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Conference Paper

Survey of Major Points of Interest About Reactions of Cholinesterases

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Three areas of current interest and activity in the study of the cholinesterases have been chosen for comment:

Aging of phosphorylated cholinesterases. Recent work indicates that aging (loss of groups attached to the phosphorus) can be brought about by at least two mechanisms. It now seems probable that aging is a more general reaction and is not restricted to the cholinesterases.

Inhibition by excess substrate and other effects. An attempt has been made to bring together into one model experimental results from a number of different approaches. These include acceleration of hydrolysis and inhibition by quaternary ammonium compounds, differences in pH-rate profiles for various substrates and various inhibitors, substrate inhibition and inhibition by haloxon.

Molecular weight of acetylcholinesterase in membranes. A recent paper indicating that acetylcholinesterase in membranes is monomeric is discussed.

For the purpose of the task stated in the title I have selected three areas which interest me and others. In a field of study as extensive as the cholinesterases it is inevitable that in a brief discussion of this kind some important contributions will be omitted. I have undertaken this task with the intention of clarifying my own thinking about these phenomena; I hope I succeed in some clarification for others.

Aging

Organophosphorus compounds on reaction with cholinesterase produce disubstituted phosphorylated, phosphonylated or phosphinylated enzyme.

In the initial reaction of acetylcholinesterase with *O,O*-dimethyl *S*-4-nitrophenyl phosphate there seems no doubt that the bond broken is that between the phosphorus atom and sulphur. This may be readily deduced from the fact that the phosphorylated enzyme derivative reactivates spontaneously at the same rate as that produced from *O,O*-dimethyl *O*-4-nitrophenyl phosphate[1]. It seems unlikely that breakage could occur at the other position.

This enzyme derivative can take part in several subsequent reactions. For example, the original active enzyme may be modified either by reactions involving H_2O, OH^- or H_3O^+ (refs. 2—4).

The rate of the spontaneous regeneration of both phosphorylated and carbamylated enzymes has a bell shaped pH-rate profile implicating participation of two groups within the active site, having pK_a's of 6.9 and 9.8[4]. As far as I know, there is no definitive information whether the break is between the phosphorus and oxygen or oxygen and carbon, although the former seems likely from experience with phosphates (*cf.* Dixon and Webb[5]).

From the work of Cohen and colleagues[6] it is clear that dialkylphosphorylated cholinesterase can lose an alkyl group and become non-reactivateable by oximes (for review *cf.* Aldridge and Reiner[7]). There seems no doubt that the mechanism of this reaction for pinacolyl methylphosphonylated acetylcholinesterase involves a carbonium ion intermediate[8]. This type of reaction is increased by a decrease in pH[8–11]. The order of the rates of dealkylation for various cycloalkyloxy and benzyloxy methylphosphonylated cholinesterases seems to be the same as that for the solvolysis of the corresponding tosylates[12]. Therefore this reaction is little influenced by the detailed structure around the active centre and is quite different to the normal enzymic processes.

However, there is in the literature an example of chymotrypsin inhibited with diphenylphosphorochloridate from which 1 mol of phenol is liberated from the inhibited enzyme[13,14]. In view of the known reluctance of phenolic leaving groups to form carbonium ions this result indicates that a breakage of the bond P—O—R occurs at a position next to the phosphorus atom. Also the fact that this reaction occurs with a phosphoryl moiety containing two aromatic rings suggested that the known substrate specificity of chymotrypsin was involved[15,16]. A recent paper[17] has appeared studying the properties of the inhibited acetylcholinesterase produced by reaction with parasubstituted phenyl methylphosphonochloridates. The phenyl, 4-methoxylphenyl and 4-cyanophenyl compounds produced inhibited enzymes, which both spontaneously reactivate and age (Table I). Both processes have bell shaped pH-rate profiles. These reactions therefore may be considered to involve processes occurring in the normal catalytic process. It seems likely that the break is occuring between the phosphorus atom and the oxygen. It is probably significant that for other phosphorylated cholinesterases containing straight chain alkyl and other groups where the potential for the formation of carbonium ions may be slight, both spontaneous reactivation and aging occurs at similar rates (*cf.* Table I)[17–20]. We thus arrive at a position shown diagrammatically in Fig. 1.

TABLE I.

Phosphorylated acetylcholinesterases undergoing spontaneous reactivation and aging

Enzyme derivative	Refs.
Methylphosphonyl- Phenyl 4-methoxyphenyl 4-cyanophenyl	17
Diphenylphosphoryl- Phenylchlorophosphoryl-	18
Dimethylphosphoryl-	19
Di 2-chloropropylphosphoryl- Di 3-chloropropylphosphoryl-	20

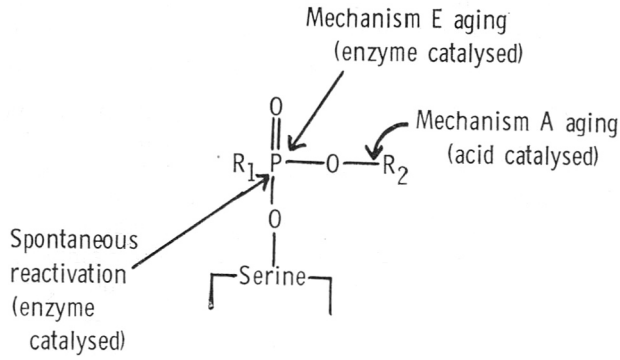

Fig. 1. Reactions of phosphorylated cholinesterases.

It seems likely if these general views are correct that the phenomenon of aging (involving both mechanism A and E) will be more prevalent than has hitherto been considered. It has now been demonstrated that these reactions can occur with acetylcholinesterase, cholinesterase, chymotrypsin[21] and probably with an enzyme from the central nervous system hydrolysing phenyl esters[22]. A paper only recently published[23] has shown that inhibition of horse, sheep and chicken liver esterase by bis (4-nitrophenyl) methylphosphate leads to the release of 2 mol of 4-nitrophenol. The rates of the aging reaction are clearly very fast. For pig liver esterase circumstantial evidence was produced indicating that spontaneous reactivation and aging were occurring at the same rate.

The time is fast approaching when we ought to qualify the use of the word 'aging' to describe what are clearly two mechanistically different phenomena. It appears that pH—rate profiles are one way of distinguishing the two mechanisms (Fig. 1). I suggest that when inability to be reactivated is demonstrated it should first be shown to be time dependent and the influence of pH over the range 5—9 examined. Mechanism A (acid catalysed) is probably by carbonium ion formation, whereas Mechanism E (enzymic) involves breakage of phosphorus-oxygen bond.

Inhibition by excess substrate and other effects

Acetylcholine can combine with acetylcholinesterase in a way which will prevent its own hydrolysis. Whether activity is totally or only partially prevented is not resolved[24].

I shall try to put together into a model some evidence from rather different kinds of measurements. These areas are as follows:

1. pH dependence of uncharged substrates such as phenylacetate and isoamylacetate when compared with acetylcholine.
2. The acceleration of the rates of hydrolysis of acetylfluoride by quaternary ammonium compounds.
3. Acceleration of rate of reaction of dimethylcarbamylfluoride and methane sulphonylfluoride by quaternary ammonium compounds.
4. pH—rate profiles for inhibition by organophosphorus compounds.

5. pH—rate profiles for spontaneous reactivation of phosphorylated enzymes when aromatic groups are attached to the phosphorus.
6. The inhibition of acetylcholinesterase by haloxon or coumarin.

My purpose is to try to put forward a simple hypothesis derived from information gained from these rather different approaches.

Acceleration of inhibition or hydrolysis only occurs with small inhibitors or substrates[25–28]. Acceleration occurs at concentrations of quaternary ammonium compounds which inhibit the hydrolysis of acetylcholine and whether it occurs depends on the size/structure of the quaternary ammonium compound. With larger inhibitors (*i. e.* methylphenylcarbamyl fluoride or diphenylcarbamyl fluoride) quaternary ammonium compounds decrease the rate of inhibition[29]. Thus it may be concluded that these small inhibitors and substrates are reacting with the active centre (esteratic site) without the necessity of direct contact with the site(s) involved in the reaction of the quaternary compounds.

Combination of the quaternary compounds with the enzyme is leading to an environment around the esteratic site which is conducive to more rapid reaction. Reaction can probably occur at the esteratic site without involvement of the binding at other sites *e. g.* hydrolysis of acetyl fluoride[26] and methylacetate[30] and inhibition by methanesulphonyl fluoride and dimethylcarbamyl fluoride[31].

Krupka[28] has recently pointed out the importance of a site which can bind aromatic groups. There is other evidence for this. The pH—rate profiles of the hydrolysis of phenylacetate[32] and for the inhibition by non charged organophosphorus compounds or carbamates[4] as well as the recent pH—rate profile for the rate of spontaneous reactivation of 4-cyanophenyl methylphosphorylated cholinesterase[17] indicates that the anionic component of binding is not associated with the binding of the aromatic ring. A similar conclusion may be reached from the fact that several uncharged aliphatic compounds are reasonable substrates[33] and that for isoamylacetate the pH—rate profile resembles that for phenylacetate[32].

I conclude that the »hydrophobic site« and the »anionic site« are different.

Some compounds (haloxon, 3-chloro-7-hydroxy-4-methyl coumarin and acetylcholine) inhibit acetylcholinesterase and competition between substrates and these inhibitors may be demonstrated at concentrations approximating to those causing substrate inhibition[34].

For the above reasons I am putting forward a scheme embodying three essential components — (1) an esteratic area, (2) an anionic site, (3) a hydrophobic area (Figs. 2 and 3). In this model there will be a three point involvement for acetylcholine. Hydrolysis can also occur with two point involvement (1 and 3) of the hydrophobic area and the esteratic site (*e. g.* phenylacetate, branched chain aliphatic esters, uncharged organophosphorus compounds and carbamates). However, good substrates and inhibitors will require a three point attachment[35]. Acceleration of inhibition and hydrolysis is brought about by quaternary ammonium compounds cross bridging the anionic site/hydrophobic area, so creating the environment in the esteratic area — *i. e.* the correct juxtaposition of reacting groups/exclusion of water *etc.* Their size will determine these factors, the optimum of which may differ, *e. g.* for hydrolysis of acetyl fluoride and inhibition by methanesulphonyl fluoride (Fig. 3) and dimethylcarbamyl fluoride.

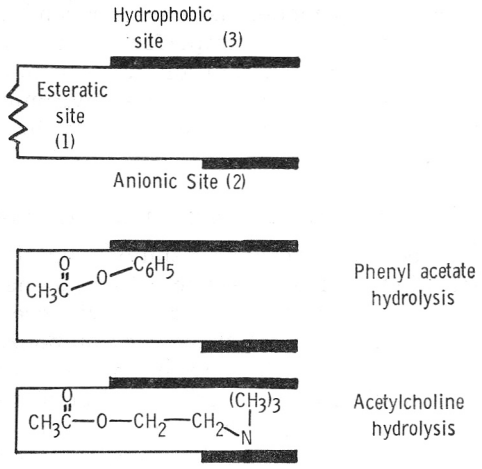

Fig. 2. Acetylcholinesterase: hydrolysis of substrates.

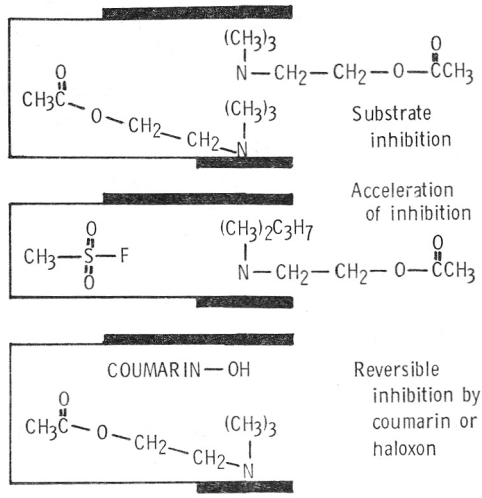

Fig. 3. Acetylcholinesterase: reaction with inhibitors.

Substrate inhibition could be brought about by combination of acetylcholine at site 3, so distorting acetylcholine to combine only with sites 1 and 2 (Fig. 3). Haloxon and the parent coumarin would be postulated to combine with site 3 only. Haloxon would have to combine in two ways: one with the phosphate moiety in the esteratic area leading to phosphorylation and the other with the phosphate moiety outside the active site.

The feature of this model is that combination with sites 2 and 3 can create the environment in the esteratic area, and I suggest that the main binding leading to hydrolysis or inhibition is by the structural requirements of the hydrophobic area; the contribution of the anionic component may be relatively small. Hydrolysis is possible without the participation of the anionic

site[32]. There are implicit in this model six ways for combinations of molecules with the enzyme. The purpose of this imperfect model is to suggest that we require at least a two point attachment of various compounds so that by changes in the conformation of the enzyme the environment of the esteratic site may be modified; it may be more conductive to reaction with some compounds and less to others.

Molecular weight of acetylcholinesterase in membranes

There has always been considerable dispute about the molecular weight of acetylcholinesterase whether from electric eel or bovine erythrocyte. Values have ranged from (13—31) $\times 10^6$ downwards[36]. Evidence has been produced that in purified preparations from eel there are components of different molecular weights[37,38]; using labelled DFP a minimum value of 63 000 was found[39]. It has been shown with solubilised preparations of bovine erythrocyte that the kinetics of reaction with some organophosphorus compounds are not simple[40]. The interpretation was that there were in the solution several different enzymic entities each with a different rate of reaction. These components appeared to be in reversible equilibrium with one another, the steady state being influenced by temperature and dilution[40]. These variable results pose serious questions when we require to link properties of enzymes with the properties of more organised biological systems in membranes, cells and organs.

A recent interesting paper has utilised a technique which may allow us to decide the size of the enzyme actually located in its natural site[41].

The basis of this technique is the use of the direct action of radiation rather than its indirect actions. The former involves interaction of radiation with molecules resulting in damage, whereas indirect action is the reaction of solute molecules with »activated« water molecules[42]. On the basis of target theory which relates the exponential loss of enzyme activity to irradiation dose, the slope of the line of a plot of log remaining activity to dose is proportional to size of the target molecule[42,43]. A relationship was developed[43]

$$\text{Mol. wt.} = 6.4 \times 10^{11}/D_{37}$$

where D_{37} is that dose of radiation in rads after which 37% of the original enzyme activity remains, or

$$\log (\text{Mol. wt.}) + \log D_{37} - 11.81 = 0$$

The graph (Fig. 4) indicates the validity of this technique for substances of molecular weight ranging from 10^2 to 10^6.

In this particular work[43] the value obtained for acetylcholinesterase in human red cell ghosts was 56 000 and for a purified eel acetylcholinesterase 170 000.

In a recent study[41] this question has been reexamined. The results (Table II) have been stated to indicate that in the membrane preparations the enzyme exists in a form which has approximately the same molecular weight as the equivalent weight determined by reaction with organophosphorus compounds with eel ChE[39,44,45] and for the minimum molecular weight determined by a gel disc electrophoresis on polyacrylamide gel[46].

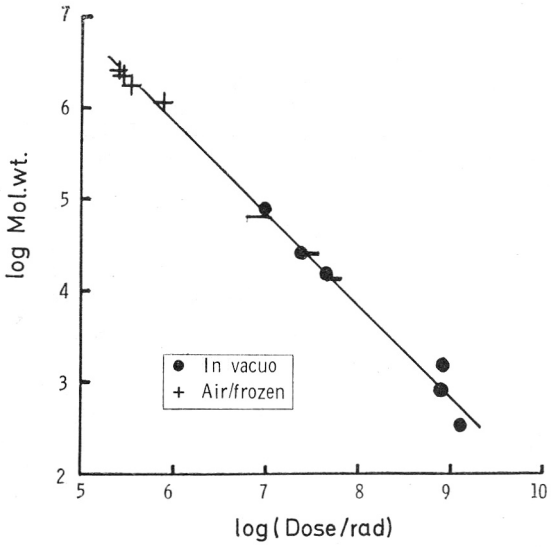

Fig. 4. Molecular weight and direct action of radiation[43].

TABLE II.

Molecular weights of acetylcholinesterase determined by irradiation inactivation[41]

PREPARATION	FREEZE DRIED	FROZEN
Membrane Bound	Mol. wt.	Mol. wt.
Electric eel particulate fraction	75 000 ± 3 000	77 000
Llama erythrocyte ghosts	75 000 ± 4 000	71 000
Purified		
Electric eel type V (Sigma)	255 000 ± 15 000	240 000
Electric eel type VI (Sigma)	71 000 ± 5 000	240 000
Bovine erythrocyte (Sigma)	72 000	—
Electric eel type V (Sigma) in 0.2% Triton X-100	—	150 000

Of interest is the finding of Levinson and Ellory[41] that when they irradiated acetylcholinesterase solutions which had been quick frozen in liquid nitrogen, they obtained decay curves which had several linear components suggesting the presence of heterogenous forms of cholinesterase. Therefore, although the results may not yet be regarded as conclusive, it appears likely that acetylcholinesterase present in the membrane of the electroplax of the electric eel and in the membranes of erythrocytes exists in a monomeric form containing one active centre. This result, if confirmed, has considerable physiological importance, but also has implications for the study of purified preparations.

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DISCUSSION

P. J. Jewess:

Do you think that there may be more than one hydrophobic binding site involved in binding of phenyl ligands in view of the difference of catalytic activity of aziridinium-inhibited acetylcholinesterase towards indoxylacetate and indophenolacetate?

W. N. Aldridge:

This is difficult to answer, but I do not consider the hydrophobic area as one with no attributes leading to specificity of a structural kind.

E. A. Barnard:

Is there any evidence known on possible differences between membrane-bound acetylcholinesterase and the solubilized acetylcholinesterase with respect to the ageing phenomenon and its enzyme-catalysed component?

W. N. Aldridge:

As far as I can recollect, all of the published experiments on ageing have been carried out with solubilized or partially purified enzymes.

M. E. Eldefrawi:

How do you explain the difference in the molecular weight of eel acetylcholinesterase determined by the irradiation and sucrose gradient techniques?

W. N. Aldridge:

As I understand the direct radiation technique is probably measuring the size of the units in the membrane which have independent catalytic activity. Thus we may have to differentiate between organization of units in clusters in membrane for some physiological purpose and catalytic activity being dependent on dimers, tetramers, etc. The direct radiation technique certainly seems to indicate that the active catalytic species in the electric eel electroplax membranes and erythrocyte membranes is the same size as that indicated by phosphorylation by DFP.

SAŽETAK

Pregled glavnih područja u istraživanjima reakcija kolinesteraza

W. N. Aldridge

Za diskusiju su odabrana tri aktualna područja istraživanja reakcija kolinesteraza. *Starenje fosforiliranih kolinesteraza:* Najnoviji radovi upućuju na to da najmanje dva reakcijska mehanizma uzrokuju starenje kolinesteraza (gubitak skupina vezanih na fosfor). Čini se vjerojatnim da je starenje općenitija reakcija i da nije vezano samo za kolinesteraze. *Inhibicija suviškom supstrata i ostali učinci:* Načinjen je pokušaj da se rezultati dobiveni nizom različitih eksperimentalnih pristupa protumače istim modelom. Eksperimentalni pristupi obuhvaćaju akceleraciju i inhibiciju hidrolize kvarternim amonijevim spojevima, razlike u zavisnosti brzine reakcije o pH za različite supstrate i inhibitore te inhibiciju supstratom i haloksonom. *Molekularna težina acetilkolinesteraze u membranama:* Načinjen je kritički osvrt na jedan novi rad prema kojem bi acetilkolinesteraza u membranama bila monomer.

SEKCIJA ZA BIOKEMIJSKE MEHANIZME,
TOKSIKOLOŠKA JEDINICA
SAVJET ZA MEDICINSKA ISTRAŽIVANJA
CARSHALTON, SURREY, VEL. BRITANIJA