THE DESIGN OF ANTICHOLINESTERASES

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ABSTRACT

The significance of new findings in two areas will be discussed with respect to the design of inhibitors of acetylcholinesterase. One area is that of enzyme structure, including the oligomeric form and the existence of allosteric binding sites, of isozymes and of a mutant enzyme. The other area is that of the forces which bind inhibitors to the enzyme prior to reacting with it, including ionic, hydrophobic and charge-transfer bonds.

Now that the chlorinated hydrocarbon insecticides are being phased out in so many parts of the world, because of environmental considerations, the carbamates and phosphates, which together make up the group which we call anticholinesterase insecticides, have come to dominate the insecticide scene. This diverse group of compounds has a number of interesting features. For instance, both the potency and the hazard vary over great extremes, all the way from the nerve gases to highly selective compounds which are almost entirely safe to man and livestock. It is not at all difficult to synthesize an entirely new anticholinesterase, for there is a tremendous diversity of permitted attachments to the basic carbamyl or phosphoryl group. Many of these new compounds would have useful insecticidal properties The problem is to develop compounds which are of modest price, excellent potency and appropriate safety to man, livestock and the environment. But since so many useful anticholinesterase insecticides are in existence already, what are the prospects for obtaining compounds new and interesting enough to encourage the substantial research outlays which are necessary for any new compound?

My own view is that we have only just begun to scratch the surface. Firstly, using the toxicity to vertebrates as an index of bioactivity, it is well known that the chemical warfare agents sarin and soman, which are anticholinesterases, are about 100 times more toxic than any of the organophosphates that are or were in commercial use. Secondly, insects can certainly be killed by different kinds of nerve poisons acting at extraordinarily low doses, as the exciting work on synthetic pyrethroids related to bioresmethrin has shown. One has a lethal dose for the housefly of 13 ng/g, or about 60 times more toxic than the best of the current commercial anticholinesterases. It seems to me entirely reasonable to set our sights upon the achievement of anticholinesterases which are about 100 times more toxic than current

anticholinesterases to insects, and to concentrate very hard on the question of selectivity in such compounds, so that the extreme potency against insects is accompanied by very little mammalian hazard.

How are such new compounds to be obtained? One possibility is that they will be turned up as a result of the ingenuity of synthetic chemists, who will attach novel substituents onto the familiar phosphoryl and carbamyl nucleus. But another possible approach lies in the attempt to understand the nature of the target enzyme, and then to use that understanding in the design of the new compounds. In particular, it is hoped that the recent growth in understanding about differences between the target in vertebrates and in invertebrates can be used as a basis of the design of selective anticholinesterases. In this presentation I shall give some of the information about differences in acetylcholinesterase, and then I shall talk about the evaluation of the forces which bind the inhibitor to the enzyme.

First let me point to the existence and importance of isozymes, that is to say different forms of acetylcholinesterase from any one organism, which can be separated by physical means. Tripathi and I[1] have studied the isozymes of the housefly, and find that by electrophoresis one can separate out four different isozymes from the head, and three additional forms (for a total of seven) from the thorax. *Figure 1* shows these seven isozymes. The experiment is done by applying the soluble isozyme mixture to a small column of polyacrylamide gel, and then applying a potential across the ends of the column. The enzyme activity is then localized by immersing the gel in a solution of acetylthiocholine, and detecting the hydrolysis product by means of a copper salt. The gel is then scanned on a spectrophotometer. Chiu and Tripathi[2] have shown that by carefully controlling the conditions one can do kinetic studies directly on the gel. For instance, one can vary the amount of substrate present during assay, and thus compute the Michaelis constant for the reaction between substrate and enzyme. Or one can expose the gel to inhibitors for varying lengths of time, after the electrophoretic separation is completed, and from the findings one can compute the rate constant for the reaction between inhibitor and enzyme. *Table 1* shows that such studies reveal measurable but quite small differences between the rate constants of inhibition for the four different isozymes, as studied *in vitro* in the way I have just described.

There is another sort of experiment one can do. One can poison the houseflies with an anticholinesterase, and at various times after poisoning one can extract the tissues and find whether the isozymes show any differences in sensitivity *in vivo*. *Figure 2* shows one of a whole series of compounds which we have explored, in this case paraoxon applied to the tip of the abdomen. As you can see, the isozymes vary a great deal in their sensitivity. For instance, head isozyme III shows very little inhibition over the whole time course, whereas thoracic isozyme VII is eliminated after 80 min. We asked the question: is any one isozyme more important than the others in determining death? Our approach was to poison houseflies with a variety of quite different organophosphates, so as to maximize the probability that differing isozymes might show differing sensitivities, and then ask if any single isozyme, when reduced to a particular level, was always associated with death. *Table 2* shows the results with four different inhibitors. The findings are expressed

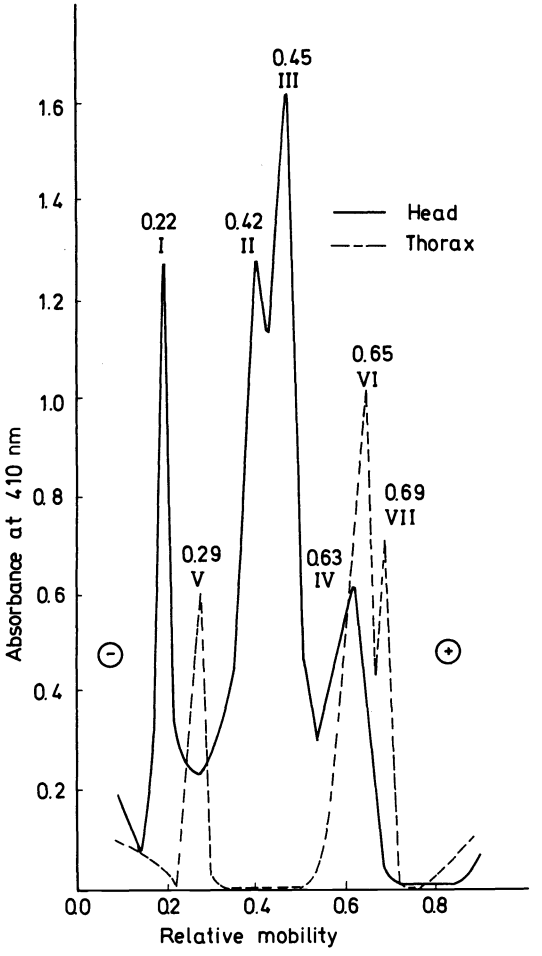


Figure 1. An electropherogram of AChE isozymes of housefly. The gels were incubated with 1×10^{-3} M ATCh for 45 min for head and 90 min for thorax. The number heading each peak is the R_m value

Table 1. Bimolecular rate constants (k_i) of AChE isozymes of housefly head with malaoxon, Tetram and eserine at 25°C, pH 6.0

Isozyme	Malaoxon	Tetram	Eserine
		$10^{-5} \times k_i$ (M^{-1} min^{-1})	
I	7.85 (±0.37)	8.80 (±0.63)	25.46 (±1.22)
II	5.34 (±0.23)	8.50 (±0.17)	22.24 (±1.59)
III	4.86 (±0.22)	7.60 (±0.29)	15.99 (±0.77)
IV	3.42 (±0.45)	5.90 (±0.21)	29.71 (±2.10)

Standard errors are shown in parentheses.

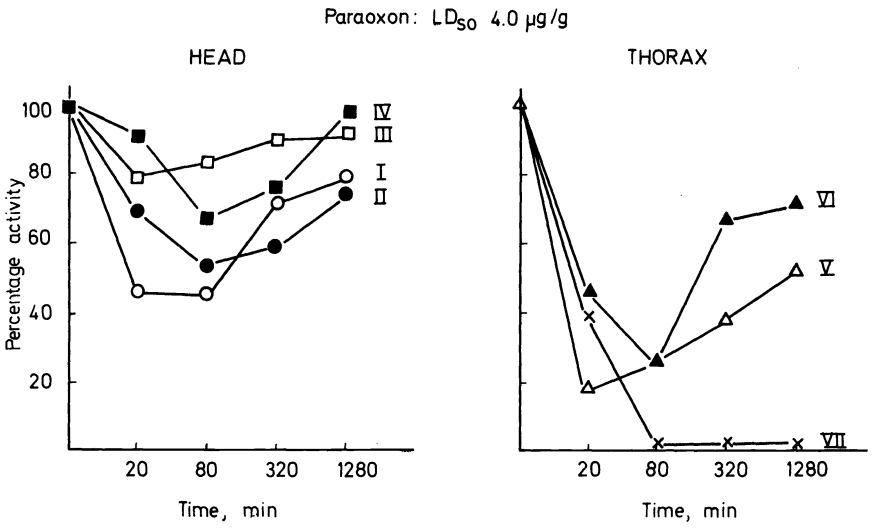


Figure 2. The effects of paraoxon on the isozymes of head and thorax

Table 2. The minimal percentage activity of AChE after poisoning with an LD_{50} dose

Isozyme	Malaoxon	Paraoxon	Diazinon	Dichlorvos	Range[a]
		Head			
I	18	45	54	39	36
II	42	53	28	72	44
III	51	78	32	57	46
IV	28	67	67	70	42
Total[b]	36	61	41	55	25
		Thorax			
V	15	18	22	21	7
VI	5	38	1	16	37
VII	1	1	1	1	0
Total[b]	6	20	7	12	14

[a] Range of minimal activities shown in table.
[b] Equals Σfm, where f is the fractional activity of each isozyme (from *Table 1*) and m is the percentage minimal activity (from this table).

as the minimal activity observed, which is to say the depth of the trough in activity. The right-hand column indicates that five of the seven isozymes showed very substantial variation in the extent of minimal activity associated with poisoning by an LD_{50} dose. For instance, the table shows that for I there was a 36 point range between the minimal activity found after malaoxon poisoning (18%) and after diazinon poisoning (54%). But thoracic isozymes V and VII showed rather little variation. When one considers that one is working with an LD_{50} dose, and therefore one-half of the population survives the experiment, it is clear that isozyme VII must be eliminated in the survivors as well as victims of poisoning. Consequently, it cannot be of physiological importance. We therefore suggest that thoracic isozyme V gives the best index of lethality. In these four quite different organophosphates

the minimal activity only varied over a 7% range, from 15% of controls in malaoxon poisoning to 22% in diazinon poisoning. These findings suggest that, under these particular conditions of treatment, thoracic isozyme V is the isozyme whose inhibition determines whether or not the insect will survive.

What are the physiological implications of the existence of acetylcholinesterase isozymes? At the moment it is too early to say, particularly in the housefly, where the relations between the isozymes, in terms of their relative molecular weights and their fine structure, have not yet been established. Working with acetylcholinesterase of *Torpedo*, Bon, Rieger and Massoulie[3] have found three isozymes in electric tissue, one of them consisting of a tetramer with a tail attached, a second of two tetramers with a single tail, and a third of three tetramers with a single tail. It may be that the tail is a long hydrophobic structure which anchors the different structures into the lipid part of the membrane of which one assumes they form a part. It is clear that heterogeneity of acetylcholinesterase is extremely common, and one needs to be aware (whether one is working *in vitro* or *in vivo*) that if the isozymes are not separated, then one is dealing with a mixture of materials, whose properties could vary substantially.

Next I should like to turn to the question of the nature of the reaction between anticholinesterases and the enzyme. In the early work it was customary to look at the over-all reaction between enzyme and inhibitor, which could be formulated thus:

$$E + PX \xrightarrow{k_i} EP + X \qquad (1)$$

In this formulation, in which an organophosphate is described as PX, with the P representing the dialkylphosphoryl group (for instance) which becomes attached to the enzyme, and X representing the group that leaves in the course of the action, the over-all reaction would seem to be governed by a single constant, k_i, which is called the bimolecular rate constant. (Incidentally, all the comments I am about to make about organophosphates are precisely comparable to what one can say about carbamates, because carbamates carbamylate the enzyme in a way absolutely analogous to the way phosphates phosphorylate the enzyme.) Many studies of organophosphates have been made in which P or X have been varied, in an attempt to find the features which give an optimum k_i; if k_i is very large, then one has a very potent inhibitor, and if all other factors are in line, one may have a basis for a good insecticide. However, in 1964, Main[4] showed quantitatively what had been implicit, but not explicit for all the earlier years. The fact is that equation (1) is an inadequate representation, because the reaction does not occur in a single step, but rather in two steps. Because these two steps are almost certainly governed by completely different factors, and therefore respond differently to changes in the nature of PX, one can understand how difficult it is to explore structure–activity relations when contemplating only the single apparent over-all step. The correct equation is equation (2), where you can see

$$E + PX \overset{K_d}{\rightleftharpoons} EPX \xrightarrow{k_2} EP + X \qquad (2)$$

there is a first step in which the inhibitor and the enzyme form a complex together. The ability to form this complex is governed by a dissociation constant, K_d, which is of course the ratio of the backward and forward steps of this reversible reaction. Once the organophosphate has 'sat down' on the surface of the enzyme, it can then engage in the second step, which is the phosphorylation of the enzyme, which is governed by a completely different constant, k_2. These two constants, K_d and k_2, are an absolute minimum number of terms required to describe the system; it is quite possible that either or both of these constants covers a series of reactions, and if so the situation would be correspondingly complex.

Why is it that so many analyses have been made of factors affecting k_i, whereas K_d has been measured on only a few occasions? The reason is that organophosphates do not have an unusually high affinity for acetylcholinesterase, a typical value for K_d being about 10^{-4} M. Good compounds compensate for this poor K_d by having excellent k_2 values. Consequently, we are all used to the fact that concentrations such as 10^{-6} M of a phosphate give quite rapid inhibition of enzyme. Now if an organophosphate has a K_d of 10^{-4} M, then if one uses it at a concentration of 10^{-6} M, the amount of reversible complex, EPX, which is formed will only be 1% of the total enzyme present. Any attempt to demonstrate that small amount of EPX is going to be very difficult. To improve the chances of seeing it, one needs to get a substantial amount of E tied up as EPX, and in order to do this one must work near the dissociation constant, i.e. at something like 10^{-4} M. But under these conditions, the reaction will go with lightning speed, and so conventional techniques will not work.

Main has attempted to solve this problem by using a variety of ingenious devices, such as tubes with multiple sidearms which one can use to flip inhibitor into enzyme, and then a second or two later can flip substrate into the mixture in order to quench the reaction. These are all rather cumbersome, and it is hard to get reaction times less than a few seconds with any accuracy. Hart and I[5,6] approached this problem at two levels. For agents with relatively slow k_2s, we developed a kinetic procedure in which one records the ongoing reaction of enzyme and substrate on a recording spectrophotometer, and then injects inhibitor into the system, which can be done (with mixing) in a second or so. By following the change in the trace on the recording spectrophotometer, one can follow very comfortably the reaction over the next 10 or 15 s. For most carbamates and many organophosphates, this new procedure is quite good enough to enable one to work with concentrations which are close to the K_d.

For compounds with larger values of k_2, we have taken the next logical step, and gone to the stopped-flow apparatus. We use the Durrum–Gibson apparatus, which involves two syringes attached to a fast mixing device. One syringe contains enzyme, and the other contains inhibitor and substrate. At the touch of a switch, a solenoid rams home the two syringes, and they squirt their contents into a mixing chamber, and the mixed sample is viewed by an optical system which is connected to an oscilloscope. With such a device one can comfortably follow reactions down to a few milliseconds, and this is quite a short enough time for the most ferocious organophosphates that have yet been discovered, including nerve gases.

It follows that the determination of K_d and k_2 separately can be readily achieved with an ordinary recording spectrophotometer for most compounds, or with a stopped-flow apparatus for highly reactive compounds. This development should permit the routine determination of these two kinds of constants, along with a better ability to analyse the reasons why some compounds are better inhibitors than others. I should like to illustrate the value of these conclusions by referring to a study on resistance mechanisms conducted by Tripathi and me[1]. In this study the recording spectrophotometric method was used.

As background, let me say that, prior to this study, resistance of houseflies to organophosphates had been known for at least 15 years, and many investigators had studied the possibility that the resistance might be related to a change in the sensitivity of the acetylcholinesterase to inhibition. Such a change had never been found. However, when Professor Matthysse of Cornell discovered a strain of houseflies which was extraordinarily resistant to the organophosphate Rabon, we discussed with him the possibility that the unusually high resistance could be due to an unusual mechanism, and indeed we found that the resistant houseflies had an extraordinarily insensitive acetylcholinesterase. The experiments were performed as follows.

Houseflies that were hard to control by Rabon were brought into the laboratory and divided into two groups. One was kept for 20 generations in the absence of insecticide. The other was treated with high concentrations of Rabon in the larval medium for 20 generations, such that 90% of each generation was killed. These two groups then made up the susceptible and resistant populations. *Table 3* shows our observations with five different

Table 3. Bimolecular reaction constants (k_i) for the inhibition of susceptible (S) and resistant (R) housefly brain AChE at 25°C, pH 7.4

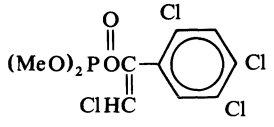
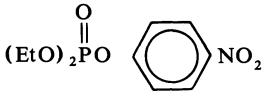
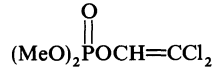
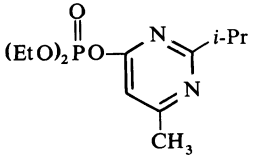
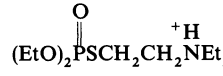
Compound	$k_i(M^{-1}\ min^{-1})$ S	R	Ratio	Resistance factor
Rabon	12.30×10^6	5.96×10^4	206	>1500
Paraoxon	3.19×10^6	0.34×10^5	94	17.0
Dichlorvos	1.16×10^7	0.99×10^5	117	16.0
Diazoxon	0.62×10^8	0.56×10^7	11	11.0
Tetram	2.40×10^7	0.345×10^7	7	—

organophosphates. There was enormous resistance to Rabon, to an extent greater than 1500-fold, and much less resistance to paraoxon, dichlorvos and diazoxon. The exciting thing to note is that the over-all sensitivity to organophosphorus inhibition of the acetylcholinesterase *in vitro* differed very substantially between the two groups. The biggest factor was in the sensitivity to Rabon *in vitro*, and the enzyme from the susceptible flies was about 200 times more sensitive to Rabon than that from the resistant flies. But Tripathi was able to carry the experiment further, by analysing the k_i into its two component parts, the k_2 (or phosphorylation constant) and the K_d (or binding constant). *Table 4* shows that, surprisingly enough, the enzyme of the resistant flies had a somewhat higher phosphorylation constant for all the organophosphates than had the susceptible flies. The paradox was

Table 4. Phosphorylation constants (k_2) of susceptible and resistant housefly brain AChE at 25°C, pH 7.4

Compound	k_2 (min^{-1}) S	R	Ratio
Rabon	0.59	1.64	2.7
Paraoxon	0.87	3.00	3.4
Dichlorvos	0.89	2.94	3.3
Diazoxon	0.83	2.57	3.0
Tetram	0.399	4.84	12.1

Table 5. Dissociation constants (K_d) of susceptible and resistant housefly brain AChE at 25°C, pH 7.4

Compound	Structure	K_d (μM) S	R	Ratio
Rabon		0.048	27.5	573
Paraoxon		0.27	86.9	322
Dichlorvos		0.077	29.5	383
Diazoxon		0.0134	0.463	36
Tetram		0.0166	1.404	84

resolved when we looked at *Table 5*, which shows that this small increase in the phosphorylation step was overwhelmed by a simply enormous decrease in affinity. For Rabon, the affinity for the susceptible enzyme was almost 600 times greater than that for the resistant enzyme. Similar large factors were found for other inhibitors, but never to the extent of Rabon.

We are anxious to find if the resistant acetylcholinesterase is a mutant form of the susceptible kind; we hope to do this by establishing the amino acid composition of both forms of enzyme. Before drawing the conclusions from the study, let me add that all of the isozymes of the resistant flies show insensitivity to the organophosphates and to a similar extent. Thus the mutation must have occurred in all of the isozymes. That must mean that all of the isozymes are synthesized under the control of a single gene, because it is extraordinarily unlikely that four simultaneous mutations of four different genes in the same direction would have occurred. It must follow that the difference in the isozymes themselves must be caused by what are called epigenetic factors, that is to say factors which operate subsequent to the original assembly of the enzyme under the control of its single gene.

Table 6. Michaelis constants (K_m) and maximum velocity (V_{max}) for ATCh hydrolysis by soluble brain AChE from susceptible and resistant strains of housefly at 25°C, pH 7.4

Constant	S	R	Ratio
K_m	$0{\cdot}95 \times 10^{-5}$ M	$3{\cdot}26 \times 10^{-5}$ M	3·4
V_{max} (μmol/h · mg protein)	18.5	68.3	3.7

The other important conclusion is as follows. *Table 6* shows that the difference in the ability of the resistant and susceptible acetylcholinesterases to react with their substrate, in this case acetylthiocholine, is extraordinarily small. The K_m values differ over a factor of three. Consequently, it is almost certain that the affinity of the substrate for the enzyme surface is substantially similar. Yet we have just seen that the affinity of compounds like Rabon for the enzyme surface is enormously changed in the development of resistance. Nor is this an unexpected discovery. A mutation which substantially changed the ability of an enzyme to react with its substrate would probably be lethal, because the enzyme would be almost useless. But you can see what a practical advantage it has given the insect to mutate to a form which is just as good at reacting with its substrate, but which has virtually lost its ability to react with an organophosphate.

This observation tells us important things about the acetylcholinesterase of the housefly. If the affinity for the phosphate can be reduced drastically with little effect upon the affinity for the substrate, these binding sites must be completely different for substrate and inhibitor. Now it might seem that such a conclusion is not surprising. There is very little in common between the relatively large and apolar organophosphate and the relatively small and ionic substrate, so that one might expect they would enjoy different binding sites, even though they could use the same catalytic site. Yet for many years, whenever compounds were found from which one wished to postulate especially good binding to the enzyme, it was customary to invoke binding to the anionic site, which at that time was the only binding site which had been proposed. *Figure 3* shows the familiar diagram found in the textbooks, and indeed the model is perhaps entirely suitable when describing the interaction with acetylcholine and closely related ionic compounds,

although these days we think of more elastic and dynamic models than the rigid structure shown in this figure. It is now widely believed that the crucial amino acid which makes up the esteratic site is a serine, whose hydroxyl is located in a microenvironment such as to make it very sensitive to phosphorylation or acetylation. Now of course this serine residue is surrounded by a

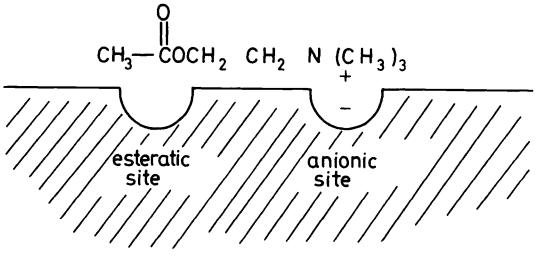


Figure 3. The original Wilson and Bergmann model

whole host of amino acids, as is true for any protein. And of course any of these amino acids could constitute a binding site. Neighbouring ionic groups could constitute ionic binding sites, and presumably this is what the anionic site is all about. But neighbouring hydrophobic areas might well constitute binding sites for hydrophobic compounds, neighbouring aromatic areas present possibilities of π–π interactions, and so on. Bearing these considerations in mind, it is not difficult to imagine how a mutant acetylcholinesterase might develop, in which a drastic change might occur in the portion of the enzyme responsible for binding compounds such as Rabon, with very little interference with the part of the enzyme binding acetylcholine.

But I have been talking as if there is no experimental evidence for additional binding sites other than the anionic site. In fact a variety of studies has suggested the existence of at least four quite different sites, and *Figure 4*

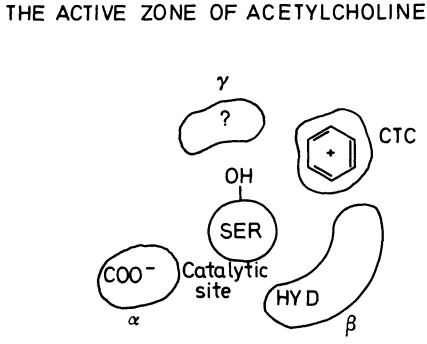


Figure 4. A speculative model of the arrangement of four binding sites around the catalytic serine of acetylcholinesterase. α, anionic site; β, hydrophobic site; γ, indophenyl site; CTC, charge-transfer site

shows a speculative model of how these might be arranged about the esteratic site. I personally prefer a somewhat different terminology. I like to speak of the serine hydroxyl as being the catalytic site, because it does not only react with esters; and I like to call the whole collection of potential binding sites along with the catalytic site the active zone of the enzyme. And I wish to stress that at this moment we are discussing only simple binding sites in the active zone, and later on we shall discuss the possibility of additional sites called 'regulatory sites' which are outside the active zone.

The only one of these supplementary binding sites that I want to discuss in detail now is the proposed charge-transfer complex (or CTC) site. It is possible that this CTC site might be also hydrophobic in character, which would simplify the rather complicated map of *Figure 4*. We have been able to explore the possibility of the existence of a CTC site only for carbamates, and only by indirect methods. However, although the existence of the site is not proven, the concept has been very helpful in designing carbamate anticholinesterases.

Let me begin by commenting upon the concept of CTC formation. Such complexes can be formed between two molecules if a number of conditions are fulfilled. One molecule, the donor, has to have an electron which is relatively easily lost, i.e. it has to have a low ionization potential. The other molecule, the acceptor, has to have a tendency to accept electrons, that is to say it has to have a high electron affinity. In addition, the two molecules need to be able to be so closely juxtaposed that their electron clouds can overlap. In such circumstances, a new molecular orbital may be created, and assuming that the electron is not entirely captured by the acceptor (in which case a free radical would be formed), a molecular complex is formed. The new orbital can be characterized by a new absorption band, and quite commonly these bands are in the visible range.

Hetnarski and I[7] soon showed that insecticidal carbamates can be good CTC donors in the sense that they form a complex with a model acceptor, TCNE or tetracyanoethylene. *Figure 5* shows the TCNE alone or phenyl-dimethylcarbamate alone has no absorption in the visible range, but when they are mixed a very substantial absorption takes place. We were able to

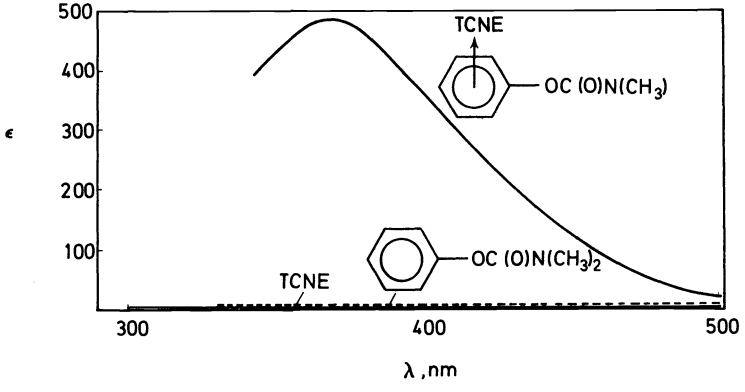


Figure 5. Absorption curve of phenyl dimethylcarbamate–TCNE complex

show that the complex had a one-to-one stoichiometry. By measuring the concentrations of reactants necessary to give half-maximal production of complex, we were able to calculate binding constants, which we call K_x, for a large number of carbamates and related compounds with our model acceptor, TCNE. We also demonstrated that these aromatic carbamates used the π-electrons of their rings to donate in complex formation.

As you would expect, ring substituents that were capable of donating electrons to the ring increased the strength of the complex, as shown by an increase in K_x. By contrast, electron-withdrawing substituents, such as the nitro group, completely abolished the ability to form CTC with the model acceptor. As *Figure 6* shows, with two different sets of carbamates, the variation in their potency against erythrocyte acetylcholinesterase can be rather well accounted for by the variations of their ability to form CTC with

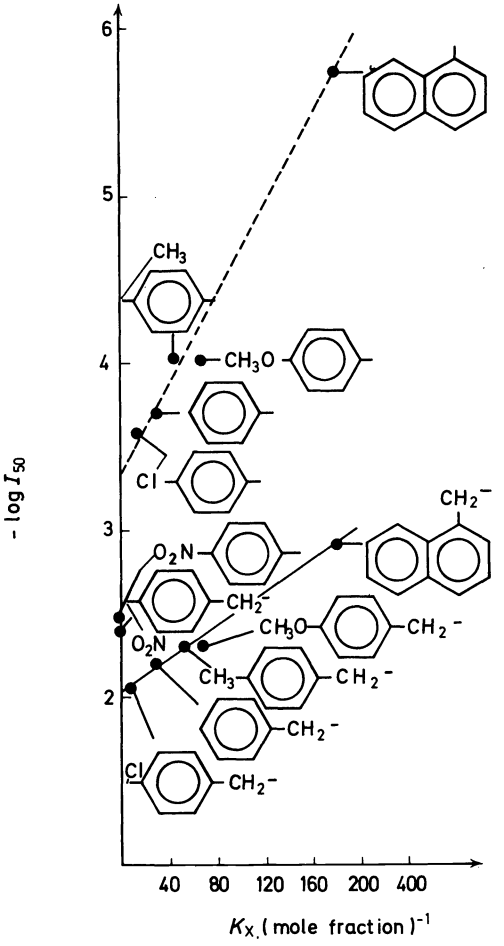


Figure 6. Relation between $-\log I_{50}$ and K_x for substituted aryl methylcarbamates (– – –) and arylmethyl methylcarbamates (———)

our model acceptor. I should like to comment briefly on the two series of compounds shown in *Figure 6*. The top group is a set of simple substituted phenyl methylcarbamates. When we had explored these, it became obvious that the over-all potency, which is measured here by I_{50} (that is, the molar concentration to inhibit by 50% in a fixed time) should have an affinity component and a carbamylating component. There was every reason to expect that electron-pushing substituents, which should improve the affinity ($1/K_d$), would have the opposite effect upon k_2, the carbamylating activity, because one anticipates that carbamylation involves an electrophilic attack upon the enzyme's catalytic site. In simple aromatic methylcarbamates any substitution upon the ring is bound to influence both the availability of the π-electrons for complex formation and also the carbamylating effectiveness of the carbamyl group. We therefore prepared a new series of carbamates, with a methylene group inserted between the ring and the carbamyl group. The result was a series of compounds, illustrated in *Figure 6*, which were such bad carbamylating agents that they did not carbamylate the enzyme at all, but only formed a reversible complex with it. These are what we call non-carbamylating carbamates. In this series, the I_{50} is simply a reflection of reversible complex formation, and once again you see that the variations in this value were well accounted for by variations in the ability to form complexes with the model acceptor.

In recent months we have been elaborating our thoughts in related directions. Firstly, we have added in a series of *meta*-substituted phenyl methylcarbamates, in addition to the *para*-substituted series which is shown at the top of the figure. Furthermore, it was easy to show that the complexing ability of aromatic carbamates was entirely due to their aromatic portions, and was in fact somewhat reduced by the carbamyl group. It should follow that simple aromatic compounds, lacking the carbamyl group, could be reversible inhibitors of acetylcholinesterase with dissociation constants quite like their derived carbamates. And *Table 7* shows that such is the case.

Table 7. Dissociation constants for acetylcholinesterase

	K_d (mM)		
	Aromatic hydrocarbon	Aryl methylcarbamate	Arylmethyl methylcarbamate
p-Chlorophenyl	3.8	4.5	3.1
Phenyl	3.9	6.0	2.8
p-Tolyl	2.3	3.9	2.4
p-Anisyl	2.7	3.5	2.3
Naphthyl	0.6	0.013	0.13

Nomenclature: The three compounds referred to in the top line are chlorobenzene, *p*-chlorophenyl methylcarbamate and *p*-chlorophenylmethyl methylcarbamate.

With these four series of compounds we have measured the dissociation constants with acetylcholinesterase, using the spectrophotometric method of Hart and O'Brien[5]. We found that it was necessary to take into account, in every case, the hydrophobicity of the compounds, which we measure by the so-called π-coefficient of Hansch[8]. This coefficient is a measure of the

tendency of the material to bind to hydrophobic areas, or to partition into apolar solvents. The relative importance of the π-value and the ability to form CTCs varied for each of the four series of compounds. But by taking both π and CTC formation into account, we were able to account fully for the variability in the dissociation constants of these four sets of compounds for the enzyme.

At this point the designer of carbamates might fairly ask the following question. Is there any value in trying to improve the affinity of aromatic carbamates for acetylcholinesterase, if we do so by attaching electron-pushing groups to the ring, because these substituents may well improve the affinity, by the effect upon K_d, but will they not have the reverse effect upon the carbamylating effectiveness, as measured by k_2? The surprising thing is that the answer to this question is 'no'. For reasons that we do not understand, the k_2 values of carbamylating carbamates are remarkably insensitive to ring substituents. This was something that we reported in 1966, and have confirmed by entirely independent studies recently. This unexpected finding may account for the fact that in aromatic organophosphates, where effects upon k_2 seem to be of major importance, excellent inhibitors are obtained by having highly electron-withdrawing groups attached to the ring. Precisely the opposite is true in the case of carbamates.

It is not hard to imagine that a more detailed knowledge of the nature of the binding sites within the active zone could be helpful in insecticide design. But now I want to turn to areas outside the active zone. In the last decade the concept of allosteric sites has become important. Very many cases are known in which inhibitors or activators bind to sites which are physically far from the active site of the enzyme, but binding to such different or allosteric sites causes changes in the configuration of the enzyme which have profound influence upon the effectiveness of the active site. I should like to discuss briefly whether such sites occur in the case of acetylcholinesterase. I like to refer to them as regulatory sites, because in some sense the non-regulatory binding sites which I have just been describing for things like organophosphates might be conceived of as being 'allosteric', since they are not the binding sites enjoyed by the substrate itself.

The most persuasive evidence for regulatory sites is the observation that certain compounds can increase the activity of the catalytic site. A particularly well worked out example deals with various aziridinium compounds, which were shown long ago by Purdie and McIvor[9] to react with acetylcholinesterase to produce an enzyme which was inhibited with respect to acetylcholine hydrolysis, but which was activated with respect to indophenyl acetate hydrolysis. I showed later[10] that this effect had nothing to do with isozymes, that it had nothing to do with changes in the affinity of indophenyl acetate for the active zone, but was entirely due to effects on the catalytic site. Other cases of activation include the ability of the compound decamethonium, which is essentially two quaternary ammonium groups separated by a ten-carbon chain, to increase the ability of acetylcholinesterase to undergo sulphonylation with methane sulphonyl fluoride[11]; and of tetramethylammonium to accelerate the hydrolysis of phenyl acetate, which it does by improving the deacetylation rate[12].

There seems to be only one known example in which a regulatory site is

directly involved with organophosphates or carbamates. Aldridge and Reiner[13] reported that organophosphates such as coroxon bind to a secondary site for acetylcholine, a site which is normally only involved when excessive acetylcholine concentrations are used, and one sees the phenomenon known as excess substrate inhibition. It appears that coroxon binds to this secondary site, and that subsequently it phosphorylates the catalytic site. I think this secondary site has to qualify as a regulatory site, because when it is occupied by acetylcholine, the catalytic effectiveness for acetylcholine is reduced. However, this is a rather complicated situation, because it is probable that the occupancy of that secondary site of acetylcholine produces its effect not upon the acetylation of the catalytic site itself but upon the secondary process of deacetylation. What can be said with some confidence is that coroxon acts very differently from the great majority of organophosphates, and that the difference is due to binding to a quite different site.

We are particularly interested in the possibility of a completely different role of regulatory sites in phosphorylation, i.e. that occupation of regulatory sites by appropriate drugs could modify the sensitivity of the catalytic site to phosphorylation by organophosphates. If such were the case, one might have very effective prophylactic agents against organophosphate poisoning.

Before summing up, I should like to recall a conversation I had with a very distinguished colleague in about 1955. At that time he was just leaving the organophosphate field, because he felt that our knowledge of the reaction between organophosphates and acetylcholinesterase had been worked out in such complete detail as to leave nothing but fine points to be cleared up. I should like to suggest that in 1955 the situation really was that we only knew the global outlines of the reaction between anticholinesterases and the enzyme. Now at least we know that isozymes do exist, that the active site has numerous non-regulatory sites which can be important in inhibition, and that there are regulatory sites which might be of even greater importance. We are able to analyse the inhibition reaction into at least two steps, and there may well be additional steps to be discovered. And although there are dozens of suggestive pieces of evidence in the literature about differences between the acetylcholinesterases of different species, this is an area where a tremendous amount remains to be done. Although we are all anxious to see the development of entirely new kinds of insecticides, it is my guess that in the next 10 years we will also see the development of dramatically improved anticholinesterase insecticides, although only time will tell whether these will emerge from deliberate design, based on enzymology, or from the ingenuity of organic chemists.

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