Biochemical Toxicology of Insecticides

Biochemical Toxicology of Insecticides

Edited by
R. D. O'Brien
Division of Biological Sciences
Cornell University
Ithaca, New York

Izuru Yamamoto
Department of Agricultural
Chemistry
Tokyo University of
Agriculture
Tokyo, Japan

Academic Press 1970 New York London

ACADEMIC PRESS, INC.
111 Fifth Avenue, New York, New York 10003

United Kingdom Edition published by
ACADEMIC PRESS, INC. (LONDON) LTD.
Berkeley Square House, London W1X 6BA

LIBRARY OF CONGRESS CATALOG CARD NUMBER: 71-117106

PRINTED IN THE UNITED STATES OF AMERICA

PREFACE

This book is comprised of papers presented at the Fifth United States-Japan Cooperative Science Program, meeting in Tokyo, June 1969. The meeting was sponsored by the Japan Society for the Promotion of Science and the U. S. National Science Foundation.

These papers represent a cross section of some of the research frontiers in the study of insecticide action and metabolism. Their most evident value will be to those whose research or teaching interests lie in this area. For these readers, the substantial amount of detail given will be appreciated. However, we hope that the book will serve other purposes. Those wishing to know what problems are of greatest current interest in this area, or the depth to which research has penetrated, should find this book useful. For instance, even a casual reading will indicate the astonishingly important role of microsomes in metabolism of very diverse toxicants and drugs, the extreme complexity of the pathways involved in breakdown of insecticides, or the great power of the polyfactorial approach to analysis of structure-activity relations.

This book is more than a symbol of the cordial relations among scientists of Japan and the United States; it is a direct product of that cordiality. Fundamental research on insecticides is an area in which Japan and the United States account together for a remarkably large share of the world's total. The participants found in their week of presentations and discussions an exceptional level of mutual friendliness and admiration. We hope that the close ties between our two countries will be strengthened by publication of this joint offering, and that the personal contacts fostered by the meeting will eventually extend well beyond the participants.

Izuru Yamamoto
R. D. O'Brien

CONTENTS

THE PROPERTIES OF ACETYLCHOLINE RECEPTOR *in vitro* FROM TORPEDO ELECTROPLAX, HOUSEFLY HEAD AND RAT BRAIN

R. D. O'Brien

Cornell University
Ithaca, New York, U.S.A.

Abstract

Evidence is presented that the binding of tritiated muscarone reflects acetylcholine receptor activity. The effect of drugs upon binding activity was compared in housefly head and in electroplax from the electric skate, *Torpedo*. The drug response of the plax was compatible with the established view that it has synapses of the neuromuscular type. In fly head, a mixed muscarinic and nicotinic character was found, and also some entirely unexpected effects were noted.

It is well known that cholinergic neurones, that is to say nerve cells whose effects are transmitted to their neighbors by release of acetylcholine, are of vital importance to insects and mammals. The best evidence of this is that disruption of cholinergic neurones leads to prompt death in these animals. There are two particular steps which involve macromolecules which are targets for poisons. The best known is acetylcholinesterase, the enzyme which destroys acetylcholine and therefore prepares the neuron for a second excitation. But there is a completely different macromolecule called the receptor, which is of even more central importance to cholinergic neurons. This is the protein with which acetylcholine normally combines in order to produce its excitatory effect.

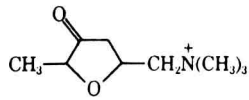
Perhaps the most remarkable thing about the acetylcholine receptor is that it has never been isolated, nor has its presence been unambiguously shown in any broken cell preparation. The reason is that we only recognize its properties by its action in the intact cell. But there is

no reason to doubt that this vital component of the machinery of the neuron is a macromolecule which can be isolated, whose properties can be explored, and for which toxicants can be designed. At the moment, it is throughly established for vertebrates, and it is assumed to be true for insects that nicotine acts by combining with the receptor. In the vertebrate, very many other agents are known which act upon the receptor; these include curare, a poison which has been used for centuries in South America, succinylcholine, and atropine. There is every reason to expect that new kinds of toxicant may be designed to operate on the receptor. We may also expect that (just as in the case of acetylcholinesterase) there will be important differences between the receptor of insects and mammals, and that these differences might be made the basis for making selective toxicants.

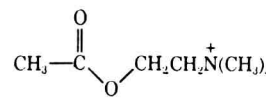
In this paper I describe what I believe is the first study upon acetylcholine receptor at the broken cell level. First let us turn to the question; if one recognizes a receptor by its physiological response, how can one measure its presence in a broken cell preparation? There is not an unambiguous reply to this question. The criteria which we have used have been as follows. One would anticipate that receptor would combine with a transmitter substance with high affinity; would be present in a suitable quantity in the tissue of interest; would be present in appropriate tissues (such as nervous tissue) and absent in inappropriate tissues (such as liver); would show reversible binding to agents which bound to it reversibly in intact systems; and above all would show an appropriate drug response. That is to say, that drugs such as curare and nicotine, which were active in combining with receptor in vivo would also compete for binding with transmitter in a broken cell preparation. Finally, it would be very pleasant if one could isolate and purify a receptor macromolecule, and try and build it back into a model system, in which one could show once more the essential property which it has in the intact system; that is to say, that when acetylcholine is brought up to it a change occurs in the permeability of that membrane, particularly with reference to the ability to conduct sodium ions.

The compound whose binding we would of course like to have studied would be acetylcholine. However, all preparations containing receptor also contain a large amount of acetylcholinesterase, which rapidly destroys added acetylcholine. This barrier to advancement was overcome when we

became aware of the work of Waser (1958, 1960, 1961) describing the compound muscarone.



Muscarone



Acetylcholine

This, quite unlike its close relative muscarine, a potent stimulant for nicotinic and muscaronic varieties of cholinergic receptor. At this point I should make it clear that data on the pharmacology of vertebrates has clearly indicated that there are at least two kinds of cholinergic receptor; both are stimulated by acetylcholine. One is called the muscarinic type and is stimulated by muscarine and blocked by atropine; the other is called the nicotinic type and is stimulated by nicotine and blocked by curare. In the vertebrate there is doubt at all that the kind of cholinergic receptor found in muscle and electroplax is nicotinic, whereas the central and ganglionic kinds are muscarinic. In the insect, we of course have very little information about the pharmacology of these neurones.

The great advantage of using muscarone rather than acetylcholine is that it has an ether in place of an ester group, and is therefore not degraded by acetylcholinesterase. In fact, our studies on a number of tissues showed us that it is completely unmetabolised.

We prepared tritiated muscarone with a specific activity of about 80 mc/mmole. At this time I went to Naples, Italy, where we studied the binding of muscarone to rat brain and were disappointed to find negligible binding. It is important to explain to you the "centrifugal technique" we used at that time. It is only suitable for studying binding to particulate material, and it involves adding a known amount of muscarone to a suspension of particulate material, which is then centrifuged. By observing the amount of muscarone left in the supernatant, one can compute how much was bound to the particles. One can repeat this experiment in a variety of muscarone concentrations, and plot, in this way, the binding of muscarone as a function of concentration. This procedure should yield a graph from which one can determine the binding constant of muscarone.

In November of 1967 we began studying the tissue for which we had come to Naples: the electroplax of the electric skate Torpedo. The reason we looked at electroplax

was that it is an extremely condensed nervous tissue which has been known since the 1930's for having a very high level of acetylcholinesterase, and therefore by implication of other components of chemical transmission. We were delighted to find immediately that there was very substantial binding to a variety of particulate preparations from electroplax. At this time we settled down on the precipitate from spinning a water homogenate at 11,500 x gravity as our standard preparation. There was substantial binding of muscarone in the concentration range of 10^{-5} to 10^{-7}M. When this data was plotted on a double reciprocal plot it gave an approximately linear response. A plot of this type indicates, just as in the case of enzymes, the saturating amount of binding to the material (given as the reciprocal of the intercept on the y axis) and it also indicates the binding constant of the material to the electroplax, (given by the negative reciprocal of the intercept on the x axis). From this data we were able to calculate an approximate binding constant of 7 x 10^{-6}M, and an amount of binding at saturation of 1.8nm per gram. I use the term "approximate" advisedly. In part there was a good deal of scatter in the data as you can see, but a much more important criticism is that there was poor linearity in the relation between the amount of tissue used and the quantity of binding.

We used very large amounts of tissue in the above assay, employing 5g of electroplax for every individual assay we did. The reason is that in binding studies, unlike enzymes studies, one only sees as much binding as there is macromolecule. In other words one is dealing with a stoichiometric rather than a catalytic phenomenon. One consequence was that the physical properties of the homogenates were poor; they were viscous because of the high quantity of mucopolysaccharide present. Nevertheless, we decided to go ahead with this particular preparation to determine some of its properties. In the first place we were glad to observe that muscarone binding was reversible, as is its pharmacological action. For instance, if we took the precipitate prepared as above and containing the muscarone which it had bound, and then re-suspended it in fresh water, the total radioactivity distributed itself in the proportions calculable from the binding constant. One of our criticisms of other workers' studies with curare was that they were looking at the irreversible binding of curare to their preparations, even though the pharmacological action of curare is reversible.

The next thing to look at was the response to variety of drugs, or the "drug profile" as I call it. Fortunately, those agents which were supposed to react with cholinergic receptors had an effectiveness against muscarone binding to our particles which was similar to their degree of effectiveness in vivo. In addition, a variety of drugs which should not have any effect upon cholinergic junctions were without action upon muscarone binding, (the detailed effects will be described below for "plax powder"). Of particular interest was the effect of acetylcholine. As I pointed out before, if one adds acetylcholine to these systems it is rapidly destroyed. But we found that paraoxon, a potent anticholinesterase, was without action upon muscarone binding. We were therefore able to use 10^{-4}M paraoxon to block all of the acetylcholinesterase in the preparation, and then add acetylcholine to observe its effect upon muscarone binding. When we did this we found substantial blockade in the concentration range 10^{-6}M of acetylcholine. A more detailed examination at various acetylcholine concentrations enabled us to calculate that the binding constant of acetylcholine was about 1.1×10^{-6}M. This was the first time that any high affinity binding of acetylcholine to any broken cell preparation had ever been seen.

Additional properties of our preparation included the fact that muscarone binding activity was destroyed by boiling the preparation. But the preparation could be frozen or freeze-dried with negligible loss of activity.

It was at this stage that we came to feel that there was good preliminary evidence that the binding that we were looking at was due to acetylcholine receptor. If we were to proceed further, it would obviously be necessary to attempt solubilization and purification. We therefore changed at this point to a simple equilibrium dialysis. The preparation under study was placed in a 1 ml dialysis bag and suspended in 100 volumes of labeled muscarone. After leaving in the cold overnight the concentration of muscarone inside and outside the bag was measured, and one could calculate the additional muscarone present in the bag which was due to binding to the material. We reconfirmed that curare and acetylcholine still blocked the preparation as shown by this kind of assay. We are also able to provide one of the several pieces of evidence that our muscarone was binding to receptor and not to acetylcholinesterase. For instance, if we spun plax homogenate at 100,000 x g, about 10% of the acetylcholinesterases remained in the

supernatant whilst all the muscarone binding activity was precipitated. The drug profile of acetylcholinesterase and muscarone binding was also completely different, as was the pH dependance. In addition, subsequent studies upon binding of muscarone to acetylcholinesterase have indicated negligible binding in the 10^{-6} molar range. Finally, one can extract acetylcholinesterase from plax with toluol, but muscarone binding is destroyed in the process. We therefore believe that the two molecules are quite unrelated.

We now had to return from Italy, and therefore began to stockpile electroplax. We obtained 264 Torpedos which gave us 36 kg of electroplax. The precipitate from a 20% homogenate of this was freeze-dried and yielded 493g "plax powder". This material was then flown back to Ithaca, New York. With our new assay technique and the plax powder, the kinetics were much improved. Above all, there was a linear relation between concentration of tissue and amount of binding. We also obtained a much improved double reciprocal plot (Fig. 1) which gave us a better estimate of the binding constant as $K = 7.19 \times 10^{-7}$M and the amount of binding as 1.01 nm/g. We were also able to study the temperature dependence of the process and to observe that the binding was somewhat increased at 38°C as compared with 25° or 4°C.

At this stage I was joined by Professors Eldefrawi and Toppozada. We explored the problems of solubilization and electrophoresis of plax preparations. To summarize a great deal of work which is not yet completed, the usual solubilizing agents, including ionic and non-ionic detergents, give rise to grave difficulties because they interfer with the assay. Particularly embarrassing is the question of SDS (sodium dodecyl sulfate). At first we thought we were getting magnificant solubilization by SDS, but it turned out that SDS in combination with any protein will give an apparent binding. This binding is curare-insensitive, and so quite unlike the real binding which one sees with normal electroplax. By contrast Triton X100 gives a severe inhibition of binding, and so is equally unsuitable. We have however, been able to get up to 25% solubilization by mechanical treatments of various sorts, including ultrasonication. However, although several techniques will permit one to obtain binding activity in a 100,000 x g supernatant, when such a supernatant is submitted to electrophoresis, numerous proteins will migrate, but the binding activity

remains on the origin. We do not know yet whether this is because the activity is still particulate, even though in particles small enough not to precipitate at 100,000 x g, or whether some sort of coagulation occurs before or during electrophoresis. This matter is under continuing study.

We have examined the effect of 18 enzymes upon our plax powder. The only enzymes which are effective in degrading it were trypsin, chymotrypsin and phospholipase C. A variety of other enzymes including lipase and snake venom (containing phospholipase A) were inactive upon the preparation. Although the enzymic data is not conclusive, it suggests that the receptor is a phospholipoprotein.

The next topic I want to discuss is the drug profile of the plax powder. It is convenient at this time to describe to you simultaneously the studies that Dr. Toppozada has been carrying out on housefly preparations. We have worked primarily with housefly head, which we selected because it has been known for many years that it has as much acetylcholinesterase as electroplax, and therefore one might expect that it also be very high in receptor. We were delighted to find that in fact homogenates had very high binding activity, and that in addition much of the activity was soluble, i.e. was in the supernatant at 100,000 x g. The homogenates were prepared in water so there was no problem of detergents. The supernatant contained 64% as much binding activity as the plax powder, expressed on a protein basis. However, its binding constant was 2.4 x 10^{-6}M, which implies an affinity of about 3 fold less than plax. In addition, like the solubilized plax material, upon electrophoresis many proteins migrated, but all the binding remained at the origin. Clearly there were several similarities between the fly and the Torpedo preparation.

A comparison of the drug profiles of the two preparations (Table 1) showed many similarities. The major nicotinic agonists and antagonists were potent blockers at 10^{-5}M; whilst transmitters not connected with cholinergic systems (such as norepinephrine and serotonim) were without effect at 10^{-4}M. But there were important differences (Table 2). Curare, which is a rather specific neuromuscular variety of nicotinic agent, was much more effective on plax than on fly, whilst several other cholinergic agents which are muscarinic and not nicotinic (e.g. atropine, pilocarpine) were much more effective on fly than on plax. These findings suggest that whereas plax receptor is a

"pure" neuromuscular type. This agrees with the view that the plax is homologous with muscle. By contrast, the receptor in housefly head is of mixed nicotinic and muscarinic type.

An unexpected finding (Table 2) was that three compounds (amphetamine, tyramine and hordenine) which are not considered cholinergic in vertebrates, proved quite potent on muscarone binding to housefly head.

This implies that the cholinergic receptor is unlike that found in any vertebrate cholinergic junction, and gives hope that poisons might be found which would be specific for the insect receptor.

We have done relatively little work upon the binding which we have now observed in rat brain. I should emphasize that the reason we can see this binding now is that our "dialysis" technique is much improved over the early "centrifugal" technique. We have looked only at the soluble activity in rat brain, and all that I can say at the moment is that in some ways it has a drug profile somewhat intermediate that of housefly and of electroplax. For instance, it is similar to electroplax in being insensitive to eserine and codeine. But it is similar to flyhead in that it is inhibited by pilocarpine and is not terribly sensitive to curare or imipramine. It also has a lower affinity for muscarone than either of the other preparations. Needless to say, we intend to explore this aspect in much more detail, and particularly to explore the binding to sub-fractions of brain particulates. We are especially interested in finding whether there is binding to postsynaptic membranes and not to the small vesicles which are primarily sites of storage. Currently the only evidence we have that binding which we see is to receptor sites rather than to storage sites is that our drug profile in the electroplax material is appropriate. However, it is possible that drugs do not normally have an opportunity to reach the storage sites so our conclusion has to be suitably tentative.

We have under development one other approach. In brief, we are trying to get a system consisting of a bimolecular layer of phospholipids whose permeability to ions we can study electrically. We have in fact successfully made use of the system of Mueller and Rudin (3) for this purpose. It is our hope that when we can obtain a purified receptor molecule, we can incorporate it into such a model membrane, and then show that whilst such a membrane

is normally relatively impermeable to sodium ions (for instance) when one adds acetylcholine to the system, the ions can suddenly permeate the membrane. If we can succeed in doing this, we will have fully modeled the essential properties of the synapse.

References

1. O'Brien, R. D., Gilmour, L. P. and Eldefrawi, M. E. Proc. Nat. Acad. Sci, in press (1970).

2. Eldefrawi, A. T. and O'Brien, R. D., J. Neurochem. submitted (1970).

3. Mueller, P. and Rudin, D. O., Nature 217, 713 (1968).

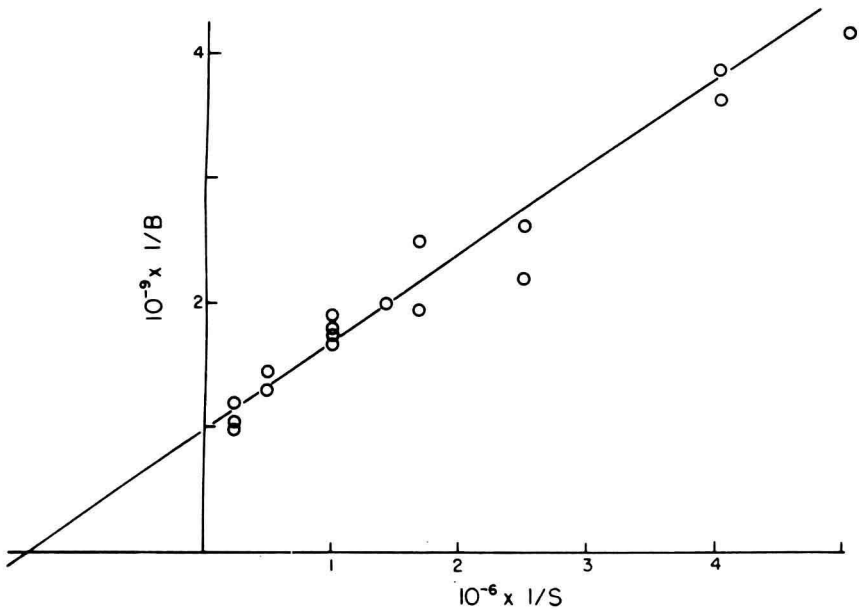
Table 1. Similarities in drug response of Torpedo plax powder and fly head.

Compound	% Blockade of 10^{-6}M Muscarone Fly	Torpedo
CHOLINERGIC COMPOUNDS		
Nicotine 10^{-5}M	39	59
Succinylcholine 10^{-5}M	63	86
Decamethonium 10^{-5}M	65	77
Acetylcholine 4 x 10^{-5}M (after paraoxon treatment)	76	82
Strychnine 10^{-4}M	60	76
OTHER COMPOUNDS		
Serotonium 10^{-4}M	(13)	0
Norepinephrine 10^{-4}M	(9)	0
γ-Aminobutyrate 10^{-4}M	(-7)	0
Glutamine	0	0

Note. Parenthetic values are not significantly different from the control value i.e. from 0% blockade. Data from O'Brien et al (1) and Eldefrawi and O'Brien (2).

Table 2. Differences in drug response of Torpedo plax powder and fly head.

Compound	% Blockade of 10^{-6}M Muscarone	
	Fly	Torpedo
CHOLINERGIC COMPOUNDS		
Curare 10^{-5}M	(19)	79
Tetraethylammonium 10^{-4}M	63	32
Atropine 10^{-4}M	72	21
Pilocarpine 10^{-4}M	84	0
Eserine 10^{-4}M	78	0
Codeine 10^{-4}M	71	0
OTHER COMPOUNDS		
Amphetamine 10^{-4}M	51	0
Tyramine 10^{-4}M	60	0
Hordenine 10^{-4}M	84	19



1. Dependance upon muscarone concentration (S) of binding (B) to freeze-dried 12,000 x g precipitate derived from 0.5g of plax.
   Dialysis method.