IV, the rates measured spectrophotometrically were subject to the greater experimental

error, but the results do show that there is no significant difference in the rates of formation of acetic acid and phenol in the presence of either alcohol. Furthermore, increasing the ethanol concentration (Table II) reduced the rate of indophenol production from indophenyl acetate. Thus, the main effect of alcohol is not transesterification.

The reversibly inhibited enzyme behaves quite differently from DPA-enzyme. This is shown most clearly by its behaviour towards indophenyl acetate whose catalysed hydrolysis is slowed by the reversibly complexed enzyme but accelerated once alkylation occurs. Thus, the site of alkylation may be different from that of the complexing.

There seemed to be no reason why DPA should not interact reversibly or irreversibly at several sites on acetylcholinesterase, although reaction at some sites might be expected to influence the kinetic properties more than at others. The conditions under which DPA-enzyme was prepared were controlled very carefully and exactly the same procedures followed each time to reduce the number of known variables to a minimum. No deviation was noticed in Lineweaver–Burk plots as would be the case if a mixture of enzymes was present with differing kinetic constants towards a given substrate. The slight inhibition of DPA-enzyme by neostigmine and its parent phenol could be due to weak interaction with a major component of DPA-enzyme or to strong interaction with a minor component or both of these interactions. If the residual hydrolysis of acetylcholine (referred to in RESULTS) is carried out by DPA-enzyme, the low activity must be due to low affinity of the major component rather than only a low catalytic constant because of the lack of interference by acetylcholine in the hydrolysis of isoamyl acetate. Alternatively, the percentage of a more active component must be quite low. Thus we were satisfied that the homogeneity of DPA-enzyme was such that we could draw valid conclusions from subsequent kinetic data. The data on the aliphatic acetate series in Table V show that there is no startling change in $K_{m(app)}$ between acetylcholinesterase and DPA-enzyme but the latter appears to have lost much of the specificity of acetylcholinesterase. Increasing chain length and branching of the side chain results in smaller changes in $K_{m(app)}$ and k_{cat} for DPA-enzyme than for acetylcholinesterase. The k_{cat} values for DPA-enzyme are rather uniformly low and may actually decrease with increasing methylation of the γ-carbon atom rather than markedly increase as with acetylcholinesterase. It is interesting that 3,3-dimethylbutyl acetate, the carbon analogue of acetylcholine, is a good substrate for acetylcholinesterase by virtue of its increased k_{cat} rather than just an increased affinity (which would show up only in $K_{m(app)}$).

With acetylcholinesterase, the trends in $K_{m(app)}$ and k_{cat} for 3,3-dimethylbutyl acetate, isoamyl acetate and *n*-butyl acetate are similar to those reported by KRUPKA[13] for acetylcholine, dimethylaminoethyl acetate and methyl aminoethyl acetate at pH 6.5, although the spread in k_{cat} and $K_{m(app)}$ is greater for the amino series. It seems that although the introduction of both a positively charged nitrogen and methyl groups at the γ-position enhances the binding to acetylcholinesterase, the effect on k_{cat} is at least as important. An increase in the number of methyl groups alone has a significant effect and this is greater when these are attached to a positively charged nitrogen. Conversely, γ-methylation appears to increase the binding to DPA-enzyme only slightly and to have a slight adverse effect on k_{cat}; the presence of a positive charge virtually abolishes any interaction.

The non-binding or very greatly reduced binding of acetylcholine and the quaternary inhibitors tested, might appear to be due to an electrostatic effect (repulsion between the quaternary head and the protonated dimethylamino group of the DPA residues), especially as the binding of 3,3-dimethylbutyl acetate is little affected. The pK of N,N-dimethyl-2-phenylethylamine is 9.4 (ref. 14), that of N,N-dimethyl-1-phenylethylamine would be lower because of the greater influence of the phenyl group. In addition, the nature of the linkage of DPA to the enzyme would affect the pK, *e.g.* an ester linkage would lower it. Thus it is probable that at pH 9.0 the dimethylamino group is only partly protonated. As the activity of DPA-enzyme towards acetylcholine was unchanged in the range of pH 7.4 to 9.0 it is unlikely that this group is responsible for the non-binding. A possibility is that reaction with DPA causes a rearrangement in the conformation of the peptide chains, exposing a group with a permanent positive charge, *e.g.* arginine, near the prospective binding site for acetylcholine. Alternatively, if a methionine residue were alkylated this would also introduce a permanent positive charge.

Although *p*-substitution in the phenyl ring has quite marked effects on the $K_{m(app)}$ and k_{cat} values for acetylcholinesterase, DPA-enzyme appears indifferent to such changes, and again the k_{cat} values are relatively low (Table VI).

Phenyl acetate is a good substrate for acetylcholinesterase, mainly by virtue of its high k_{cat} although the intrinsic binding ability may not be very high. This ester possesses none of the features of acetylcholine which could cause the postulated favourable conformational changes leading to acetylation, and its efficiency may be primarily due to its electronic structure. The hypothesis first put forward by WILSON *et al.*[15] that acetylcholinesterase-catalysed hydrolyses proceed *via* two kinetically significant intermediates (the Michaelis complex and the acyl enzyme) has been widely accepted. Steady-state data on acetylcholine and other substrates have been interpreted on this basis[15,16]. Using similar reasoning, from a comparison of the k_{cat} values for acetylcholine and the phenyl acetates deacetylation would be largely rate-controlling for the unsubstituted and *p*-methoxy ester. Hence it is reasonable to conclude that the drop in k_{cat} through the *p*-chloro to the *p*-nitro analogue is due to a decrease in acetylation rate; *i.e.*, electron-withdrawing substituents hinder acetylation. This is in contrast to the action of α-chymotrypsin on *p*-substituted phenyl acetates for which a positive Hammett ϱ constant for acylation is obtained[17], similar in value to that of the non-enzymic nucleophilic reactions of these esters. The $K_{m(app)}$ values for acetylcholinesterase and the phenyl acetate series may indicate some variation in affinity for the enzyme as the values for phenyl acetate and its *p*-methoxy derivative differ by a factor of seven although their k_{cat} values are very similar. By contrast, for DPA-enzyme the $K_{m(app)}$ and the k_{cat} values are identical with the k_{cat} values being only slightly greater than those for aliphatic substrates. The similarity of these values, and the lack of electronic influence, may indicate that for DPA-enzyme deacetylation is largely rate-determining for the substrates studied. However, the general validity of this hypothesis of two intermediates has been questioned by GUTFREUND *et al.*[18,19]. Using rapid-flow techniques they have produced experimental evidence that for trypsin and α-chymotrypsin and certain substrates, a significant intermediate occurs between the formation of Michaelis complex and acyl enzyme. If this is so for acetylcholinesterase and the substrates in question, then the above conclusions cannot be drawn from steady-state data.

There is some evidence that spontaneous decarbamylation is hindered in DPA-enzyme (R. A. McIVOR, personal communication). It would be interesting to know what effect nucleophiles other than water would have on this stage, *i.e.* whether the approach is sterically hindered or whether a necessary rearrangement of peptide chains to bring catalytic group(s) into action, is hampered.

Most of the above discussion has been concerned with specificity as regards leaving group, *i.e.* the alcohol produced from the substrate, but it seems that, although DPA-enzyme is indifferent to rather large structural changes in the latter, it will not act on 2′,6′-dichloroindophenyl butyrate, suggesting that it is primarily still an acetylesterase.

If the non-competitive kinetics indicate different binding sites for substrate and DPA, and possible simultaneous binding of these, protection by substrate against the irreversible reaction could be due to steric blocking, or to conformational changes unfavourable for alkylation. The relevant groups may be made inaccessible or a necessary re-arrangement of the peptide chains to accommodate the alkylation could be prevented. In any event, acetylcholinesterase, in the presence of acetylcholine, does not appear to react irreversibly with DPA at as fast a rate, or else it does not give the same product as the free enzyme. In this connection, it is interesting that the analogues of DPA containing *m*-groups, usually considered to be esteratic site orienting, act as purely competitive inhibitors and do not irreversibly react with the enzyme[5].

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