Mechanism of Pesticide Action

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G. K. Kohn, *Editor*

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Robert F. Gould, *Series Editor*

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FOREWORD

The ACS SYMPOSIUM SERIES was founded in 1974 to provide a medium for publishing symposia quickly in book form. The format of the SERIES parallels that of its predecessor, ADVANCES IN CHEMISTRY SERIES, except that in order to save time the papers are not typeset but are reproduced as they are submitted by the authors in camera-ready form. As a further means of saving time, the papers are not edited or reviewed except by the symposium chairman, who becomes editor of the book. Papers published in the ACS SYMPOSIUM SERIES are original contributions not published elsewhere in whole or major part and include reports of research as well as reviews since symposia may embrace both types of presentation.

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PREFACE

Improved pesticidal compositions require a better fundamental understanding of the complex interactions of organism (including man), host plant, environment, and pesticidal composition. This, is the subject of this symposium entitled, "Mechanism of Pesticidal Action: Cellular and Model Systems." Its origin was predicated on the assumption that a more fundamental understanding of the mechanisms of existing pesticides would lead to the better pesticides of the future. The papers in this volume are divided into three sections, proceeding from mechanisms that relate to broad groups of pesticides, to those that have significance to a single group, and then to single pesticides or unique mechanisms or novel model systems.

The simplicity of the Nachmanson-Wilson model of a two-point landing of the inhibitor or the normal substrate on the anionic and esteratic sites of the enzyme, cholinesterase, has unfortunately given way to a far more complex view of that enzyme. R. D. O'Brien's "Recent Studies on Acetylcholinesterase Inhibition" continues to explore portions of this complex system which applies to all insecticidal phosphate and carbamate compositions and as well to the perplexing problems of insect resistance to these substances.

"Interactions of Pesticides with Cytochrome P-450" by E. Hodgson and A. B. Kulkarni extends the studies of the senior author on one of the most significant mechanisms applicable to the metabolism of pesticides by both arthropods and mammals. Again this oxidative process relates to resistance by arthropod species and to safety of pesticides (or lack of it) to man. R. J. Kuhr explores, particularly in insects, various mechanisms by which the organism rids itself of the offending toxicant.

The problems of the metabolism of chlorinated hydrocarbons in mammals is the subject of the chapter by H. B. Matthews and associates. It contains the scientific facts of that metabolism much needed in an area clouded by public controversy. Finally, a more general mechanistic paper is the one relating to herbicides and plant interactions entitled, "Herbicide–Lipid Interactions" by J. B. St. John and J. L. Hilton.

The following series of papers treats more specific classes of compounds and their mechanism of action. Great organic chemists have been associated historically with pyrethroid chemistry. M. Elliott and his collaborators at the Rothamstead Experiment Station, England, have trans-

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formed this field over the past few years from one of mild academic interest to one of acute practical consideration. Some of the newer conformations of these chrysanthemum acid esters are enormously more potent and at the same time less UV degradable than their natural relatives. They may be among the practical agricultural and home pesticidal compositions of the near future particularly if their toxicology and manufacturing economics continue to look favorable.

One of the more interestingly practical acaricidal and insecticidal groups to appear during the last decade have been the formamidines. C. O. Knowles and associates examine some of their biochemical interactions, particularly the biogenic amines that relate to nervous system activity. Those familiar with recent practical aspects of weed control in agriculture are familiar with the heterocyclic photosynthesis inhibitors developed in the Bayer Laboratories in West Germany. Extensive regression analysis along the Hansch model reveals the structure–activity relationship that has generated one of the more significant new preemergent herbicides with a bright future of practical application in world agriculture.

G. G. Still develops further his study of plant interaction with an older and still important group of herbicides—the carbanilates (IPC and CIPC). His use of luciferase as a model enzyme system is one of the earliest, if not the first, for that enzyme in studying plant pesticide interactions.

Some years ago chemosterilization of insects appeared to be a highly promising new avenue for insect control. Most of the more effective sterilizing materials so far developed—except for certain complex hormonal chemicals—have proved to be alkylating agents. As such they are potentially carcinogenic, and this has been the greatest bar to their practical development. A. B. Borkovec has been examining their compositions in the USDA laboratories for several decades, and he discusses the present status of the "Mechanism of Action of Alkylating and Nonalkylating Chemosterilants."

A recent discovery of considerable theoretical interest is the totally unexpected insect growth regulant activity of structures roughly similar to conventional herbicide compositions. This discovery by scientists at Philips-Duphar of Holland is the subject of the chapter by L. C. Post and R. Mulder. A model system for studying such activity and other processes has been developed independently at the USDA laboratories in Fargo, N. D. E. P. Marks and B. A. Sowa describe the culturing of insect cuticle and use of this tissue culture for estimating ecdysone-like activity. As a model system for the fundamental study of interaction of certain chemicals with insect cuticle, it is undoubtedly significant.

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Phosphoramidates have provided some interesting herbicidal compositions in the past. S. Sumida and M. Ueda describe the use of the algal species *Chlorella ellipsoidea* as a model system to explore the mode of action of a new preemergent herbicide. Melvin Calvin used a similar system in his classical studies in the biochemistry of photosynthesis, and it is interesting to note this pesticide extension of those earlier studies.

One of the most useful chemicals for combating plant disease is the fungicide captan. Its agricultural significance has increased since its discovery several decades ago. J. R. DeBaun and associates in *Xenobiotica* [(1974) 4 (2), 101] describe (for the first time) the fate in mammals of the $SCCl_3$ group and identify heretofore undetected metabolites. A mechanism of growing significance is the pesticidal biochemistry related to the uncoupling of oxidative phosphorylation. Dr. Nishizawa and associates at Sumitamo discovered a new class of potent uncouplers. Certain representatives of this class possess interesting acaricidal and fungicidal activity.

This volume does not include one paper given at the Los Angeles meeting. The content of that talk by Dr. Jaworski was published prior to the meeting in *Journal of Agricultural and Food Chemistry* [(1972) 20, 1195]. The mechanism of interference with plant amino acid synthesis, the interesting phosphonate structure, and the potential agricultural usefulness merit inclusion here if only by reference.

The papers here deal with insecticides, herbicides, fungicides, acaricides, and sterilants. They derived from universities, government experiment stations, and industrial laboratories and include contributions from Germany, Japan, Holland, and England as well as the United States. They provide a picture typical but not all inclusive of the state of the art and science. It is hoped that these contributions will provoke the thought and action needed to create the improved pesticides of the future.

G. K. KOHN

Richmond, Calif.
September 3, 1974

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Recent Studies on Acetylcholinesterase Inhibition

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There is now general agreement that the toxic action of organophosphates and carbamates, upon insects as well as vertebrates, is caused by their ability to inhibit acetylcholinesterase in various parts of the nervous system, and thereby disrupt nervous transmission at that location. Today I would like to cover three different but related topics. The first deals with a relatively recent discovery that acetylcholinesterase almost invariably occurs with multiple molecular forms. We will then move to discuss the inhibition of the enzyme, and a recent new development in the analysis of the several steps involved in inhibition; and I will finish up with a discussion of the topography of the active site at the enzyme, with special interest in the forces which bind substrates and inhibitors at or near the active site.

Until recent years, those of us working with acetylcholinesterase from any given source have treated it as a single kind of enzyme. Meanwhile, workers with very different enzymes showed that it was not at all unusual for these enzymes to occur in multiple molecular forms, which under certain circumstances we can refer to as isozymes. For the purposes of this talk, I shall use the term "isozymes" to refer to a particular enzyme from a particular source, such as housefly head acetylcholinesterase, which can be separated by purely physical means (most commonly electrophoresis) into different physical forms, all of which have the essential properties of the parent-enzyme mixture, but which may show subtle and potentially important variations on the parent's basic theme. The classic enzyme on which the isozyme system was worked out was the enzyme called lactic dehydrogenase, for which it was shown that different forms occurred in the skeletal muscle and the heart muscle of the rat. The two forms differed in the pattern they showed in electrophoresis, a technique which involves putting a mixture of material on a jelly-like column made by polymerizing acrylamide, and then applying a high voltage across the little column (typically about 5 cm long) and subsequently staining or otherwise assaying the gel column to find the migration velocity of the different components. To summarize the findings of the lactic dehydrogenase work, let me say that the various

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forms which were encountered were explained as being due to simple combinations of two different kinds of building block or protomer. If we call the protomer most abundant in heart muscle H, and if we call the form most abundant in skeletal muscle M, then the actual enzyme was a combination of four protomers, that is to say an oligomer, made up of various combinations of H and M. In skeletal muscle, the oligomer was MMMM, in heart it was HHHH, and in other tissues there were different mixtures such as MMHH. Each different mix was a different isozyme, and each differed in the details of its kinetics.

The existence of enzymes in oligomeric forms is now known to be very common indeed, particularly when those enzymes play a crucial role in controlling the function of a cell. Acetylcholinesterase is no exception, and recently there have been abundant papers pointing to the different isozymes of acetylcholinesterase, although there is still a great deal of uncertainty about their physiological significance. One should always be aware of the possibility that one might create apparent isozymes artificially. For instance, if proteinases present in a tissue cause partial degradation of the enzyme without loss of function, one is quite likely to create an artificial isozyme, which might be interesting enzymologically but tells one nothing of events in the intact organism.

Dr. Tripathi and I started the exploration of this situation in houseflies, and indeed we soon showed that there was a minimum of seven isozymes in houseflies, four in the head and three in the thorax. Fig. 1 shows data on the soluble components of housefly head acetylcholinesterase, and also on the soluble component of housefly thorax acetylcholinesterase. The crude mixture was run on the gel, and the location of the enzyme was established by staining the gel by the procedure of Karnovsky and Roots (1), in which the hydrolysis of acetylthiocholine at the location is detected by making a copper salt which precipitates out as a dark band. You will see that the bands are well separated, and that none of the four head isozymes appears to correspond to any of the three thoracic isozymes. Chiu and Tripathi (2) were able to work out conditions for the development of these colors which were so precisely controlled that one could make kinetic measurements right on these analytical gels. This is an immense convenience over the other alternative approach, which involves large-scale preparative separation of isozymes, taking them off their columns, and then performing classic kinetic studies. We showed that good Michaelis-Menton plots can be obtained from studies involving different substrate concentrations, from which one can compute the Michaelis constant of the enzymes upon the gels. One could also incubate them with inhibitors for varying lengths of time, and calculate the inhibition constants. Consequently Tripathi was able to establish the substrate and inhibition kinetics for the various housefly isozymes (3) and found that the Michaelis constants for the four head isozymes varied modestly, over a range of 4.2-fold. It was of even greater importance to note that the

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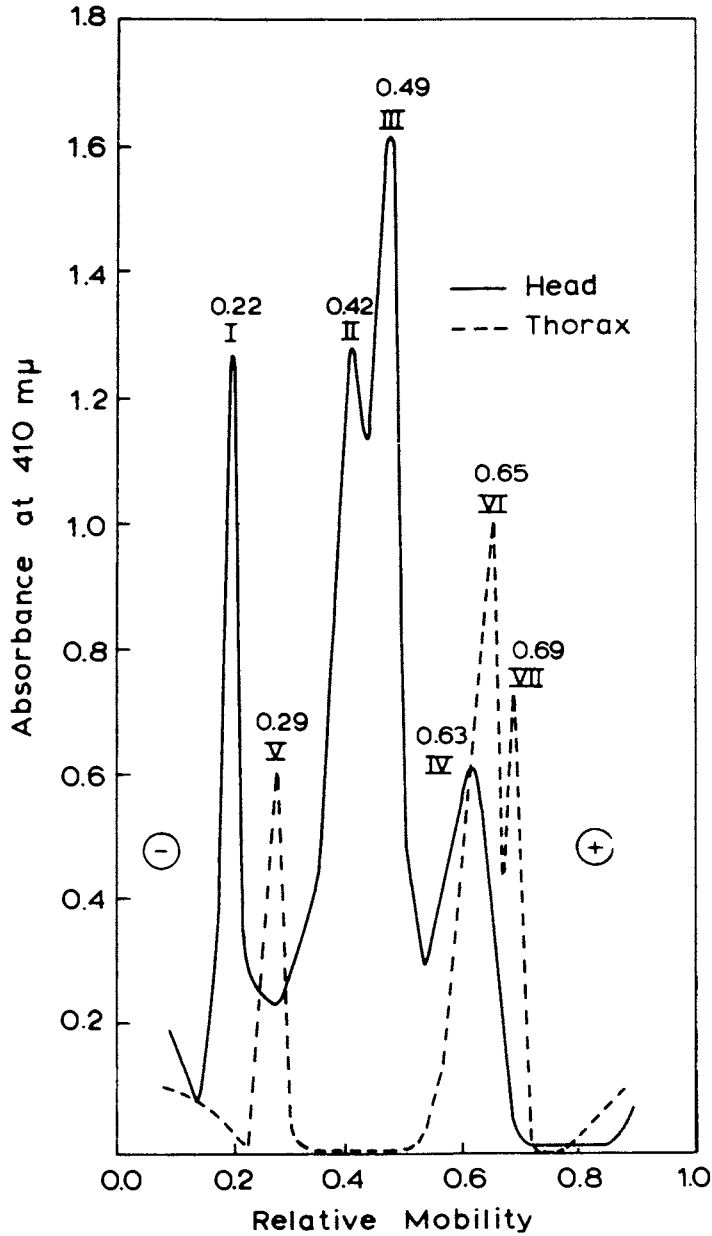

Figure 1. An electropherogram of AChE isozymes of housefly. The gels were incubated with 1 × 10⁻³ M ATCh for 45 min for head and 90 min for thorax. The number heading each peak is the R_m *value.*

isolated isozymes differed significantly in their sensitivity to inhibition by various organophosphates *in vitro*. The range of sensitivities was not very large, the biggest variation being 2.3-fold in the case of malaoxon. We next turned (4) to the question of whether these differences had any significance in the intact insect. We poisoned houseflies by various inhibitors, and studied the time-course of poisoning of each of the isozymes. Figure 2 shows just one example, demonstrating the inhibition of the head and thoracic isozymes in the course of poisoning the housefly by an LD_{50} dose of paraoxon. You will see that the isozymes vary tremendously in their response, some being only transiently inhibited and rapidly returning to normal, whereas others are profoundly inhibited and stay totally inhibited. You should note that these data are from groups of houseflies, so that what you are seeing is the average effect in a population, of which at 24 hours half were killed by the LD_{50} and half survived. We must now ask ourselves the physiological significance of these observations. Table 1 contains the summary of the results of all the inhibitors upon all the isozymes, the point we have picked being the trough of activity of that particular isozyme, i.e. the maximal inhibition (or minimum activity) which is seen for that isozyme. We then

TABLE 1
Minimal Percent 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 and m is the percentage minimal activity (from this Table).

asked ourselves the question, can it be that death of the insect occurs when a particular isozyme reaches a particular degree of inhibition? You will see that this is a much more meaningful question now than it was in those days when we used to pose it for

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the whole acetylcholinesterase of, for instance, the head. It would not be expected that a mixture like that would provide much information.

Note first that thoracic isozyme VII is always maximally inhibited, and that it is totally inhibited in the whole population, including survivors and victims. Consequently it cannot be that this is an important isozyme, since the survivors get by very nicely without having any of it. Next we ask, is there any one isozyme which reaches a particular maximum of inhibition with all these quite diverse organophosphates? First you should notice that most isozymes show a great deal of variation amongst the different phosphates. But you will note that thoracic isozyme V shows remarkably little variation with these different inhibitors. It would appear that the LD_{50} dose of any one of these is just correct to produce a minimal activity of about 19%. If our argument is correct, and there are obviously plenty of loopholes, it should follow that isozyme V is the true target of organophosphorus poisoning, and that studies of any of the others (or the whole mixture) throw rather little light upon the significant poisoning process.

Whilst on the subject of housefly acetylcholinesterase I want to report to you a recent exciting finding (5). Dr. Matthysse at Cornell had found a local population of houseflies in the field which was remarkably resistant to the phosphorus insecticide Rabon We wondered whether this especially high resistance could be due to an unusual mechanism, and particularly we wondered whether it could be due to a mutation of the acetylcholinesterase. Although such a resistance is a fairly obvious one, it has been looked for without success very many times in houseflies for the last fifteen years. I am delighted to tell you that in this case we were very fortunate, and found that indeed the resistant houseflies had developed an enzyme, by mutation, or by selection of a variant gene, which had a profound insensitivity to Rabon. When this field housefly was brought into the laboratory, and subjected to selection by high concentrations of Rabon over twenty generations, it was more than 1500-fold more resistant to Rabon, in comparison with a group obtained from the same parent population, without Rabon selection. Table 2 shows that the mutant enzyme was not much different from the wild enzyme as far as its K_m for acetylthiocholine was concerned, although in fact its quantity was rather greater in the resistants as judged by the increase in maximum velocity of substrate hydrolysis given by a particular concentration of enzyme. But the Table shows that there was an enormous difference in the sensitivity of the mutant enzyme to Rabon. Using a method I shall describe later, we were able to measure separately K_d, the dissociation constant, which is the reciprocal of the affinity of the inhibitor for the enzyme; k_2, the phosphorylation constant, which measures the ability of the inhibitor, once bound to the enzyme, to react with it to give the phosphorylated inactive form. As you can see, the affinity was reduced about 500-fold, whereas the ability to phosphorylate

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TABLE 2
Soluble AChE of Rabon-Resistant (R) and Sensitive (S) Houseflies

	S	R	Ratio
Acetylthiocholine			
K_m	0.95×10^{-5}M	3.26×10^{-5}M	3.4
V_{max}	18.5	68.3	3.7
Rabon			
$10^7 \times K_d$ (M)	0.48	275.0	573
k_2 (min^{-1})	0.59	1.64	2.8
$10^{-6} \times k_i$ ($M^{-1}min^{-1}$)	12.3	0.06	205

the enzyme subsequent to binding was in fact somewhat greater in the mutant than in the wild enzyme. There can be little doubt that this enormous reduction in sensitivity to the inhibitor is fully adequate to account for all or most of the observed resistance of the housefly to poisoning. We have now explored a number of other phosphates and carbamates, and in every case the mutant enzyme is unusually insensitive to inhibition, in every case it is due to a loss in affinity rather than reactivity, and in every case the housefly is resistant to poisoning by the inhibitor.

Quite apart from telling us something about a new way in which insects can fight back against poisoning, the finding tells us a good deal about the enzyme acetylcholinesterase. Firstly, it is apparent that the mutant enzyme can bind substrate almost as well as the wild enzyme, but that it binds phosphates and carbamates extremely poorly. This finding must mean that the binding sites for phosphates and carbamates are quite different from the binding sites for the substrate. Although that does not seem a surprising conclusion, very much of the discussion of the relations between structure and activity of inhibitors has been based upon the assumption that the binding sites are similar for substrates and inhibitors, although I hope to provide quite different evidence later in this lecture to show you that the assumption is quite unwarranted.

A completely different point is provided by a study of the isozymes of the mutant enzyme. A scan of the isozymes looks remarkably similar to a scan of the wild enzyme. This, and the fact that all of the acetylcholinesterase activity of the head shows insensitivity to organophosphates, demonstrated that the mutation which occurred was such as to bestow insensitivity upon all the isozymes. Since it is extraordinarily unlikely that a whole series of mutations occurred simultaneously, this makes it very likely that all the head isozymes are under the control of a single gene, whose mutation affected them all similarly. This

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rules out the possibility that each isozyme was synthesized under the control of a different gene. Instead, a single gene must have had at least a part to play in the case of all head isozymes. One possibility is that the subsequent building up of the basic protomers was under the control of quite different genes, each of which could build up its own favored isozyme. A more likely possibility is that any given isozyme is made up of a combination of catalytically-active and catalytically-inactive protomers, and that the different isozymes are made up of a common catalytic protomer, with differing quantities or types of inactive protomer, to change the physical properties and no doubt the cellular locations quite profoundly. Thus the physical properties and location would vary, but the catalytic component would always be the same.

Now I want to turn to a completely different acetylcholinesterase, that derived from bovine red blood cells. It has been very popular because of its easy commercial availability, and by pure good luck it turns out to have almost exclusively one isozyme, so that the data from years past does not have to be reevaluated. Let me begin by going back to the question of the two quite different steps involved when a phosphate or carbamate attacks acetylcholinesterase. As already mentioned, there are two discrete steps, the first being the binding of the inhibitor to the surface of the enzyme, which is governed by a dissociation constant (K_d); when the constant is very small the affinity is very high. The second (k_2) step involves the actual phosphorylation or carbamylation of the enzyme to lead to the inhibited form. For many years it was extremely difficult to sort out these steps, and we all worked experimentally as if the reaction could be correctly described by a one-step process, in which enzyme plus inhibitor was converted to inhibited enzyme. This progressive reaction could be described by a rate constant k_i. Hundreds of papers have been written describing the influence of various changes in inhibitor structure upon the size of k_i, and therefore upon the overall potency of the inhibitor. Ten years ago, Main pointed out kinetic and experimental tricks which could be done to separate out these two steps which were combined in the k_i step; and he demonstrated that $k_i = k_2/K_d$. The reason it is so important to separate out these steps is that changes in the molecule which improve the K_d step may very well have an adverse effect upon the k_2 step. What insecticide designers need to know is the factors which will improve K_d and also improve k_2, and this can only be done by separate evaluation of structure-activity relationships upon the two steps.

The reason why it took such a long time to appreciate this point was as follows. Because of the extreme reactivity to the organophosphates, and because normal laboratory experiments on inhibition are conveniently carried out over a span of ten or twenty minutes, it was natural to work with concentrations of inhibitor that gave inhibitions in this time. Thus concentrations in the order of 10^{-6} - 10^{-8} M were commonly used, and if much

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higher concentrations were employed, the enzyme was inhibited so rapidly that one could not follow it conveniently.

Now as we shall soon see, the binding constants of phosphates are in the order of 10^{-4} M, which means that if one adds 10^{-4} M inhibitor to the enzyme, at that very moment about 50% of the enzyme will be converted to the complexed form. If we work with an inhibitor concentration of 10^{-6} M, we will get an amount of enzyme converted to enzyme-inhibitor complex which is only 1% of the total enzyme. This is an extraordinarily difficult amount to detect experimentally. Therefore the problem reduced to doing experiments in very short times, so that one could employ very high inhibitor concentrations. Very recently we have solved this problem experimentally in a convenient way. One procedure which is good enough for most inhibitors has already been published (6). It involves adding the inhibitor to an ongoing reaction between substrate and enzyme, which is being recorded on a recording spectrophotometer. One can use very high inhibitor concentrations, and the reaction goes to completion in a minute or two, and can comfortably be followed on a recording spectrophotometer. More recently, we have used the stopped-flow technique to measure this reaction (7) The enzyme is held in one hypodermic syringe and a mixture of substrate and inhibitor is held in another. One presses a button, and a solenoid rams the two hypodermic plungers down, and their contents are mixed almost instantly in a special vortex chamber, and propelled out into a reaction chamber. while this is going on, the rate of color change in the mixture is monitored by a photomultiplier tube hitched up to an oscilloscope, and in this way one can trace out the course of the reaction over a few milliseconds. The actual equipment is moderately expensive, the stopped-flow instrument itself costing about $12,000, and the oscilloscope being an added cost. Nevertheless, with this instrument one can easily measure the dissociation constants and the phosphorylation constants of even the most reactive inhibitors. Figure 3 shows an oscilloscope trace of an actual reaction. If no inhibition had occurred, the line would continue almost vertically over the time-course of the system. In fact the line falls away steadily, and the reaction is almost complete within about one second.

Finally I want to turn to questions of the topography around the active site of acetylcholinesterase, and the kinds of forces which bind inhibitors to the surface of the enzyme. In effect I want to discuss the K_d step, and demonstrate that the situation is a great deal more complex than we have previously thought. Textbooks commonly show the early Wilson model which implies that there are only two sites of importance on the enzyme. One is the esteratic site, which probably contains a serine hydroxyl, and is the one which is acetylated by acetylcholine, carbamylated by carbamates and phosphorylated by organophosphates. The other site is now known as the anionic site, and undoubtedly plays a big role in the binding of the quaternary nitrogen of acetylcholine onto

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the enzyme surface. But very often people have assumed that any kind of special binding of inhibitors or other substrates that occurs must involve binding to the anionic site. This is extraordinarily unlikely, particularly when the substrate or inhibitor does not have an ionic group on it, and very few organophosphates or carbamates do have such ionic groups. What I have said is fully confirmed by the experiments I described earlier, showing that the binding site for acetylcholine in the mutant enzyme is little changed, whereas the binding sites for all phosphates and carbamates so far studied are profoundly altered. In recent years we have produced a good deal of evidence for at least three kinds of additional binding sites: these are a hydrophobic site, an indophenyl site, and a charge-transfer complex site. I shall discuss only the charge-transfer complex (CTC) site today.

Let us begin by defining CTC. In order to form such a complex, it is required that one of the components (the donor) can rather easily lose an electron from its outermost orbital, that is to say that it has a low ionization potential. It is also required that the other compound (the acceptor) can readily accept an electron into its outermost orbital, that is to say it has a high electron affinity. If these two conditions are met, and also there is an ability of the molecules to approach very close to each other, then an electron from the one which can easily lose one may become involved in a new bonding orbital, so that the electron is shared between the two molecules. There are two consequences. One is that a complex is formed between the two molecules, whose affinity can be measured by an association or a dissociation constant. The other is that a new spectrophotometric absorption band appears, related to the new orbital. In recent years Dr. Hetnarski and I have been exploring the question of whether such complex formation can occur between carbamates and acetylcholinesterase, and I would like to present the evidence, which is at the moment indirect.

Figure 4 provides direct evidence that aromatic carbamates act as electron donors to complexes with a model acceptor, in this case tetracyanoethylene. The figure shows that phenyl methylcarbamate and tetracyanoethylene when present by themselves have no absorption in the visible spectrum. But when they are mixed together, a new absorption band is formed, which is excellent evidence of a new complex with a new molecular orbital. We have now performed studies such as these with a very large number of carbamates, and we find the expected result. That is, if these aromatic compounds have no substituents on the ring or else have electron pushing substituents, they can act as very good donors in the formation of CTC. But if they have strong electron-withdrawing groups, such as the paranitro group, then they are very poor donors and do not make complexes.

To summarize several years' work (8,9) we believe that the variations in anticholinesterase activity of aromatic methylcarbamates can be fully accounted for in terms of only two factors

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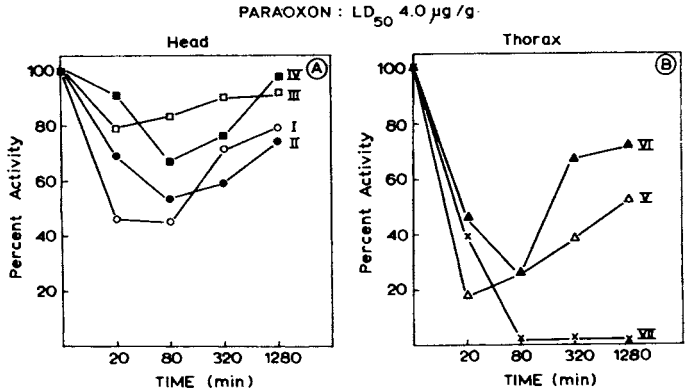

Figure 2. The effects of paraoxon on the isozymes of head (A) and thorax (B)

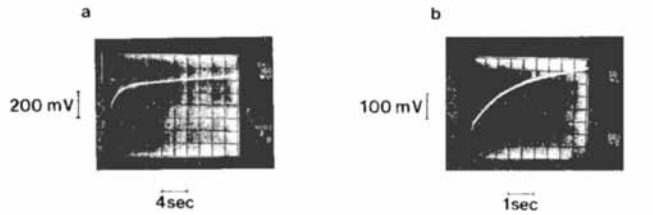

Figure 3. Typical photographic records of oscilloscope traces showing time–course of the reaction during inhibition of acetylcholinesterase (100 µ/ml) by paraoxon (2.5×10^{-4}M) in the presence of p*-nitrophenyl acetate (1 mM) in 2.1% ethanol — 0.1*M *phosphate buffer at pH 7.*

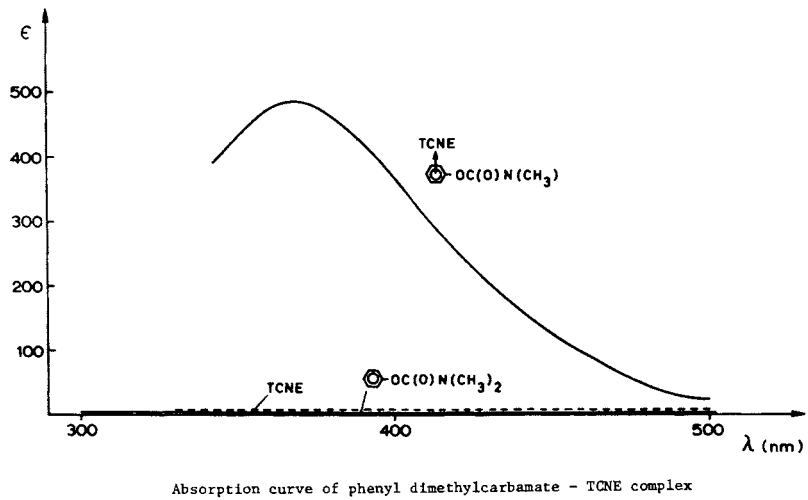

Figure 4. Absorption curve of phenyl dimethylcarbamate–TCNE complex

One is the ability to act as a donor in CTC formation, which we measure as the ability to form CTC with a model acceptor. We also need to consider the hydrophobic character of the carbamate, which we measure by the pi value for the substituted carbamate. The pi value is defined by Hansch (10) as the logarithm of the partition coefficient of the compound of interest, minus that of its unsubstituted parent compound. This pi value is, under ideal conditions, unrelated to the nature of the organic solvent involved in the determination of the partition coefficient.

Perhaps the most persuasive argument that the two factors I have mentioned can account for the activity, is the demonstration that the predicted affinities, based upon the formulae which we have developed, give remarkably fine agreement with the measures which we have actually observed. Let me stress that we are talking only about the first step of the reaction between the carbamate and the enzyme, that is the formation of the carbamate-enzyme complex, characterized by the dissociation constant K_d. We have explored four different series of anticholinesterases. One is the phenyl methylcarbamates which are substituted in the para position. The second is the comparable group of compounds substituted in the meta position. The third series is a novel one, that of arylmethyl methylcarbamates. We found that these were noncarbamylating carbamates, and owed all their inhibitory activity to simple complex formation without carbamylation.

When we observed that these arylmethyl methylcarbamates had quite good complexing activity with the enzyme, we argued that in view of the fact that the complexing activity was entirely due to the aromatic ring, then compounds which only contained an aromatic ring should be anticholinesterases also. We therefore looked at the fourth group of anticholinesterases, which were simple aromatic compounds, such as benzene, chlorbenzene and naphthalene. And indeed we observed that these simple aromatic hydrocarbons were perfectly good reversible inhibitors of acetylcholinesterase. Once again, their ability to act as inhibitors was precisely paralleled by their ability to form charge-transfer complexes with our model acceptor, tetracyanoethylene.

The evidence I have presented suggests that in addition to the anionic binding site, the hydrophobic site and the indophenyl site, there is a CTC site as well. Figure 5 shows a diagram of how these binding sites could be arrayed around the catalytic site itself. It should be no surprise that the catalytic site is surrounded by a whole variety of potential binding sites. Acetylcholinesterase, like any other enzyme, is a polypeptide; its catalytic site is therefore surrounded by a whole sea of amino acids. Each of these amino acids provides a potential binding site. Any carboxylate ion could bind cations, any protonated site could form hydrogen bonds or could bind to an anionic group; any aromatic group could enter into pi-pi complex formation, and so on. The model suggests only four fairly well characterized binding groups around the catalytic site, but there are probably many more, as one

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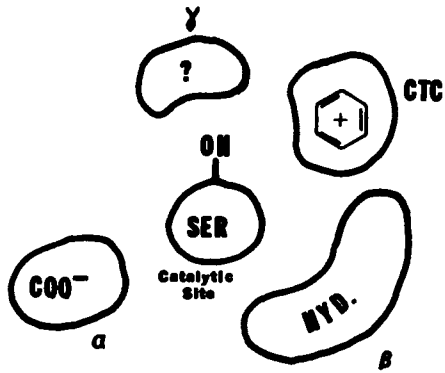

Figure 5. A speculative model of the arrangement of four binding sites around the catalytic serine of acetylcholinesterase. α: anionic site; β: hydrophobic site; γ: indophenyl site; CTC: charge transfer complex site.

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allows oneself to wander further from the catalytic site. All these binding sites present potential points of attachment for inhibitors. If compounds can be designed with extremely high affinity for binding sites which are close to the catalytic site, then they can be excellent inhibitors even with fairly moderate reactivity towards the catalytic site per se. In all cases where a compound first binds and then reacts with the enzyme, the overall reactivity k_i can be described by the relation described above: $k_i = k_2/K_d$; that is to say that the overall reactivity, as measured by k_i, is the ratio of the chemical reactivity divided by the association constant. If we can come up with a compound which has a terrific superiority in terms of its association constant, then it can be a fine inhibitor in spite of having only a modest reactivity, as measured by k_2.

In conclusion, acetylcholinesterase is seen to be a much more complex enzyme than the early work implied. Quite apart from the additional binding sites for which I have presented evidence, you should recall the work on the mutant acetylcholinesterase in the resistant insect implies that the binding site for the natural substrate, acetylcholine, is on a quite different part of the molecule from the binding site for the inhibitors which we have explored in this regard. It may therefore be that we have only just begun to explore the potential sites which we can utilize in inventing new anticholinesterases. And we have not even begun to explore in a serious way the differences between the enzyme in the vertebrate and in the insect, so that we can expand greatly the number of selective compounds, whose selectivity is based upon differences in the nature of the target in vertebrates and in insects.

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Interactions of Pesticides with Cytochrome P-450

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Introduction

During the 1950's intense interest was generated in the oxidation of xenobiotics by microsomal enzymes prepared from mammalian liver. Much of this work was summarized, in an important review, by Brodie et al. in 1958 (1). At about the same time Klingenberg (2) and Garfinkel (3) independently described a carbon monoxide (CO)-binding pigment in mammalian liver, subsequently called cytochrome P-450 as a consequence of the prominent peak at 450 nm in the CO-reduced cytochrome optical difference spectrum. Cytochrome P-450 was established as the pigment necessary for oxygen activation in drug and steroid oxidation by Estabrook et al. (4) and since that time has been shown to be the key element in the microsomal electron transport system responsible for a wide range of mixed-function oxidations of both xenobiotic and endogenous substrates. A personal and incidentally, highly amusing, account of the discovery of the function of cytochrome P-450 has recently been published (5). Many reviews and conference proceedings have considered various aspects of cytochrome P-450, the most recent being the proceedings of a conference held in Palo Alto, California (6).

Biochemical investigations have been complicated by the particulate nature of cytochrome P-450 and the other enzymes associated with it and it has proven particularly refractory to solubilization and purification. Spectroscopy of microsomal preparations is complicated by the fact that turbid samples scatter light and that light scattering is a function of wavelength. This being the case, changes in the absolute spectra due to ligand addition are seen as variations in a constantly changing baseline. Optical difference spectroscopy avoids this problem by recording only the changes in the spectrum rather than the absolute spectrum itself. This is done by placing the microsomal preparation in both reference and sample cuvettes of a split beam spectrophotometer, thus balancing both light-scattering and the absolute spectra of all pigments in the preparation and thereby obtaining a flat baseline.

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The ligand of interest is then added to the sample cuvette and the extent to which it perturbs the absolute spectrum recorded as a difference spectrum. Difference spectroscopy is subject to many problems which will not be discussed here. These problems have been discussed, and guidelines for the proper interpretation of cytochrome P-450 difference spectra proposed, by Mailman et al. (7).

The most important difference spectrum of cytochrome P-450 is that formed by the interaction of CO with the reduced cytochrome, since this spectrum is the basis for the determination of cytochrome P-450 (8,9). Type I and II spectra (10) are formed by the addition of various ligands to the oxidized form of cytochrome P-450. Type I has a peak at 385 nm and a trough at 420 nm while type II has a peak at about 430 nm and a trough between 390 nm and 410 nm. The former is caused by a large number of ligands and is believed to be a result of binding to a lipophilic site somewhat removed from the heme iron, while the latter, formed primarily by organic nitrogen compounds, is believed to be caused by binding to the heme iron. The modified type II (415-420 nm peak, 390 nm trough) has also been called the reverse type I (11,12,13) since one hypothesis holds that it is the result of the displacement of an endogenous type I substrate in the sample cuvette. It is possible, however, that this spectrum may represent the binding of a nucleophilic oxygen atom to the heme iron (7). The type II spectrum formed by n-octylamine is of particular interest since it occurs in two forms, one with a single trough at 390 nm and the other with a double trough at 394 and 410 nm. These forms have been used in the characterization of qualitatively different forms of cytochrome P-450, both in mammals (14) and in insects (15,16).

Ethyl isocyanide and type III spectra are the result of interactions with the reduced form of the cytochrome and consist of two peaks in the Soret region, these peaks being in pH dependent equilibrium. Following the demonstration that these spectra were formed by methylenedioxyphenyl compounds the term type III was proposed as a general term for all pH-dependent double Soret spectra (17).

Although solubilization and complete purification of cytochrome P-450 has been an elusive goal, a number of methods recently developed have allowed partial purification (18,19,20). They involve detergent solubilization, with or without sonication, followed by such well-known techniques as ammonium sulfate precipitation, absorption on calcium phosphate gel and DEAE-cellulose chromatography. Glycerol is usually added to stabilize cytochrome P-450. Using these methods some purification and reconstitution have been achieved, but it is clear that the best technique for either solubilization or purification has not yet been found.

Most is known about the cytochrome P-450 of mammalian liver and adrenal gland, but more recently, cytochrome P-450 of other mammalian tissues has been examined, including lung, small intestine, kidney and testes. It is apparently ubiquitous, its

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occurrence having been noted in at least 14 species of mammal, 17 species of insect and one or more species of birds, reptiles, amphibians, fish, bacteria, fungi and higher plants (21).

Since the initial observations of Brown et al. (22) it has become clear that mixed-function oxidase activity of mammalian liver can be induced by many xenobiotics, including many pesticides. The early work was summarized in an important review by Conney (23). All inducers do not induce the same enzyme activities. Some, such as phenobarbital, are more general, while others, such as the polycyclic hydrocarbons, induce a much narrower ranger of enzyme activities. Differences are also apparent in the cytochrome P-450 spectra, being seen in λ max of the CO difference spectrum as well as in the pH equilibrium of the ethyl isocyanide difference spectrum (24). Recently, Lu et al. (18) have shown, using enzyme systems reconstituted from partially purified enzymes, that these differences reside in the cytochrome P-450 moiety of the electron transport chain. Induction is also known to occur in other mammalian organs, and in insects, but is less well understood in these cases.

Qualitative differences are also of interest from the comparative point of view since they indicate the degree of difference or similarity between the cytochrome P-450's of different species. Comparisons of type I, type II (including n-octylamine), type III (including ethyl isocyanide) and CO spectra from a number of different species of mammals, insects and fish (25) indicate that qualitative differences between species are common. In a series of studies on the cytochrome P-450 of insecticide susceptible and resistant strains of the housefly (15,16,26) it has been shown that marked qualitative differences exist between the cytochromes and, moreover, that these differences are under genetic control.

Evolutionary considerations (21) led to the conclusion that the mixed-function oxidation of xenobiotics was an adaptation, in animals, for the detoxication of dietary lipophilic toxicants of low, but appreciable, toxicity. Induction serves to provide an additional level of flexibility permitting adaptation to conditions of continuing intake. The more substrate specific cytochrome P-450's of microorganisms or of mammalian organs mixed-function oxidase not involved in detoxication, would appear to be more specific evolutionary developments from the same common progenitor that gave rise to the cytochrome P-450 of low substrate specificity which is used by animals for the detoxication of xenobiotics.

Pesticides may interact with cytochrome P-450 in a number of ways, either directly as substrates or as inhibitors of mixed-function oxidase activity, or indirectly, as inducers. In the former case a spectral perturbation, which can be measured by difference spectroscopy, usually occurs, although exceptions to this will be noted.

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Ligand Binding to Cytochrome P-450

General Considerations. Type I spectra are caused by many compounds, including drugs (10,27), pesticides (28-30) and steroid hormones (31). Other reports have concluded that type II binding is caused by aromatic amines (32), aliphatic amines (14), isocyanides (33), certain steroids (14,31) and alcohols (11).

Schenkman et al., (10) suggested that type II spectra were due to the formation of a ferrihemochrome from the interaction of a basic amine and a ferrihemoprotein. The above references suggest that other compounds may also play a similar role. Recent reports (7,30,34-36) support the contention that steric and basic features of nitrogen are important in the formation of type II spectra.

We have recently carried out a study of structure-function relationships in the binding of ligands to oxidized cytchrome P-450 in hepatic microsomes from the mouse (7). Using derivatives of pyridine, pyrrolidine and piperidine as well as benzonitriles, phenols, alcohols and other compounds, it was concluded that a type II spectrum was associated with ligands with a nitrogen atom in which sp^2 or sp^3 non-bonded electrons are sterically accessible. Using, for example, methyl derivatives of pyridine it can be seen that accessibility is important since 3 and 4 substitutions have no effect while 2 or particularly 2 and 6 substitution causes a significant decrease in the size of the spectral shift. Similar findings are seen with carboxyl derivatives of pyridine except that the size of all spectra are reduced, due, presumably, to the decrease in lipophilicity.

The results with oxygen compounds are of importance in the interpretation of the so-called modified type II or reverse type I spectrum. Without going into any detail, the simplest explanation of the data (7) is that oxygen atoms may act like nitrogen atoms - that is, as nucleophiles replacing another ligand at the fifth or sixth ligand position of the heme groups of cytochrome P-450 causing a bathochromic shift. It is of lower intensity and shorter wavelength due to the lower nucleophilicity of the groups involved. Although this argues against the 'endogenous substrate displacement' (11-13) explanation of this spectral type, it is entirely possible that both hypotheses may be true, depending on the ligand and the cytochrome P-450 involved. More recent evidence (37) based on lipid extracted microsomes has supported the contention that the modified type II spectrum is not due to the displacement of endogenous substrates.

In the companion study (35) on the binding spectra of cytochrome P-450 from the abdomens of insecticide susceptible (CSMA strain) and insecticide resistant (Fc strain) houseflies it became apparent that, although the same basic requirements held true for the housefly cytochrome as for the mouse cytochrome, there were interesting differences between insect and mammal and also between one insect strain and another.

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The lack of type I spectra in susceptible or wild-type flies previously noted (15,16,26) was confirmed in the case of 18 more ligands which gave clearly discernible type I spectra with the resistant Fc strain but not with the susceptible CSMA strain. This difference is probably the most dramatic one which has been recorded between the cytochrome P-450's of different animals.

Many of the differences between housefly and mouse cytochrome P-450 indicate that the heme may be more accessible to ligand binding in the former. Such differences include:

1. Some compounds such as 2-ethyl piperidine, propionitrile and n-hexanol give mixed type I and II spectra with mouse microsomes but a type II with housefly microsomes.
2. Some compounds such as 1-cyclohexenyl pyrollidine and sec-butanol are type II with housefly microsomes but type I with mouse microsomes.
3. Compounds such as tetrahydrofuran and tryptophane give type II spectra with housefly microsomes but give no detectable spectra with mouse microsomes.

Jefcoate et al., (14,38) noted a double trough in the n-octylamine spectrum with rabbit liver microsomes and related this to high and low spin forms of cytochrome P-450. They also speculated that alkylamines were bound to two sites on each form of the cytochrome. A double trough occurs in the n-octylamine spectrum of CSMA microsomes but a single trough in the case of Fc microsomes (15,16,26,35).

In houseflies (35), benzimidazole exhibited a concentration dependent double trough in which a single minimum at 410 nm became a double trough at 410 and 392 nm as the concentration was increased, and finally, a single minimum at 392 nm. If these findings are interpreted to represent two binding sites for which benzimidazole has either a high affinity (perturbation at 410 nm) or a low affinity (perturbation at 392 nm) then they may help to explain the observation that the range of peak and trough positions in the type II difference spectrum of houseflies is much greater than that in mouse hepatic microsomes.

The response of both Fc and CSMA microsomes was similar, but certain differences could be noted. Spectral magnitudes, relative to the CO-spectrum were always smaller in the CSMA than in the Fc microsomes. Other differences appear to be related to the fact that there is no type I component in the CSMA spectrum e.g. 1-cyclohexyl-2-pyrrolidinone is type I with Fc microsomes but type II with CSMA microsomes, 2,6-lutidine gives a mixture of types I and II with Fc but type II with CSMA. Whether this is also responsible for the fact that spectral maxima and minima, if they are not identical, are always at a shorter wavelength in Fc microsomes than in CSMA, is not yet apparent (35).

Pesticides. The involvement of the microsomal mixed-function oxidase system in the metabolism of pesticides is well documented (39-41). The spectral perturbations caused by the first step in this process, binding to oxidized cytochrome P-450 can, as

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indicated above, be studied by optical difference spectroscopy. Most studies to date (10,29,30,42,43) have involved only a small number of compounds and/or species, making comprehensive comparisons difficult. We (44) have recently studied the spectral interactions of a large number of pesticides with hepatic microsomes from the rat, mouse, rabbit and sheep as well as abdominal microsomes from insecticide-resistant (Fc) and susceptible (CSMA) houseflies.

Only three of the 48 insecticides tested caused type II spectra with microsomes from all species investigated, namely nicotine, anabasine and zinophos. These spectra are typical, being characterized by a peak at around 425 nm and a trough between 390 and 410 nm (Figure 1A). Most of the insecticides caused a type I spectral change (Figure 1C) with all microsomes except those prepared from the CSMA susceptible strain, thus confirming previous reports (15,16,26,35) of the absence of type I spectra in this strain.

Spectral magnitude was observed to vary not only with the ligand but also with the species. Thus in the closely related group DDT, kelthane and TDE, although DDT was found to give the smallest type I spectrum in all species tested, kelthane gave the largest spectrum with microsomes from sheep, rat and Fc houseflies and TDE gave the largest spectrum with microsomes from mouse and rabbit.

Some of the organophosphates tested (dimethoate, trichlorfon, azodrin) and a carbamate (methomyl) caused either mixed type I and type II spectra or showed concentration dependent changes from one spectral type to another, raising the possibility of their binding to both type I and type II sites on the cytochrome P-450 molecule. Menazon did not cause any spectrum with microsomes from CSMA flies, evoked a type I response with microsomes from Fc flies, a type II response with microsomes from mouse liver and a mixed response with hepatic microsomes from sheep, rabbit and rat. It seems clear that qualitative differences between species, either in the cytochrome P-450 or in the microsomal membrane, are common.

In addition to type I and type II interactions, some unusual difference spectra were observed. The difference spectra of pyrethrins, allethrin and bioallethrin with housefly microsomes from either Fc or CSMA strains has a peak at 415-418 nm and a trough at 443-445 nm (Figure 1D). In mammals, all of these compounds give rise to type I spectra (Figure 1C).

Rotenone did not yield any detectable difference spectrum with microsomes from mouse, rat or either Fc or CSMA houseflies. However, with microsomes from either sheep or rabbit, a spectrum with a peak at 418 nm and a distinct trough at 390 nm was produced (Figure 1B). Carbaryl, a type I ligand with the other species, produced an unusual spectrum with hepatic microsomes from the rabbit, with two peaks at 385 and 407 nm and a trough at 427 nm (Figure 1E).

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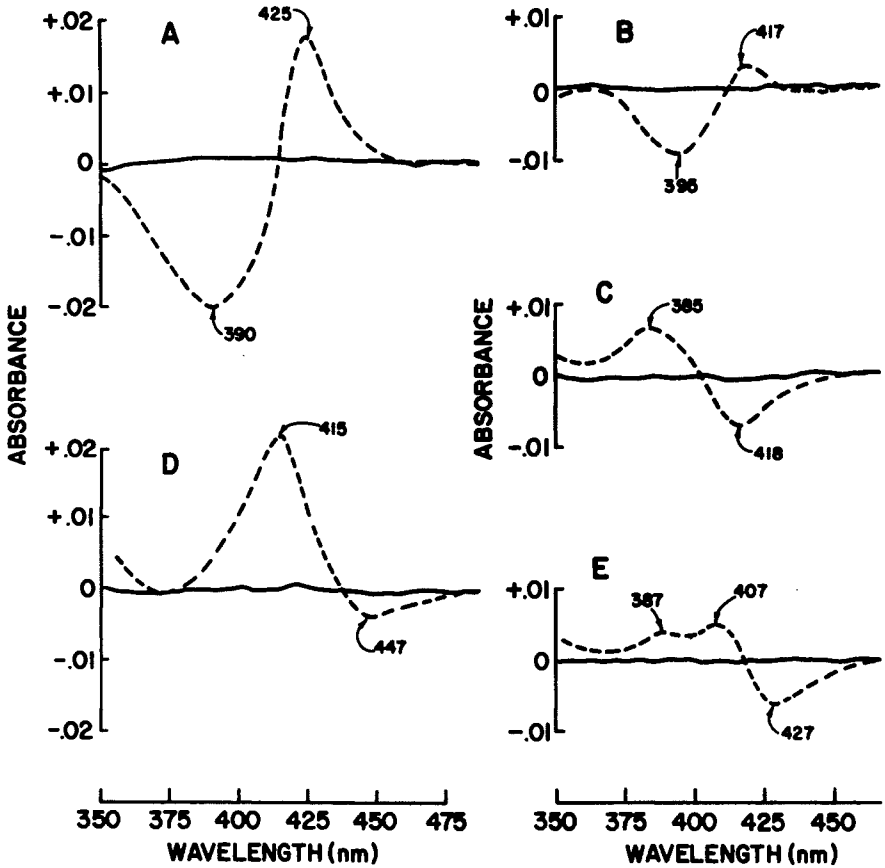

Figure 1. Optical difference spectra of selected pesticides. A is the type II spectrum of nicotine obtained with mouse liver microsomes, B is the spectrum of rotenone obtained with rat liver microsomes, C is the type I spectrum of pyrethrins obtained with mouse liver microsomes, D is the spectrum of pyrethrins obtained with abdominal microsomes of Fc houseflies and E is the carbaryl spectrum obtained with rabbit liver microsomes (44).

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The data generated by this study is in agreement with the previous report on mouse hepatic microsomes (30) and confirms the studies of the structure-function relationships necessary for type II spectrum formation (7,35).

Synergists. Perry and Bucknor (45) and Matthews and Casida (46) using houseflies and Matthews *et al.*, (47) using mice, first demonstrated that treatment, *in vivo*, with piperonyl butoxide caused an apparent reduction in the cytochrome P-450 level. Subsequently, Philpot and Hodgson (17,48,49) demonstrated that piperonyl butoxide reacted with the reduced form of the cytochrome to form a spectrally observable complex which prevented CO binding, thus explaining the apparent reduction in cytochrome P-450 level. The type III spectrum observed, following the incubation of piperonyl butoxide and microsomes in the presence of NADPH, has two peaks in the Soret region, one at 455 nm and one at 427 nm. These peaks are in pH dependent equilibrium and the spectrum cannot be displaced by CO or any other ligand tested. Franklin (50) has shown that the stability of the type III complex is unaffected by oxidative and conjugative reactions, mixed-function oxidase inhibitors, free radical scavengers and mixed-function oxidase cofactors.

With few exceptions (51) most of the methylenedioxyphenyl compounds caused type I spectra with oxidized microsomes which become type III on incubation with NADPH. The formation of a type I spectrum with oxidized microsomes is not obligatory for the subsequent type III interaction, however, since piperonyl alcohol, 3,4-methylenedioxyphenylacetonitrile and 3,4-methylenedioxyphenylacetanilide all cause type II spectra with oxidized microsomes, becoming type III in the presnce of NADPH.

Some compounds, such as cis and trans-methylenedioxycyclohexane give rise to a single peak at 427 nm when incubated with microsomes and NADPH and others, such as the bromo-, dibromo-, dichloro- and 1-bromo-6-methoxy derivatives of 3,4-methylenedioxybenzene caused the appearance of a peak at 450 nm, similar to that caused by CO, on the addition of dithionite (51).

The spectral interactions of methylenedioxyphenyl compounds with abdominal microsomes from Fc and CSMA houseflies (52) are essentially similar to those of mice hepatic microsomes (57). The presence of an apparent type I spectrum with CSMA microsomes and sulfoxide is of interest, as are the much smaller spectra of similar forms seen with other methylenedioxyphenyl compounds, since this spectrum was not formed with CSMA microsomes and any other ligand tested, including examples from many different chemical classes (35,44). Whether this represents a binding to a different site, which, by coincidence, perturbs the spectrum at the same wavelengths as do type I ligands in other organisms or a type I binding site which is much less accessible in CSMA housefly microsomes than in those from other organisms, is not known.

The cytochrome P-450 difference spectra of synergists other than the methylenedioxyphenyl compounds have not been studied in

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detail. Matthews and Casida (46) showed that MGK 264, 2-(2,4,5-trichlorophenyl)-propynyl ether, and 2-methylpropyl 2-propynyl phenylphosphonate gave type I spectra with mouse microsomes, while 5,6-dichlorobenzothiadiazole gave a type II spectrum at low concentrations which shifted to a mixed type I/type II at higher concentrations. Furthermore, the latter compound gave an unusual spectrum with a peak at 444-446 nm with reduced microsomes. The substituted imidazoles, a new class of synergists, are type II ligands which bind very tightly to the cytochrome (53). One of these, isopropylphenylimidazole, is reported to yield a type III spectrum with reduced microsomes (54).

The initial observation that methylenedioxyphenyl synergists affected oxidative metabolism was made by Sun and Johnson (55) on the basis of *in vivo* toxicity tests on houseflies. Hodgson and Casida (56,57) then demonstrated that piperonyl butoxide and sesamex inhibited the oxidative metabolism of carbamates by rat liver microsomes *in vitro*. When the available information on inhibition of mixed-function oxidases by methylenedioxyphenyl compounds is compiled, it is clear that this is a general phenomenon: over 50 such compounds inhibit the oxidation of almost as many substrates in numerous species of both mammal and insect (58). The spectral interaction referred to above represents the formation of a stable non-competitive inhibitor complex and both Philpot and Hodgson (49) and Franklin (59) have suggested that this complex is important in synergistic action. The kinetic complexities of methylenedioxyphenyl inhibition of microsomal mixed-function oxidase reactions are due, at least in part, to a transition from competitive to noncompetitive inhibition which accompanies complex formation. Franklin (59) has shown that piperonyl butoxide inhibition of ethylmorphine N-demethylation is competitive before complex formation and noncompetitive afterwards.

Suggestions by Casida *et al.* (60) that methylenedioxyphenyl compounds act as alternative substrates implies a competitive mechanism, while proposals by Hansch (61), Hennessy (62) and Ullrich and Schnabel (63), suggesting that methylenedioxyphenyl compounds form free radicals, carboxolium ions or carbanions respectively, all suggest a noncompetitive mechanism. The stable complex described above also indicates a noncompetitive mechanism, however, since the complex does not form with all of the cytochrome P-450 present, it is possible that competitive inhibition also plays a role in the synergistic process.

Although the mechanism by which the complex is formed has not been fully elucidated proposed mechanisms should account for the known facts. Structure-function studies have shown that an unsubstituted methylene group in the 2-position is necessary and it appears that interaction with this group involves one of its C-H bonds. The requirement for NADPH and oxygen suggests an active oxygen complex of cytochrome P-450 is also involved (see 58 for references).

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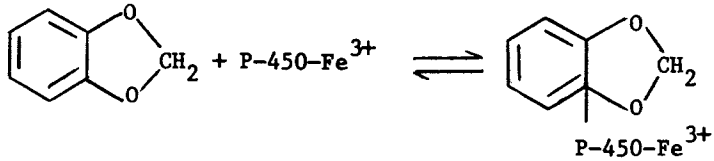
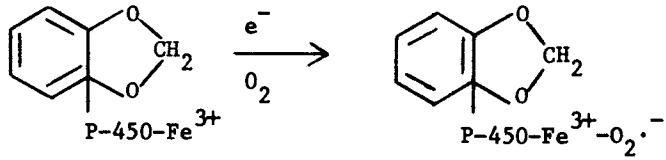
The merits of the proposals by Hansch (61), Hennessy (62), and Ullrich and Schnabel (63) have been discussed at length by Hodgson and Philpot (58) who concluded that present data do not permit a precise resolution of the differences between them but that production of free radicals, via homolytic cleavage of a C-H bond, is the most plausible.

One of the difficulties in assigning a mechanism to the interaction of methylenedioxyphenyl compounds with cytochrome P-450 is the lack of knowledge surrounding the nature of the active oxygen species involved. Wilkinson (64) suggested that an ion analogous to the percupryl ion complex $(Cu^{+}O_2)^{2+}$ implicated in phenolase action by Bright *et al.*, (65) was involved. The structure of such an ion would be $(Fe^{3+}O\cdot^{-})^{2+}$. It is of interest in this regard that phenolase is also known to be inhibited by methylenedioxyphenyl compounds (66).

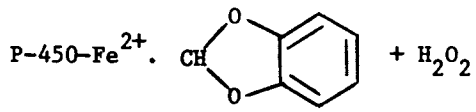
The following mechanism is a summary consistent with the above requirements. The first step involves the binding of the methylene dioxyphenyl compound to oxidized cytochrome P-450 to form a type I spectral complex:



This is followed by the incorporation of one electron, from NADPH, and oxygen to form an activated oxygen complex:



This complex abstracts a hydrogen from the methylenedioxy carbon and in the presence of excess NADPH the following products are produced:



Upon complete oxidation of the NADPH the ferrous form of the complex is formed.

Little is known of the mode of action of other types of synergists which inhibit mixed function oxidase reactions. Although

they may be competitive inhibitors, this would be a viable mechanism only if they bind tightly to the cytochrome or if the enzyme-substrate complex is relatively stable. In the case of some of the substituted imidazoles it is clear that the former conditions exists since the Ks values are in the range of 1 x 10^{-6} to 1 x 10^{-5} M (53). The possibility of some mode of action other than, or in addition to, competitive inhibition is raised by the demonstration of a type III spectrum for 1-phenylpropylimidazole (54) and the unusual reduced spectrum with a peak at 444-446 nm, for 5,6-dichlorobenzothiadiazole (47).

Relationship of Spectra to Metabolism

If the difference spectrum is a reflection of enzyme-substrate complex formation a correlation between spectral binding and rate of metabolism might be expected. Although almost all type I ligands are substrates for microsomal mixed-function oxidation, previous workers have seldom been able to demonstrate a relationship between Ks (ligand concentration at half maximum spectral size) and Km (Michaelis constant for the enzyme reaction). Exceptions to this include Schenkman *et al.*, (10) and Remmer *et al.*, (67), who demonstrated that using hexobarbital and aminopyrine with rat liver microsomes, Ks was approximately equal to Km.

Although few attempts have been made to correlate spectral characteristics with metabolism in the case of pesticides, it is apparent that the same generalizations can be made. Most type I pesticides are substrates but it is not known whether Ks is related in any meaningful way to Km. Mirex, a type I compound with microsomes from rat and mouse liver (28) as well as microsomes from sheep and rabbit liver and Fc housefly abdomens, does not undergo any known biotransformation in mammals or insects (68). On the other hand, many compounds which cause type I binding in mammals or in Fc houseflies do not give rise to any detectable spectrum in CSMA houseflies (15,16,26,35,44) but are known to be substrates for the microsomal oxidases of this strain.

Induction of Mixed-Function Oxidase Activity

More than 200 chemical compounds of diverse chemical classes have been documented as inducers of microsomal mixed-function oxidase activity and among these are represented such pesticide groups as insecticides (23), herbicides (69), fungicides (70), miticides (71) and insecticide synergists (51). Studies have been centered on the effects of the inducers on the *in vivo* or *in vitro* oxidation of drugs and cytochrome P-450, the key element in the microsomal electron transport system, has received little specific attention.

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Insecticides. Increase in liver size, which frequently occurs concurrently with induction of microsomal enzymes, was first observed by Laug and Fitzhugh in 1946 (72).

Thirty-five or more insecticides and/or their metabolites have been shown to induce mixed-function oxidase activity (Table 1).

Both vertebrates and invertebrates are susceptible to induction following exposure to insecticides such as DDT, chlordane, dieldrin, Mirex, etc. Increases up to 2-fold in the cytochrome P-450 levels are usually observed and the induction level seems to depend not only on the dosage, exposure time, species and sex but also varies between strains. Recently stress factors have also been implicated (154). As compared to DDT and its analogs, cyclodienes appear to be more potent inducers and up to 5.5-fold increases in cytochrome P-450 were reported in squirrel monkeys subjected to pretreatment with chlordane (138). Mirex is the most potent inducer yet reported among pesticides and caused a 6-fold increase in mouse hepatic cytochrome P-450 (28). Buhler (114) reported a selective induction of drug metabolizing enzymes when rainbow trout were pretreated with DDT. However, in a later communication (115) they reported that hepatic microsomal activity was not affected consistently by feeding or injecting DDT or chlordane.

Most of the organophosphate insecticides are reported to be inhibitors of microsomal mixed-function oxidase activity (23,82, 89, 147,148,155-157). However, significant induction in cytochrome P-450 levels and other microsomal enzymes was observed when mice were administered subacute doses (1/2 LD_{50}) of parathion disulfoton, paraoxon and carbaryl for a period of five days (43, 150,151).

Although insecticides of botanical origin such as pyrethrum were reported to be non-inducers in mammals (143), Springfield *et al.*, (144) recently demonstrated induction of rat hepatic microsomal enzymes, including cytochrome P-450, by pyrethrum, although the doses employed in this study were high and would not be encountered by animals under normal conditions. In addition to pyrethrum, other botanicals such as nicotine, cotinine, rotenone and dimethrin are also known to have some inducing ability when administered to the rat, mouse, mosquito fish and other animals (14,142,145-146).

Most studies on pesticide induction of cytochrome P-450 have reported only the reduced CO difference spectra as a measurement of induction (150,43,158,109,144,73,102,133), few workers have investigated the qualitative characteristics of the induced cytochrome.

Pretreatment of Japanese quail (107) and mouse (96) with DDT and the mouse with low doses of Mirex (28) did not cause any discernible differences in the absorption maximum of the reduced CO difference spectrum or the ethylisocyanide-reduced cytochrome P-450 difference spectra. However, higher doses of dietary Mirex

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Table I

Bibliography of Investigations Concerning Insecticides and Their Metabolites as Potential Inducers*

DDT	Rat	(72-95)
	Mouse	(96-100)
	Guinea Pigs	(101)
	Dog	(102)
	Man	(103-105)
	Japanese Quail	(74-76,80,106,107)
	Chicken	(108-111)
	Pigeon	(112,113)
	Fish	(114-116)
	Cell Culture	(117,118)
	Insects	(16,119-128)
DDT analogs and related compounds	Rat	(74-76,78,80,83,106,109, 111,129)
	Mouse	(83,96,97,130)
	Dog	(129)
	Man	(103)
	Quail	(74-76,80,106,107)
Aldrin/Dieldrin	Rat	(42,89,131,132)
	Mouse	(42,133,134)
	Guinea Pig	(101)
	Hog	(42)
	Monkey	(42)
	Man	(103)
	Quail	(107)
	Pigeon	(113)
	Chicken	(110,111)
Chlordane	Rat	(77,82,100,135-137)
	Mouse	(99)
	Dog	(77)
	Squirrel monkey	(138,139)
Toxaphene	Rat	(85)
Heptachlor	Rat	(131,140)
Lindane	Rat	(79,87,152,153)
	Man	(104)
	Guinea Pig	(101)
Mirex	Mouse	(28)
	Rat	(28)
Nicotine	Mouse	(141,142)
Cotinine	?	(142)
Pyrethrum	Rat	(143,144)
Rotenone	Fish	(145)
Dimethrin	Rat	(146)
Organophosphates	-	(82,89,147-151)

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seem to induce the synthesis of a different cytochrome P-450 as inferred from the differences in the ratios of type II to type I difference spectra in treated microsomes (28). A similar study in rat with chlordane or DDT pretreatment revealed changes in the spectral binding of ligands (88). These authors observed a loss of type I binding of diphenylhydantoin and an increase in Δ A max of the difference spectra caused by benzphetamine and aniline with microsomes from both DDT and chlordane treated animals.

Synergists. Methylene dioxyphenyl synergists in addition to their role as inhibitors of hepatic microsomal-mixed function oxidases may also function as inducers. This has been the subject of a recent review (58). The interaction of these compounds is a biphasic process consisting of an initial inhibition followed by induction (48).

Apparently, the mechanism of induction due to methylenedioxyphenyl compounds differs from compound to compound. Administration of a single dose of piperonyl butoxide to the mouse stimulates the synthesis of a qualitatively different cytochrome P-450. This induced cytochrome shows significant differences in the ethylisocyanide equilibrium point as well as in the spectral binding of several ligands. In contrast to these findings, n-propylisome-induced microsomes do not show qualitative changes in spectral characteristics although cytochrome P-450 is elevated (48). Reports of the induction of a new hemoprotein (159-161) by safrole and isosafrole may in fact be due to the formation of the type III complex (58) with the induced cytochrome P-450.

Induction in insects. Several insecticides such as: DDT and DDE; the cyclodienes, aldrin, dieldrin, endrin, isodrin, chlordane, heptachlor, heptachlor epoxide; baygon and malathion have been studies as potential inducers of the microsomal mixed function oxidase system in different species of insects (41,46,91,122,124-128,162-165).

Increased titers of cytochrome P-450 have been reported by Perry et al. (125) when diazinon-R houseflies were subjected to DDT exposure. However, in the Fc strain of housefly Oppenoorth and Houx (124) were unable to demonstrate any increased oxidative activity due to DDT treatment. Increase in cytochrome P-450 levels and change in the ethylisocyanide-cytochrome P-450 difference spectra were observed by Matthews and Casida (46) when houseflies of Orlando DDT strain were topically treated with dieldrin. Recently, Tate et al. (16) studied induction due to DDT in the Orlando DDT strain of houseflies and reported on increase in the cytochrome P-450 in treated insects. However, spectral studies, including ETNC-cytochrome P-450 difference spectra, failed to reveal induction of a qualitatively different cytochrome P-450 species.

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Induction in relation to combined pretreatments. Several workers have studied the effects of insecticides and other agents administered sequentially, or as a mixture, on the hepatic mixed-function oxidase system with interesting results (89,94,113,140, 166). For example, a simultaneous treatment of rats with chlordane and 3-methylcholanthrene or phenobarbital showed an additive effect on cytochrome P-450 levels and *in vitro* zoxazolamine metabolism, indicating that these enzymes were induced by different mechanisms with different inducers. However, the same pretreatments did not yield an additive effect on benzpyrene hydroxylase and NADPH oxidase, apparently indicating that the same mechanism was involved in the induction of these enzymes. Similarly, no additive effects on aminopyrene N-demethylase were evident when rats received both chlordane and methyltestosterone treatments (137). Although DDT, phenobarbital, and hydrocortisone all elevated *in vitro* DDT metabolism by microsomes, combinations of phenobarbital and hydrocortisone with DDT produced antagonism since the increase in liver DDT metabolizing enzymes was less than that expected with either compound alone (94).

Rats fed diets containing a mixture of dieldrin and DDT or methoxychlor, showed an increase in *in vivo* hexabarbital metabolism and *in vitro* aniline hydroxylation as compared to rats receiving dieldrin alone (166). An increase in aniline hydroxylase and nitroreductase activity was reported by Williams and Casterline (167) in all treatments to rats with aldrin, dieldrin and piperonyl butoxide alone or in combination. In white pigeons (113) higher rates of testosterone and progesterone metabolism were observed in birds pretreated with a mixture of dieldrin and DDT than either compound alone.

Carbaryl and carbofuran metabolism was stimulated by chlordane and inhibited by methylmercury-hydroxide pretreatment in rats. However, simultaneous treatment by these agents produced results similar to controls indicating the effects of the individual agents were balanced out (158).

A single subacute oral dose of parathion to female rats resulted in mild inhibition of O-demethylase (I), O-dearylase (II), N-demethylase (III), azoreductase (IV) and nitroreductase (V). DDT alone stimulated I and II while aldrin or dieldrin elevated all the enzymes except IV. Combined treatement of parathion with organochlorine insecticides produced the following results. Up to 50% inhibition of reactions I, II, III and IV was observed with aldrin, of reaction II, III and V with chlordane, of reaction II with DDT and of reaction I and IV with methoxychlor. Fifty to 100% stimulation of reaction III was noticed with aldrin, of reaction IV with chlordane, or of reaction I with methoxychlor. In the authors' opinion the net result of administration of two compounds on the hepatic microsomal mixed-function oxidase system cannot be predicted, particularly when one of them is an inhibitor and the other an inducer (89).

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In fetal rat liver cell culture, DDT was found to be a potent inducer of arylhydrocarbon (117) and benzpyrene (118) hydroxylases. Addition of DDT in a mixture with many lipophilic compounds including phenobarbital and 3-methylcholanthrene caused induction. Gielen and Nebert (117) observed an additive effect and suggested that at least two different mechanisms of microsomal induction exist in cultured fetal hepatocytes. However, in a later study (118) the authors were unable to identify discrete classes of inducers when two or more compounds were added either sequentially or simultaneously.

Yu and Terriere (164) studied the induction of heptachlor epoxidation and naphthalene hydroxylation in houseflies of the Isolan-B strain by combination of dieldrin with several other cyclodiene insecticides. The data obtained showed no evidence of an additive effect indicating that the same mechanism was involved in the induction processes.

Effect of Addition of Insecticides to the Mixed-Function Oxidase System In Vitro

Inclusion of DDT in an incubation mixture containing rat microsomes was found to cause marked inhibition of 6β-, 7α- and 16α-hydroxylation of testosterone (156), diphenylhydantoin metabolism (88) and aminopyrine N-demethylation (98). DDT did not alter the rates of aldrin epoxidation (80) and aniline hydroxylation (98) but caused slight stimulation of benzpyrene hydroxylase when higher concentrations were used (98). Nakatsugawa *et al.*, (168) reported that epoxidation of heptachlor and aldrin was inhibited 44-47% in the presence of γ-BHC.

Marked species and sex differences were noticed when the *in vitro* effects of aldrin and dieldrin addition on mixed-function oxidase system were studied (42,169). With the exception of a slight stimulation due to dieldrin in male and female dogs and male mice, both aldrin and dieldrin caused slight to marked inhibition of NADPH cytochrome c-reductase in both sexes of rat, mouse, dog and monkey. These insecticides, however, stimulated NADPH oxidase in all species. *In vitro* ethylmorphine metabolism was significantly inhibited by aldrin while a similar effect due to dieldrin was observed only in male and female rats. Male and female dogs and male mice showed no effect while slight stimulation was noticed in monkey and female mice. It is interesting to note that aldrin inhibited aniline hydroxylation in all species studied whereas dieldrin, except for male rats and dogs, was stimulatory.

Nakatsugawa *et al.* (168) and Gillett and Chan (131) observed a mutual inhibition of epoxidase activity when microsomes were treated *in vitro* with some combinations of cyclodiene insecticides.

Chlordane, another member of the cyclodiene family, caused no significant change in the rates of aminopyrine and hexabarbital

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metabolism by rat microsomes (170). However, chlordane was found to be a potent inhibitor of testosterone metabolism (156). Recently, Kutt and Fouts (88) have reported 20-50% inhibition of diphenylhydantoin metabolism due to addition of chlordane to rat microsomes.

Welch et al., (155) first reported the inhibition of in vitro hexabarbital metabolism by organophosphate insecticides. Reduction in the rates of in vitro metabolism of testosterone by the addition of parathion, malathion, chlorthion and paraoxon were later reported (156,157,171). These experiments also revealed that 16α-hydroxylation of testosterone was more readily inhibited than 6β-hydroxylation while 7α-hydroxylation was not affected. Chlorthion was found to be the most potent inhibitor of testosterone metabolism while paraoxon was least effective. Essentially similar results were observed by Stevens (172) with parathion except that the 6β-hydroxylation was inhibited to a greater extent than the 16α-hydroxylation of testosterone.

Addition of parathion to the incubation mixture resulted in 21 and 33% reduction in epoxide formation from heptachlor and aldrin respectively (168) and 64 and 33% inhibition in NADH and NADPH cytochrome c reductase respectively (173). In a series of papers Stevens and co-workers (43,172,174-176) reported that addition of 1.0 mM, malathion, and carbaryl inhibited in vitro metabolism of hexabarbital in mouse, rat, rabbit, and dog. Malathion was also a potent inhibitor of aniline hydroxylase. Carbaryl, however, although inhibitory in rat and mouse did not affect aniline hydroxylation in the rabbit and dog. Side chain oxidation of hexabarbital was inhibited by paraoxon in mouse and rat, unaffected in dog and stimulated in rabbit. OMPA had no effect on aniline hydroxylation but enhanced hexabarbital oxidation in rat and rabbit. Malathion, parathion, disulfoton, and carbaryl all inhibited aniline hydroxylation in man, whereas it was stimulated by paraoxon in all species studied. In addition to carbaryl, Kuwatsuka (29) reported inhibition of aniline hydroxylation by allethrin and rotenone.

Several combinations of cyclodiene insecticides were found to be inhibitory and endrin appears to be the most potent inhibitor of aldrin epoxidation in vitro both in mouse and bluegill sunfish (177).

Conclusions

Cytochrome P-450 and the electron transport system associated with it form a mixed-function oxidase system which, in many organisms and tissues, functions in the detoxication of toxic compounds. Evolutionary considerations lead to the conclusion that cytochrome P-450 has been adapted, in certain tissues of animals, for the detoxication of lipophilic dietary toxicants of relatively low toxicity and that induction provides an additional level of flexibility permitting adaptation to chronic intake of

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these toxicants. As a result of the lack of substrate specificity required for such a function, this system also metabolizes such synthetic organic compounds as pesticides.

Pesticides can clearly interact with microsomal enzymes, particularly cytochrome P-450, in a variety of ways. They can function as substrates, as inhibitors or as inducers. Certain compounds may function first either as inhibitors or substrates, then, on prolonged treatment, as inducers. The effects of pesticides administered either together or sequentially, frequently vary from the effects of the same compounds administered alone, and these combined effects are not predictable.

There are many reasons why it has been difficult either to ascribe mechanisms to these interactions or to see systematic relationships between the many variables. Many of these reasons are experimental. The reactions of particulate enzymes with insoluble substrates and/or inhibitors seldom follow classical Michaelis-Menton kinetics and, due to the problems of solubilization and purification, this difficulty is far from resolution. The use of optical difference spectroscopy, although it has been the most valuable technique to date for the investigation of cytochrome P-450, is an indirect measure of binding and cannot discriminate between the binding of substrate to enzyme and other less functional binding. Other problems are physiological. It is still not clear whether there is more than one cytochrome P-450 in normal mammals, although evidence from a variety of different types of experiments suggest there is. If this is the case, we need to know how they differ from one another, how their substrate specificities vary and how they function together in the microsomal membrane.

The importance of this enzyme system cannot seriously be challenged: almost every pesticide used interacts with it in both target and nontarget organisms. The best hope for understanding the complex interactions involved seems to lie in purification of the components of the system and reconstitution of simplified but functionally active systems, free from the microsomal membrane. A similar dissection of the induction process will also ultimately be necessary.

Investigations of the type proposed are being carried out in a number of laboratories. However, the complexity of the task and the relatively small number of investigators interested in the findings as they apply to agricultural chemicals, suggests that it will be some time before the necessary knowledge is acquired.

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3

Mechanisms of Insecticide Metabolism in Insects: Tissue, Cellular, and Subcellular Studies

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Introduction

The metabolism of insecticide chemicals by insects has been a part of several recent review articles (1-7). However, most of these articles either deal with insecticide metabolism from a comparative standpoint or refer only to one particular metabolic mechanism or class of compounds. Thus, in the following "review of reviews", data pertaining only to insects is presented with selected examples which represent all of the major insecticide metabolic mechanisms operative within insects. Emphasis has been placed on some of the methods used for *in vitro* insect enzyme studies, on the nature of the enzymes responsible for insecticide metabolism, on the cellular and subcellular distribution of these enzymes, and on the variation of enzyme activity with age, strain, sex, etc. More complete biochemical information available on these enzyme systems can be found in the listed review articles.

Methods of Study

After agreeing to work on insect metabolism, one of the first decisions to be made is which species to work with. This may sound trivial, but I remind you that there are an estimated 2.5 to 10 million different insect species (1). At the present time, only about 20-30 species have received any significant attention with respect to insecticide metabolism. These were chosen for a variety of reasons including: ease of laboratory or greenhouse rearing; length of generation; size; agricultural or health significance; genetic makeup; and previous biochemical knowledge. Often the choice is based on a combination of these factors, but they should not be considered as limiting factors.

Having chosen the insect, it is necessary to determine which life stage to study. Little has been done with insecticide metabolism by eggs or pupae, mostly because results to date have shown these stages to be poor metabolic subjects. Most research has centered around either the immature insect forms or the adults,

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but in only a few instances have comparative studies between young adults been conducted. Generally, insect control practices are aimed at the egg and immature forms of agricultural insect pests and at the adult forms of insects affecting health, but there are exceptions. More attention should be given to these field aspects when considering which species and which life-stage to work with. Whether adult or immature is chosen, parameters such as age, sex, and diet must also be considered. Often their role can only be evaluated throughout the course of the study, but their influence on metabolism should not be overlooked.

Although considerable information about insecticide metabolism can and should be obtained from living insects, specific details about the nature of the enzymes involved and the formation and structure of primary or intermediary metabolites requires the use of cellular and subcellular preparations, either from whole insects or, preferably, from individual insect tissues. Just as mammalian biochemists work with *in vitro* preparations of liver, lung, gut and muscle, insect biochemists have recently turned to homogenates of gut, fat body, muscle and Malpighian tubules. The metabolic significance of each tissue can then be evaluated with respect to its size, its location within the body, its probability of contact with a poison, etc.

Of course the insect's size and ease of dissection can limit tissue studies. For example, removing the gut from an aphid is tedious and time-consuming but removing enough aphid guts to make a microsomal preparation is almost out of the question. On the other hand, microsomal studies have been conducted with the midgut from one Cecropia caterpillar. Removal of adult tissues usually takes place through the use of classical insect dissection methods whereby a series of ventral incisions are made after which the outer skeleton is pinned aside and the desired tissues are extracted. With larvae, a squeeze technique, in which the head is removed and the body contents are eased out of the cuticle casing and then teased apart into separate tissues, has allowed for the rapid dissection of many immature insects, even though small in size. Finally, another method which is often used involves separating insects into different body regions, such as heads or abdomens, which tends to concentrate certain tissues.

Once the tissues have been separated, metabolism studies can be performed with the intact tissue, or with tissue homogenates. Because insects of the same age may vary considerably in size and because of difficulties in weighing certain tissues, comparisons of enzymatic activity among different ages, tissues and species can be frustrating. Thus, homogenates, which allow for comparisons based on total nitrogen or protein, are generally preferred. However, disruption of the cells can release endogenous inhibitors which may seriously inhibit enzymatic metabolism. Therefore, if homogenates are used, care should be taken to ensure that the homogenate activity reflects the activity of the intact tissue. In some cases, inhibitors can be removed before homogenization.

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For example, the gut contents of several insects have been shown to contain an inhibitor of oxidative metabolism (1). If the guts are cleaned before homogenization, no inhibition results. Others have found that the addition of bovine serum albumin (BSA) protects the enzyme from inhibition by endogenous materials. However, my experience with BSA and carbamate metabolism in insect preparations indicates that BSA can cause erratic and misleading results. Thus, I urge that if BSA is used, careful control experiments should be included to ensure that BSA does not alter metabolism of the substrate by the tissue under study.

Problems with inhibitors can sometimes be solved by subcellular fractionation. This procedure also concentrates the enzymes for more intensive biochemical investigations. However, fractionation requires more insects and more time and may not always be necessary. The isolated tissue or whole insect is macerated in saline, buffer, sucrose or a combination of these solutions, which may or may not be the same as the tissue dissection medium. Homogenization is achieved with a blender, tissue grinder, or mortar and pestle. Other factors which need to be considered are the concentration of insects in the homogenate, length of homogenization, and degree of abrasiveness employed. It has been found that the choice of procedure can severely alter enzyme activity.

Although some investigators use the resulting whole homogenate as an enzyme source, most prefer at least one centrifugation at about 800g for 10 min to remove unbroken cells, cell debris, and nuclei. Further centrifugation at 10,000g for 30 min and finally at 100,000g for 60 min yields a mitochondria, microsome and soluble fraction. Although this scheme has successfully divided subcellular constituents of mammalian tissues and a few insect preparations, notably adult housefly abdomens (8), other insect tissues require drastic changes in centrifugal forces to sediment desired constituents. For example, electron microscopic examination of American cockroach fat body homogenates indicated that mitochondria were sedimented at 650g for 120 min and microsomes at 55,000g for 45 min (9). Other variations with other insects illustrate the need for careful examination of homogenization and centrifugation techniques which may vary from insect to insect and from tissue to tissue within the same insect. One possible solution to some of these problems is the use of sucrose density gradient centrifugation (1).

Whether whole tissue, homogenate or subcellular fraction, the next procedure is incubation of the enzyme with substrate. Once again the medium must be considered. Buffer pH, strength and type, and saline content are important factors. Generally phosphate or Tris buffers are used with or without KCl or NaCl, with pH optimum usually between 7.0 and 8.5. Temperature in early insect enzyme studies was generally set at 37°C based on mammalian body temperature. However, more recently, enzyme preparations from insects have been shown to optimize at lower temperatures,

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some as low as 20-25°C (7,10). In some cases BSA is also added, but care must be taken as discussed earlier. Time of incubation is also important and every study should examine this parameter. Often linear enzymatic reactions proceed for only 10-15 min with insect preparations.

Cofactors are usually necessary to obtain maximum *in vitro* activity. The nature of these cofactors often helps characterize the enzyme system involved in metabolism by the tissues. If mixed-function oxidase activity is being measured, NADPH and oxygen are required, unless present in sufficient quantities as endogenous materials. Substitution of NADPH by other cofactors such as NADH, FMN or FAD does not increase activity, and addition of the latter two often causes inhibition of activity. Glutathione transferase activity is tested for by addition of GSH. Nicotinamide and EDTA, which are often added to mammalian enzyme preparations, are not generally needed for maximum insect *in vitro* metabolism. Various metal ions have been incubated with insect preparations, and in some cases inhibition or stimulation have resulted. In a few instances there are marked requirements for metals, but these vary from insect to insect and tissue to tissue.

The nature of the enzymatic reaction in insect preparations often can be clarified by addition of materials which inhibit the reaction. These include methylenedioxyphenyl synergists, WARF antiresistant, non-toxic insecticide analogs, various metals and carbon monoxide. The response of the enzyme to each of these additions depends, of course, on the level at which they are added. Few test for inhibition at concentrations higher than 10^{-3}M, and few get excited unless response is elicited by concentrations lower than 10^{-5}M.

Finally, the level of enzyme and substrate added to the incubation flask is important and sometimes overlooked. Included in the examination of these factors should be a study of possible enzymatic interaction with metabolic products formed from the substrate (product inhibition).

Upon completion of the incubation, products are extracted with an organic solvent to remove primary unconjugated metabolites which are identified by various chromatographic and spectrophotometric techniques. In some cases, water-soluble products are also characterized, especially if whole homogenates or preparations containing soluble enzymes are used. The nature of these products, together with the information gained through the study of cofactors, inhibitors, etc., can help in assessing the enzymatic mechanism utilized by the insect to metabolize insecticides. From such studies by numerous investigators in the past 20 years, many metabolic pathways in insects have been described and these will be summarized on the next few pages.

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Mechanisms of Reaction

Hydrolases. Carbamate and organophosphorus insecticides are subject to hydrolysis in insects. For carbamates, this is considered to be a minor metabolic pathway (7) and thus very little work has been conducted with the enzymatic mechanism in insects. An example of the reaction is shown in Fig. 1A for carbaryl (11). Presumably a carbamate esterase is involved, although chemical hydrolysis cannot be discounted. A certain amount of the latter often occurs when using *in vitro* systems where control flasks without enzyme can give 1-5% hydrolytic degradation.

With respect to organophosphorus compounds, considerably more is known about enzymatic hydrolysis. From the information available, it appears that a number of different phosphatases with varied substrate spectra exist within insects. For example, dichlorvos, TEPP and DFP were hydroylzed by the soluble fraction of adult houseflies (Fig. 1B), but compounds such as malathion, parathion, phosdrin and dipterex were not (12). Various activators and inhibitors of the phosphatase activity were found and division of the fly into different sections indicated that the abdomen was three times more active in hydrolysis than the head or thorax (based on wet weight). A similar study with Culex mosquito larval homogenates indicated that phosphatase activity towards malathion in this insect was enhanced, not inhibited, by Cu^{++} and that Co^{++} and Mn^{++} had no effect on activity (13). However, the abdomen was again the most active tissue.

Ester hydrolysis can also occur on another position of the malathion molecule. The reaction catalyst, carboxyesterase, has been isolated from several insects and is illustrated on Fig. 1C by that from Culex mosquito larvae (13). Comparison studies between different mosquito strains showed that the titre of carboxyesterase was higher in malathion resistant mosquitoes, and that enzymes from resistant and susceptible strains had different pH optima, different turnover numbers, and different heat and acid lability. Most of the activity on a per-insect basis was in the abdomen, particularly the gut. This tissue has also been implicated as the primary source of carboxyesterase in other insects (14).

Glutathione Transferases. Hydrolysis of organophosphorus insecticides also takes place via a glutathione transferase which is present in insects as a soluble enzyme system. An example of this reaction is the demethylation of methyl parathion in larval soluble fractions from horn beetles and silkworms (15, Fig. 2A). Interestingly, ethyl parathion was not degraded by this system implying an enzyme specific for demethylation. Soluble preparations from different larval tissues fortified with GSH showed that the midgut was the most active tissue in the horn beetle while the fat body was most active in the silkworm on a fresh-weight basis. Azinphosmethyl demethylation was mediated

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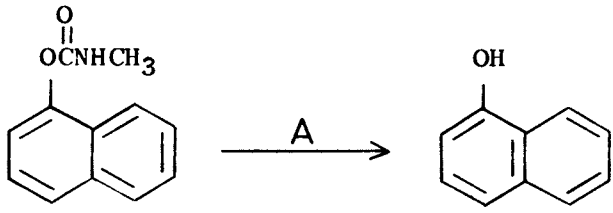
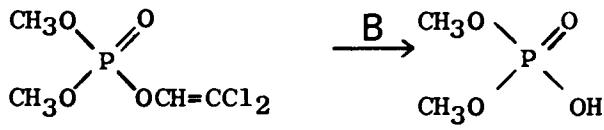


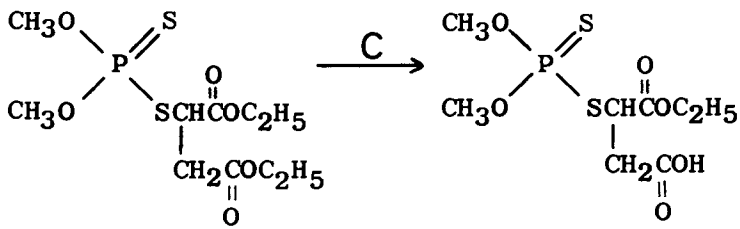
carbaryl

Enzyme: Carbamate esterase (11)
Source: Cabbage looper larva, gut and fat body, 800g homogenate



dichlorvos

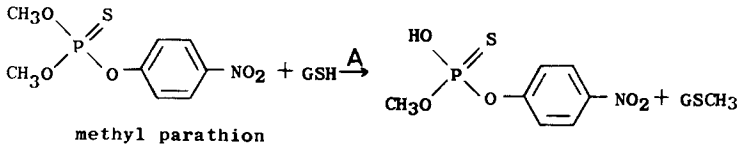
Enzyme: Phosphatase (12)
Source: Housefly adult, whole insect, soluble
Activator: Mn^{++}, Co^{++}
Inhibitor: Hg^{++}, Cu^{++}, p-chloromercuribenzoic acid
Tissue: Abdomen> thorax, head=0 (fresh weight)



malathion

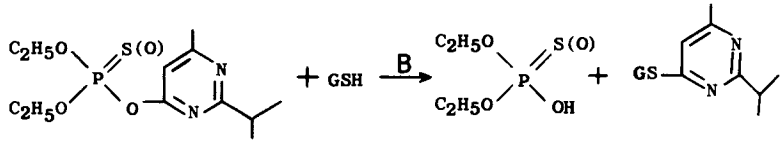
Enzyme: Carboxyesterase (13)
Source: Mosquito larva, whole insect, 10,000g pellet + soluble
Inhibitor: EPN
Tissue: Abdomen> gut> thorax> head (per insect)

Figure 1. Insecticide metabolism by insect hydrolases



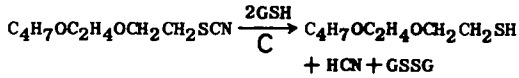
methyl parathion

Enzyme: Glutathione transferase (15)
Source: Horn beetle & silkworm larva, midgut, soluble
Cofactor: Glutathione
Tissue: Horn beetle: midgut≫fat body > cuticle & muscle > blood (fresh weight)
Silkworm: fat body > midgut > blood > cuticle & muscle (fresh weight)



diazinon

Enzyme: Glutathione transferase (18)
Source: American cochroach, fat body, soluble
Cofactor: Glutathione
Inhibitor: Phenylmercuric acetate, p-chloromercuribenzoate, $K_3Fe(CN)_6$
Tissue: Fat body > gut > muscle & cuticle ≫ blood (fresh weight)



Enzyme: Glutathione transferase (19)
Source: Housefly adults, whole insect, soluble
Cofactor: Glutathione

Figure 2. Insecticide metabolism by insect glutathione transferases

by soluble enzymes from whole adult houseflies (16) and deethylation has been demonstrated with diazinon and diazoxon (17).

In addition to dealkylation, glutathione transferases catalyze removal of the leaving group of organophosphorus insecticides (Fig. 2B). Thus, diazinon and diazoxon were degraded by glutathione-activated soluble enzymes from several tissues of the American cockroach (18). Maximum activity for both compounds was found in the fat body and very little GSH-stimulated activity was in the mitochondria and microsome fractions of gut and fat body.

A recent investigation has illustrated that glutathione transferases are involved in insect metabolism of insecticidal organothiocyanates such as Lethane 384 (19). The soluble enzyme system is responsible in this instance for activation of the insecticide rather than detoxication since HCN is released which is believed to be partially responsible for the toxicity of these insecticides (Fig. 2C).

Glutathione also plays a role in the metabolism of organochlorine insecticides by insects, particularly reactions characterized by the loss of chlorine atoms (Fig. 3A). The dechlorination of various isomers of HCH has been studied using the soluble fraction or acetone powders of whole houseflies fortified with GSH (20,21). Homogenates of many other larval and adult forms of insects contain a glutathione-dependent enzyme capable of HCH dechlorination (20,22,23). In studies where age was considered, newly emerged blowfly adults were not able to metabolize)-HCH or PCCH (23). However, as the adults aged, dechlorinase activity increased steadily up to 10 days. When houseflies were examined, a similar increase in enzyme activity occurred with increasing age of adult while much lower levels of dechlorinase were detected in eggs, larvae and pupae. Variation in substrate specificity was also found.

Results from some of these studies indicated that the same enzyme system, or one closely related, is responsible for the dehydrochlorination of DDT (23,24). This reaction, catalyzed by glutathione-dependent DDTase, is probably the most well-known insecticide detoxication mechanism in insects (Fig. 3B). The enzyme complex has been purified from houseflies and studied extensively, the results of which have been summarized in a review (25). The enzyme also dehydrochlorinates DDD, but does show species differences with respect to other DDT isomers. High titers of the enzyme have been found in DDT-resistant strains of insects and in DDT-tolerant species of insects. Enzyme levels from whole insect preparations vary with age, being high in larval and adult stages , but low near, through and shortly after pupation of Mexican bean beetles (26) and houseflies (27). Two studies using different insects showed different DDTase distribution among tissues with fat body having most activity in houseflies (28) and least in Mexican bean beetles (26). Perhaps most surprising is the high level of dehydrochlorinase found in the reproductive

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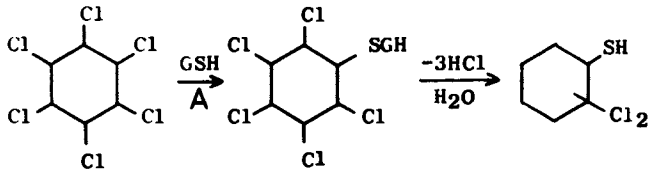
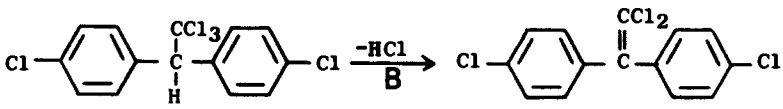


HCH

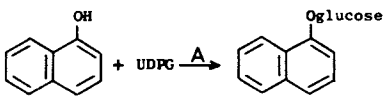
Enzyme: HCH dechlorinase (20-21,24)
Source: Housefly adult, whole insect, soluble
Cofactor: Glutathione
Inhibitor: Bromphenol blue, WARF antiresistant



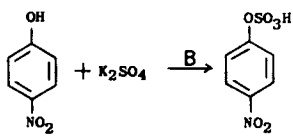
DDT DDE

Enzyme: DDT dehydrochlorinase (25)
Source: Housefly adults, whole insects, homogenate
Cofactor: Glutathione
Inhibitor: WARF antiresistant, bis(p-chlorophenyl)ethane
Tissue: Housefly: fat body > nerve > cuticle > muscle > reproductive > gut (protein) (28)
Bean beetle: reproductive > cuticle > gut > muscle > nerve > fat body (dry wgt.) (26

Figure 3. Insecticide dechlorination by insect glutathione transferases



Enzyme: Glucosyl transferase (29)
Source: Tobacco hornworm larva, midgut, soluble
Cofactor: Uridine diphosphoglucose
Inhibitor: Cd^{++}, Zn^{++}, Hg
Tissue: Fat body > midgut > hindgut, foregut, blood (fresh weight)



Enzyme: Sulphotransferase (30)
Source: Southern armyworm larva, gut, soluble
Cofactor: ATP, Mg^{++}, SO_4^{--}
Tissue: Gut > Malpighian tubules ≫ fat body

Figure 4. Insecticide metabolism by other insect transferases

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organs of the naturally tolerant bean beetle.

Other Transferases. Other transferases have recently received some attention with respect to insect metabolism of insecticides. Unlike GSH transferases, these enzymes are primarily involved with the formation of secondary metabolites and do not generally operate on the parent compound. One of these secondary metabolic pathways in insects results in the formation of glucoside conjugates of various oxidative and hydrolytic insecticide metabolites. The enzymatic nature of glucoside formation has been examined using in vitro preparations of tobacco hornworms and houseflies (29). The substrate used, 1-naphthol, is a hydrolytic metabolite of the insecticide carbaryl (Fig. 4A). Glucosyl transferase activity was found to be maximum in fat body tissues, but also high in midgut homogenates of the hornworm larvae. Although subcellular fractionation separated the UDPG-dependent enzyme into the soluble fraction of whole hornworm homogenates, adult housefly fractionation yielded maximum activity in the microsome pellet.

The same primary insecticide metabolites are also subject to conjugation in insects as sulphates. In vitro studies with p-nitrophenol demonstrated the presence of a soluble sulphotransferase mainly in the gut, but also in the Malpighian tubules and fat body of southern armyworm larvae (30, Fig. 4B). The enzyme was able to catalyze the sulphation of a wide variety of substrates other than p-nitrophenol and has also been found in a number of other adult and larval insects (31). Age studies indicated that the enzyme levels of the gut increased with larval age with peak activities between moults (32). Another conjugate of p-nitrophenol formed in insect soluble preparations was shown to be the phosphate derivative which was believed to have been catalyzed by a phosphotransferase requiring ATP and Mg^{++} (31).

Epoxide Hydrases. Another enzyme which might possibly play a role in the degradation of insecticides, particularly cyclodienes, in insects has been recently described. The epoxide hydrase, which transforms an epoxide group into a trans diol, has been found in adult housefly microsomes (33) and homogenates of blowfly and mealworm pupae (34).

Oxidases. By far the greatest amount of information about insect metabolism of insecticides has been gathered for those reactions involving an oxidation. A very thorough review of this field written by Wilkinson and Brattsten included 300 references (1). Other reviews supplement this paper (2-7,35). Very detailed information is available with respect to differences in enzymatic activity with age, sex, diet, tissue, temperature, pH, etc. Where subcellular fractionation has been performed, oxidative activity has been associated primarily with a microsome fraction, although this fraction may sediment at different forces with different

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insect preparations. The enzyme(s) is a mixed-function oxidase (mfo), requires NADPH and oxygen, and is inhibited by methylenedioxyphenyl compounds and carbon monoxide.

One form of oxidation, namely hydroxylation, takes part in insect metabolism of most classes of insecticides. Microsomal mixed-function oxidases of various insects catalyze the hydroxylation of pyrethroids, rotenone, DDT, dihydroisodrin, and several carbamates (Fig. 5). These hydroxylated products usually serve as intermediates for dealkylated or conjugated products, particularly *in vivo*. Another oxidation, which may involve a hydroxylated intermediate, results in ester cleavage of certain organophosphorus compounds such as parathion (Fig. 6A). In addition, these insecticides are also subject to oxidative desulfuration which yields the P = S to P = O activation (Fig. 6B). Epoxidation of cyclodiene insecticides, particularly aldrin, has been demonstrated with a number of insect subcellular preparations (Fig. 6C), while sulfoxidation has been examined with housefly microsomes and methiocarb (Fig. 6D).

Considerable work has been conducted with respect to tissue distribution of mfo systems in insects. Fig. 7 compares some of these studies. Azinphosmethyl desulfuration occurred in almost all tissues of the American cockroach, with most activity in Malpighian tubules (36). Hydroxylation was found to be greatest in cockroach gut tissue with dihydroisodrin as substrate (37) while hydroxylation of carbaryl was shown to be maximum in cabbage looper fat body (7). However, other tissues also contained active enzymes. Aldrin epoxidation took place predominantly in gut tissues of southern armyworm and several other lepidopterous insects (38).

Regardless of the substrate, tissue, or insect examined, effects of age on oxidation have been found to be very similar (1). With a few exceptions, mfo activity is low in eggs, increases as young larvae proceed through instars, and drops dramatically at pupation. Then activity may or may not rise again as the adult ages. The level of activity or titre of enzyme may vary between sexes, with females having the highest activity, but several studies have shown similar activity in male and female insects. Perhaps the largest variations occur between resistant and susceptible strains of the same insect species where substantial differences in substrate oxidation have been demonstrated. Other factors such as diet and enzyme induction have been considered in only a few instances but they probably have some influence on mfo activity in insects.

Conclusions

Understanding the biochemical mechanisms which insects utilize to metabolize insecticide chemicals is essential if we are to continue in our efforts to control those species which are considered pests of man, his food, fibre and domestic animals. In

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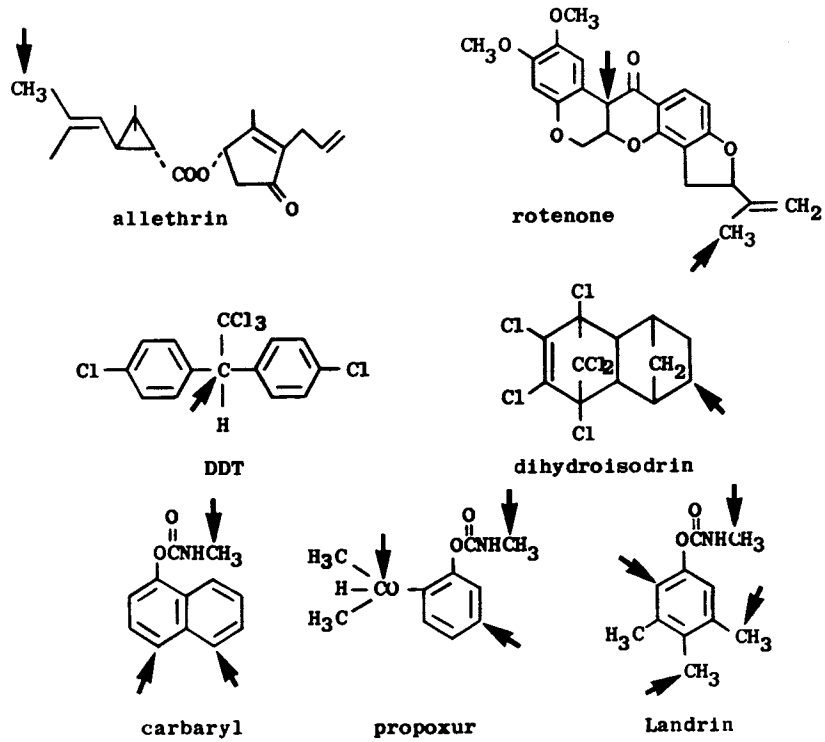

Figure 5. Insecticide metabolism by insect oxidases. Arrows indicate carbon atoms hydroxylated.

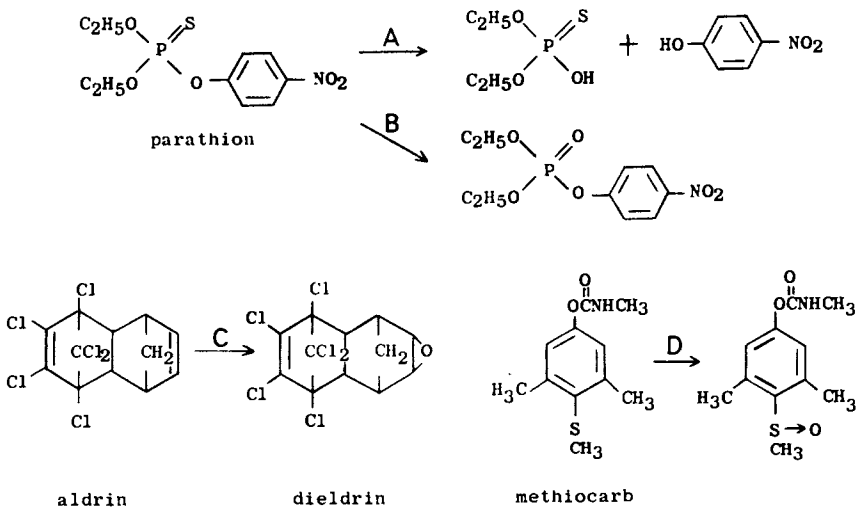

Figure 6. Insecticide metabolism by insect oxidases

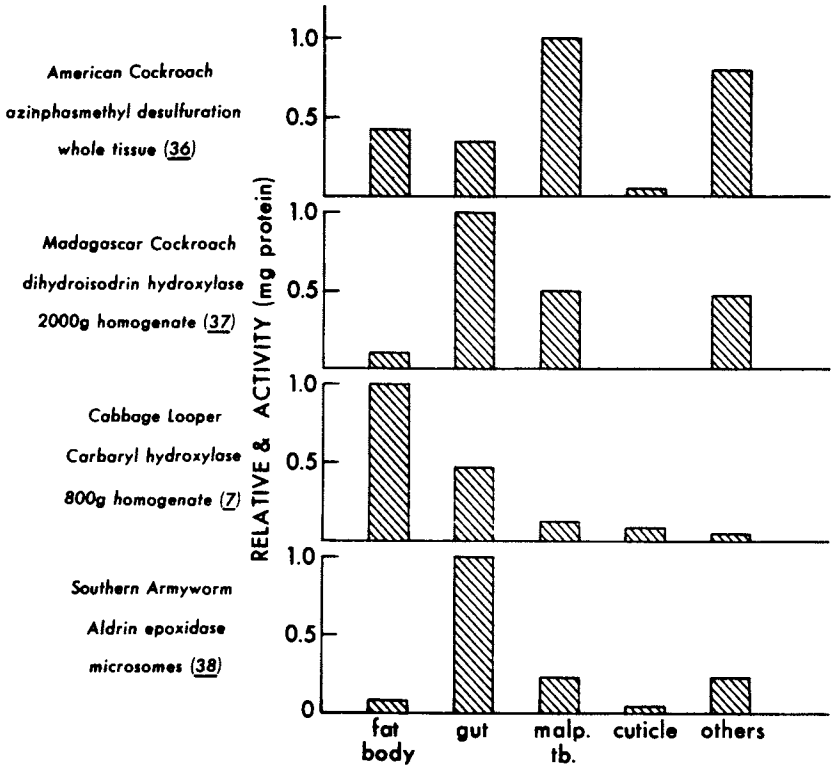

Figure 7. Tissue distribution of insect oxidases.

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the last several years, a number of investigators interested in insect metabolism have broadened our knowledge base through careful examination of tissue distribution of metabolic activity; age, sex and diet influence on metabolic activity; subcellular distribution of the enzymes responsible for metabolism; and extensive biochemical characterization of some of these enzymes. Studies have expanded to include a wider variety of insect species from several orders, many of which are agriculturally important species. And although considerable effort is spent defining the metabolic processes involved in oxidation reactions, efforts have also grown toward an understanding of the mechanisms of glucose, sulphate, phosphate and glutathione conjugation. Only through further study of these enzymatic mechanisms, without losing sight of their significance in the whole living organism, can we ensure a thorough understanding of insecticide metabolism in insects.

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4

The Metabolism, Storage, and Excretion of Highly Chlorinated Compounds by Mammals

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Abstract

The distribution and excretion of 4-chloro-,4,4'-dichloro-, and 2,4,5,2',5'-pentachlorobiphenyl have been studied in the rat. The half-lives of these compounds in the blood, liver, muscle, skin and particularly the fat were correlated with their degree of chlorination, whereas their overall rate of metabolism and excretion was inversely correlated with chlorination. In fat, the half-life of these compounds showed an exponential relationship to their degree of chlorination by weight. In the rat, this exponential relationship indicates that the half-life of chlorinated biphenyls increases by approximately two-fold with each 10 percent increase in chlorination. The effect of chlorination on the biological half-life of these compounds has been related to the problems, both anticipated and realized, arising from the use of these and other lipophylic chlorinated hydrocarbons.

Chlorinated hydrocarbons are a diverse group of compounds which have a variety of molecular structures, degrees of chlorination, uses and associated problems. Their structures vary from simple benzene rings with varying degrees of chlorination to complex mixtures of chlorinated compounds. Their uses range from various types of pesticides to hydrolic fluids and electric insulators. The value of their use is undeniable; DDT has saved millions from malaria (1) and the polychlorinated biphenyls (PCB's) have become almost invaluable industrial tools (2). However, the problems associated with their use is also equally undeniable, since they range from environmental contamination (1,2,3,4) to outbreaks of human disease in which numerous people died (5). Thus, depending upon your source of information, the chlorinated hydrocarbons are a group of compounds which we cannot live with or we cannot live without.

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Chlorinated hydrocarbons, with the exception of the dibenzodioxins and dibenzofurans (6), do not have a high acute toxicity. Most of the problems associated with the use of these compounds are related to their persistence in the environment and their tendency to accumulate in the tissues of virtually every type of exposed organism. However, the diverse molecular structure of these compounds and the lack of adequate assay techniques have complicated comparative studies of their metabolism, distribution and excretion. Thus, to date, it has been possible only to assume, from the existing data, that the rate of metabolism and excretion of chlorinated hydrocarbons was in some way inversely correlated to their degree of chlorination. Thus, we saw the PCB's as a unique opportunity to study a series of chlorinated hydrocarbons having the same basic carbon skeleton and differing only in their degree of chlorination. Such a study would allow us to relate the metabolism, storage and excretion of a series of chlorinated hydrocarbons directly to their chlorination.

The adult male white rat was chosen as the subject of the study because of its convenience and availability. Also many of the previous metabolism, distribution and excretion studies with chlorinated hydrocarbons have used this animal. Any variability in intestinal absorption was circumvented by injecting the material into the tail vein. The numerous rapid and accurate assays of tissue concentrations necessary for such a study were facilitated by the use of carbon-14 labeled PCB's and a Biological Material Oxidizer (R. J. Harvey Instrument Corp.).

PCB formulations are marketed according to their percent chlorine by weight. The compounds studied to date, a mono-, di- and pentachlorobiphenyl, (Fig. 1) contain 18.8, 31.8, and 54.5 percent chlorine by weight, respectively. Thus their degrees of chlorination compare to those of Arochlors 1221, 1232 and 1254 which contain approximately 21, 32 and 54 percent chlorine by weight, respectively. Studies of the pentachlorobiphenyl at doses of 0.06, 0.6 and 6.0 mg/kg showed that the distribution and excretion of this compound over a 100-fold dose range was similar (7). Mono- and dichlorobiphenyl were studied at doses of 0.6 mg/kg. The data presented here for pentachlorobiphenyl represents the 0.6 mg/kg dose only. Three animals were sacrificed at each of the time points which varied from 5 min to 7 days post treatment; however, in order to simplify the figures only those time points which were most relevant are presented. Initially the PCB content of every major organ and tissue was determined. This list was eventually narrowed down to the blood, liver, muscle, skin and fat as being most important in the distribution of these compounds. Animals held for 24 hrs or longer were held in metabolism cages and urine and feces were collected daily.

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PCB removal from blood with time is shown in Fig. 2. The time points shown are 30 min, 1, 2, 4 and 8 hr, 1 and 2 days, and the curves are extrapolated to the next point in both directions. Greater than 90 percent of each of the PCB's was removed from the blood within the first 30 min post treatment. The initial rate of PCB removal from the blood appeared to be related to the degree of chlorination (Fig. 2). However, this situation reversed itself at approximately 12 hrs and thereafter.

In any study in which the distribution of a compound is studied by intravenous injection 100 percent of the total dose must be in the blood at zero time. In order to prove this point and in an effort to observe the initial very fast removal of a PCB from the blood, we injected pentachlorobiphenyl into the right lateral tail vein, as usual, and withdrew blood samples from the left lateral tail vein at short time periods thereafter. The results of this study are presented in Fig. 3. A straight line through the points obtained with three animals treated in this way comes quite close to 100 percent of the total dose at zero time and quite close to the value obtained by drawing blood from the hearts of three other animals sacrificed 10 min post treatment. Thus, the removal of PCB's from the blood appears to follow a decay curve having three components. The initial "very fast" component represents PCB distribution throughout the body. The second component represents redistribution of the PCB's from tissues of high perfusion but low affinity to tissues of lower perfusion but higher affinity. Transport of metabolites to the excretory organs may also be involved in the second component. The third component of the blood decay curve primarily represents a general elimination of the PCB's from the body.

The major deposition sites at the early time points were the liver and muscle (Figs. 4 and 5). Again, we obtained data for times varying from 5 min to 7 days post treatment but only the 30 min, 1, 2, 4, and 8 hr and 1 and 2 day points were plotted and the curve was extrapolated to the next point if the PCB had not been effectively removed from the given tissue. PCB removal from the blood and accumulation in these tissues initially appeared to be related to the degree of chlorination, and could account for the fact that pentachlorobiphenyl was more rapidly removed from the blood than either the mono- or dichlorobiphenyl. The initial peak, which in the case of pentachlorobiphenyl exceeded 30 percent of the total dose in each of these tissues at the earliest time points, was most impressive in the liver since this tissue accounts for only about 5 percent of the total body weight. The muscle, of course, accounted for a large percentage of the total dose by virtue of its relative mass which in the rat is 50 percent of the total body weight. Following an initial peak concentration, the amount of PCB in each of these tissues decreased

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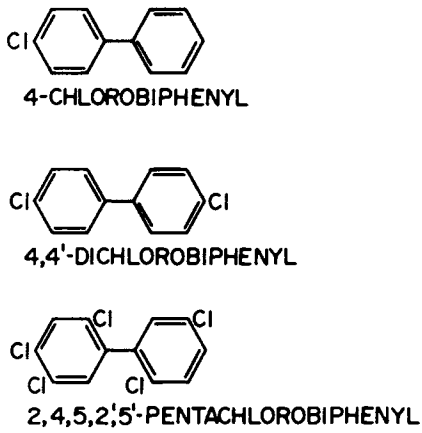

Figure 1. Structural formulas for 4-chlorobiphenyl, 4-4'-dichlorobiphenyl and 2,4,5,-2',5'-pentachlorobiphenyl

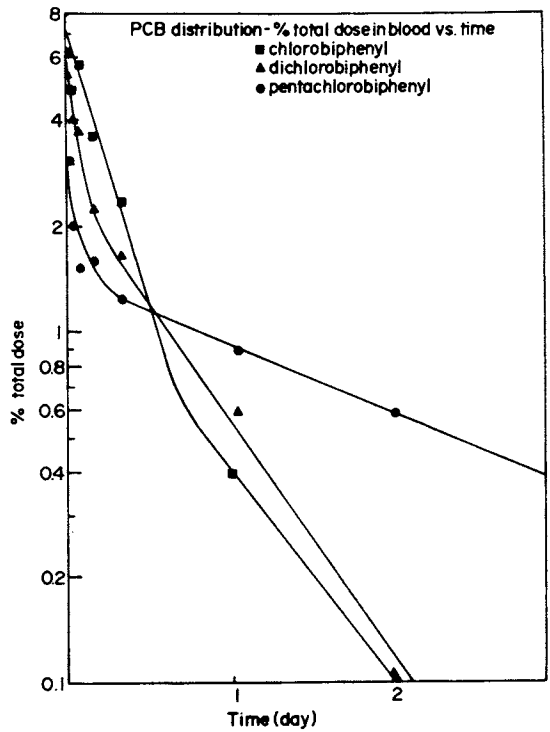

Figure 2. Percent of the total PCB dose in blood at 30 min, 1, 2, 4 and 8 hrs, and 1 and 2 days after intravenous administration

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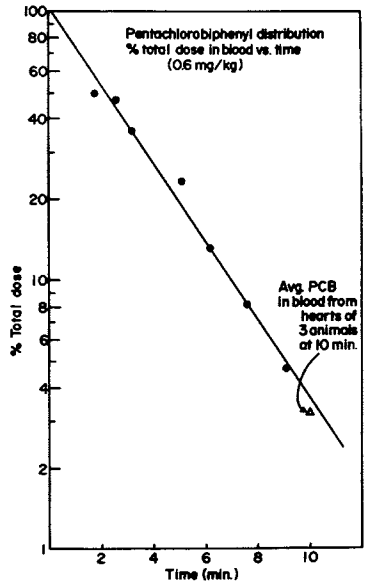

Figure 3. Pentachlorobiphenyl removal from the blood during the first 10 min after intravenous administration. The data obtained from three animals by withdrawing blood from the tail veins is compared with that obtained from the hearts of three other animals.

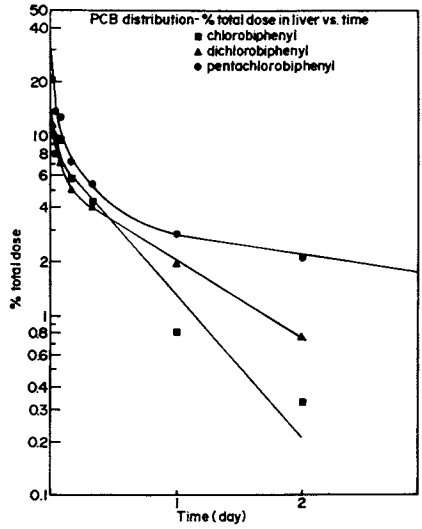

Figure 4. Percent of the total PCB dose in liver at 30 min, 1, 2, 4 and 8 hrs, and 1 and 2 days after intravenous administration

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rapidly. The removal of the PCB's from the liver at the early time points was accounted for by biliary excretion and some redistribution of the pentachlorobiphenyl to other tissues. The latter component of the exponential decay curve in liver could be accounted for by removal of the PCB's from the blood and excretion in the bile. On the other hand, PCB removal from the muscle was accounted for entirely by blood redistribution to tissues of higher affinity. Significant amounts of pentachlorobiphenyl were retained by the muscle and liver for the full 7-day study, whereas the mono- and dichlorobiphenyls were effectively eliminated by the second day after administration.

Skin accounts for about 16 percent of the total body weight of a rat and has a relatively low perfusion rate. However, a significant portion of each of the PCB's was deposited in the skin (Fig. 6). Since PCB removal from the skin was slower than PCB removal from the liver and muscle, only the 2 hr through 7-day time points were plotted. However, the peak accumulation of mono- and dichlorobiphenyl occurred at less than 1 hour and represented 15 to 16 percent of the total dose in each case. The peak concentration of pentachlorobiphenyl occurred at approximately 4 hours and also represented 15 percent of the total dose. Once a peak concentration was reached PCB removal from the skin followed a two component exponential decay for the mono- and dichlorobiphenyls. Only a single component exponential decay was observed for pentachlorobiphenyl through the course of the 7-day study; however, studies are currently underway to determine if a second slower component of this curve exists. The initial, and possibly the second, half-lives of these compounds in the skin suggest a relationship to their degree of chlorination. PCB storage in the skin takes on increased significance when one considers that certain disorders of the skin, particularly chloracne, are characteristic of chronic intoxication by chlorinated hydrocarbons, including PCB's (5).

In any study of chlorinated hydrocarbon distribution, storage in the fat is anticipated. These studies of the PCB's were no exception (Fig. 7). Due to the slow removal of the PCB's from fat, only the 1 hr to 7-day time points were plotted. Peak concentrations for mono-, di- and pentachlorobiphenyl in fat occurred at 1, 2 and 4 hrs after administration, respectively. Our preselected sample times may have caused us to miss an early spike in the 4-chlorobiphenyl concentration in fat such as we observed for the other two PCB's; but, in each case, once an equilibrium was established, the PCB concentration in fat decreased as a single component exponential, the half-life of which was exponentially related to the degree of chlorination, as will be discussed later. At present it is thought that the

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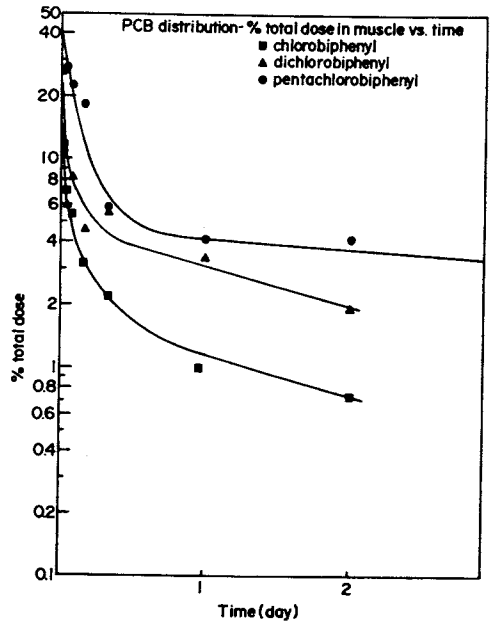

Figure 5. Percent of the total PCB dose in muscle at 30 min, 1, 2, 4 and 8 hrs, and 1 and 2 days after intravenous administration

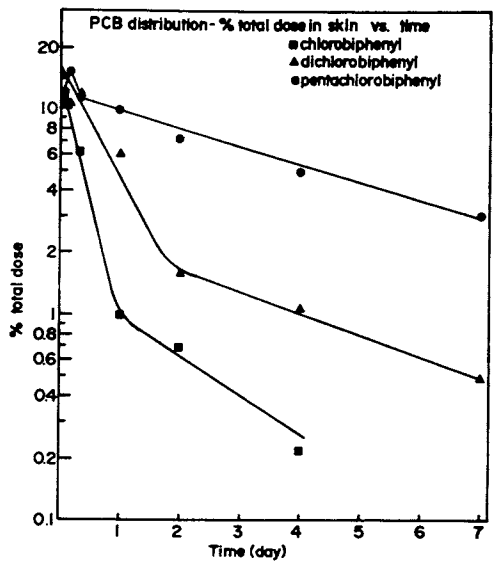

Figure 6. Percent of the total PCB dose in skin at 2, 4, and 8 hrs and 1, 2, 4 and 7 days after intravenous administration

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plateau effect seen in the removal of pentachlorobiphenyl from the fat is an artifact and that the true rate is an exponential decay represented by the dashed line. However, this is being investigated further.

PCB excretion in urine during the first 24 hours was inversely related to the degree of chlorination of the biphenyl molecule (Fig. 8). Excretion of PCB's in the urine decreased markedly during the second day and accounted for a negligible amount of the total dose after the third day. Cumulative excretion of the PCB's in the urine during the 7-day holding period accounted for approximately 57, 34 and 6.5 percent of the total dose of mono-, di- and pentachlorobiphenyl, respectively.

On the other hand, PCB excretion in the feces during the first 24 hr post-treatment was not apparently affected by chlorination (Fig. 9). By the end of the second day the excretion of the mono- and dichlorobiphenyl was limited by the fact that most of the dose had already been excreted, whereas the excretion of the pentachlorobiphenyl continued at a significant, but much slower, rate for the full 7 days.

In an effort to determine when, or if, the rats could effectively excrete the total pentachlorobiphenyl dose, three rats were treated in the usual manner and held for 21 days. Feces and urine were collected daily and the percent of the total dose excreted in the feces daily was plotted vs. time and the result was a two-component exponential decay curve for pentachlorobiphenyl excretion in the feces (Fig. 10). The fast and slow components, which are represented by the dashed lines, had half-lives of 26 hrs and 7.6 days respectively. During this time approximately 75 percent of the total dose was excreted in the feces, 6.6 percent was excreted in the urine and approximately 15 percent of the dose which remained was in the fat and 2.2 percent was still in the skin. Thus, since we cannot determine if there is another still slower component of this decay curve, we are hesitant to predict if, or when, complete clearance of pentachlorobiphenyl would occur.

Studies in our laboratory and elsewhere have shown that the overall rate of PCB elimination from the body is associated with the rate of metabolism which is inversely correlated with the degree of chlorination (8,9,10). In the rat, less than 5 percent of the pentachlorobiphenyl dose was excreted as the parent compound (11). The major metabolites were 3'-hydroxy-, 3',4'-dihydroxy- and 3',4'-dihydrodihydroxypentachlorobiphenyls. Hydroxylated metabolites have also been shown to be the major metabolites of a 4-chloro- and 4,4'-dichlorobiphenyl (9). We have also shown that pretreatment of rats with phenobarbital in an effort to induce an increased rate of metabolism increased the rate of pentachlorobiphenyl excretion in the feces by

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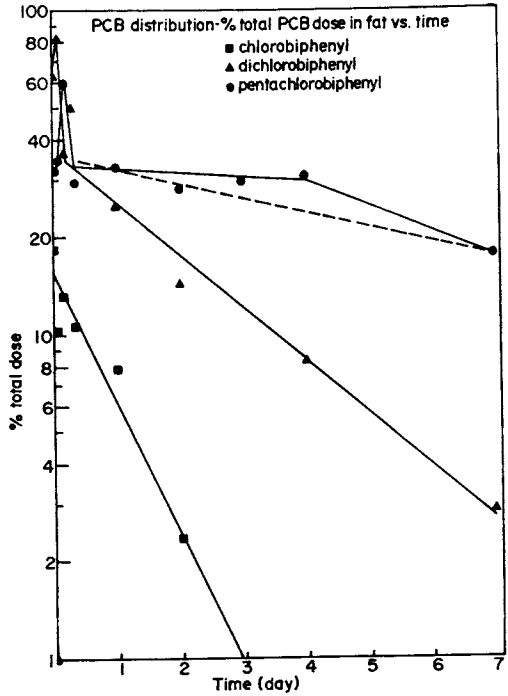

Figure 7. Percent of the total PCB dose in fat at 1, 2, 4 and 8 hrs and 1, 2, 4 and 7 days after intravenous administration

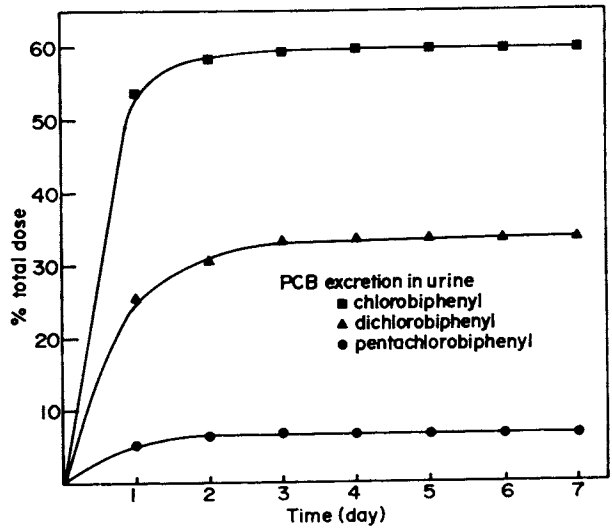

Figure 8. Cumulative daily excretion of PCBs in urine for 7 days

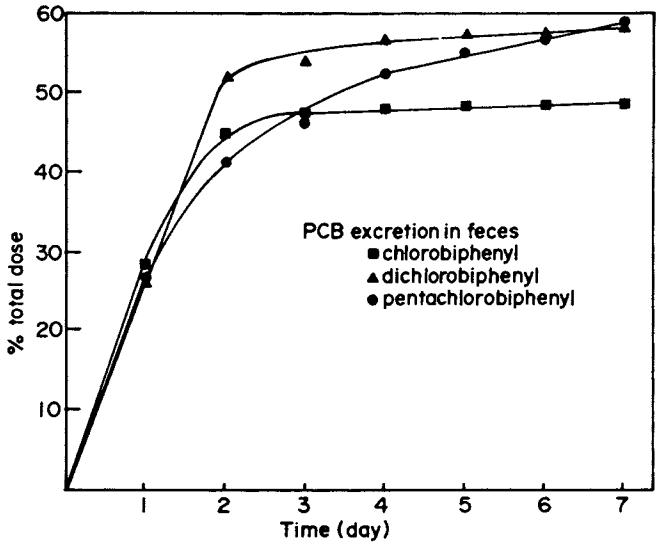

Figure 9. Cumulative daily excretion of PCBs in feces for 7 days

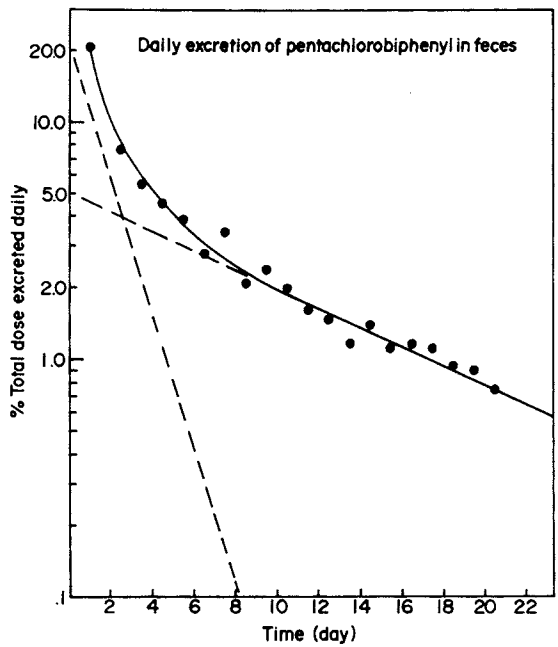

Figure 10. Daily excretion pattern of pentachlorobiphenyl in the feces for 21 days. The two components of the exponential decay are represented by dashed lines.

approximately 50 percent and resulted in a corresponding decrease in fat storage. Whereas, treatment with SKF-525 A in an effort to inhibit metabolism decreased the rate of pentachlorobiphenyl excretion in the feces by approximately 50 percent and resulted in a corresponding increased storage in fat (7).

As mentioned earlier, the polychlorinated biphenyls were chosen as the subjects of this study not only because of their importance as environmental contaminants, but also because they allowed the metabolism, distribution and excretion data obtained to be related directly to the chlorination of the same carbon skeleton. The data just presented showed that all of the compounds were rapidly removed from the blood and initially stored in the liver and muscle. Redistribution, by the blood, to the fat and skin occurred rapidly and these tissues became the major storage sites within 1 hr after PCB administration. The rates of distribution into the fat and skin, removal from these tissues and excretion in the urine was apparently inversely related to the degree of chlorination of each of the biphenyls. Excretion in the urine effectively ceased by the third day after administration for all of the compounds studied. However, excretion of pentachlorobiphenyl in the feces continued for as long as 21 days and the rate of excretion followed a two-component exponential decay curve. The slower component of the pentachlorobiphenyl excretion curve had a half-life which was similar to the half-life of this compound in fat. Only a single component for excretion in the feces was observed for the mono- and dichlorobiphenyls; however, the half-life of that component was also similar to the half-lives of these compounds in fat. Thus, the slow component of the exponential decay for fecal excretion probably represents removal of the given PCB from the fat and excretion in the bile.

Fat is well known as the major long-term storage site for chlorinated hydrocarbons, and with the PCB's used in this study the half-life in fat was directly proportional to their percent chlorine by weight (Fig. 11). Mono-, di- and pentachlorobiphenyl which have percents chlorine by weight of 18.8, 31.8 and 54.5, respectively, have half-lives in the fat of 19.8, 44.4 and 163 hours respectively. Thus, in the male rat a 10 percent increase in the chlorination of a biphenyl increased its half-life in the fat by approximately two-fold.

Among mammals the rate of clearance of xenobiotic is generally inversely related to size. Other species, such as birds and fish, are known to clear chlorinated hydrocarbons more slowly than mammals. In addition, the rate of clearance is also related to age, sex and the activity of certain enzymes as mentioned earlier. Thus, even though we expect the relationship between degree of chlorination and increased half-life

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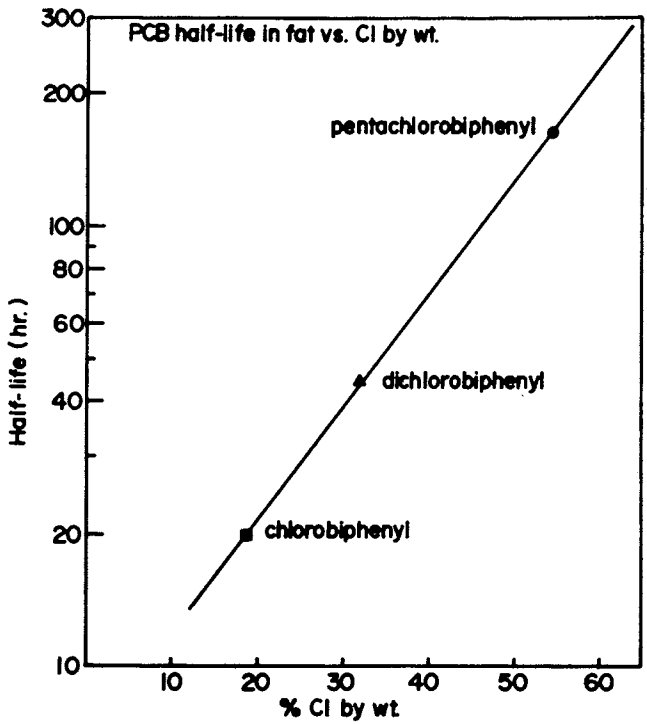

Figure 11. Effect of increased chlorination by weight on PCB halflife in fat

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in fat to extrapolate from species to species, we do not expect the slope or the level of the line shown in Fig. 11 to apply to any species other than the adult, male laboratory rat. That is, in species which metabolize and clear PCB's more slowly than the rat, we expect the increased half-life and/or level of these compounds in fat, with every 10 percent increase in chlorine by weight, to be proportionally greater than two-fold.

The PCB's are an important group of environmental contaminants and a better understanding of the factors controlling their rates of metabolism and excretion and their consequent persistence in the environment is important to the recommendations as to the safe use of these compounds. However, the factors which control the biological half-lives of these compounds probably also have a similar effect on the half-lives of numerous other chlorinated hydrocarbons. For example, the PCB's are lipophilic compounds which must be metabolized prior to their excretion, whereas the chlorinated herbicide 2,4,5-T which contains a hydrophilic group can be excreted without prior metabolism and thus has a relatively short biological half-life (12) and does not accumulate to a significant extent in animal tissues. Also the PCB's are cyclic compounds which offer no readily available site for enzymatic attack and increasing chlorination further decreases their accessibility. An example of the effect a readily metabolized site can have on the half-life of a highly chlorinated compound is seen in the insecticide endrin which has the same molecular formula and many of the same uses as the insecticide dieldrin. However, due to a different stereochemical structure, endrin is more readily metabolized and has a half-life of approximately 12 hours in the rat vs. a half-life of approximately 60 hrs for dieldrin in the same animal (13). This fact alone could account for Robinson's observation (3) that residues of endrin have never been reported in samples derived from the general human population, whereas residues of dieldrin are routinely reported.

The PCB's may offer considerable insight into the effect of chlorination on compounds which are not readily excreted or metabolized. Among these compounds are numerous chlorinated pesticides and several classes of chlorinated industrial compounds including the chlorinated naphthalenes, triphenyls and biphenyls. In these classes of compounds we anticipate an exponential increase in half-life in response to increased chlorination. And, since most of these compounds are metabolized by the same enzymes, stored in the same tissues and excreted primarily via the same route, it might also be anticipated that in many cases a prediction of their biological half-lives could be made based upon known half-lives of other chlorinated hydrocarbons. For example, dieldrin and penta-

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chlorobiphenyl have similar degrees of chlorination, neither is very rapidly metabolized, neither contains a hydrophilic group and both have very similar half-lives in the rat. If the same relationship holds for mirex and hexachlorobenzene, then the half-lives of these compounds in fat would be in excess of 30 days or approximately 5 times that of pentachlorobiphenyl. Research in our laboratory indicates that mirex is poorly absorbed from the gut, but that portion of the dose which is absorbed is excreted so slowly that a half-life could not be predicted from a 7-day holding period (14). A similar study of hexachlorobenzene has shown this compound to be more readily absorbed from the gut than mirex, and trace amounts of several metabolites have been detected. However, excretion was still so slow that the half-life could not be predicted from a 7-day study (15). We believe that two basic conclusions concerning lipophilic chlorinated hydrocarbons can be drawn from those observations. They are: 1) The rate of metabolism, with exception of those compounds which contain a hydrophylic group, controls the biological half-life of these compounds, and 2) The degree of chlorination, with exception of those compounds having a readily metabolized group, controls the rate of metabolism.

Thus, it is only reasonable to anticipate that continued contamination of the environment with such highly chlorinated compounds as mirex, hexachlorobenzene and the more highly chlorinated biphenyl, triphenyl and naphthalene formulations may eventually lead to serious residue problems. What we believe we should do is take advantage of the exceptions to the rules. That is, if highly chlorinated compounds must be used, then every effort should be made to synthesize compounds of comparable properties which also contain hydrophylic groups or groups that can be readily metabolized to hydrophylic groups. An example would be a methyl group in one or both of the four positions of the biphenyl molecule. Dr. Metcalf (1) has already shown that similar modifications of the DDT molecule can greatly reduce the biological half-life of DDT without seriously effecting its properties as an insecticide. We may have to pay more for such a product today, but the price might well prove to be cheaper in the long run.

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5

Herbicide–Lipid Interactions

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Introduction

Numerous herbicides are more soluble in lipids than in water. Therefore, we suspected that herbicide-lipid interactions might play a regulatory role in the biological expression of action of highly lipid-soluble herbicides. Enhancement of herbicide activity by surfactants also should involve interplay with lipids. In the present paper we present evidence that herbicide-lipid interactions include: (a) alterations in the phytotoxic action of some herbicides; (b) involvement in the selective phytotoxicity of some herbicides; and (c) alterations in plant lipid metabolism *in vitro* and *in vivo*. We also correlate surfactant phytotoxicity with alterations in membrane permeability of isolated plant cells and with structural alterations in artificial phospholipid bilayer membranes.

Herbicide-Lipid Interactions Leading to Alteration in Phytotoxic Action*

Studies (1) on the mode of action of the lipid-soluble pyridazinone herbicides revealed that the most potent of several known inhibitors of chloroplast pigment formation is San 6706 [4-chloro-5-(dimethylamino)-2-(α,α,α-trifluoro-*m*-tolyl)-3(2*H*)-pyridazinone]. Efforts to determine a physiological basis for this inhibition led to evaluation of certain chloroplast constituents for the circumvention of San 6706 action (2). We observed that the protective metabolites were lipoidal materials (Table I).

*This is a report on the current status of research concerning use of chemicals that require registration under the Federal Insecticide, Fungicide, and Rodenticide Act, as amended by the Federal Environmental Pesticide Control Act. Not all of the chemicals mentioned here are presently so registered with the Environmental Protection Agency. No recommendations for use of these chemicals are implied in this report.

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Table I

Lipids that protect seedlings against phytotoxicity of San 6706 and trifluralin herbicides.

Lipid	San 6706	Trifluralin
[Isoprenoid compounds]		
Tocopherol acetate	+	+
Phytol	+	+
Squalene	+	+
Ubiquinone 30	-	+
Vitamin K_1	-	+
[Alkyl chains]		
Oleic acid	+	+
Methyl oleate	+	+
Methyl linoleate	+	+
Methyl linolenate	+	+
Methyl palmitate	-	+
Methyl stearate	-	+

While various lipids will circumvent the action of San 6706 (Table I is a partial listing), we have been most interested in the interaction between San 6706 and tocopherol acetate. Because of the high concentration of lipid relative to San 6706 (>1000 to 1) required for protective action, we suggested that San 6706 partitioned into the lipid external to the plant and thereby became less available to the seedling (2). This hypothesis was substantiated with [14_C]-San 6706. Thus most of the protection occurs outside of the plant. Part of the internal protection may involve a similar trapping mechanism, because some of the treatment lipid is taken inside the plant. Also, treatment with tocopherol acetate increases the total bound fatty acids; thereby creating additional lipids to trap the herbicide.

However, lipid protection cannot be explained solely on the basis of preventing the inhibitor from reaching the chloroplast. Tocopherol acetate-treated shoots are green, even when shoots contain 3 or 4 times the amount of San 6706 required to produce white foliage in unprotected shoots. Furthermore, tocopherol treatment prevented San 6706-induced reduction in chlorophyll content without exerting a similar protective effect against induced increase in fatty acid content. This implies still another action by tocopherol, presumably physiological in nature and related to the phytotoxic action of San 6706. Tocopherols are known to function as antioxidants to lipid peroxidation (3) in both plant (4) and

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animal (3, 5) systems. In such systems, numerous compounds such as synthetic antioxidants, methylene blue, ascorbic acid, $Na_2SeO_3.5H_2O$, and various other salts may be effectively substituted for tocopherol. The failure of any of these compounds to substitute for α-tocopherol in our system indicates that tocopherol does not exert its protective action through its function as an antioxidant.

Our results with San 6706 suggested that lipids would protect plants against other lipid-soluble herbicides. One of the most lipid-soluble herbicides is trifluralin (α,α,α-trifluoro-2,6-dinitro-N,N-dipropyl-p-toluidine). Lipids were just as effective against trifluralin as against San 6706 (Table I), even though trifluralin and San 6706 have different sites and mechanisms of action. Trifluralin inhibits cell division in the growing points of roots and shoots, presumably by interfering with an early step in the formation of microtubular protein (6). The site of San 6706 action is in a lipid-rich structure, the chloroplast, where it is a potent inhibitor of the photolysis of water (Hill reaction) (1), of chloroplast pigment formation (1, 2, 7), and of membrane lipids formation (2). The data in Table I show that there are also differences in the lipoidal compounds effective against these two herbicides. With San 6706, the effective compounds are generally chloroplast lipids, whereas lipids that protect against trifluralin include those characteristic of other structures (such as ubiquinone and saturated fatty acids) in addition to chloroplast lipids.

Data in Table II show the circumvention by lipids of the trifluralin inhibition of lateral root development on cotton seedlings.

Table II

Circumvention by lipids of the trifluralin inhibition of lateral root development on cotton seedlings.

Soil treatment	Average number of roots/plant in trifluralin-treated soil (upper 5cm)		
	Control	Trifluralin	
		EC 10 ppm[a]	TG 10 ppm[a]
None	36	0	1
α-Tocopherol[b]	38	42	37
Cotton oil[b]	30	15	21

a/EC = emulsifiable concentrate; TG = technical grade.

b/Dissolved in petroleum ether and applied as a drench over the planted seeds.

Similar results have been obtained with barley and wheat seedlings (8). Also, lipid-trifluralin interactions on greenhouse-cultured cotton have substantiated laboratory findings (9).

The initial visible symptom of San 6706 toxicity to plants is strikingly similar to one of those associated with phytotoxicity of amitrol (3-amino-s-triazole). New foliage produced after treatment with San 6706 or amitrole is either a bleached white or anthocyanin red, as determined by species capacity for anthocyanin production (1). Amitrole, in contrast to San 6706 and trifluralin, is highly water-soluble. None of the lipids effective against either San 6706 or trifluralin will in any way protect against the phytotoxic action of amitrole.

Herbicide-Lipid Interactions Involved in Selective Phytotoxicity

Results from the above studies led to the hypothesis that endogenous lipids might contribute to the selective action of lipid-soluble herbicides (8). Trifluralin was selected for these studies because of its extreme lipid-solubility and because oilseed crops are generally the most trifluralin-tolerant species. A log-log plot of the linear regression of sensitivity of germinating seeds of 11 species to trifluralin on the percentage lipid content of the dry seeds showed that plants with high lipid content, such as jimson weed, are 10 times more tolerant than low-lipid seeds, such as ryegrass and millet (8). The correlation coefficient was significant at the 1% level. Therefore, the hypothesis is proposed that selective phytotoxicity of some of the most lipid-soluble herbicides is determined in part by the amount of stored lipids available to trap the herbicides and prevent them from reaching their sites of action in the plant.

A similar lipid entrapment mechanism also seems to explain the relative tolerance of cotton to San 6706. When San 6706 was applied to the roots of young seedlings, CO_2 fixation in the shoots of the sensitive species, barley and corn, was markedly inhibited after 3 and 24 hr, respectively; whereas inhibition was barely detectable in tolerant cotton after 4 days (1). We suggested that the relative tolerance of cotton to San 6706 was due to limited uptake or movement of San 6706 to its site of action. The subsequent work of Strang and Rogers (10) lends validity to this contention. [^{14}C]-San 6706 and the techniques of microautoradiograph were used to study the localization of root-applied San 6706 in tolerant cotton and in the more susceptible soybean and corn plants. San 6706 was readily accumulated in the leaves of the more susceptible soybean and corn plants. In cotton plants, the herbicide accumulated in striking concentrations in the lysigenous glands and trichomes, which are lipid-rich structures. The tolerance of cotton to another lipid-soluble herbicide, diuron [3-(3,4-dichlorophenyl)-1,1-dimethylurea], has been explained on the same basis (11). Also, the tolerance of safflower to trifluralin has been proposed to result from entrapment of the

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herbicide in oil ducts (12).

These findings support the hypothesis that the selective phytotoxicity of lipid-soluble herbicides is determined in part by the availability of endogenous lipids and/or lipoidal structures to trap the herbicide and prevent access to the site of action.

Herbicide-Lipid Interactions Related to Mode of Action

While the herbicide-lipid interactions discussed up to this point are of biological significance, they are largely of a physical nature. Evidence is beginning to accumulate, however, relating to herbicidal effects on lipid synthesis in plants. Penner and Meggitt (13) were unable to demonstrate any significant alterations in the fatty acid composition of soybean oil as a result of field treatment with 13 herbicides. However, Mann and Pu (14) demonstrated inhibition of lipogenesis by 7 of 30 herbicides tested, based on reduced incorporation of radioactive malonic acid into lipids by excised hypocotyls of hemp sesbania. Sikka et al. (15) and Zweig et al. (16) found that treatment of *Chlorella* cells with some quinone pesticides reduced acetate incorporation into lipids. More recently, Sumida and Ueda (17) showed that herbicides that are potent inhibitors of the Hill reaction substantially decreased acetate incorporation into complex lipids of *Chlorella*. The galactolipids were affected to a greater extent than other complex lipids. It was felt that the effect on these complex lipids was secondary, resulting from the inhibition of the Hill reaction, although a direct effect on enzymes responsible for galactolipid metabolism was not ruled out.

We have used the spinach leaf microsome system of Chenaie (18) to test for herbicide inhibition of triglyceride synthesis *in vitro*. In this system, L-α-^{14}C-glycerol phosphate is incorporated into phosphatidic acid, mono-, di-, and triglycerides, with phospatidic acid serving as the precursor for synthesis of the glycerides. Herbicides found to inhibit triglyceride synthesis *in vitro* are listed in Table III. Herbicides that did not inhibit this *in vitro* system include trifluralin, San 6706, nitralin [4-(methylsulfonyl)-2,6-dinitro-*N*,*N*-dipropylaniline], amitrole, picloram (4-amino-3,5,6-trichloropicolinic acid), dalapon (2,2-dichloropropionic acid), and oryzalin (3,5-dinitro-N^4,N^4-dipropylsulfanilamide).

We selected MBR 8251 [1,1,1-trifluoro-4'-(phenylsulfonyl)-methanesulfono-o-toluridide] and dinoseb (2-*sec*-butyl-4,6-dinitrophenol) to ascertain the physiological significance of inhibited glyceride synthesis *in vivo*. The physiological significance of the inhibition demonstrated *in vitro* was confirmed in intact wheat seedlings; dinoseb and MBR 8251 inhibition of glyceride synthesis *in vivo* was evidenced by a buildup in free fatty acids and a decrease in neutral and polar lipids (19). The most striking alteration effected by dinoseb and MBR 8251 was the decrease in polar lipid levels. Dinoseb and MBR 8251 reduced total polar

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lipids by 72 and 46%, respectively. Polar lipids from shoot tissue are predominantly glycolipids, which are almost exclusively confined to chloroplast membranes. Another class of polar lipids, the phospholipids, are the main lipids found in mitochondrial membranes. Thus our results suggest that MBR 8251 and dinoseb may affect membrane formation by limiting the synthesis of the structural lipids required for membrane formation. Because polar lipids were reduced more severely than growth, polar lipids could be growth-limiting.

Table III

Inhibition of L-α-^{14}C-glycerol phosphate incorporation into glycerides *in vitro*

Herbicide tested	% Inhibition at herbicide concentration (M)	
	1×10^{-4}	5×10^{-4}
PCP	31	93
MBR 8251	57	87
Dinoseb	25	71
Ioxynil	15	55

San 6706 is another herbicide with pronounced effects on polar lipid levels *in vivo*. We found that San 6706 inhibited chloroplast polar lipid formation by 74.3%, compared to 74.2% inhibition of glactolipids in the same experiment (2). The final light-mediated process of chloroplast development includes simultaneous large increases in chlorophyll and galactolipids. San 6706 inhibition of galactolipid formation is related to a deficiency of linolenic acid. Data in Table IV show that San 6706 negates the light-induced increase in linolenic acid content of polar lipids. Thus a major action of San 6706 is an inhibition of the formation of galactolipids required for chloroplast lamellar structure.

The carotenoid pigments represent a second class of lipoidal materials present in the chloroplast. Bartels and Hyde (7) discovered that the *in vivo* San 6706 inhibition of carotenoid formation was accompanied by accumulation of more saturated carotenoid precursors. They hypothesize that in the absence of the carotenoid pigments that protect chlorophyll against photooxidation, chlorophyll or a precursor of chlorophyll is an unstable photosensitizing molecule, which destroys 70 S ribosomes and thylakoids.

The data on San 6706 inhibition of galactolipid and carotenoid formation led us to consider the hypothesis that San 6706 acts as a general inhibitor of the chloroplast desaturase enzyme

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systems responsible for formation of highly unsaturated lipoidal constituents of the chloroplast.

Table IV

San 6706 effect on fatty acid composition of wheat lipids

Fatty acid	Control		San 6706	
	Dark	Light	Dark	Light
	µg/g DW	µg/g DW	µg/g DW	µg/g DW
C_{16}	3,546	4,070	4,275	4,305
C_{18}	215	205	245	270
$C_{18:1}$	1,140	1,235	3,680	3,940
$C_{18:2}$	9,310	11,145	31,140	29,300
$C_{18:3}$	14,985	22,385	7,530	7,640
18:2/18:3	0.62	0.50	4.14	3.84

Surfactant-Lipid Interactions

In practical applications, surfactants are widely used as constituents of herbicidal formulations to enhance herbicidal activities. Some surfactants show an inherent phytotoxicity in the absence of any herbicide. In fact, selective phytotoxicity of soapy solutions was reported as early as 1890. In a more recent report by Jansen et al. (20), 63 surfactants were evaluated for effects on the herbicidal activity of 4 herbicides on 2 plant species. The inherent phytotoxicity of some surfactants was again noted. We related surfactant phytotoxicity to effects on permeability of plant cells (21) by use of a model system, namely: enzymatically isolated single cells from leaf tissue of soybean and wild onion, a dicot and a monocot, respectively. The surfactants evaluated (Table V) were selected on the basis of the report of Jansen et al.(20). Tween 20 and Daxad 21 were selected to represent surfactants with low inherent phytotoxicity, and Sterox SK and AHCO DD 50 were selected as phytotoxic surfactants. Changes in cell permeability were studied by following the loss of ^{14}C-labeled material from the cells.

Treatment of cells with Daxad 21 or Tween 20, surfactants with low inherent phytotoxicity, only slightly increased the efflux of ^{14}C-labeled material (Table VI). However, treatment with Sterox SK or AHCO DD 50 markedly increased the release of intracellular material from both soybean and wild onion cells.

Treatment of cells with Daxad 21 or Tween 20, surfactants with low inherent phytotoxicity, only slightly increased the

Table V

List of surfactants studied*/

Surfactant number a/	Chemical description	Type b/	Trade name
S-145	Polyoxyethylene sorbitan monolaurate	N	Tween 20
S-102	Polyoxyethylene thioether	N	Sterox SK
S-064	Mono-calcium salt of polymerized aryl alkyl sulfonic acids	A	Daxad 21
S-029	Alkylbenzyl quaternary ammonium halide	C	AHCO DD 50

a/ Information in this table is taken from Jansen et al. (20).

b/ A = anionic; C = cationic; N = nonionic.

efflux of ^{14}C-labeled material (Table VI). However, treatment with Sterox SK or AHCO DD 50 markedly increased the release of intracellular material from both soybean and wild onion cells.

Table VI

Release of intracellular ^{14}C-material from soybean and wild onion cells in the presence of surfactants

Surfactant (0.01% concentration)	% of total incorporated radioactivity leaked in 45 min	
	Soybean	Wild Onion
Untreated Control	4	21
AHCO DD 50	82	93
Sterox SK	72	64
Daxad 21	12	14
Tween 20	11	20

*/ Mention of a trade name or proprietary product does not constitute a guarantee or warranty of the product by the U. S. Department of Agriculture and does not imply its approval to the exclusion of other products that may also be suitable.

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Based on the report of Jansen et al. (20), these surfactants can be ranked AHCO DD 50 > Sterox SK >> Daxad 21 = Tween 20 with respect to phytotoxicity. The surfactants rank in the same order with respect to their action in altering cell permeability (Table VI, and ref. 21). Additional correlations between phytotoxicity of surfactants and their effects on permeability can be drawn from reports in the literature. Phytotoxicity of surfactants reported by Jansen et al. (20) corresponds to surfactant effects on permeability of red beet root tissue reported by Sutton and Foy (22).

Temple and Hilton (23) studied the foliar toxicity of a number of surfactants applied to cucumber plants. They found that, in general, cationic surfactants showed the greatest toxicity, non-ionics were intermediate, and anionics were low in toxicity. The four surfactants we have evaluated also conform to this general order in their effect on cell permeability.

The significance of our observations of surfactant effects on isolated leaf cell permeability depends on the ability of surfactants to penetrate leaf surfaces to affect cells inside the leaf. The phytotoxicity of some surfactants is in itself circumstantial evidence of penetration. Because of limited availability of radioactive surfactant, there is only limited direct evidence of surfactant penetration. The data of Smith and Foy (24) concerning foliarly applied [^{14}C]-Tween-20 show that 3.2% of the radioactivity moved out from the treated spot in a 4-day period. If only 3.2% of a 1% solution of AHCO DD 50 penetrated to the internal cells, more than 10 times as much surfactant would be present as would be required to affect membrane permeability. We believe our data support the hypothesis that the phytotoxicity of some surfactants is exerted through an alteration in cell membranes.

The most commonly accepted structure of biological membranes involves a bimolecular thickness of lipids, which serve as a fluid supportive phase for the membrane proteins. In the last analysis, it is the lipid portion of the membrane that accounts for the permeability barrier, and its structure is an important feature of cellular transport. Therefore, we have studied the effects of these surfactants on artificial lipid bilayer membranes.

Phospholipid micelles are ordered structures, containing aqueous inner compartments, bounded by single bilayer walls. These micelles show many of the functional properties of biological membranes, especially those related to permeability. 1-Anilino-8-naphthalenesulfonate (ANS) is representative of a class of compounds known as fluorescent probes. These compounds are virtually nonfluorescent in highly polar (i.e. aqueous) solution, but are strongly fluorescent in nonpolar solvents or when bound to hydrophobic molecules.

We have correlated the fluorescence changes of ANS bound to lipid micelles with surfactant interactions at the membrane

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surface (25). Surfactant effects are observable for concentrations as low as 0.0001%. The order for ANS fluorescent enhancement is AHCO DD 50 > Sterox SK > Tween 20 > Daxad 21. Again, the more phytotoxic the surfactant, the more pronounced the effect. Results obtained with lipid micelles are significantly independent of any membrane protein components or cellular metabolic functions. Our results provide evidence that the surfactant interactions are manifested through the lipid regions of biological membranes. However, they do not rule out the possibility for other types of reactions with metabolizing biological systems. We also believe our results strongly suggest that the enhancement of herbicide activity by surfactants involves more than the simple wetting of the leaf surface.

It is interesting to note that the mode of action of numerous antibiotics also involves alterations in the permeability of cell membranes (26, 27). Many of these antibiotics are, in effect, naturally occurring surfactants. Surfactin is a proteolipid cationic detergent that induces leakiness in membranes, apparently as a result of interaction with membrane phospholipids (27).

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6

Insecticidally Active Conformations of Pyrethroids

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The most active pyrethroids are effective at 0.03 mg/kg insect bodyweight (0.002 mg/kg with synergist), and small changes in structure and configuration greatly affect their potency. Therefore, the mechanism of their action as insecticides must involve very specific interference with the biological system. The information available on the nature of the site of action from a study of molecular conformations of active compounds is reviewed. The factors considered are (a) the essential requirements for activity (b) how modifications of substituents affect activity and speed of action (c) the influence of polarity (d) the influence of asymmetric substituents.

Many powerful toxicants act by interfering with a specific component essential for normal functioning of the organism. Effectiveness at doses less than 25 mg/kg is considered to indicate such specificity (1). Table 1 shows the range of concentrations at which some mammalian poisons and insecticides act. The diversity of organisms and routes of administration necessarily limits the value of such comparisons but the figures illustrate that, as a group, pyrethroids are at least as active as other insecticides. Further, some recently developed pyrethroids, discussed here, are more potent than other insecticides, of whatever class, under our test conditions and act at concentrations comparable with other categories of outstandingly active poisons.

The lethal action of a toxic compound may be interpreted as a dynamic process at the target, in which an important factor is the strength of bonding with the receptor. This depends on the extent to which the chemical and stereochemical features of the poison and site of action complement one another. Knowing the conformation of very active biological

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agents should help locate and identify such sites of action. The purpose of this communication is to discuss these factors for the pyrethroids, especially the most potent members of the class.

To compare results from many different tests, insecticidal potency is here expressed relative to bioresmethrin, given the value of 100, as standard. The relative potency of different pyrethroids depends particularly on the insect species tested, so that generalised structure-activity relationships cannot be deduced from tests against one species. Therefore, here broad trends are indicated by approximate relative activities to both houseflies (HF) (Musca domestica L.) and mustard beetles (MB) (Phaedon cochleariae Fab.) determined by topical application of measured drops in acetone, as described in detail elsewhere (2).

The results are appropriately considered with reference to pyrethrins I and II, (Figure 1) the most important constituents of pyrethrum extract (3, 4). Both these compounds contain the basic structural features required for the most active compounds. These are methyl groups held by the carboxylic ester function of the cyclopropane ring and the cyclopentenolone in the required steric relation to an unsaturated centre (in pyrethrins I and II, the conjugated double bonds) in the alcohol side chain. In every instance investigated so far, removing any of these features decreases activity (5). A variety of groups and structures cis and trans to the carboxyl function, some greatly enhancing activity, can replace the isobutenyl side chain of chrysanthemic acid, and so direct involvement of this group in precise fit at the site of action is unlikely. However, these side chains influence the overall properties, including polarity, of the molecules, and in the active conformations of the esters are oriented so as not to impede interaction of the dimethyl group with the site of action when the unsaturated side chain is correctly positioned. Recently, (6) many of the most active pyrethroids, including pyrethrin I, have been found to have polarities (expressed by their octanol-water partition coefficients) within a narrow range of values. An important contribution of the acid side chain, therefore, appears to be to influence the polarity of the molecule from this sterically unobtrusive position.

This concept is illustrated by comparing pyrethrin I with pyrethrin II, in which a methoxycarbonyl group replaces methyl, where shown. Pyrethrin II is more polar than pyrethin I, having a partition coefficient similar to other pyrethroids which are effective knockdown agents (7). Killing power to most species is diminished by the substitution.

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The most direct evidence about the structural features of the pyrethroids important for high activity, and concerning their conformations at the site of action, comes from bioassay results with esters of symmetrical alcohols such as 5-benzyl-3-furylmethyl alcohol (8, 9) and 3-phenoxy-benzyl alcohol (5, 10) (Figure 2). Esters such as pyrethrin I, and S-bioallethrin, of the asymmetric alcohols pyrethrolone and allethrolone (11) give results more difficult to interpret, especially when only houseflies, atypical in their response to pyrethroids, are considered.

Steric requirements for the acid components of the esters are clarified by considering esters of [1R]- and [1S]-2, 2-dimethylcyclopropane carboxylic acids (Figure 3) which have similar insecticidal activity. Replacing either hydrogen atom at C-3 in the [1R], but not in the [1S] form with an isobutenyl substituent gives much more potent esters (2); this substitution is possibly at a location remote from the region on the receptor accessible to the unsaturated alcoholic side chain and the methyl groups on the cyclopropane ring. The overall properties of the ester are thus modified to give improved insecticidal efficiency without interfering with the essential access to the methyl groups. In contrast, by similar operations on the [1S] ester access to the methyl groups is impeded and insecticidal activities are much decreased.

Only unsaturated side chains in the [1R, trans] esters give high activity (Figure 4). The isobutyl compound is much less potent. Re-arranging the isobutenyl (natural) group to but-1(Z)-enyl increases potency and, with appropriate alcohols, the butadienyl acid gives almost the most potent esters known (12). All these changes are variations of a C-4 unit and illustrate the great influence of diverse types of substituent at this position in the molecule. An ester (not shown) with an isobutenyl side chain, but no methyl groups on the cyclopropane ring, is completely inactive. This evidence supports the conclusion that the dimethyl group is an essential feature of the conformation of very active pyrethroids. This group is also present in the six-carbon side chain of the ethanochrysanthemate RU 11,679 (13) and in the halovinyl compounds (12) (Figure 5). Here, with appropriate alcohols, exceptional insecticidal activity is attained, greater than in any other compound reported so far (14). As in the previous series described [1R] stereochemistry at C-1 is important.

The steric relationship of the methyl groups on the cyclopropane ring with respect to the alcohol in the potent conformations will now be considered. In the esters of Figure 2, pyrethrolone, 5-benzyl-3-furylmethyl alcohol and

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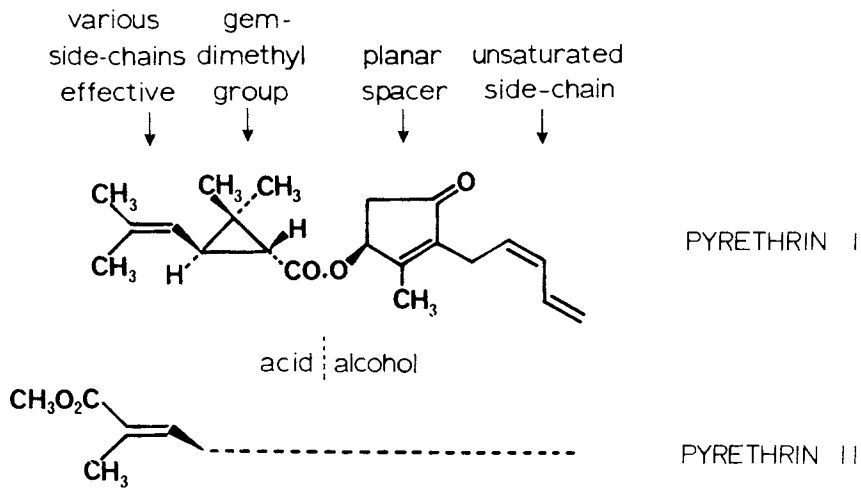

Figure 1. Structural requirements for activity in pyrethroid insecticides

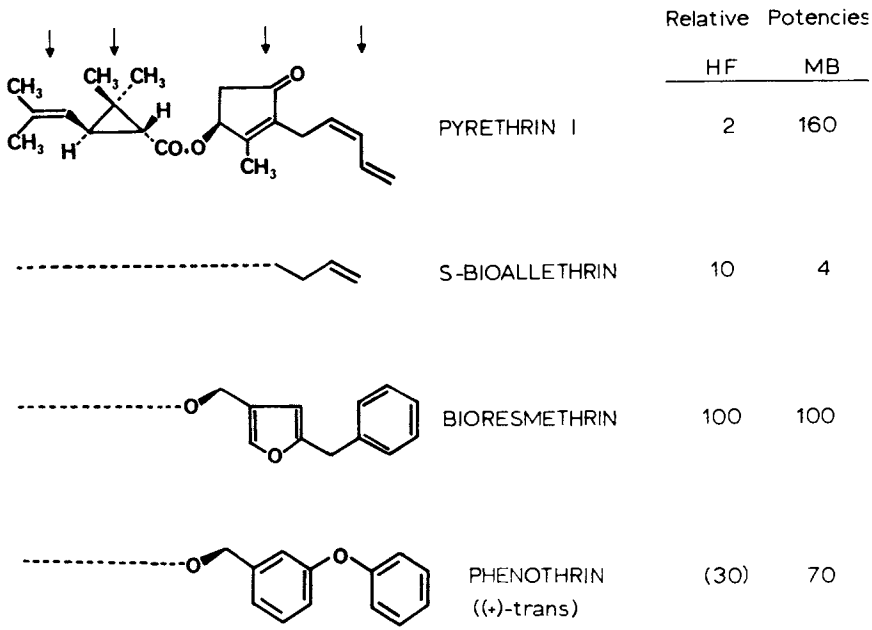

Figure 2. Some active pyrethroids

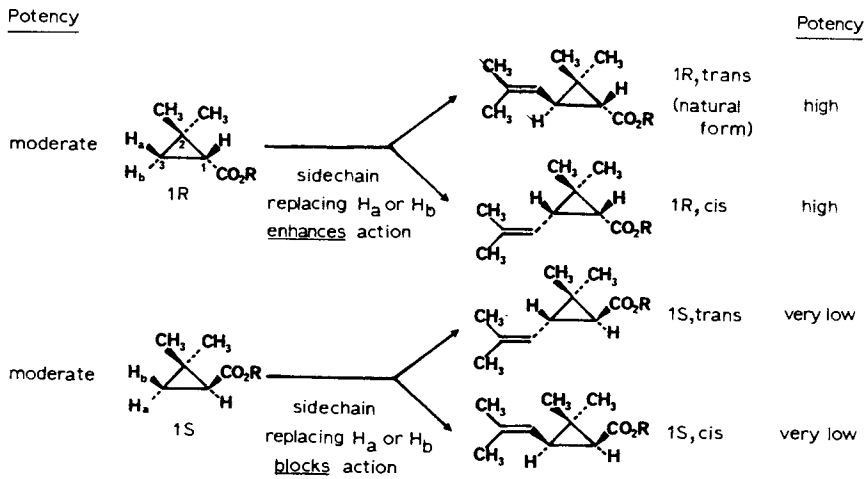

Figure 3. Effect of introducing C-3 substituent

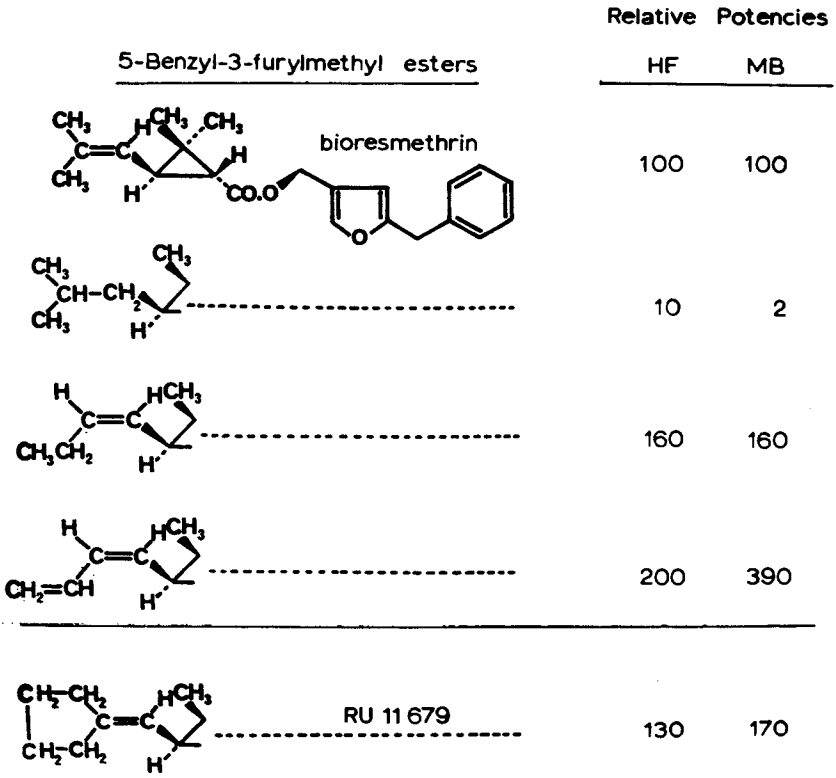

Figure 4. Various C-3 sidechains

3-phenoxy-benzyl alcohol have in common an unsaturated centre (pentadienyl, benzyl, or phenoxy) held by the cyclopentenolone ring in pyrethrolone, the 3,5-substituted furan or the m-substituted benzene ring in a very similar relationship to the alcohol link and thence, via the cyclopropane carboxylate function, to the dimethyl groups.

There is evidence that an important feature of all three alcohols, and of others giving potent esters, is the ability of the unsaturated side chain to adopt a conformation not coplanar with the ring (Figure 6). For example, the ester of the xanthene alcohol is quite inactive. Rotation about the bond shown in 3-phenoxybenzyl alcohol, and at corresponding positions in 5-benzyl-3-furylmethyl alcohol and in pyrethrolone can occur freely, but is precluded in the xanthene.

To gain further insight into the structural requirements for effective alcoholic components, numerous substituted 5-benzyl-3-furylmethyl and 3-phenoxybenzyl alcohols were investigated. Nearly all nuclear substituents on both alcohols decreased activity, as did α-methyl (Figure 7) and α-cyano groups on 5-benzyl-3-furylmethyl and 5-benzylfurfuryl esters (Figure 8). However, increased activity of the α-cyano-3-phenoxybenzylchrysanthemate prompted an examination of esters of this cyanohydrin with other effective acids, and with the [1R, cis]dibromovinyl acid it was possible to compare the esters of the two optical enantiomers (Figure 9). One of the cyanohydrins was obtained by addition of hydrogen cyanide to the aldehyde in the presence of the enzyme D-oxynitrilase, and was thus available for esterification. The crystalline ester of the other enantiomer separated on standing (Figure 10). The 5-benzyl-3-furylmethyl and 3-phenoxybenzyl esters of this acid were themselves more potent than the chrysanthemates, but the crystalline ester (NRDC 161,Figure 11) was quite exceptional and much more active than the liquid enantiomer. The latter was little, if any, more active than the unsubstituted compound, so the remarkable increase in activity is produced by replacing one of the prochiral hydrogen atoms of the α-methylene group, whilst substituting the other has little influence on toxicity. The absolute configuration of the crystalline isomer has been tentatively deduced to be S. On a molar basis the crystalline isomer is 20-30 times more active than bioresmethrin, itself more potent than many other established insecticides. From the considerations discussed at the beginning of this communication, such activity should indicate a very specific interaction with a receptor site, which other work suggests is probably in the central nervous system of the insect (15). This compound may therefore be the most effective agent yet available to investigate the mechanism of action of this group of

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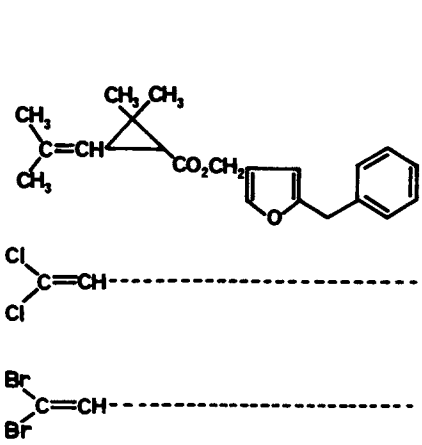


	Relative Potencies of Isomers to houseflies			
	1R,trans	1S,trans	1R,cis	1S,cis
	100	0.5	40	low
	250	3	260	14
	110	-	170	-

Figure 5. Replacement of methyl by halogen

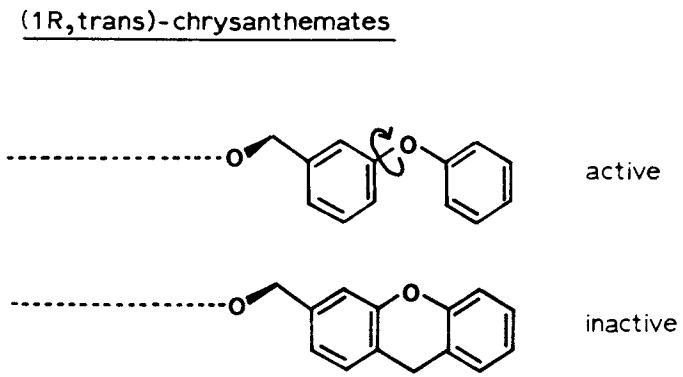


Figure 6. Conformation of alcohol sidechain

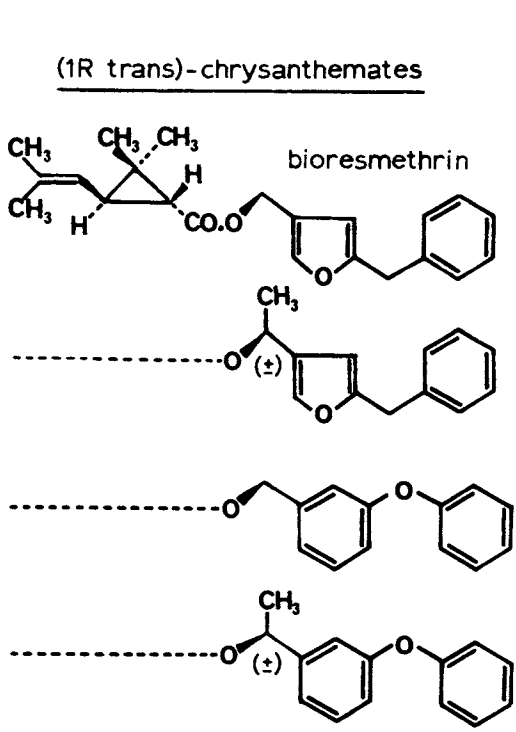


Figure 7. Effect of α-methyl group

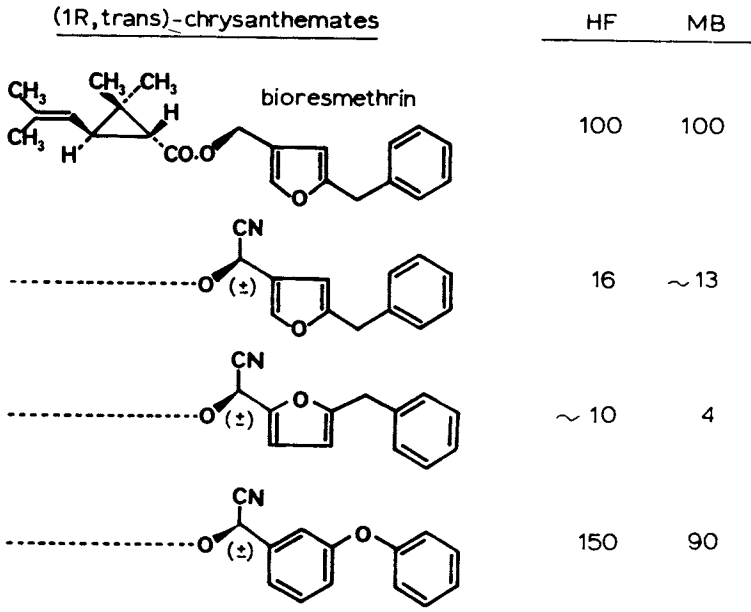
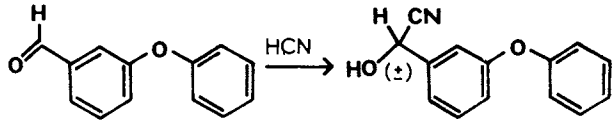


Figure 8. Effect of α-cyano group



R-form: Selective destruction of S-isomer with D-oxynitrilase, then esterification

S-form: Crystallisation from mixture of esters

Figure 9. Synthesis of isomeric cyano–esters

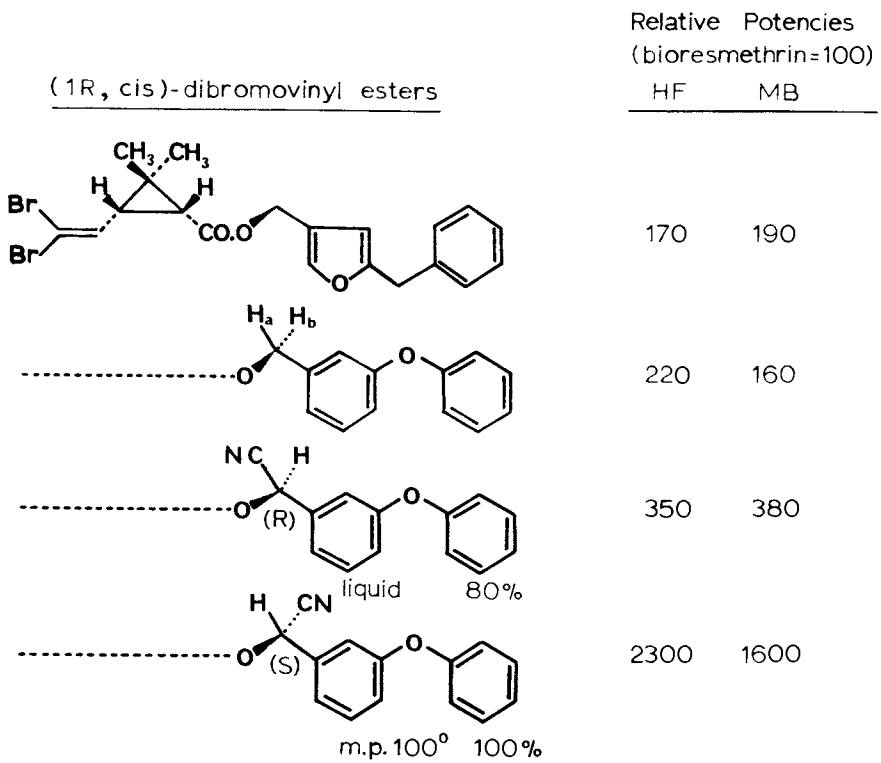


Figure 10. Effect of cyano group in each a position

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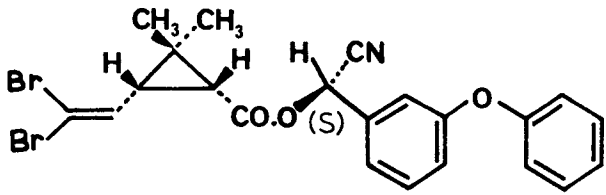

NRDC 161

M.Wt. 505

M.p. 100°

$[\alpha]_D$ +15.6°

	LD_{50} (µg/insect)
Mustard beetles	0.0003 (≡ 0.03 mg/kg)
Houseflies	0.0003 " "
Houseflies pretreated with synergist	0.00002

Figure 11. Properties of most active compound

insecticides, and to indicate their active conformations and hence the nature of their site of action.

Acknowledgements:

We thank Dr. I. J. Graham-Bryce for encouragement and critical interest and the National Research Development Corporation for financial support.

Table 1

COMPARISON OF SOME TOXICANTS

	Test species	LD_{50} (mg/kg)
Mammalian poisons:		
Cyanide	Mice	10
Curare	"	0.5
Tetrodotoxin	"	0.008
Tetrachlorodioxin	Guinea pigs	0.0006
Insecticides:		
DDT & analogues	Houseflies	7-14
Lindane	"	2
Parathion	"	2
Dieldrin	"	1
Zectran	Bees	0.6
Dimethoate	Houseflies	0.5
Pyrethroids	"	down to 0.03

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7

Interaction of Formamidines with Components of the Biogenic Amine System

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Introduction

Chlordimeform or N-(4-chloro-o-tolyl)-N,N-dimethylformamidine, a member of a new class of agricultural chemicals, is active as an adulticide, larvicide, and ovicide to ticks and mites and as a larvicide and ovicide to certain insects (1,2). It possesses moderate to low toxicity to mammals (3). Symptoms manifested by rats and mice poisoned with chlordimeform are sympathomimetic and resemble those described previously for the biogenic amine 5-hydroxytryptamine or serotonin (4,5). Beeman and Matsumura (5) administered chlordimeform intraperitoneally to rats and mice at a level of 200 mg/kg, an approximate LD_{90} dosage level. Hyperexcitation was apparent in 5 to 10 minutes, and animals exhibited tremors and became hypersensitive to external stimuli. Rats showed gradual dilation of pupils. Poisoned animals also displayed locomotor difficulties which were attributed to frequent hyperextension of the hind legs (5).

As a result of the sympathomimetic symptoms observed with chlordimeform-poisoned animals it seemed plausible that this formamidine or certain of its metabolites were interacting with components of the biogenic amine system, probably resulting in the accumulation of endogenous amines. Two types of interaction seemed likely and others also are possible. The formamidine could be interfering at the receptor, thus blocking the normal interaction of transmitter amine with its tissue receptor. Alternatively, the formamidine could be interfering with biogenic amine degradative mechanisms. Biogenic amines are inactivated or removed *in vivo* by two enzymes. Catecholamines, such as norepinephrine and dopamine, undergo O-methylation, the reaction being effected by catechol-O-methyltransferase (6). The indoleamine serotonin and the catecholamines undergo oxidative deamination; the reaction is catalyzed by monoamine oxidase (MAO) which probably acts as a scavenger enzyme to prevent excessive accumulation of amines (6).

In 1972 Knowles and Roulston suggested that demethylchlor-

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dimeform, the N-demethyl analog of chlordimeform, might be interacting with the biogenic amine system (7). This was based on the sympathomimetic symptoms displayed by formamidine-poisoned animals and on the structural similarity between formamidines and some amidine compounds previously reported as inhibitors of MAO. In 1973 Aziz and Knowles (4) and Beeman and Matsumura (5) independently reported inhibition of rat liver MAO by chlordimeform and related compounds. These latter workers made the additional observation that chlordimeform-poisoned rats accumulated norepinephrine and serotonin. This present paper elaborates on the anti-MAO activity of formamidines and related compounds and also considers their interaction with certain receptor proteins.

Experimental

Interaction with MAO. The MAO enzyme source consisted of the 500 g supernatant prepared from a 20% homogenate of rat liver. For MAO assay the spectrophotometric technique described by Weissbach et al. (8) was used. This method is based on the oxidative deamination of kynuramine by MAO at 27°C; disappearance of substrate is followed at 360 nm. The inhibitory potency of chlordimeform and related compounds was examined by adding solutions of these materials at various concentrations to the enzyme preparation 30 minutes before addition of the kynuramine (4).

Interaction with Receptor. Ventricles (1 g) from hearts of male rats were placed in cold Ringer's solution (3 ml) buffered at pH 7.4, and a homogenate was prepared. The homogenate was centrifuged for 20 minutes at 18,400 g, and a microsomal pellet was obtained by centrifuging the 18,400 g supernatant for 4 hours. The microsomes were resuspended in one half of the original volume of Ringer's solution containing 0.1% Lubrol PX. This preparation was used immediately for binding studies or was frozen at -20°C for a maximum of two weeks. Binding of norepinephrine-^{3}H (specific activity 6.41 Ci/mmole, New England Nuclear Corp.) to rat cardiac microsomes was determined by the equilibrium dialysis technique (9,10). The rat cardiac microsomal suspension (0.5 ml) was added to a small dialysis tube, and the tube was placed in 100 ml of Ringer's buffer solution containing norepinephrine-^{3}H at the desired concentration. Dialysis was allowed to continue for 12 hours at 4°C with slow shaking. Three 0.1-ml aliquots from the bath and tube, respectively, were radioassayed, and the amount of norepinephrine bound to the microsomes was calculated. Compounds examined as potential blockers of norepinephrine-^{3}H binding were added to the bath prior to introduction of the dialysis tube. The protein concentration of the rat cardiac microsomes was determined by the method of Lowry et al. (11).

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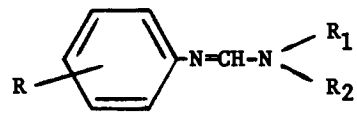
Results and Discussion

Table 1 gives the potency of formamidines and related compounds as inhibitors of rat liver MAO. Compounds with I_{50} values of 10^{-4}M or less included chlordimeform, Hokko-20013, demethylchlordimeform or C-8520, C-22840, BTS-27271, and C-10405. I_{50} values for iproniazid and tranylcypromine, two classical MAO inhibitors, are included for comparison. There was no obvious relationship between formamidine structure and anti-MAO activity; however, the most active inhibitors were N'-arylformamidines with N,N-dimethyl, N-methyl N-methylthiomethyl, N-methyl, or N-ethyl moieties. We have not studied the toxicity of each of these formamidines to rats in an attempt to correlate toxicity with MAO inhibition.

The anti-MAO activity of BTS-27419 and BTS-23376 also is given in Table 1. BTS-27419 with an I_{50} value of 6.6×10^{-7}M was the most potent formamidine-like compound examined comparing favorably with tranylcypromine. BTS-27419 and BTS-23376 are not formamidines in the strict sense. However, there is evidence that BTS-27419 converts to BTS-27271 or N'-(2,4-dimethylphenyl)-N-methylformamidine and that BTS-23376 converts to demethylchlordimeform (2).

TABLE 1

INHIBITION OF RAT LIVER MONOAMINE OXIDASE BY FORMAMIDINE COMPOUNDS



Compound	R	R_1	R_2	I_{50}, M
Chlordimeform	2-CH_3,4-Cl	CH_3	CH_3	1.4×10^{-5}
C-8519	2-CH_3,4-Cl	n-C_3H_7	n-C_3H_7	5.2×10^{-2}
H-20013	2-CH_3,4-Cl	CH_3	CH_2SCH_3	1.1×10^{-5}
C-8520	2-CH_3,4-Cl	H	CH_3	4.7×10^{-6}
C-22840	2-CH_3,4-Cl	H	C_2H_5	5.6×10^{-6}
C-22511	2-CH_3,4-Cl	H	i-C_3H_7	8.8×10^{-4}
C-8515	2-CH_3,4-Cl	H	n-C_4H_9	1.1×10^{-4}
C-9496	2-CH_3,4-Cl	H	t-C_4H_9	1.7×10^{-4}
BTS-27271	2-CH_3,4-CH_3	H	CH_3	2.7×10^{-5}
C-14640	2-CH_3	CH_3	CH_3	7.2×10^{-4}
C-10405	2-CH_3,4-Br	CH_3	CH_3	2.2×10^{-5}
C-17294	2-C_2H_5,4-Br	CH_3	CH_3	5.9×10^{-4}
C-17296	2-Cl,4-Br,6-CH_3	CH_3	CH_3	5.0×10^{-4}
BTS-27419	2-CH_3,4-CH_3	CH_3	-CH=N-Ph-CH_3-2,4	6.6×10^{-7}
BTS-23376	2-CH_3,4-Cl	CH_3	-CH=N-Ph-CH_3-2,Cl-4	6.8×10^{-5}
Iproniazid				6.3×10^{-6}
Tranylcypromine				5.8×10^{-7}

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Table 2 presents the anti-MAO activity of chlordimeform metabolites. Each of these metabolites possessed some anti-MAO activity, but, other than chlordimeform and demethylchlordimeform, only the formotoluidide had an I_{50} value of less than 10^{-4}M.

TABLE 2

INHIBITION OF RAT LIVER MONOAMINE OXIDASE BY CHLORDIMEFORM AND METABOLITES

Compound	I_{50}, M
Chlordimeform	1.4×10^{-5}
Demethylchlordimeform	4.7×10^{-6}
4'-Chloro-o-formotoluidide	2.5×10^{-5}
4-Chloro-o-toluidine	1.2×10^{-4}
N-Formyl-5-chloroanthranilic acid	8.6×10^{-4}
5-Chloroanthranilic acid	1.9×10^{-4}

Table 3 presents the influence of norepinephrine concentrations, ranging from 0.76 nanomolar to 5.84 nanomolar, on binding to rat cardiac microsomes. Binding varied directly with norepinephrine concentration and was generally irreversible as, on the average, more than 90% of the original bound amine remained with the microsomal fraction upon redialysis.

TABLE 3

BINDING OF NOREPINEPHRINE-^{3}H TO RAT CARDIAC MICROSOMES

Norepinephrine concn, nM	Binding pmoles/mg protein	Reversibility %
0.76	0.51	13.6
1.99	1.15	9.1
2.12	1.59	6.1
2.94	1.67	7.6
3.22	2.21	10.3
3.65	2.39	5.3
4.21	2.67	7.7
5.49	3.02	8.1
5.84	4.28	6.4

The effect of catecholamines and related compounds on norepinephrine-^{3}H binding to rat cardiac microsomes is given in Table 4. The concentration of norepinephrine-^{3}H was 2 nanomolar,

and 100% control binding corresponded to 1.6 picomoles of norepinephrine bound per mg of microsomal protein. The concentration of compounds examined as potential blockers was 10 micromolar. The beta-adrenergic agents epinephrine, norepinephrine, isoproterenol, DOPA, and dopamine appreciably reduced norepinephrine-^{3}H binding. Phenylephrine and ephedrine, two latent beta-adrenergic agents, were inactive as was propranolol, a beta-adrenergic blocking agent. Dihydroxymandelic acid, a dihydroxyphenyl compound, reduced binding. Normetanephrine, metanephrine, vanillylmandelic acid, and homovanillic acid, all 3-*O*-methylated metabolites of adrenergic agents, were inactive, as was beta-phenethylamine. The sulfhydryl reagents *p*-chloromercuribenzoic acid and *N*-ethyl maleimide reduced control norepinephrine binding to 64 and 54%, respectively. We recognize that rat cardiac microsomes might contain several different binding sites for norepinephrine; however, with one exception, the drug profile given here is consistent with that of the beta-adrenergic receptor isolated from cardiac tissue of dogs by Lefkowitz and associates (12,13,14). Propranolol was the exception. This beta-adrenergic blocker inhibited norepinephrine binding to dog beta-adrenergic receptor protein approximately 30% at a concentration of 1 X 10^{-4}M (12). We observed no inhibition with this compound; however, the highest concentration examined by us was 1 X 10^{-5}M, and this may account for the apparent disparity.

TABLE 4

EFFECT OF CATECHOLAMINES AND RELATED COMPOUNDS ON NOREPINEPHRINE-^{3}H BINDING TO RAT CARDIAC MICROSOMES

Compound	% Control Binding
Epinephrine	52
Norepinephrine	32
Isoproterenol	67
DOPA	19
Dopamine	23
Phenylephrine	100
Ephedrine	100
Propranolol	100
Dihydroxymandelic acid	89
Normetanephrine	100
Metanephrine	100
Vanillylmandelic acid	100
Homovanillic acid	100
beta-Phenethylamine	100
p-Chloromercuribenzoic acid	64
N-Ethyl maleimide	54

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Table 5 gives the effects of formamidines and aryl amines on the binding of norepinephrine-^{3}H to rat cardiac microsomes. N-methyl, N-ethyl, N-i-propyl, N-n-butyl, and N-t-butyl N'-(4-chloro-o-tolyl) formamidines did not significantly decrease norepinephrine binding to rat cardiac microsomes since control binding was greater than 90% in every case. However, N-methyl, N-ethyl, and N-t-butyl N'-(1-naphthyl) formamidines appreciably reduced norepinephrine binding. Norepinephrine binding in the presence of these compounds ranged from 65 to 75% of the control (Table 5).

Arylamines also reduced binding of norepinephrine to rat cardiac microsomes (Table 5). 4-Chloro-o-toluidine, the chlordimeform metabolite, reduced norepinephrine binding to 58.3% of the control. 3-Chloro-o-toluidine, 3-chloro-p-toluidine, 4-chloroaniline and 3,4-dichloroaniline also were active. 1-Naphthylamine was the most active reducing binding to only 30.3% of the control (Table 5).

TABLE 5

EFFECT OF FORMAMIDINES AND ARYL AMINES ON NOREPINEPHRINE-^{3}H BINDING TO RAT CARDIAC MICROSOMES

Phenylformamidines	% Control Binding
C-8520, R=CH_3	91.3
C-22840, R=C_2H_5	94.2
C-22511, R=i-C_3H_7	94.2
C-8515, R=n-C_4H_9	92.1
C-9496, R=t-C_4H_9	92.8
Naphthylformamidines	
R=CH_3	67.4
R=C_2H_5	65.4
R=t-C_4H_9	75.1
Aryl Amines	
4-Chloro-o-toluidine	58.3
3-Chloro-o-toluidine	49.8
3-Chloro-p-toluidine	40.0
4-Chloroaniline	46.4
3,4-Dichloroaniline	52.9
1-Naphthylamine	30.3

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Conclusions

Chlordimeform and its metabolites demethylchlordimeform (C-8520), 4'-chloro-o-formotoluidide, 4-chloro-o-toluidine, N-formyl-5-chloroanthranilic acid, and 5-chloroanthranilic acid each inhibit rat liver monoamine oxidase with demethylchlordimeform being the most potent. Demethylchlordimeform did not significantly inhibit binding of norepinephrine to rat cardiac microsomes (possibly the beta-adrenergic receptor); however, 4-chloro-o-toluidine did appreciably inhibit norepinephrine binding. Other formamidines and related compounds also inhibited rat liver monoamine oxidase, and several N-(1-naphthyl) N-monoalkylformamidines and aryl amines blocked the binding of norepinephrine to rat cardiac microsomes.

Clearly, chlordimeform, certain of its metabolites, and other formamidines and related compounds are interacting with components of the biogenic amine system. These interactions are likely involved, at least in part, in the toxic action of formamidines. Further studies are obviously required to understand the toxicological significance of these interactions. What remains to be demonstrated is a high degree of target specificity by chlordimeform itself or by one of its metabolites.

Contribution from the Missouri Agricultural Experiment Station. Journal Series No. 7013.

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8

Structure, Activity, and Selectivity of 1,2,4-Triazinone Photosynthesis Inhibitors. Quantitative Studies

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Summary

1,2,4-Triazinones are a recently developed class of potent herbicides whose commercial exploitation has just begun (product: Metribuzin). Their principal mode of action is an inhibition of the electron transport in photosystem II (Hill reaction). Chemical variations in all the three free positions of the triazinone heterocyclus have led to active compounds. Regression equations of the Hansch type, correlating pI_{50}-values from photosynthesis inhibition and several physico-chemical parameters, were successfully used to direct synthesis. Interactions of the substituents in 3-, 4-, and 6-position of the heterocyclus are not additive, therefore several triazinone classes had to be analysed seperately. As an approach towards the problem of herbicide selectivity, in vivo data obtained from several plant species were correlated with pI_{50}-values and partition coefficients. Significant relationships resulted with some plants, indicating a dependance of the herbicidal activity on penetration and transport processes.

A decade ago, the synthesis of a new class of heterocyclic compounds, 4-amino-1,2,4-triazin-5-ones was first described in the literature (1). Shortly after that, their herbicidal properties were discovered. One product has been developed so far, the soy bean herbicide metribuzine (Sencor®) (2,3). The herbicidal potential which was found in this class of heterocycles made them worthy of rather thorough structure-activity studies with the objective of broadening the range of useful compounds.

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Mode of action and structural requirements of photosynthesis inhibitors

1,2,4-Triazinones are photosynthesis inhibitors (4,5), thus belonging to that large group of herbicides which act upon the most important energy-conserving process of the living plant. Fig. 1 shows a recent scheme of the photosynthetic electron transport and the sites of electron flow blocking by several common inhibitors (6).

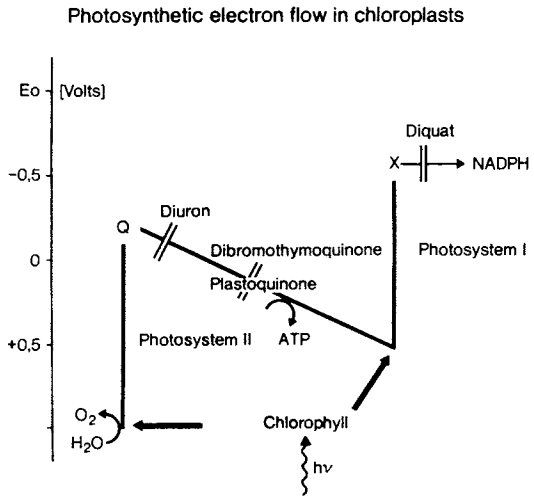

Fig. 1

Numerous compounds of diverse chemical structure act on the same site as diuron. Among them are about 50% of all commercially important herbicides. The chemical structure of the receptor within the electron transport chain is still unknown. Known, however, is the ratio of chlorophyll molecules to inhibition sites. The figure has been determined by several methods to be 2500 (7,8). It corresponds well with the value of the photosynthetic unit which produces one molecule of O_2 per step (9). The binding of the diuron type inhibitors is reversible as could be shown by restoring the photosynthetic activity of inhibited chloroplasts through simple rinsing with water (7). Reversibility of inhibition is a necessary requirement when quantitative structure activity analyses are considered, since the activity data are measured under equilibrium conditions. Photosynthesis inhibitors belong to different chemical classes as shown

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in Fig. 2.

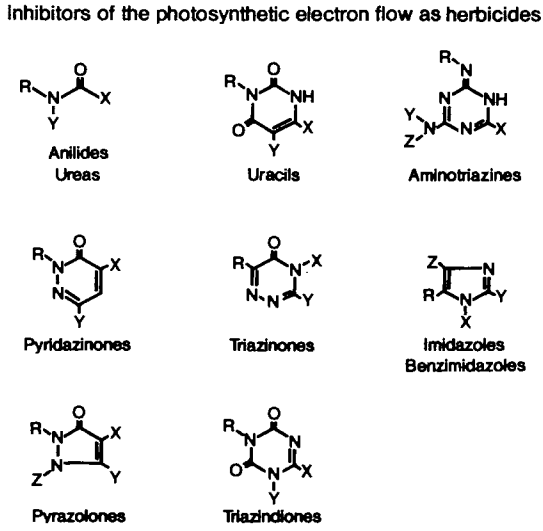

Fig. 2

All these molecules, however, have two common features:

1. the substructure $N-\overset{\overset{X}{\|}}{C}-$ with
   X = O or N (but not S)

2. a lipophilic group in the close vicinity of the essential substructure.

To fit into this scheme the amino-triazines have to be written in a somewhat unusual way which, however, is not in disagreement with their chemical and physical properties. It follows from this general structure that a free NH-group is not essential, a statement which is in contrast to earlier proposals in the literature (10,11), and which is confirmed by a number of examples.

Photosynthesis inhibition by triazinones

Among the triazinones, many of the most active members contain a N-amino group although this is not necessary for activity. The general structure and a few examples are shown in fig. 3.

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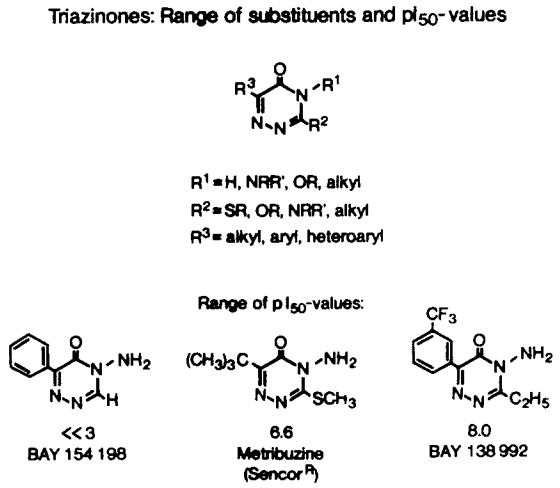


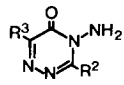
Fig. 3

One of the examples shown in fig. 3, the compound BAY 138992, is the most active photosynthesis inhibitor we have ever found. Its pI_{50}-value of 8.0 is about the highest that can be expected under our experimental conditions on theoretical grounds. Calculations show that nearly all molecules of inhibitor must be bound to the receptor.
Broad variations in all positions of the basic structure can be carried out without loss of the inhibitory activity, although sometimes apparently minor variations like putting a H into position R^1 lead to completely inactive compounds.

Structure-activity correlations with in-vitro data

Structure-activity correlations in the triazinone group were performed as soon as their mode of action was discovered and thus biological in-vitro data became available (4,5).
They were successfully used as a guide to further synthesis and have initiated interesting developments. To elucidate structure-activity relationships, four different sets of triazinones were analysed separately. They differed from each other with regard to variations of R^1 , R^2, and R^3.
Set 1 was mainly varied in position R^3 with small variations of R^2 (SCH_3, OCH_3, $NHCH_3$).

Set 1: Quadratic regression equation



No.	R_2	R_3	log P	obs. pI_{50}	calc. pI_{50}	ΔpI_{50}
1	SCH_3	CH_3	−0.16	3.88	4.28	0.40
2	OCH_3	i-C_3H_7	−0.06	4.57	4.49	0.08
3	$NHCH_3$	i-C_3H_7	+0.30	5.79	5.15	0.64
4	SCH_3		0.38	5.38	5.28	0.10
5	SCH_3	C_2H_5	0.46	5.27	5.40	0.13
6	SCH_3	n-C_3H_7	0.93	5.70	6.01	0.31
7	SCH_3	i-C_3H_7	1.01	6.24	6.09	0.15
8	SCH_3	i-C_4H_9	1.39	6.15	6.39	0.24
9	SCH_3	C_6H_5	1.66	6.63	6.52	0.11
10	SCH_3	t-C_4H_9	1.70	6.63	6.54	0.10
11	SCH_3	i-C_5H_{11}	1.85	6.40	6.56	0.16
12	SCH_3	c-C_6H_{11}	2.14	6.60	6.57	0.03
13	SCH_3	n-C_6H_{13}	2.68	6.43	6.37	0.07

$pI_{50} = 4.608 + 1.959 \log P - 0.486 (\log P)^2$ $r = 0.953$ $s = 0.259$

Table 1

A quadratic regression equation was found to give a good fit of the experimental data. The octanol-water partition coefficient was the only parameter used in this correlation.

Set 1: parabolic regression curve

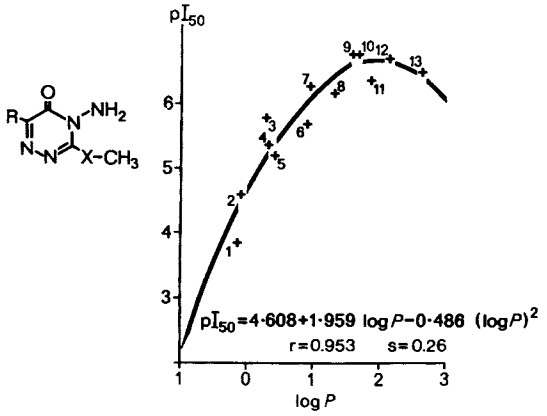


Fig. 4

This simple application of the Hansch approach had been carried out in retrospection in 1968. From the activity data of triazinones with R^2-groups other than SCH_3, $NHCH_3$ it was inferred that R^2 contributed to inhibition mainly by steric interaction with the binding site. As a consequence, the synthesis of compounds with further variations of R^2 was suggested. Set 2 consists of a number of compounds which have resulted from this suggestion, among others that did not meet the narrow range of R^2-variations of set 1. A quantitative correlation of the activity data with physico-chemical parameters presented a formidable problem. Hansch equations with the parameters π, π^2 and σ, σ^2 were of rather low significance (correlation coefficients < 0.8). Some improvement could be gained by a novel parameter, δ, which was experimentally derived from partitioning the compounds between water and Sephadex G-10 gel (12).

$$pI_{50} = 5{,}23 + 2{,}1\ \Delta R_{M1} - 3{,}4\ \Delta R_{M1}^2 + 2{,}2\ \Delta R_{M2} - 0{,}5\ \Delta R_{M2}^2 - 16{,}6\,\delta + 45{,}0\,\delta^2$$

$$n = 28$$
$$r = 0{,}94$$
$$s = 0{,}41$$

This equation contains linear and squared terms of ΔR_M-values which are hydrophobic substituent parameters obtained by reversed-phase thin-layer chromatography, and in addition δ, which probably reflects a mostly steric effect on binding to a surface. Correlations with the molecule parameters logP and R_M, and with Taft's steric constant E_s were insignificant. The occurence of squared terms of the hydrophobic parameters in structure-activity correlations has been explained by Hansch and co-workers in two ways. Both explanations rest on the assumption that the compound has to penetrate several lipophilic-hydrophilic barriers or compartments on its way to the site of action.
In the case of the triazinone set 2, however, these explanations do not seem to be valid since correlations with hydrophobic molecule parameters (logP or R_M) were not significant, and the coefficients

of the hydrophobic substituent parameters in the regression equation were different for positions R^2 and R^3. Hence we conclude that the hydrophobic parameters are reflecting the binding to the receptor rather than penetration. R^1 seems to play a specific role in the interaction with the receptor. The δ-values which refer to R^1 only, may bear some relevance to that particular type of interaction although the only moderate fit of the calculated data points (fig. 5) shows that Sephadex gel might not be the best model system.

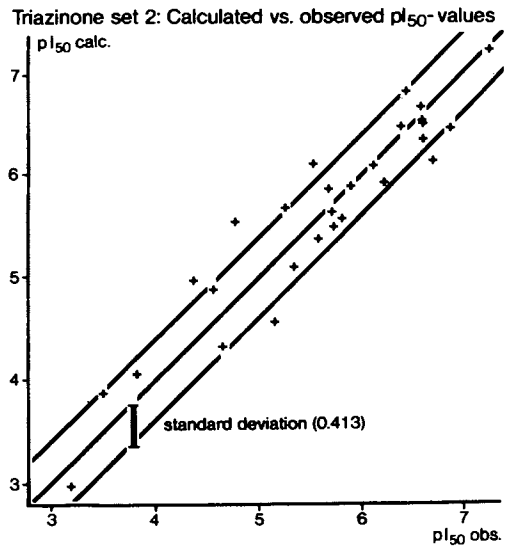
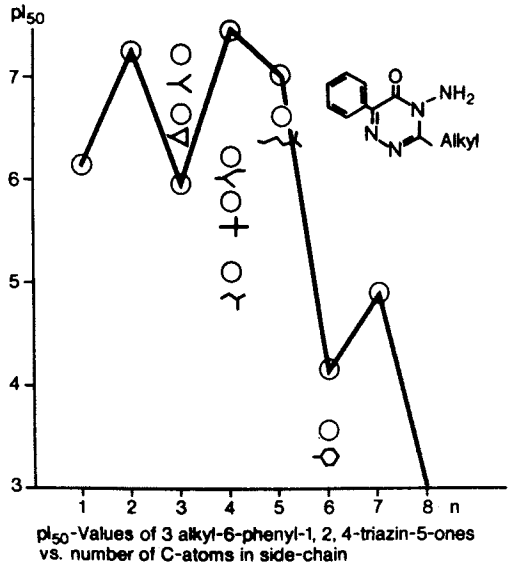


Fig. 5

The very complex effect of R^2 on the biological activity becomes obvious by an inspection of set 3, where R^2 is alkyl from C_1 to C_8 and R^1 and R^3 are kept constant.



pI_{50}-Values of 3 alkyl-6-phenyl-1, 2, 4-triazin-5-ones vs. number of C-atoms in side-chain

Fig. 6

In the n-alkyl-groups whose pI_{50}-values are connected by a line, electronic substituent effects are nearly constant, and the hydrophobic parameters increase monotonously with the number of C-atoms. The Taft constant E_s does not vary much from C_3 to C_8. Thus it is evident that none of these parameters is correlated with pI_{50} in an obvious way. So far, no satisfactory solution of the structure-activity problem presented by this set of compounds has been found.

In a further set, major variations of R^1, the substituent at the ring nitrogen, were included.

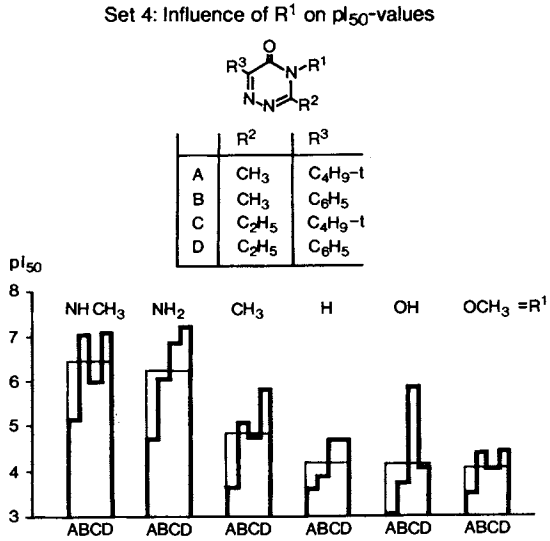


Fig. 7

The average photosynthesis inhibition was about the same with $R^1 = NH_2$ and $NHCH_3$. Triazinones with $R^1 = CH_3$, H, OH, and OCH_3 show decreasing levels of activity. On the other hand, even in these groups pI_{50}-values of nearly 6 are reached. This clearly shows that in the triazinone class a free NH-group is not necessary for activity, although the most active triazinones contain one.
A quantitative analysis of the set 4 data could not be achieved with the usual molecule and substituent constants. In addition, parameters obtained by HMO-calculations were included in the regression equations. Of the quantum-chemical data, especially E_{HOMO} and E_{LUMO} show considerablbe variance within this set, but no significant correlations could be obtained by using them.

A summary of the structure-activity relationships within the triazinone sets 1-4 is shown in fig. 8.

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Triazinone sets 1-4: Results of structure-activity relationships

Set	Variations of R^1	R^2	R^3	Parameters used in correlations	N	r	s
1	I	I	■	log P	13	0.953	0.26
2	I	■	■	$\Delta R_M{}^1, \Delta R_M{}^2, \delta$	28	0.938	0.41
3	I	■	I	log P, R_M, Es, δ	18	no correlation	
4	■	I	I	log P, R_M, δ, E_{HOMO} etc.	24	no correlation	

Fig. 8

Problems of correlations with in-vivo data

From this analysis it became evident that formidable problems of correlating structure and activity occur when wide variations of the molecule are carried out, even when biological in-vitro data can be used. The final aim of this study, however, is not an active photosynthesis inhibitor, but a herbicide, preferable a selective one. Any quantitative study in this direction would require the use of in-vivo data which can, however, only be measured with much lower accuracy than in-vitro data. It appeared to be without a chance of success, therefore, to correlate herbicidal activity with molecule or substituent parameters in the rather complex triazinone sets 2-4. A more promising approach might be to treat the in-vitro pI_{50}-value as an independant variable and use it together with physico-chemical molecule parameters in regression equations. Still, a number of requirements have to be met if any success is expected. The problems involved become obvious when a photosynthesis inhibitor is followed on its path from external application to the final site of action within the thylacoid membrane of the chloroplast (fig. 9).

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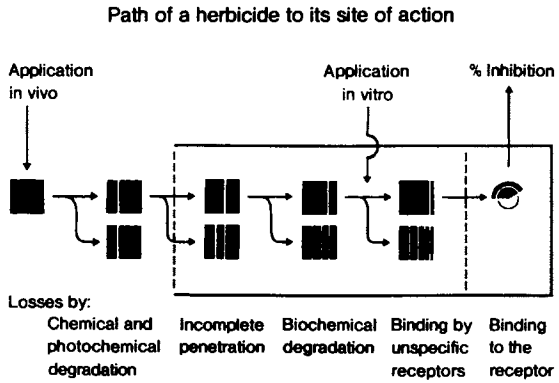

Fig. 9

Provided all compounds included in an analysis have the same mode of action, it appears feasible to account for the penetration process and unspecific binding by a hydrophobic parameter. In case of H-bonding groups, an electronic parameter might be required. Chemical, photochemical, and biochemical degradation will probably make a quantitative analysis impossible when they occur to a greater extent with some members of a given set.

Selectivity of herbicides

Selectivity, one of the most important properties of a modern herbicide, can be detected by comparing the in-vivo results from typical crop and weed species. Thus a rational approach to the problem of herbicide selectivity might be achieved if significant in-vivo correlations for several plants are obtained. We tested this possibility with seven crop plants from the herbicide screening.
ED_{50}-values were calculated from regression lines obtained with glass-house screening data.
R_M-values from thin-layer chromatography on polyamide were taken as hydrophobic parameters. They were shown previously to be a good substitute of partition coefficients measured in the octanol-water system within in this class of compounds. Regression equations were calculated with the data of a 10-compound set of triazinones. Equations of the form

$$(1)\quad \log(1/ED_{50}) = a_0 + a_1 \cdot pI_{50}$$

and

$$(2)\quad \log(1/ED_{50}) = a_0 + a_1 \cdot R_M$$

were insignificant. Good to fair correlations, how-

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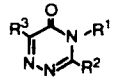
ever, were obtained with the equation:

$$(3)\ \log(1/ED_{50}) = a_0 + a_1 \cdot pI_{50} + a_2 \cdot R_M$$

A quadratic term gave no improvement. The biological data refer to post-emergence tests. With pre-emergence data no significant correlations were obtained. Results are shown in table 2.

10 Triazinones: In-vivo correlations (post-emergence data)



R^1 = H, NH_2
R^2 = C_2H_5, C_3H_7, C_3H_7-i, C_3H_5-c, C_4H_9, C_4H_9-s, C_4H_9-t
R^3 = C_3H_7-i, C_6H_5, 4-Cl-C_6H_4

Table 2

log (1/ED_{50}) post-emergence	$\log(1/ED_{50}) = a_0 + a_1 \cdot pI_{50} + a_2 R_M$				
	a_0	a_1	a_2	r	s
Sorghum	1.78	0.34	−0.74	0.74	0.35
Oat	2.91	0.19	−1.40	0.77	0.43
Wheat	2.15	0.27	−1.04	0.81	0.31
Cotton	0.53	0.57	−1.48	0.95	0.22
Sugarbeet	1.74	0.45	−0.99	0.71	0.50
Beans	2.47	0.21	−0.99	0.84	0.25
Mustard	2.32	0.40	−0.25	0.62	0.49

An equation of good significance was obtained with cotton, followed by beans and wheat. The bean values lie in a rather narrow range, but the cotton and wheat values cover two powers of ten. Some consideration of the corresponding equations seems appropriate.

Plots of the measured and calculated $\log(1/ED_{50})$-values of the cotton and wheat equations are shown in fig. 10.

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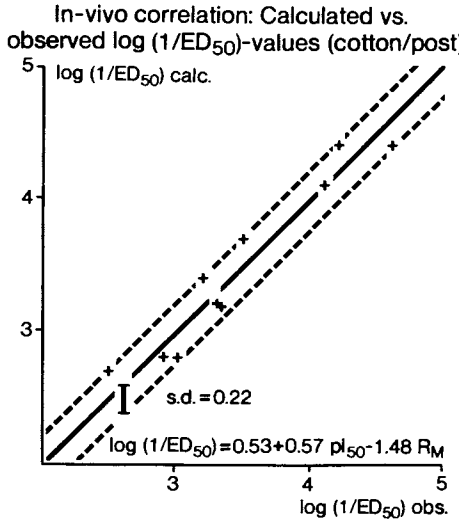
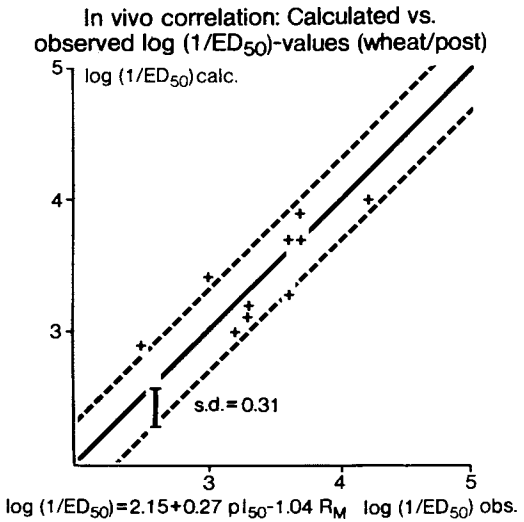


Fig. 10



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In the cotton equation, the coefficient a_2 is the most negative of all. This would imply that cotton may tolerate relatively high rates of strongly lipophilic compounds even when they are active photosynthesis inhibitors. This could be confirmed by further examples, e.g. the compound BAY 138992 mentioned before to possess a $pI_{50} = 8$. It is highly lipophilic and is tolerated very well by cotton.
In the wheat equation, a_0 has a very large value which indicates that much of the variance in the data is not accounted for by the two variables. The same holds true for the other equations, which do not allow any valid conclusions to be drawn.
It appears interesting that several plants have completely resisted the attempt to correlate their sensitivity to photosynthesis inhibitors with pI_{50} and a hydrophobic parameter. An explanation might be that in these plants some compounds of the series are detoxified by specific mechanisms at a greater rate than others. This means that one of the prerequisites of any successful quantitative analysis - identical conditions of transport for every member of the set - would have been invalidated.
For a number of herbicides it is known that selective detoxification is the clue to their selectivity. A well-known example is atrazine whose chlorine atom on the triazine ring is removed by nucleophilic displacement by glutathione in corn but not in many weeds. Other detoxification mechanisms imply hydrolysis of ester bonds, glucoside formation, and oxidative hydroxylation. Several of these mechanisms may operate with the 4-amino-triazinones although no biochemical evidence is at present available.
Further support to the importance of selective metabolic detoxification was lent by the results of in-vivo correlations with pre-emergence data of a set of 30 triazinones. With corn no correlation at all was obtained, whereas with sorghum an equation of fair significance resulted (table 3).

30 Triazinones:
In-vivo correlations (pre-emergence data)

	r	s	a_0	a_1	a_2	a_3
Corn	0.28	0.62	2.30	0.10	-0.55	0.82
Sorghum	0.71	0.38	2.36	0.28	-1.03	1.59

Table 3

$$\log (1/ED_{50}) = a_0 + a_1 \cdot pI_{50} + a_2 \cdot R_M + a_3 \cdot R_M^2$$

These equations should not be over-interpreted on statistical grounds, but nevertheless they confirm the conclusion that specific detoxification reactions are operating in corn to a greater extent than in sorghum.

Conclusions

The light reaction II of the photosynthetic electron transport has several features which make it an ideal model to study structure activity relationships of its inhibitors:

1. It is operating with subcellular particles (broken chloroplasts) in essentially the same way as in the living plant, provided some artificial donors and acceptors (ADP, potassium ferricyanide or anthraquinone, NADP) are added.

2. The inhibition of the photosynthetic electron flow dy diuron-type inhibitors is almost completely reversible. Thus under equilibrium conditions the kinetics of binding does not enter the inhibition constants which is not frequently the case with biological data.

Considering this, one might infer that pI_{50}-values of photosynthesis inhibition present a true picture of the interaction of inhibitor and receptor. This would be, however, a rather naive interpretation. Transport and penetration may in fact play a minor role in this system, but most certainly this cannot be said of binding to unspecific receptors. We suspect that simple correlations which describe activity as a linear or quadratic function of a hydrophobic parameter, are often nothing but a representation of the competition of specific and unspecific binding sites. This may hold true for our set-1 regression equation as well as for many examples in the literature. The activity data of our set 2 probably contain some variance from inhibitor-receptor interaction, which to a great extent determines the activity within sets 3 and 4. In the latter cases, correlations were not obtained due to the lack of parameters depicting with sufficient subtlety the properties of substituents, even of alkyl groups.

These considerations do not generally invalidate the use of structure-activity correlations of the Hansch type as a tool in drug synthesis, since in many instances penetration and binding to competitive un-

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specific receptors are probably the factors that impose limitations on activity.

A great risk, however, is involved in drawing pictures of the inhibitor-receptor interaction which are exclusively based on apparently significant regression equations. With regard to the triazinones as inhibitors of the photosynthetic electron flow, the contributions of the three substitutions to the over-all inhibitors activity can tentatively be described in the following way:

1. R^3 is important for the lipophilicity of the whole molecule and thus essentially influencing penetration properties and unspecific binding. A certain but not very critical lipophilicity of R^3 is also required to ensure a good fit to the receptor.

2. Some not fully understood steric property of R^2 is very critical for a good fit; hydrophobic and electronic properties seem to be less important although it might be difficult to separate them from each other.

3. R^1 is of great importance in binding to the receptor, possibly through inflicting a certain favourable electron distribution on the heterocyclic system, but perhaps also by direct interaction with some part of the receptor.

The in-vivo correlations with triazinones which have only partly led to significant equations, may serve as a tool to distinguish between herbicide tolerance based on penetration and transport and that based on biochemical detoxification.

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9

Carbanilate Herbicides and Their Metabolic Products—Their Effect on Plant Metabolism

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Abstract

In resistant soybean, the major chlorpropham metabolite was isopropyl 5-chloro-2-hydroxycarbanilate (2-hydroxychlorpropham). In susceptible plants, the predominant chlorpropham metabolite was isopropyl 4-hydroxy-3-chlorocarbanilate (4-hydroxychlorpropham). Both resistant and sensitive species conjugate the phenolic chlorpropham metabolites. Respiration and phosphorylation activities of plant mitochondria were affected by chlorpropham and its phenolic metabolites. Both chlorpropham and 2-hydroxychlorpropham inhibited NADH oxidation by 30%, whereas only the 2-hydroxychlorpropham metabolite inhibited NADH-linked ATP formation (85-100%). 2-Hydroxychlorpropham and dinitrophenol inhibition were similar. The 4-hydroxychlorpropham metabolite exerted no effect upon respiration, phosphorylation, or ATPase activity. Conjugation of the phenolic metabolites reversed the inhibition. When firefly luciferase was used as a model enzyme system, the 4-hydroxychlorpropham metabolite was shown to be a linear competitive inhibitor with respect to ATP and D-luciferin. This inhibition was not observed when 4-O-glucosylchlorpropham was used in place of 4-hydroxychlorpropham. Several factors appear to be involved in the chlorpropham detoxification mechanism. Susceptibility to chlorpropham could be a function of the rate of aryl-hydroxylation or the rate of conjugation of the aryl-hydroxylated products.

Introduction

Carbanilate herbicides have been used for two decades and no definitive mechanism of action has been proposed. Two separate toxic responses to chlorpropham (isopropyl 3-chlorocarbanilate) have been reported. Eshel and Warren (1) suggested that chlorpropham was metabolized differently by sensitive and resistant plants. Ennis (2) showed that propham (isopropyl carbanilate) inhibited root and shoot elongation, with concurrent swelling of

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these tissues. Interruption of the mitotic cycle and increased chromosome number were among the abnormalities observed. Storey and Mann (3) reported that the phenyl carbamates had an effect on chromosome contraction and the production of granulated interphase nuclei. They observed that the phenyl carbamate herbicides had the ability to inhibit the metabolic reactions which were dependent on the synthesis of specific proteins. Recently, Bartels and Hilton (4) compared the effects of propham and colchicine treatments on microtubules. They concluded that colchicine inhibited polymerization of subunits into microtubules whereas propham appeared to interfere with the arrangement of the microtubules in the cell. This report confirmed earlier observations by Hepler, Jackson, and others (5). Mann et al. (6) and recently Yung et al. (7) have observed that the phenyl carbamate herbicides cause inhibition of amino acid incorporation into protein and phosphate incorporation into both RNA and DNA. Yung et al. (8, 9) also demonstrated that phenyl carbamate herbicides altered the expression of gibberellic acid and IAA in plants. Chlorpropham has also been shown to inhibit the Hill reaction (10) and oxidative phosphorylation. St. John (11) has demonstrated the inhibition of ATP synthesis in Chlorella by chlorpropham. In all of these investigations it was assumed that the parent compound, propham or chlorpropham, was the active agent. Recent investigations in our laboratory (12, 13, 14, 15) have demonstrated that the half-life of propham or chlorpropham is short, and that there is a precursor-product relationship between the parent herbicides and their metabolic products. The question then arises as to which are the active toxic components; the parent herbicides, or the metabolic products.

RESULTS AND DISCUSSION

This paper will present some of our data on chlorpropham metabolism in plants and will report our studies to ascertain the biological significance of the chlorpropham plant metabolites. We have investigated the metabolic fate of chlorpropham in soybean, alfalfa, orchardgrass, and cucumber. An example is chlorpropham metabolism in soybean. The inventory of radiolabel from chlorpropham-^{14}C is shown in Figure 1. Note the disappearance of radiolabel from the exogenous root nutrient solution (B) with the concommitant increase of radiolabel into the root and shoot tissues (A). Curves C and D represent either the radiolabel left in a nutrient solution in the absence of plants, demonstrating the volatility of chlorpropham, or the sum of the radiolabel in the exogenous treating solution plus the radiolabel in the root or shoot tissues. These curves were identical whether the radiolabel was in the isopropyl moiety or the phenyl nucleus of chlorpropham (12).

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Pulse time course experiments (Table I) demonstrated that there was little, if any, acropetal translocation of chlorpropham metabolites. At zero day posttreatment soybean plants had been root-treated for 3 days with chlorpropham-phenyl-^{14}C or chlorpropham-2-isopropyl-^{14}C. At this point in the experiment, the exogenous radiolabel was replaced with nutrient solution. At zero day posttreatment, trifoliate group 5 had just begun to emerge and trifoliate groups 6, 7, and 8 did not exist. As the plant grew, very little radiolabel appeared in trifoliates 5, 6, and 7, and no radiolabel was found in trifoliate group 8, the seed pod, or the fruit. From these data, we conclude that though large quantities of radiolabeled materials resided in the root, leaves, and stems of the plant, the newly emerging leaves and the fruit were not labeled by acropetal translocation of these materials (12).

The nature of the radiolabel in these tissues was determined by Bligh-Dyer extractions which showed that the parent chlorpropham ($CHCl_3$) was rapidly converted in root, stem, primary leaf, and trifoliate tissues to polar metabolites (H_2O) and nonextractable residues (Figure 2). Therefore, in soybean, as well as the other plants we studied, there was a rapid conversion of chlorpropham to polar metabolites and, in some tissues, to bound residues.

Figure 3 is a summary of the polar metabolites isolated and characterized from resistant and susceptible plants. Isopropyl 5-chloro-2-hydroxycarbanilate (2-hydroxychlorpropham) was found as the glucoside in soybeans (13) and as an unidentified glycoside in alfalfa shoot and root. 2-Hydroxychlorpropham was not found in the susceptible orchardgrass or cucumber plants (15). Isopropyl 3-chloro-4-hydroxycarbanilate (4-hydroxychlorpropham) was found in soybean shoots (13) and was the major component in alfalfa shoots. 4-Hydroxychlorpropham was present in alfalfa root and was the predominant component found in the roots and shoots of orchardgrass and cucumber, the susceptible species studied. The question then arises, does the site of aryl hydroxylation of chlorpropham result in susceptibility or resistance, and a second question, what is the biological significance of the aryl hydroxylated chlorpropham metabolites upon plant metabolism?

Initial studies were with phosphorylating plant mitochondria. Figure 4 summarizes the effect of chlorpropham metabolites upon phosphorylating plant mitochondria. The results indicate that 0.1 mM chlorpropham and 2-hydroxychlorpropham inhibited NADH oxidation by 30%, whereas only 2-hydroxychlorpropham inhibited ATP formation (85-100%). Dinitrophenol and 2-hydroxychlorpropham exerted similar results upon respiration, phosphorylation, and ATPase activity. 4-Hydroxychlorpropham (0.1 mM) exerted no effect upon respiration, phosphorylation, or ATPase activity. β-O-glucosides of the hydroxy metabolites of chlorpropham did not inhibit NADH-linked respiration or phosphorylation at 0.1 mM concentrations. Comparative studies with corn, cucumber, and

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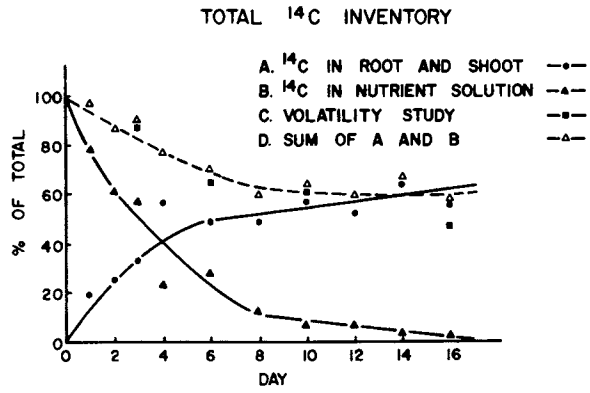

Figure 1.

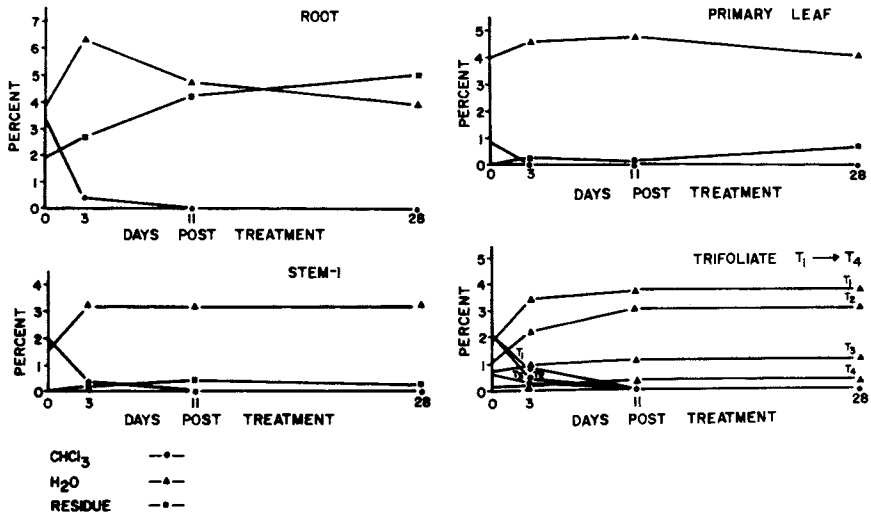

Figure 2. Normalized Bligh-Dyer data average of chlorpropham ring and ^{14}C-isopropyl

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soybean mitochondria indicated that the parent herbicide and its aryl hydroxylated metabolites affected respiration and phosphorylation activities in a similar manner. These results indicate that previous observations of oxidative phosphorylation inhibition by chlorpropham may have been the result, not of the parent chlorpropham, but the 2-hydroxychlorpropham plant metabolite.

We next investigated the model enzyme system, firefly luciferase (17). Figure 5 compares some of the adenylate activation reactions; firefly luciferin activation, fatty acid activation, and amino acid activation. It was our contention that firefly luciferin activation would be an appropriate test system for the comparative study of the activity of chlorpropham and its aryl hydroxylated polar metabolites. Figure 6 compares the effects of chlorpropham, 2,4-dinitrophenol, 2-hydroxychlorpropham, 4-hydroxychlorpropham, and the glucose conjugates of the hydroxychlorpropham analogs on luciferin activation. Dinitrophenol and the glucosyl conjugate of 2-hydroxychlorpropham had no effect upon the ATP-luciferin light activation system. Inhibition by the 4-glucosylchlorpropham may have been the result of the glycoside itself or a result of the luciferase system's ability to hydrolyze the glucoside to yield 4-hydroxychlorpropham, the most potent inhibitor studied. 2-Hydroxychlorpropham and chlorpropham also inhibited this test system. Table 2 compares the inhibition of luciferase by chlorpropham metabolites and reports the inhibitor constants with respect to ATP and luciferin. When D-luciferin was held constant and the concentration of ATP was varied, chlorpropham and 2-hydroxychlorpropham demonstrated noncompetitive inhibition kinetics, while 4-hydroxychlorpropham or a mixture of 4-hydroxychlorpropham and 100 μM AMP showed competitive inhibition kinetics. Note that the Ki for 4-hydroxychlorpropham dropped from 1×10^{-6}M to 2×10^{-7}M with the addition of AMP.

4-Hydroxychlorpropham was the only competitive inhibitor with respect to ATP and luciferin in nondialyzed and dialyzed firefly lantern extract systems. Moreover, the inhibition constants for 4-hydroxychlorpropham in the presence of AMP are quite similar, with respect to ATP and D-luciferin. This observation suggests that 4-hydroxychlorpropham and ATP may form an enzyme-adenylate complex.

The enzyme systems responsible for firefly luciferase light emission, amino acid activation, and fatty acid activation have been compared in the literature (18). Each system forms an appropriate enzyme-adenylate complex in the presence of ATP and Mg^{+2}. The luciferin-adenylate complex reacts with oxygen, emitting light and releasing AMP; the fatty acid adenylate complex reacts with CoASH, forming fatty acid-CoASH and AMP; and the amino acid-adenylate complex reacts with t-RNA, forming an amino acid-t-RNA complex plus AMP. If the inhibition of the firefly luciferase system by 4-hydroxychlorpropham can be correlated with amino acid activation in plants, a possible mode of action of chlorpropham might be suggested. Our data have demonstrated that not only

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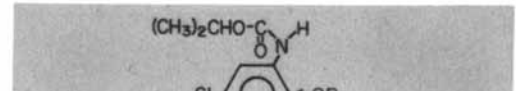

2-HYDROXYCHLORPROPHAM
4-HYDROXYCHLORPROPHAM

PLANT TISSUE	HYDROXYCHLORPROPHAM CONJUGATES	
	2-OH	4-OH
RESISTANT:		
SOYBEANS		
SHOOT	+	+
ROOT	+	0
ALFALFA		
SHOOT	+	+
ROOT	+	+
SUSCEPTIBLE:		
ORCHARDGRASS		
SHOOT	0	+
ROOT	0	+
CUCUMBER		
SHOOT	0	+
ROOT	0	+

Figure 3.

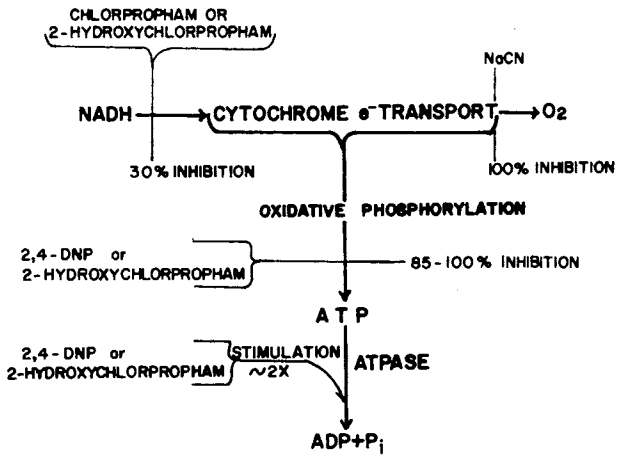

Figure 4. Summary of clorpropham metabolite effects on phosphorylating plant mitochondria

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Table 1. Average Total Dpm X 10^4 Found in Soybean Segments, Root-Treated Chlorpropham-phenyl-^{14}C or Chlorpropham-2-isopropyl-^{14}C for a 3-Day Pretreatment[a]

Plant Segment Assayed	Days after Pretreatment			
	0	3	11	28
Root	20.40	16.75	16.22	15.20
Primary leaf	7.40	10.35	7.48	7.80
Stem 1	10.75	5.40	5.87	5.10
Stem 2			0.16	0.05
Trifoliate 1	7.85	7.18	7.50	5.82
Trifoliate 2	11.10	3.80	6.03	2.74
Trifoliate 3	2.25	1.77	2.25	1.64
Trifoliate 4	0.18	0.24	0.42	1.12
Trifoliate 5		0.12	0.15	0.22
Trifoliate 6			0.00	0.12
Trifoliate 7			0.00	0.04
Trifoliate 8				0.00
Seed pod				0.00
Fruit				0.00

[a] All dpm data from oxygen combustion of each sample.

Table 2. Inhibition of Luciferase by Chlorpropham Metabolites: Inhibitor Constants in Respect to ATP and Luciferin

Inhibitor	Ki [μM][a]		
	Luciferase	Dialyzed Luciferase	
	Endogenous Constant LH_2 Vary ATP	Synthetic Constant LH_2 Vary ATP	Vary LH_2 Constant ATP
Chlorpropham	23 [NC][b]	25 [NC]	6 [C]
2-Hydroxychlorpropham	23 [NC]	17 [NC]	7 [C]
4-Hydroxychlorpropham	1 [C]	1.5 [C]	6 [C]
4-Hydroxychlorpropham plus AMP [100 μM]	0.2 [C]	0.5 [C]	0.6 [C]

[a] Determined from plots of reciprocal velocity vs. inhibitor [μM].

[b] [NC] = noncompetitive inhibition.
[C] = competitive inhibition.

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does the parent compound, chlorpropham, have an inhibiting effect, but the metabolite, 4-hydroxychlorpropham, also inhibits 20 to 100 times more effectively than the parent herbicide. Further, when 4-hydroxychlorpropham was conjugated with glucose, the inhibitory properties were lost.

The next approach was to study the ability of etiolated tissues to metabolize the hydroxychlorpropham analogues. It is recalled (Figure 3) that soybean shoot converted chlorpropham to 2-hydroxy and 4-hydroxychlorpropham while soybean roots yielded only 2-hydroxychlorpropham. Because of the high susceptibility of oat to chlorpropham, metabolism studies were not possible. However, if orchardgrass can be compared to oat it might be anticipated that oat tip or root tissues would convert chlorpropham only to 4-hydroxychlorpropham. Further, it should be recalled that the nature of the conjugate of the orchardgrass 4-hydroxychlorpropham metabolite is not known.

Portions of etiolated soybean and oat plants were used to study the effect of the hydroxychlorpropham metabolites (Figure 7). The disappearance of 2-hydroxychlorpropham from the exogenous buffer and in soybean and oat tissues, is compared in Figure 8. Soybean hypocotyl arch and root rapidly converted 2-hydroxychlorpropham to polar metabolites and nonextractable residues. In soybean shoot, the glycoside rapidly appeared and then disappeared, while in soybean root the glycosyl derivatives reached a stable plateau. Resistant soybean rapidly converted the exogenous 2-hydroxychlorpropham to new polar materials. In susceptible oat tissues the concentration of 2-hydroxychlorpropham fell slowly to a concentration of 20% in shoot and 40% in root. In both root and shoot there was a concommitant rise and fall of the 2-hydroxychlorpropham glycoside with the formation of other polar metabolites and nonextractable materials. However, the rate of 2-hydroxychlorpropham conjugation was much slower in these susceptible plant tissues.

The same comparison was made between soybean and oat tissues with 4-hydroxychlorpropham as substrate (Figure 9). Recall that the 4-hydroxychlorpropham was found in soybean shoot tissues but not in the roots. Both soybean root and shoot showed the ability to convert the phenolic derivatives to the glycoside, nonextractable materials, and other polar metabolites. In oat roots, 4-hydroxychlorpropham was rapidly converted to the glycoside and other polar metabolites and a high concentration of nonextractable materials. In susceptible oat shoot tips, the natural metabolite, 4-hydroxychlorpropham, was slowly removed from the tissue and media so that, after 25 hours, greater than 40% of the 4-hydroxychlorpropham remained in the system. The glycosylated derivative was formed, as were nonextractable and polar materials. These data suggest that oat shoot tissue sensitivity to chlorpropham could be the result of the tissue's inability to conjugate or further metabolize 4-hydroxychlorpropham.

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I. FIREFLY LUCIFERIN ACTIVATION

$$LH_2 + MgATP + E \rightleftharpoons E{\cdot}LH_2{\cdot}AMP \xrightarrow{MgPPi,\ O_2,\ AMP} PRODUCTS + LIGHT$$

II. FATTY ACID ACTIVATION (DEHYDROLUCIFERIN ACTIVATION)

$$\underset{(L)}{FA} + MgATP + E \rightleftharpoons \underset{(L)}{E{\cdot}FA{\cdot}AMP} \xrightarrow{MgPPi,\ COA,\ AMP} \underset{(L)}{E{\cdot}FA{\cdot}COA}$$

III. AMINO ACID ACTIVATION

$$AA + MgATP + E \rightleftharpoons E{\cdot}AA{\cdot}AMP \xrightarrow{MgPPi,\ tRNA,\ AMP} E{\cdot}AA{\cdot}tRNA$$

Figure 5. Comparison of adenylate activation reactions

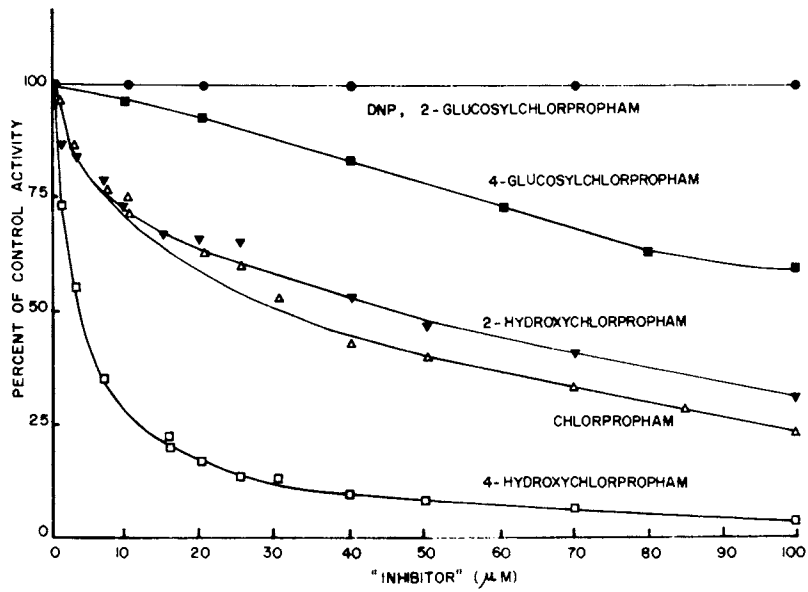

Figure 6.

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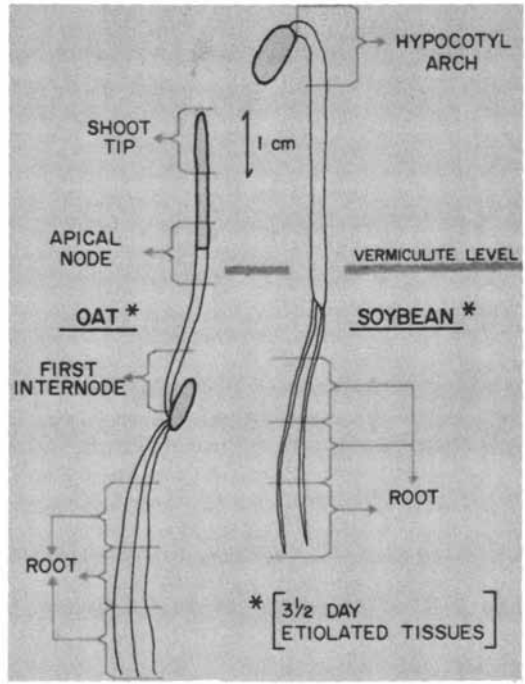

Figure 7.

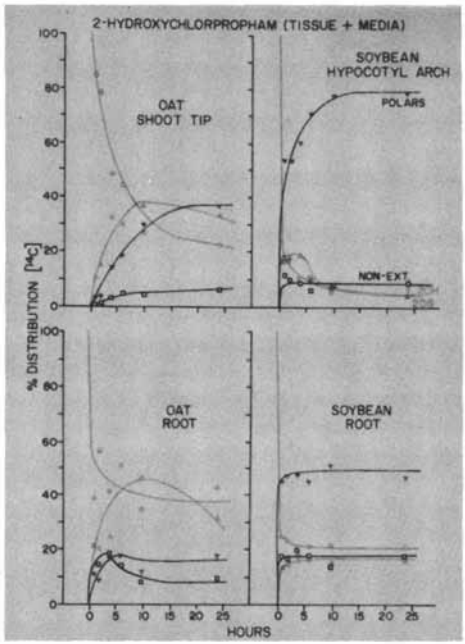

Figure 8.

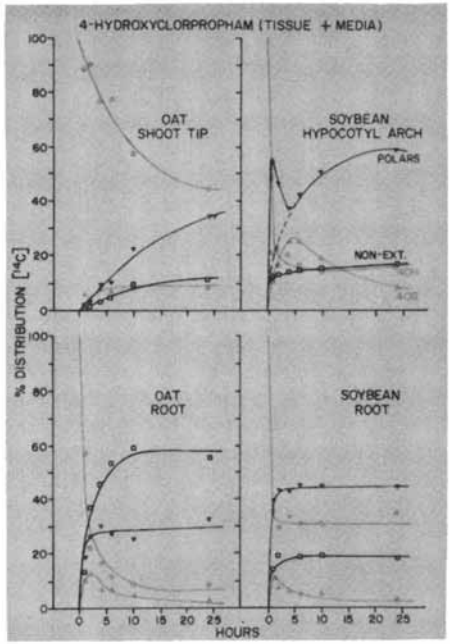

Figure 9.

Metabolism of the hydroxylated chlorpropham derivatives was comparable in both oat shoot tips and oat apical nodes (Figure 7). Using 4-hydroxychlorpropham as substrate, a soluble enzyme was isolated from oat tip. This enzyme was partially purified by fractionation with ammonium sulfate and acetic acid at pH 5. Tables 3 and 4 report the substrate specificities for the dialyzed oat shoot "pH 5 enzyme." Specific activity is in terms of nmoles of Rf 0.3 product/mg enzyme/4 hours, minus background (TLC with $CHCl_3$:CH_3OH:H_2O; 13:7:1). 4-Hydroxychlorpropham was the aryl substrate for this enzyme (Table 3). Chlorpropham, 2-hydroxychlorpropham, and 4-methoxychlorpropham were not substrates. Table 4 shows the amino acid substrate specificity for the oat shoot "pH 5 enzyme." Of all the amino acid substrates that were tested, the enzyme was specific for cysteine. From these data it appears that the carboxylic acid and the SH group of cysteine must be available to form the oat Rf 0.3 conjugate. Rice and corn shoots contained less enzyme activity, and cucumber and soybean hypocotyl arch yielded only small amounts of activity (Table 5). The product of the soluble "pH 5 enzyme" has properties similar to products of the amino acid activating enzymes and is presently being characterized.

Summary

In light of data from chlorpropham plant studies and considering the results of our investigations on the significance of the 2-hydroxy and 4-hydroxychlorpropham metabolites, we must conclude that the primary mechanism of action of the carbanilate herbicides still remains to be explained. However, the data presented in this report suggest that plant susceptibility or resistance to chlorpropham may be the result of (a) the site of aryl hydroxylation; (b) the rate of aryl hydroxylation; (c) the rate of conjugation of the aryl hydroxylated metabolites; (d) the type of conjugate formed; and/or (e) the metabolic stability of the various aryl hydroxylated conjugates.

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Table 3. Substrate Specificity for Dialyzed Oat Shoot "pH 5" Enzyme: Effect of Chlorpropham-Related Substrates[a]

Substrate	Specific Activity[b]
4-Hydroxychlorpropham	7.84
4-Hydroxychlorpropham plus 4-Methoxychlorpropham	7.32
4-Methoxychlorpropham	0.00
2-Hydroxy-5-chlorpropham	0.00
Chlorpropham	0.00

[a] The chlorpropham-related substrates are at 26 to 30 μM. The reaction also contains 5 mM L-cysteine, 120 mM P_i at pH 6.0, and 0.76 mg dialyzed oat shoot "pH 5" enzyme. Incubation is at 30° for 4 hr.

[b] Specific activity = nmole Rf $0.3_{13:7:1}$ product/mg enzyme/4 hr minus background.

Table 4. Substrate Specificity for Oat Shoot "pH 5" Enzyme: Effect of Cysteine-Related Substrates

Substrate	Relative Rate[a]
L-Cysteine	100
D-Cysteine	86
N-Acetyl-L-cysteine	27
Cysteamine	10
S-Me-L-Cysteine	9
L-Cysteine-ethyl-ester	4
L-Serine	3
L-Cysteic acid	3
L-Methionine	1
L-Homocysteine thiolactone	1
L-Alanine	1
L-Phenylalanine	1

[a] Relative rate of 100 is equivalent to 12.5 nmole $Rf_{13:7:1}$ product/mg enzyme/4 hr minus background.

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Table 5. Species Specificity for Dialyzed "pH 5" Enzyme[a]

Tissue	Relative Rate[b]
Oat shoot	100
Rice shoot	18
Corn shoot	16
Cucumber hypocotyl arch	15
Soybean hypocotyl arch	3

[a] The reaction contains 5 mM L-cysteine, 5 mM MgATP, 6 μM 4-hydroxychlorpropham, and 0.5 to 1.0 mg respective dialyzed "pH 5" enzyme at 30°.

[b] Relative rate of 100 is equivalent to 2.1 nmole $Rf_{13:7:1}$ product/mg enzyme/4 hr.

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10

Mechanism of Action of Alkylating and Nonalkylating Insect Chemosterilants

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Since the ultimate effect of pesticides is the containment or elimination of pests, chemosterilants properly belong to this class of biologically active chemicals. Nevertheless, with respect to individual pest organisms, chemosterilants do not kill, and their effects must be restricted to damaging only one function: reproduction. The potential advantages of the sterilization technique, in which chemicals are used to decrease the birth rate of a population, over the pesticidal technique, in which chemicals are used to increase the death rate of a population, were recognized first by Knipling (1). If a certain proportion of a population of sexually reproducing organisms is killed, the survivors will continue to reproduce with undiminished or even increased vigor. However, if the same proportion is sterilized, the sterile organisms will compete with the fertile ones for mates, and the reproductive rate of the population will decrease. Subsequent research has demonstrated that a great variety of chemical compounds (chemosterilants) can destroy the reproductive capacity of insects, without impairing their sexual competitiveness (2, 3). The search for vertebrate chemosterilants has been less intense but, until recently, the main goals of vertebrate sterilization was birth control of humans rather than of pests (4). Although all prototypes of the various groups of insect chemosterilants were discovered empirically, their mode of action and their apparent specificity are still being studied. Physiological effects of the most important classes of chemosterilants, i.e., alkylating agents, nonalkylating phosphorus amides, nonalkylating triazines, and antimetabolites have been determined in many species of insects but the investigation of their biochemical effects is far from complete. Of course, there are several reasons for this discrepancy. Most of the research on chemosterilants is conducted by Government laboratories where the emphasis is placed on potentially practical and useful results that are seldom attainable by biochemical studies. An equally important reason, however, is the inherent difficulty of the biochemical research on mode of action.

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The most important category of insect chemosterilants, biological alkylating agents, is the same group of compounds that have been so widely investigated during the last 25 years as cancer chemotherapeutic agents. For example, thiotepa, tretamine, and busulfan, all effective chemosterilants, are still among the best known carcinostatic drugs. Biological alkylating agents (5) include sulfur and nitrogen mustards, aziridines, epoxides, lactones, alkanesulfonates, and other structural types that react with various nucleophiles in presence of water. However, since aziridines are the most important representatives of insect chemosterilants, I shall use them as examples in illustrating the physiological and biochemical events leading to sexual sterility of insects.

In sexually reproducing organisms, fertility can be interrupted selectively in the male and in the female, but the following general mechanisms are applicable to both sexes:

(a) Direct interaction of the agent (chemosterilant) with gametes, excluding cytotoxicity. In genetic terms, this activity is designated as mutagenicity if the gamete does not lose its capacity for forming a zygote.

(b) Interaction of the chemosterilant with gonadal tissues leading to production of abnormal gametes. Again, if the abnormal gamete can still form a zygote, the overall effect is termed mutagenic.

(c) Interaction of the chemosterilant with regulatory control centers leading to malfunctioning of gonads. Although the hormonal regulation of ovarian activity is well established in female insects, no comparable mechanism is known for male insects. However, since hormonal regulatory mechanisms operate in both sexes of mammals, the existence of such mechanisms in male insects cannot be excluded.

(d) Induction of nutritional deficiencies leading to malfunctioning of gonads. If the chemosterilant interacts directly with an essential nutrient, the effects may be immediate, but an indirect action is also possible when the sterilant incapacitates digestive processes or mechanisms.

(e) Selective cytotoxic action in which the chemosterilant kills the gametes or the cells in gonadal tissues.

One of the more serious difficulties in research on physiological action of chemosterilants is that none of the five mentioned mechanisms is exclusive. In fact, alkylating agents can and frequently do exert their sterilizing effects by several of these mechanisms simultaneously. Specifically, the aziridinyl chemosterilant tepa (tris(1-aziridinyl)phosphine oxide) induces dominant lethal mutations in the sperm and ova of many species of insects, and it attacks the insect's gonads killing some cells and incapacitating others.

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The physiological effects of aziridinyl chemosterilants are undoubtedly complex but they are more or less easily observable under a microscope. On the other hand, the biochemical effects are extremely difficult to detect even with the most advanced analytical instruments. The reason for this difficulty becomes apparent when we consider the possible targets with which a chemosterilant may react. There is little doubt that when the chemosterilant is an aziridinyl alkylating agent, a true chemical reaction occurs, i.e., a covalent bond is formed between the electrophilic center of the aziridine and the nucleophilic center of the acceptor HX, which may be a constituent of a chromosome, a protein, or an enzyme complex. In aqueous media, the reactions of aziridines are complicated by at least two side reactions: hydrolysis and polymerization (Fig. 1). Polymerization may be

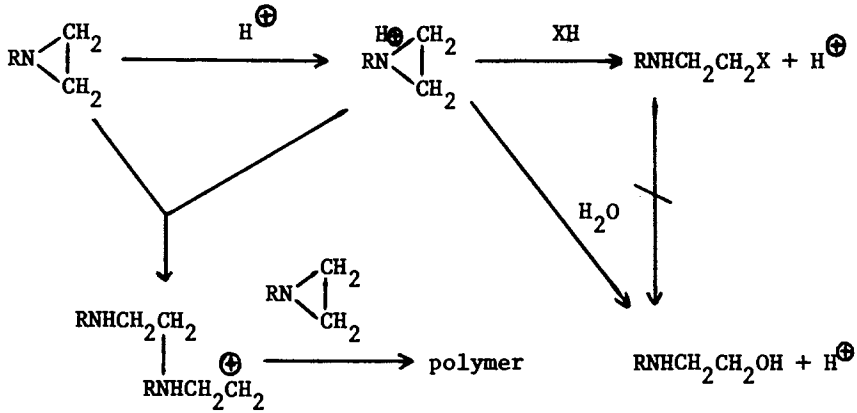

Fig. 1 - Alkylation of critical target molecule HX by N-substituted aziridines. Side reactions are polymerization and hydrolysis.

insignificant at the low concentrations that chemosterilants reach in cells but it does interfere with attempts for replicating the critical reactions in vitro.

The main reaction, i.e., the alkylation of the biological component HX, is not necessarily quantitatively important, and this strong possibility is the main obstacle that research on mechanism of action must overcome. The target nucleophilic center X may be nitrogen in nucleotides, nucleic acids or proteins, sulfur in proteins, and oxygen in nucleotides and nucleic acids. Numerous studies deal with the nucleophilic strength of these centers in various potential target molecules but the nitrogen in the seventh position in guanine and the oxygen in phosphoric hydroxyl appear to be the most probable sites for electrophilic attack in nucleic acids (6). No definite conclusion can be reached about proteins because of their extreme variability and

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our lack of knowledge of their structure. The reason why nucleic acids, particularly DNA, are the main center of attention is a logical deduction based on the Watson-Crick theory of cell replication. Presumably, a single hit on the DNA chain of a single chromosome could bring about a mutation that could change or even destroy the reproductive potential of the cell. In other words, it is the uniqueness of the chromosomal macro-molecules that accounts for the high sensitivity of a cell toward a diffuse and nonspecific reagent. To a chemist, this possibility rings a bell of disaster: how does one isolate and identify a few molecules? At present the only hope is that in reality the number of hits per cell far exceeds the minimum, and that the resulting changes will be sufficient for isolating and identifying the reaction products.

Let us now turn to another class of chemosterilants exemplified by hexamethylphosphoric triamide (hempa). Although hempa is chemically entirely different from tepa, particularly in that it is not an alkylating agent, its physiological effects in some insects are qualitatively indistinguishable from those of tepa (7). In other words, the physiological mode of action of these two compounds appears identical. Two explanations can be advanced for this similarity. One is that the response of a reproducing cell to any attack is limited, and thus the physiological and microscopically or genetically observable effects are identical even if the biochemical pathways that produced them were different. As yet, this possibility cannot be entirely rejected. However, the second explanation, i.e., that the biochemical pathways are similar, received some support in our work on the metabolism of hempa in insects (8-11). Though the chemical and thermal stability of hempa is high the compound is rapidly demethylated in cells or cellular homogenates via a highly reactive and unstable hydroxmethyl intermediate (Fig. 2). It is this intermediate that could function as an alkylating agent and perform a role similar to that of the aziridinium ion resulting from protonation of tepa, Because of its instability, the N-(hydroxmethyl)-N,N',N',N'',N''-pentamethylphosphoric triamide could not be isolated as a metabolite of hempa but it was synthesized (12), and its sterilizing activity in the house fly was equal to that of hempa.

A possible alternative is the decomposition of the hydroxymethyl intermediate to formaldehyde which is a mild alkylating agent (Fig. 2, pathway b). However formaldehyde had no sterilizing effect when injected into house flies (12). Sterically, tepa and hempa are very similar, both have excellent solubility properties, and both can be expected to diffuse into a cell rather easily.

The direct sterilizing activity of tepa on insect sperm *in vitro* was confirmed by Taber and Bořkovec (13). More recently we conducted a similar experiment with boar sperm, and the results indicated again that the alkylating agent interacted with the genetic material in the gamete (unpublished). Hempa and other

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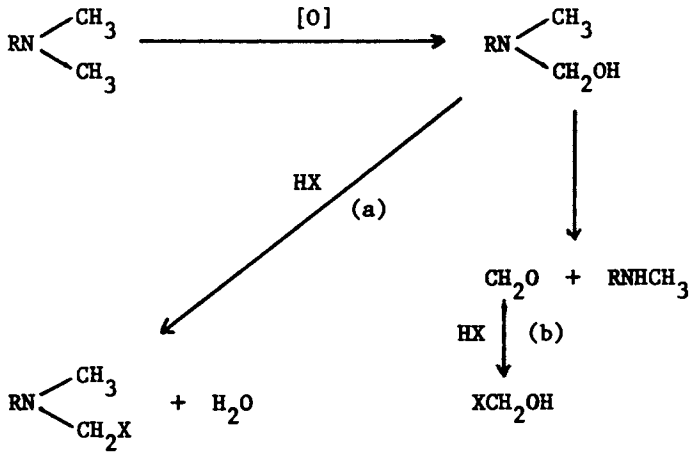

Fig. 2 - Alkylation of critical target molecule HX by metabolically activated dimethyl amide. A hydroxmethyl intermediate (a) or formaldehyde (b) are the alkylating agents.

nonalkylating chemosterilants have not been used in sperm sterilization experiments but since there is no evidence for the presence of oxidizing enzymes in sperm, the required activation of the N-methyl group may not be possible in vitro.

Because of the similarity in structure and effects of tepa and hempa, we prepared analogs of other aziridinyl chemosterilants (14,15). In phosphorothioic amides and s-triazines both analogs were active as chemosterilants but in cyclic phosphonitriles, sulfones, and carboxylic or carbamic acid amides the dimethyl analogs were inactive. Since the metabolism of the inactive compounds in insects was not studied it is impossible to decide whether the observed lack of activity was inherent to these compounds or whether their metabolism was only quantitatively unfavorable. The latter possibility may be illustrated by the differences between the sterilizing activities of hempa and its demethylated homolog pentamethylphosphoric triamide. Both compounds are metabolized in the male house fly (11) but the rate of demethylation of hempa, an effective sterilant, is much higher than that of the ineffective pentamethyl compound.

Metabolic studies present a clue but not a complete answer for resolving the problem of mode of action. Since the most promising direction for future investigations points to the interaction of an isolated sperm cell with the sterilant, insects may not be the most suitable organisms for such studies. Abundance of sperm and reliable method of artificial insemination are the two conditions that may be easily found in vertebrates and the growing interest in sterilization of such animals should provide

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the necessary impetus and motivation for research on mode of action of chemosterilants.

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11

Insecticidal Properties and Mode of Action of 1-(2,6-Dihalogenbenzoyl)-3-phenylureas

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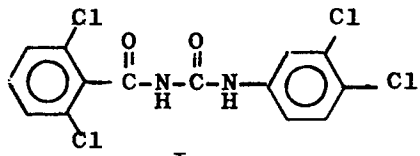
This paper is a survey of the work done at our Boekesteyn laboratory with the new type of insecticide indicated in the title. Its main topics are firstly the work done to assess the scope and limitations of this new type of insecticide, and secondly an account of more recent work aimed at the elucidation of the mode of action of these unique compounds. Most of the work reviewed here has been published, or is in press.



I

The first compound of the series to be tested for insecticidal activity was DU 19111 (I). Perhaps it is of interest to relate briefly the origin of this compound which would probably not be associated at once with insecticidal activity. In fact the compound was synthetized in the course of a program designed to exploit the manufacturing process of the pre-emergence herbicide dichlobenil.

In the primary screening DU 19111 was by no means effective against weeds. It seemed also completely ineffective towards the insect species tested, at least at first sight. But a few days after the first evaluation it was noticed that larvae of Pieris brassicae treated with DU 19111 died while moulting. Soon afterwards it turned out that larval stages of several other insect species, viz.flies, the yellow fever mosquito, and the colorado potato beetle, after ingestion of DU 19111 all died showing similar symptoms. These initial results have been published by

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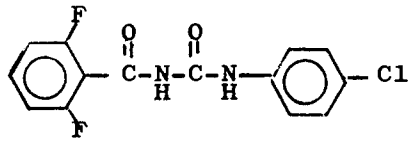
Van Daalen et al. (1)

Of course, immediately after this finding a program was launched to assess the structural borderline conditions for this peculiar larvicidal effect. Guided by structure-activity relations, to be published by Tipker and Verloop, hundreds of structural analogues were synthetized, and among these several dozens proved more or less effective in the same manner as DU 19111. The substance of this work has been published in two papers by Wellinga et al. (2), (3)



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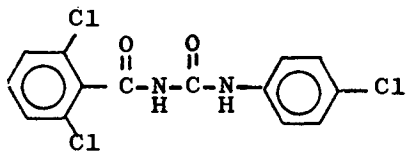

III

For further development the most favourable candidate, by practical standards, is PH 60-40 (II). PH 60-38 (III), which also was a serious candidate, appeared to have a considerable half life in water and in most soil types tested and was therefore rejected. But PH 60-40 is relatively rapidly biodegradable, and hence more acceptable from an environmental point of view. In addition it is more effective with most larval species tested, as demonstrated by Mulder and Gijswijt, (4). That this is also the case under field conditions has been reported by Mulder and Swennen (5).

The compounds are effective with larval stages only. But some of the species tested appeared altogether insusceptible, notably aphids and spider mites. This suggests that arthropods feeding selectively on plant juices do not ingest the larvicides in sufficient quantity to be affected. This inference was substantiated in an experiment with the cotton bug, another sucking insect. When nymphs of this insect were fed on cotton seed imbibed with a microsuspension of DU 19111 they survived, but when they were fed dry cotton seed, and the insecticide was added to their drinking water the nymphs would die during their moulting process.

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These results convey the impression that the compounds are unable to penetrate leaf surface. Meanwhile this has been proved with the labelled material (Nimmo, unpublished). In addition it has been confirmed experimentally that the compounds do not penetrate insect cuticle when applied topically.

As regards the mode of action of our larvicides, initially death of the larvae seemed invariably connected with moult. The more susceptible species, such as Pieris brassicae did not, as a rule, ecdyse at all, unless at marginal dosages, when larvae succeeded in partially shedding their exuviae, but always would the larvae die, loosing moisture and blackening. Less susceptible species, as for instance Barathra brassicae as a rule partly ecdysed, and at marginal dosages even fully so; but even when fully ecdysed the larvae remained pale and immobile, and eventually perished, probably of starvation.

With larvae of the yellow fever mosquito a strange effect was seen. At all concentrations of the larvicide between 1 and 0.01 ppm only about half the larvae died within the first few days, the remainder surviving much longer. But never would adult insects develop. Microscopical examination revealed that all the larvae, alive or dead, were affected at an early stage. But probably the fact that they are suspended in the water enables a substantial portion to survive this condition for a while, in contrast to the terrestial larvae that have to move actively in order to feed themselves.

All in all it looked as if we had solid evidence to associate the larvicidal effect of this new type of compound with the moulting process. The idea therefore suggested itself of interferenc with some hormonal system governing this process. Now two hormones are known to play a role in the moulting process, namely ecdysone and bursicon. Ecdyson is known to control the conditioning of the epidermal cells for the deposition of new cuticle; bursicon has been found to control cuticle hardening and darkening. As regards ecdyson we dismissed the idea of its involvement at an early stage, because obviously the formation of exocuticular tissue was unaffected (see ref. (4), Fig.1); moreover in certain instances the larvicidal effect manifested itself at stages where an ecdyson antagonism was unlikely to occur. For instance, when a Pieris larva was injected with 1 µg of PH 60-40 during its apolysis, it did not ecdyse but died instead. But at apolysis the epidermal cells are already fully conditioned for cuticle synthesis.

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This then left bursicon, a peptide hormone known to function very acutely around the ecdysial process.

We therefore set out to investigate whether our larvicides interfered with either the secretion or the functioning of bursicon. We could do this relatively easily after we had been fortunate enough to find an *in vitro* bioassay for this hormone. This method, involving the determination of the rate of decline of the tyrosine level in an isotonic suspension of *Pieris* haemocytes, was based on the hypothesis of Mills and Whitehead (6) that the function of bursicon was to render the haemocyte membrane permeable to tyrisone, thus enabling this amino acid to be converted to N-acetyl dopamine, the actual cuticle hardening agent. I will not elaborate further on this; the method has been published (7).

By applying this essay method it became clear that our larvicides did not interfere either with the functioning of bursicon in *Pieris* haemolymph or with its secretion in newly ecdysed *Pieris* larvae. Ultimately the association of the larvicidal action of these compounds with the moulting process turned out to be of a different nature.

When larvae of the Colorado potato beetle were treated with the larvicidal compounds immediately after ecdysis they showed ill effects long before their moult was due. The larvae became balloon shaped, like ripe berries about to burst. In fact they did burst when dropped from some height. Obviously their internal pressure was barely balanced by the strength of their cuticles. The question thus arose of whether the pressure was abnormally high or whether the cuticle was abnormally weak. This question could be answered with the aid of the results of histological work(4). In this work it was demonstrated that the larvicides cause extensive degradation of the endocuticular matrix. Nothing remains but an amorphous mass that coagulates in the fixation process. By contrast exocuticles appear intact. Now it is known that exocuticles consist mainly of sclerotized protein, but that the endocuticular matrix consists of roughly half protein, half chitin, the latter forming the fibrillar backbone of the endocuticular structure. Hence we surmized that the defective process was the construction of this chitinous backbone.

A promising way of testing this inference is the approach of Condoulis and Locke (8), who have made a microautoradiographic study of the incorporation of glucose and of certain amino acids in soft endocuticle of *Calpodes ethlius* larvae, using the tritiated

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compounds. We have applied their experimental approach to study the effect of DU 19111 on the incorporation of the same compounds in endocuticular tissue of *Pieris brassicae* larvae.

This work is in the press (9); its principal results were that the incorporation of label from glucose, which follows a distinct zonal pattern in endocuticle of normal larvae, was completely blocked upon treatment with DU 19111. However, with the amino acids the incorporation pattern changed only qualitatively.

From these, and other (10) results we have concluded that at any rate the synthesis of endocuticular chitin is blocked by the larvicides, the anomalities in the incorporation patterns of the amino acids probably being due to the absence of the chitinous backbone.

At that state it became of interest to find out which step in the biosynthetic pathway from glucose to chitin (Fig.1) was inhibited by the larvicides.

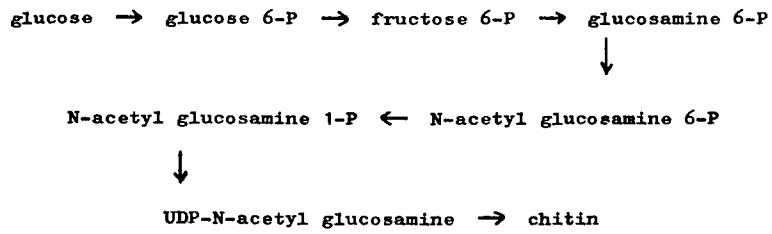

Fig.1. Biosynthetic pathway of chitin synthesis from glucose.

A logical approach to this problem is to see in what way the ultimate precursor, uridine diphosphate N-acetyl glucosamine (UDPAG) is affected by treatment with the larvicides. If a reaction between glucose and UDPAG were inhibited this would necessarily result in the depletion of the UDPAG. If, on the other hand, the polycondensation step were blocked one would expect UDPAG to accumulate, leaving aside possible regulatory mechanisms that could distort such a simple picture. We therefore injected both normal and DU 19111-treated *Pieris* larvae with ^{14}C-glucose and homogenized them one hour later, after removal of their guts and haemolymph. Paper histograms of the deproteinized homogenates, developed in three different solvent systems, revealed that no significant differences in UDPAG-levels occurred between normal and DU 19111-treated larvae. In fact, the only significant difference between histograms from treated

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as compared to normal larvae was a large peak of radioactivity in the former that was almost completely absent in extracts from normal larvae. In all the solvent systems tried this peak had the same R_F-value as acetylglucosamine (AGA).

What explanation could there be for the occurrence of relatively large amounts of AGA in homogenates of DU 19111-treated larvae, as compared to little or none in homogenates of normal ones? AGA as such (Fig.1) has no part in the biosynthetic chain from glucose to chitin. In normal larvae it is only likely to occur during apolysis, when the larva breaks down its old endocuticle and uses the building blocks to form a new one, as has been demonstrated by Bade and Wyatt (11). But our larvae were not apolysing and indeed they did not contain appreciable amounts of AGA at that stage. But in the treated larvae, with their chitin synthesis blocked, it did occur in considerable amount. In our opinion the concurrence of the inhibition of chitin synthesis, on the one hand, and of AGA accumulation, on the other, can best be explained as follows: under the influence of DU 19111 the chitin synthetase continues to accept precursor molecules of UDPAG. But in the process of passing the N-acetylglucose moiety on to the chitin primer the enzyme apparently drops the monomer so that it accumulates in the medium. This would mean that the chitin synthetase is not really blocked but, as it were, uncoupled.

This by itself may be looked upon as an interesting finding. It becomes the more surprising when compared with our preliminary results with the DU 19111-analogue PH 60-40. While we were engaged in repeating the DU 19111 experiments we deemed it unwise to stick to the arbitrarily chosen set up with the larvicide applied 24 h before injection of the labelled glucose. So we performed an additional experiment with simultaneous injection of the larvicide and the labelled glucose, killing the larvae 1 h later. With DU 19111 this experiment yielded no new insights; only, not unexpectedly, the accumulation of AGA with respect to the control was less than in the experiments where the larvae were pretreated with the larvicide.

Next we did a twin series of experiments with PH 60-40, one involving prior treatment, the other with simultaneous injection of the larvicide and the glucose. At several time intervals between 15 min. and 4 h the larvae were sampled, and deproteinized extracts from them were chromatographed. The results of this preliminary experiment will in due course be published by Deul *et al.* The salient results were that

in the larvae pretreated with PH 60-40 virtually no differences from the controls were apparent, at any rate not in the early samplings. But after 1 h the UDPAG-level gradually rose in the treated larvae with respect to the controls. By contrast, when the larvicide and the labelled glucose were injected at the same time, the UDPAG-level in the treated larvae was about 2.5 times that in the controls. This difference was fully manifest as early as 15 min. after the injection of PH 60-40. Remarkably, in neither of the PH 60-40 experiments did any accumulation of AGA occur.

What can be inferred from these results ? For one thing, that PH 60-40 in effect blocks the chitin synthetase. This outcome would not be really surprising had we not before been confronted with the curious uncoupling effect of DU 19111. Now it looks as if we have two structurally similar compounds with identical effects in insect larvae, one uncoupling chitin synthetase, the other completely blocking it.

A second inference from our results is that the inhibition is immediate, the shortness of the interval between application and effect leaving practically no room for an indirect effect.

Finally, the apparent fact that no further accumulation of UDPAG occurred than about three times the control level, suggests that UDPAG regulates its own synthesis from glucose. This notion is not new: a similar effect has been found with UDPAG in rat liver.

In conclusion I would remark that even at this stage of the work our findings encourage us to trust that the effect of these larvicies directs itself very specifically towards the epidermal chitin synthetase of arthropods. Not even the chitinous linings of insect gut and tracheae appear to be affected by these compounds.

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12

An *in vitro* Model System for the Production of Insect Cuticle

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Processes involved in the production of cuticle by an insect may be divided into two general areas: activation of the epidermis and deposition of a chitin-bearing cuticle. These general areas can, in turn, be further subdivided into the biosynthesis of ecdysone, cell activation, biosynthesis of chitin, and deposition of the lamellate cuticle.

All these processes have been intensively studied in living insects, but there are certain advantages to using an in vitro system, namely a closed system that provides the opportunity to study a single organ in isolation and to visualize changes at the cellular level as they occur. These advantages are such that five in vitro systems have been developed in which at least some work on the production of cuticle was carried out. These include the systems of Agui et al. (1) (2) and Oberlander et al. (3) that utilized wing discs of lepidopteran larvae; the systems of Mandaron (4) and Fristrom et al. (5) that utilized wing discs from Drosophila, and the system of Marks and Leopold (6) that utilized cockroach leg regenerates, Leucophaea maderae (Fab.).

The cockroach leg regenerate system is unique among those described. Since the cockroach is paurometabolous with no imaginal discs, the regenerating leg was used to provide a source of rapidly growing tissue that completes its development within a single stadium. This development is controlled by the same hormonal events that control the molting cycle. Starting from the time the nymphal leg was removed, a regenerate leg developed from undifferentiated (or dedifferentiated) cells that were present in the coxotrochanteral region of the old leg (7). By the end of the stadium, the leg had developed a cuticle and was ready to molt with the rest of the insect. By removing the mesothoracic legs of freshly molted nymphs at the coxotrochanteral joint and maintaining the operated insects in dated containers, it was possible to provide a supply of insects with leg regenerates in various stages of development. When we explanted the tissue into tubes or tissue chambers containing a threshold dose of molting hormone, a seta-bearing, chitin-containing cuticle

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appeared on the surface of the explanted tissue in about 10 days (8), (9). Labeled precursors or inhibitors were added to or washed out of the tissues, and samples of the medium were taken at various times. Changes in the treated explants were followed either by chemical analysis or by visual observation.

Activation of Epidermal Tissues

Research on the biosynthesis of ecdysone and on the action of ecdysone at the cellular level utilizing organ culture systems has been conducted by Oberlander (10), Chihara et al. (11), Ohmori and Ohtaki (12) and Marks (13), (14). The cockroach leg regenerate system has been used to investigate the conversion of inactive ecdysone analogs into β-ecdysone and to corroborate the hypothesis of Ohtaki et al. (15) that cell activation results from the accumulation by the epidermal cells of a series of covert events that eventually cause molting.

Little is known of the early part of the biosynthetic pathway of ecdysone, but the portion between 22,25-dideoxyecdysone to β-ecdysone has been worked out by Robbins et al. (16) (Fig. 1). More recently, King (17) demonstrated that various tissues from the tobacco hornworm, Manduca sexta (L.), could convert α- to β-ecdysone in vitro. In the leg regenerate system, we demonstrated a similar conversion and showed that the effectiveness of α-ecdysone in initiating cuticle deposition was dependent on the length of time that the tissue was exposed to the hormone rather than on the amount of hormone used. The effectiveness of β-ecdysone was dependent on both the concentration of the hormone and the length of exposure to it (Fig. 2,3). Furthermore, only β-ecdysone stimulated cuticle production in the presence of puromycin (Table 1).

Table 1. Effect of puromycin on the induction of cuticle deposition by α- and β-ecdysone.

Number tested	Treatment (μg/ml)[a]		Frequency of response (%)
	Ecdysone	Puromycin	
20	2.5β	none	100
20	2.5β	0.5	100
16	1.0β	none	31
16	1.0β	0.5	25
20	10.0α	none	40
20	10.0α	0.5	10[b]

a/ All chambers were exposed to the various treatments for 3 days and then scored for cuticle deposition.

b/ Decrease significant at the 95% level of confidence.

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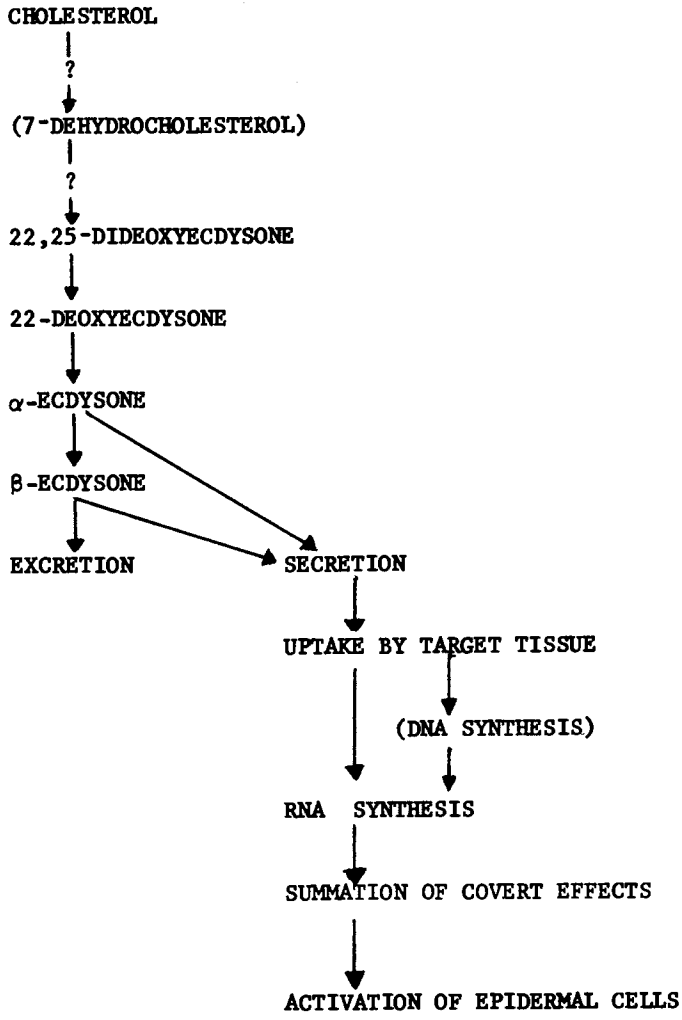

Figure 1. Flow chart of events leading to the biosynthesis of ecdysone and activation of the epidermal cells. (Tentative schema)

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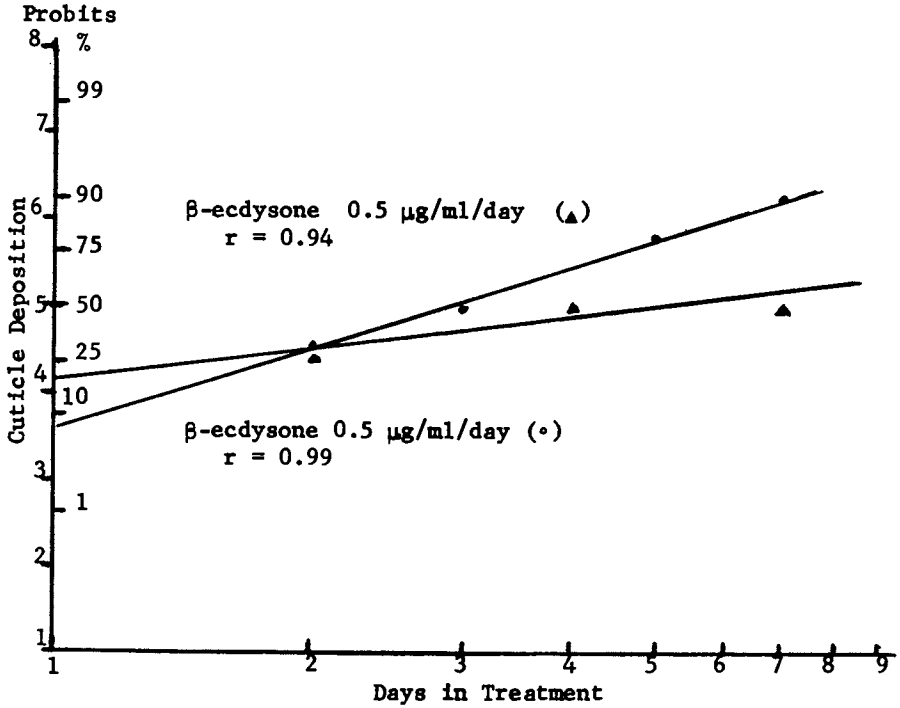

Figure 2. Graph depicting relationship between time of exposure and concentration of β-ecdysone in the induction of cuticle deposition in vitro.

▲ = The total dose was held constant by reducing the concentration of β-ecdysone as the length of exposure was increased.

• = The concentration of hormone remained constant, and the total dose was increased with the number of days of exposure.

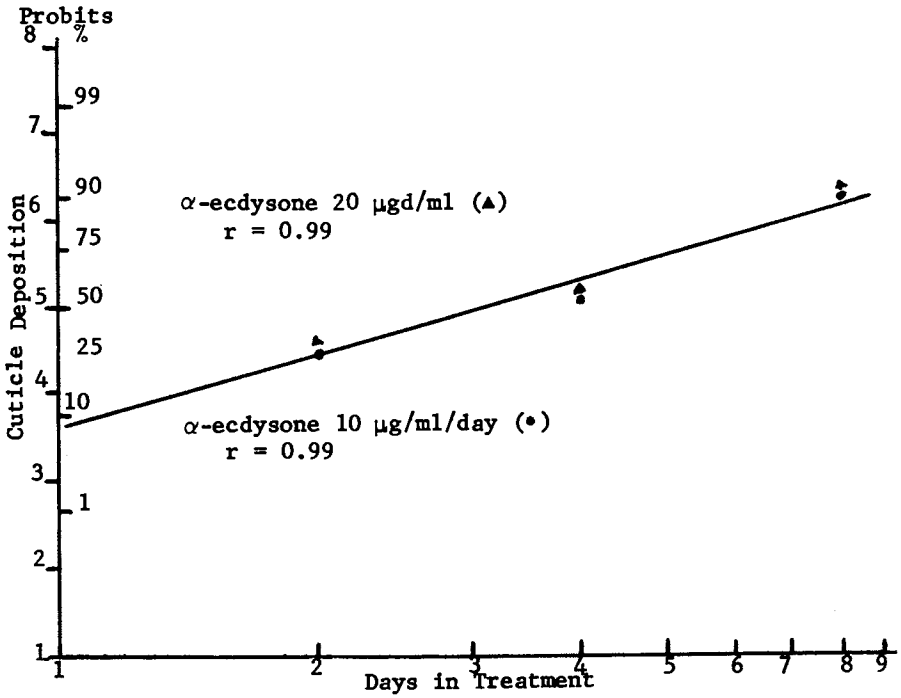

Figure 3. Graph depicting relationship between time of exposure and concentration of α-ecdysone. (Symbols are as in Fig. 2.) Increasing the total dose had no effect on the frequency of cuticle deposition. The only factor that affected cuticle deposition was the number of days of exposure. Changes in the concentration of the hormone had no apparent effect.

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We concluded that the effectiveness of α-ecdysone was dependent on a rate-limited process that required simultaneous protein synthesis. This indicated that α-ecdysone could not stimulate cuticle deposition unless it was first converted to β-ecdysone by the leg regenerate tissues. This conversion was recently confirmed by King (Zoecon Corp., unpublished results), who showed that about 20 percent of the α-ecdysone present in leg regenerate tissues is converted to β-ecdysone over a period of 6 days.

Our work on cell activation by β-ecdysone involved a series of time-dose studies in which we demonstrated a rough equivalence between the amount of hormone used and the length of exposure to it. The relationship was such that one more day of exposure gave roughly the same effect as an additional 1.3 μg/ml of hormone. Also, we conducted a series of experiments using discontinuous exposures of one day each followed by washing out the hormone and a 3-day rest before the next exposure. The results demonstrated that during the period of exposure, events resulting from the action of the hormone accumulated in the tissues and that this effect was additive and eventually resulted in cuticle deposition (Table 2).

Table 2. Comparison of responses obtained by administering a 1 μgd dose of ecdysterone in different ways

Exposures			Total	Total days		Frequency
Number	Length (days)	Dose (μg/ml)	dose (μgd)	Exposed	Elapsed	of response (%)
1	1	1.00	1	1	1	16
2	1	0.50	1	2	5	25
4	1	0.25	1	4	13	20

Our results give strong support to the hypothesis of Ohtaki et al. (15) that molting results from the accumulation of a series of covert events. Fristrom et al. (5) suggested that the covert events represent the syntheses of specific RNAs that accumulate until molting occurs.

Deposition of a Chitin-Bearing Cuticle

Cockroach leg regenerates respond to the presence of β-ecdysone by producing a chitin-bearing cuticle (8). Furthermore, leg regenerates are capable of producing as many as five such cuticles in response to separate pulses of the hormone.

The basic outlines of the processes of chitin synthesis and cuticle deposition have been known for some time (see Fig. 4). Glucose undergoes phosphorylation and amination to form D-glucos-

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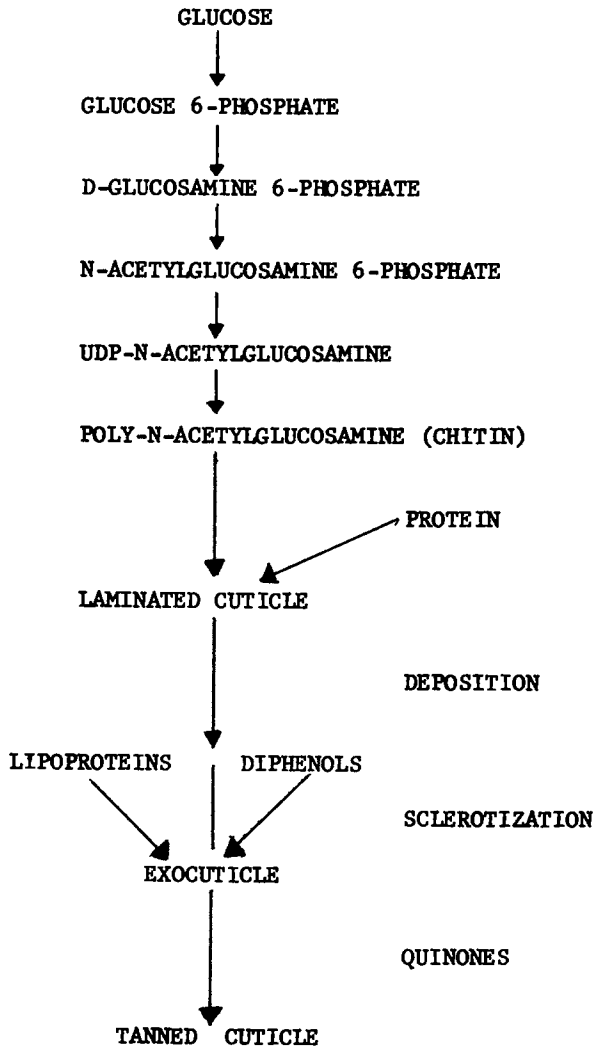

Figure 4. Flow chart depicting the events leading up to the deposition of a chitin-bearing cuticle.

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amine-6-phosphate, which is then acetylated and conjugated with UDP to form UDP-N-acetylglucosamine. This water-soluble compound is polymerized by the action of chitin synthetase to form a procuticle, the outer portion of which becomes sclerotized with the addition of diphenols and with tanning. Oberlander and Leach (18) reported that in the presence of β-ecdysone, D-glucosamine ^{3}H is taken up by incubated Plodia wing discs and that a tanned cuticle is formed. The cuticles that form on cultured cockroach leg regenerates never reach this state; the endocuticle remains only partially completed and while some pigment deposition occurs, there is no evidence of tanning.

Recently, two very interesting compounds became available, both of which affect the processes of chitin synthesis and cuticle formation. The first of these is a fungicidal compound developed at the Institute of Physical and Chemical Research, Saitama, Japan, by a group headed by Dr. Saburo Suzuki, who sent us the material used in these studies. The compound is Polyoxin D (Fig. 5), and its mode of action is as a competitive inhibitor of chitin synthetase, for which it has a greater affinity than the normal substrate UDP-N-acetylglucosamine (19). When Polyoxin D was added to the culture medium at the rate of 1 mg/ml, we observed no effect on the tissues and no visible cuticle was deposited, even after 21 days of exposure. However, when we added β-ecdysone (2 μg/ml), cuticle deposition occurred (Table 3).

Table 3. Effects of Polyoxin D on deposition of cuticle by cockroach leg regenerates in vitro.

Treatment and dose	Challenge and dose	N	Frequency of cuticle deposition (%)
Polyoxin D (1 mg/ml)	β-ecdysone (2 μg/ml)	8	100
Polyoxin D (1 mg/ml)	Control (none)	4	0
Control (none)	β-ecdysone (2 μg/ml)	8	100

Thus, Polyoxin D neither stimulated nor inhibited the deposition of a visible cuticle. However, when we repeated the experiment with D-glucosamine-^{14}C in the medium, incorporation of ^{14}C into the Hyamine Hydroxide (Rohm and Haas Co.*) insoluble fraction of the leg regenerate was inhibited (Table 4). Thus, the cuticle deposited in the presence of Polyoxin D is probably devoid of chitin since this is the only portion of a leg regenerate that

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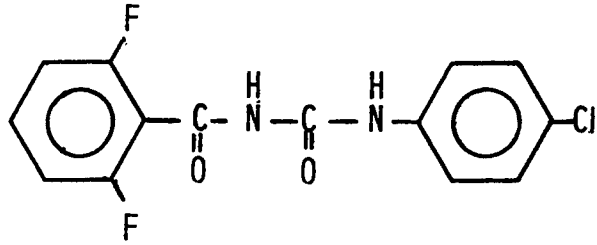
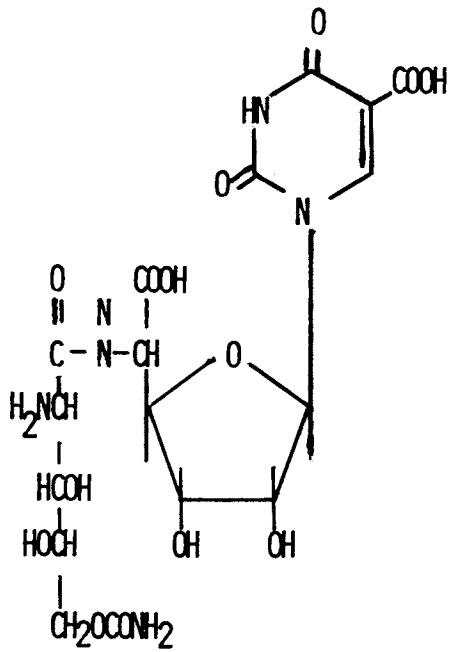


TH 6040



POLYOXIN D

FIGURE 5

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does not dissolve in a strong base. This may account for the finding of Oberlander (personal communication) that Polyoxin D completely inhibits cuticle formation in Plodia wing discs.

Table 4. Incorporation of D-glucosamine ^{14}C into Hyamine insoluble fraction of cockroach leg regenerate tissue in vitro.

Treatment and dose	Challenge and dose	Replicates	Incorporation (cpm) ± S.D.
TH 6040[a] (2.5 μg/ml)	β-ecdysone (2 μg/ml)	4	76 ± 55
Polyoxin D (1 mg/ml)	β-ecdysone (2 μg/ml)	3	52 ± 24
Control (none)	β-ecdysone (2 μg/ml)	3	951 ± 253
Control (none)	Control (none)	3	55 ± 16

a/ TH 6040 added to the culture tube in 0.5 μl DMSO. Analysis of the treated medium showed that under such conditions, the amount available in the medium was 0.75 μg/ml.

The mode of action of Polyoxin D in fungi is known (18), and it may act in the same manner in our test system (i.e., it prevents polymerizaion of UDP-N-acetylglucosamine into chitin).

The second compound tested was TH 6040 [(1-(4-chlorophenyl)-3-(2,6-difluorobenzoyl)urea], obtained from Thompson Hayward Chemical Co., Kansas City, Ka., (Fig. 5). It has good potential as an insecticide and has also been implicated in the cuticle formation process (20). Since this compound is only slightly soluble in water, we checked the effects of two solvent systems on our leg regenerate system. When we analyzed the medium from these cultures by the method of Oehler and Holman (unpublished data), we found that when 95% ethanol was used as the carrier, only 0.1 μg/ml was available in the medium, but when DMSO was used as the carrier, more than 7 times as much was available to the tissue. Like Polyoxin D, TH 6040 neither stimulated cuticle deposition itself nor interfered with the production of a visible cuticle by β-ecdysone (Table 5). Incorporation studies with D-glucosamine-^{14}C were performed in the same manner as Polyoxin D. Each 1 ml of test culture medium was treated with 2.5 μg of TH 6040 dissolved in 0.5 μl of DMSO. As with Polyoxin D, ^{14}C incorporation into the Hyamine insoluble fraction of the cuticle was almost completely inhibited (Table 4).

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Table 5. Effects of TH 6040 on the deposition of cuticle by cockroach leg regenerates in vitro.

Treatment and dose	Solvent (μl/ml)	Challenge and dose (2 μg/ml)	N	Frequency of cuticle deposition (%)
TH 6040 (2.5 μg/ml)[a]	50% ETOH	β-ecdysone	14	100
Control	50% ETOH	β-ecdysone	16	100
TH 6040 (2.5 μg/ml)[b]	DMSO	β-ecdysone	16	100
Control	DMSO	β-ecdysone	6	100
TH 6040 (5.0 μg/ml)	DMSO	Control	4	0

a/ Amount added to chamber. Analysis of medium showed that the amount available in the medium was 0.10 μg/ml.
b/ Amount added to chamber. Analysis of medium showed that the amount available in the medium was 0.75 μg/ml.

With this information, it is obvious that the visible cuticle formed in the earlier experiments with Polyoxin D and TH 6040 lacked chitin and consisted of an epicuticle and the proteinaceous portion of the procuticle only. The difference between this condition and the normal chitin-bearing cuticle would be detectable only by histochemical analysis or radiotracer studies. Thus, we determined that while Polyoxin D and TH 6040 inhibited the synthesis of chitin, they did not appear to upset the other processes involved in cuticle deposition. These findings closely support the results obtained from in vivo studies by Post and Vincent (20).

The advantages of an in vitro system provide previously unavailable opportunities for mode of action studies. We are continuing our investigations in both the areas of ecdysone biosynthesis and in the areas of cuticle formation with compounds that inhibit chitin synthesis.

* Mention of a proprietary product or company name in this paper does not constitute an endorsement by the United States Department of Agriculture.

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13

Chlorella as a Model System to Study Herbicidal Mode of Action and its Application to a New Herbicide, *O*-Ethyl-*O*-(3-methyl-6-nitrophenyl)-*N*-*sec*-butyl Phosphorothioamidate (S-2846)

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S-2846 (Fig 1) is an organophosphoramidate herbicide for annual weeds with low mammalian toxicity (1). This herbicide acts on growing points of susceptible plants and causes severe radial enlargement of the affected tissues. Microscopic inspection of a treated germinating seed of barnyardgrass revealed that meristematic cells were swollen several times in length without change in their number (2). Chlorella ellipsoidea is a unicellular green alga which is taxonomically classified as a plant with relative similarity to higher plants. When Chlorella was treated with S-2846, a considerable portion of the cells were swollen noticeably, but cell number ceased to increase. Based upon these observations, it was assumed that dividing cells of higher plants and Chlorella were affected with S-2846 by a common mode of action. Chlorella was chosen as a model system because of its rapid growth and relative ease of manipulation to obtain quantitative results.

Materials and Methods

Chlorella ellipsoidea was cultured essentially in the same manner as reported previously (3) except that 2% glucose was included in the medium for shaking culture under room light in most experiments. Respiration and photosynthesis were measured with a Gilson differential respirometer. Protein fraction was prepared by Schneider's method (4). DNA and RNA were assayed by a modification of Schmidt-Thannhauser's method (5). Cellulose fraction was prepared by a modification of Northcote and Goulding's method (6). Lipid was extracted by Bligh and Dyer's method (7), and analyzed in Nichols' TLC system (8). Water-soluble compounds were separated by the method of Benson et al (9) Radioactivity was measured with a liquid scintillation counter. ^{32}P-labeled S-2846 was synthesized by Yoshitake et al (10). Inhibition of fly-head acetylcholine esterase was assayed by pH-stat method by K. Kamoshita. Radioactive precursors were purchased from Daiichi Pure Chemicals.

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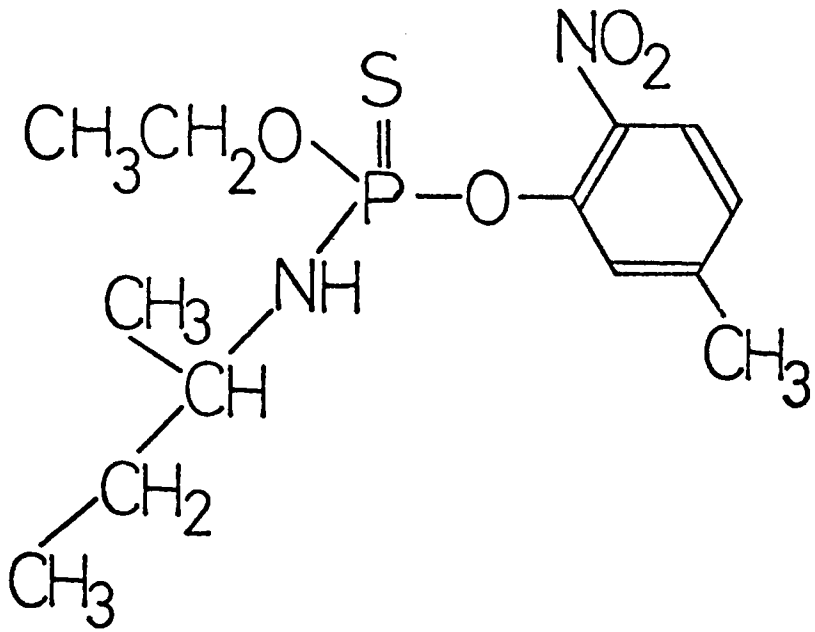

Figure 1. S–2846

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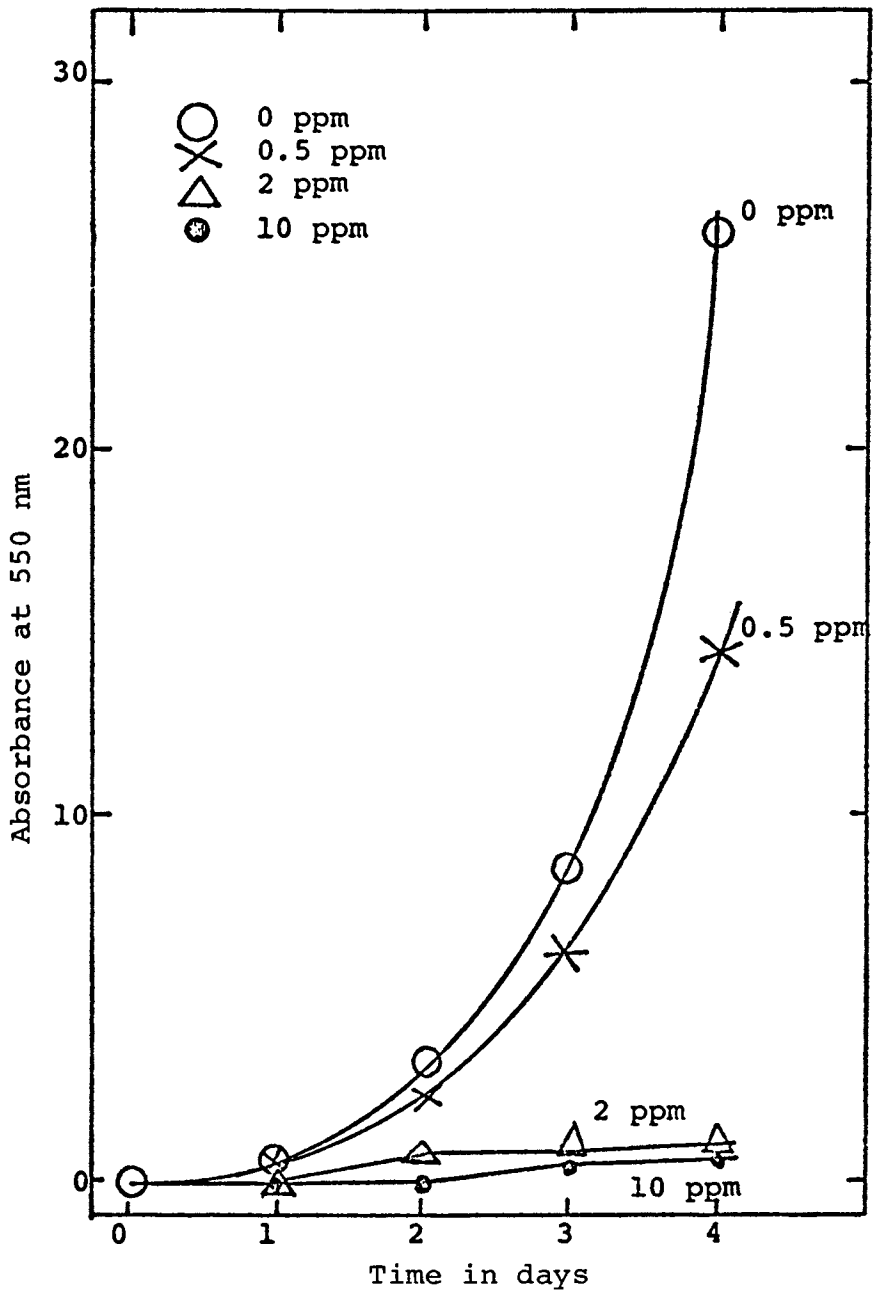

Figure 2. Effect of S–2846 on Chlorella *growth*

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Results

When Chlorella was treated at early logarithmic stage, growth was nearly completely suppressed at 2 ppm (Fig 2). Fifty percent inhibition was observed at about 0.7 ppm as calculated by provit analysis. Inhibition of growth in terms of cell number became apparent in less than 7 hours after treatment at 2 ppm. In peptone-NaCl medium, Escherichia coli grew normally in the presence of 100 ppm of S-2846 at 37°C. Viability of the bacterium was not affected after treatment of the resting cells at 1000 ppm for 20 hr (11). Respiration of Chlorella with glucose as a substrate was not significantly affected at 0.2 to 50 ppm (Fig 3). Photosynthesis was not affected in the same range of concentration (Fig 4). During five-hour treatment period at the concentrations ranging from 0.5 to 50 ppm, no significant leakage of radioactivity occurred which had been incorporated from ^{14}C-$NaHCO_3$ into the cells (Fig 5). Permeability of Chlorella cytoplasmicmembrane does not seem to be drastically affected by the chemical. Then effects of S-2846 on biosynthesis of major classes of cellular constituents were studied. After 60 min treatment, incorporation of ^{14}C-amino acids mixture into protein fraction was not inhibited even at 50 ppm. Lipid synthesis from ^{14}C-acetate was not affected at 2 ppm, but rather stimulated at 10 ppm. The compositions of complex lipids and water-soluble compounds were not significantly altered. Incorporation of ^{14}C-thymine or ^{14}C-thymidine into DNA fraction was not significantly affected. ^{14}C-Uracil incorporation into RNA fraction was not affected at 2 ppm, and moderately inhibited at 10 ppm. Incorporation of ^{14}C-glucose into cell wall fraction was significantly inhibited at 10 ppm after longer treatment in some experiments, but inhibition was not noticeable in other experiments. The variability of the results led to the idea that effects on biosynthetic processes could be interpreted more systematically on one-cell basis rather than on the basis of the whole culture. Gross biosyntheses of protein, DNA, RNA, cell wall and lipids were found to be stimulated on this basis even after 20 hours treatment at 2 ppm (Table 2). Average dry weight per cell increased in proportion to increase in average cell volume after treatment, which is consistent with the results of stimulated biosynthesis on one-cell basis. DNA content also increased on one-cell basis after treatment (Table 3). Isopropyl N-(3-chlorophenyl)carbamate as well as S-2846 caused swelling of cells (Table 4). The oxon derivative of S-2846 exhibited potent anticholine esterase activity with tentative I_{50}= 7 x 10^{-8} M. The oxon derivative was detected in chloroform extracts of cells treated with ^{32}P-S-2846. Preliminary results indicate that nondialysable compounds, probably protein, became radioactive after treatment of synchronously grown cells of Chlorella with ^{32}P-S-2846.

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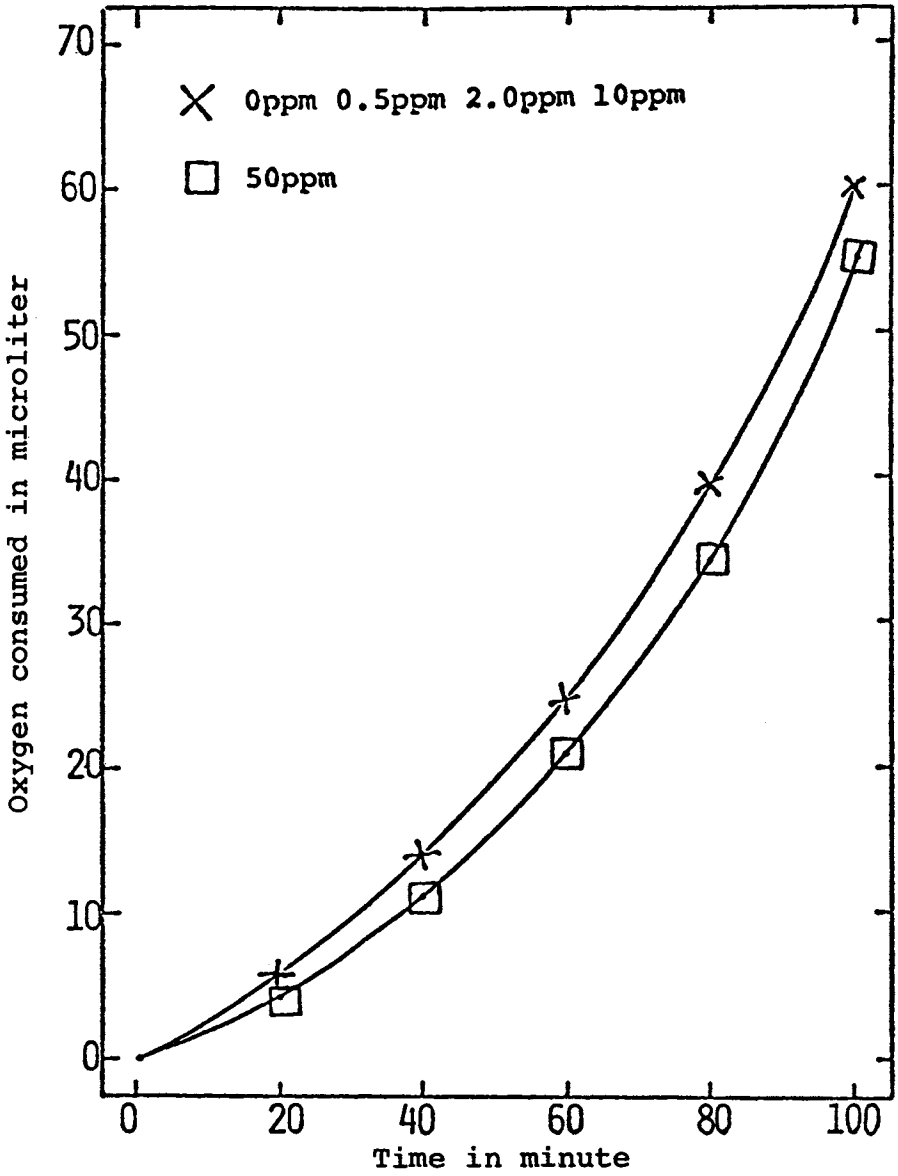

Figure 3. Effect of S–2846 on Chlorella *respiration*

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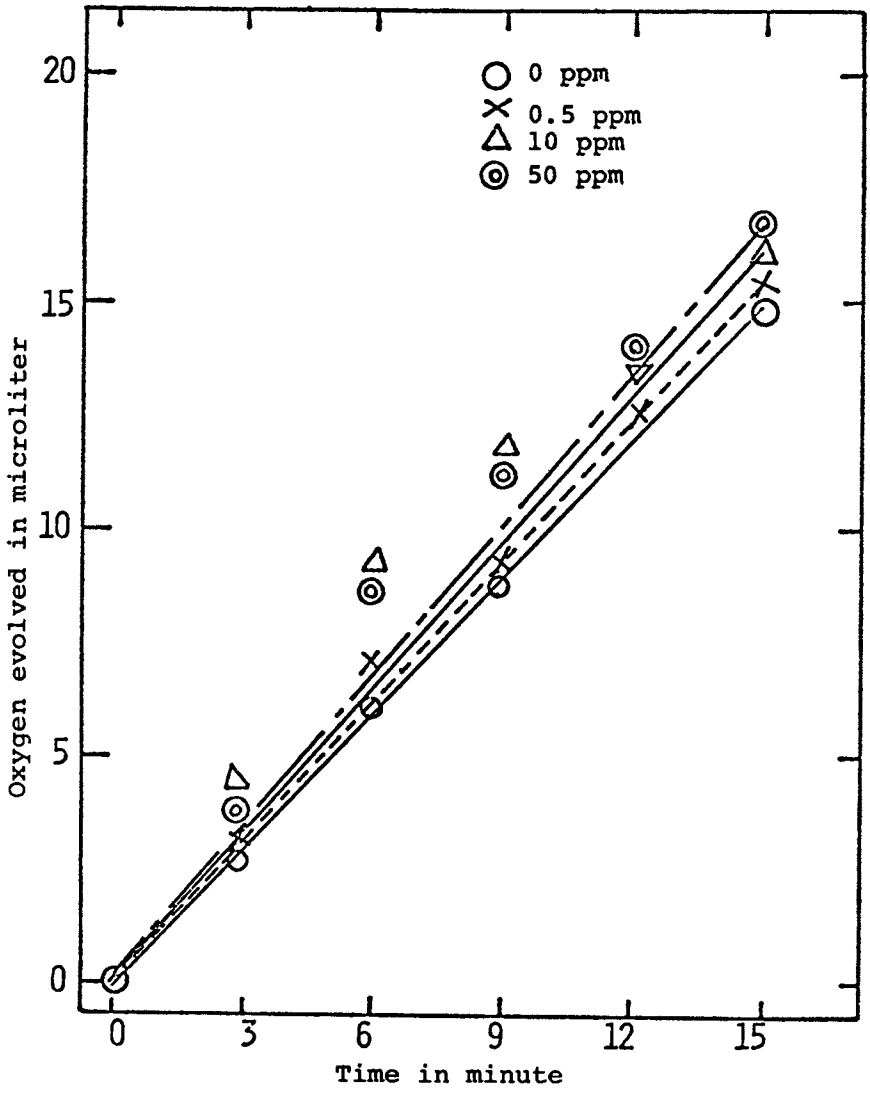

Figure 4. Effect of S–2846 on Chlorella *photosynthesis*

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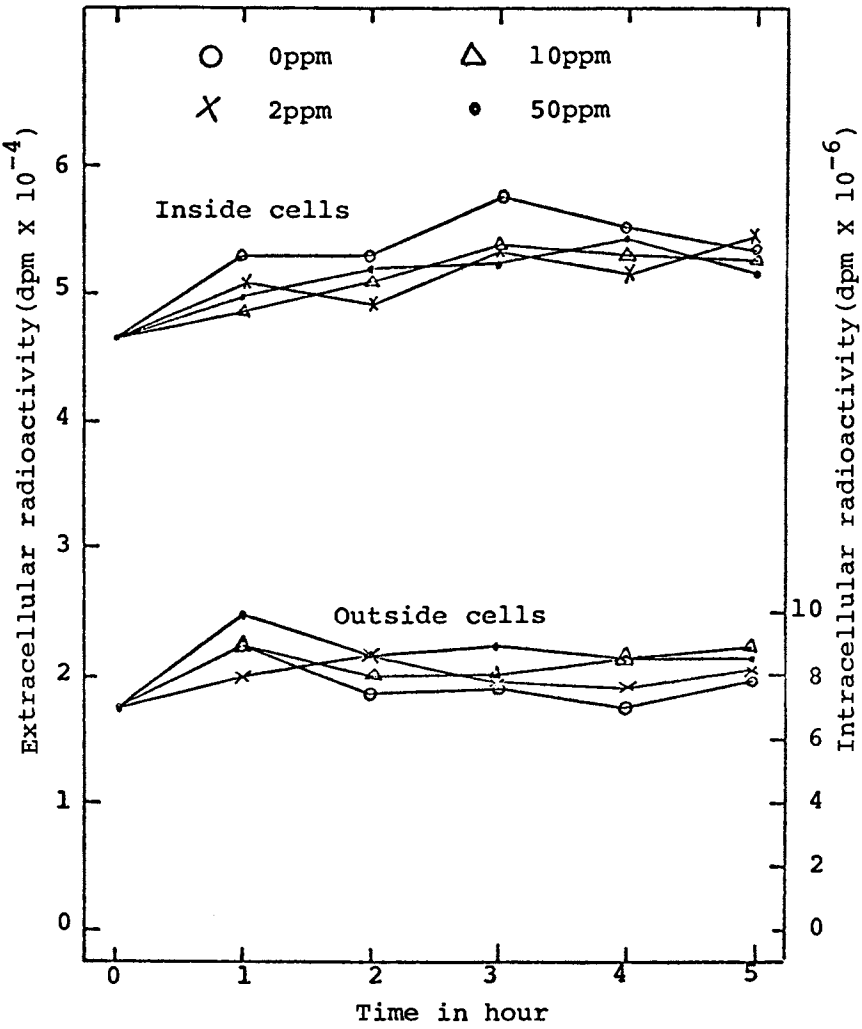

Figure 5. Effect of S–2846 on the leakage of ^{14}C from Chlorella *cells*

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Table 1. Effect of S-2846 on biosyntheses of major classes of cellular constituents (short-term treatment).

Fraction	Per cent of control on one-cell basis		
	2 ppm	10 ppm	50 ppm
protein from ^{14}C-amino acid mixture (^{14}C-U-protein hydrolysate from Chlorella)	103	133	120
lipid from ^{14}C-U-acetate	112	166	185
cell wall from ^{14}C-U-glucose	105	81	72
RNA from ^{14}C-2-uracil	94	81	75

Chlorella grown under heterotrophic conditions was preincubated with S-2846 at 2, 10, and 50 ppm at 28°C for 60 min. Biosynthesis was then assayed by further incubating the cells with an appropriate radioactive substrate for 60 min.

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Table 2. Effect of S-2846 on gross biosyntheses of major classes of cellular constituents (long-term treatment).

Fraction	Per cent of control on one-cell basis
protein	204
lipid	300
cell wall	197
RNA	159
DNA from ^{14}C-2-thymine	169

Chlorella was treated with S-2846 at 2 ppm for 20 hrs at 28°C under heterotrophic conditions. Biosynthesis was assayed by incubating the cells with an appropriate radioactive substrate for 60 min at 28°C (see Table 1 for radioactive precursors).

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Table 3. Effect of S-2846 on DNA and RNA contents in Chlorella cells

	Relative amount per one cell	
	DNA	RNA
control	1.0	1.0
treated (10 ppm, 24 hrs)	1.3	1.5

Table 4. Effect of S-2846 and CIPC on packed cell volume of Chlorella

	Packed cell volume ul/ml	Cell number/ml	PCV/Cell ul
control	1.3	5.2×10^{7}	2.5×10^{-8}
S-2846-treated (3 ppm)	1.1	1.1×10^{7}	10×10^{-8}
CIPC-treated (1 ppm)	0.92	0.84×10^{7}	11×10^{-8}

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Discussion

Neither energy metabolism, nor membrane permeability, nor gross biosynthesis of major classes of cellular constitutents was inhibited at a concentration of S-2846 (2 ppm) where cell multiplication was completely inhibited. Inhibition of cell multiplication was accompanied by cell swelling which was in turn accompanied by proportional increase in dry weight per cell. These observations would be rationalized by postulating that inhibition of cell division *per se* is one of the primary sites of action. It is interesting to note that *Escherichia coli*, a bacterium which does not go through elaborate mitosis during cell division, was highly resistant to this chemical.

During the process of cell division, microtubules are considered to play a vital role. Mitotic spindle fibers have been identified as bundles of microtubules, and seem to be highly labile structures existing in a dynamic equilibrium with a large pool of unassociated subunits (12). The subunit of microtubular protein has quanosine nucleotide binding sites (13), and a serine hydroxyl group which was phosphorylated by ^{32}P-ATP (14). This reaction was stimulated by 3', 5'-cyclic AMP. Organophosphorus and carbamate insecticides are well known to inhibit acetylcholine esterase by binding to the active site of the enzyme (15). Considering the fact that the oxon derivative of S-2846 exhibited potent anticholine esterase activity, it might possibly attack subunit of microtubular or related protein.

Morphological and cytological investigations indicate a similar mode of action for a number of carbamate herbicides studied in detail (16). These studies suggested that those carbamates acted as antimitotic agents. Isopropyl N-phenyl-carbamate (IPC) completely inhibited cell division at 1 ppm (17) where protein synthesis was inhibited by 11 % (18). Methyl benzimidazol-2-yl carbamate, a fungicidal principle, has also been reported to have antimitotic activity, causing disruption of spindle formation without direct inhibition of DNA synthesis (19, 20). A more recent report suggests that the mode of action of IPC appeared to be on the microtubular organizing centers rather than on microtubules *per se* (21).

Based upon these pieces of facts, it might be possible that the organophosphoramidate (S-2846) and those carbamate herbicides attack microtubular organizing apparatus as a common site of action, as in the case of acetylcholine esterase which is a common target for carbamate and organophosphorus insecticides.

Direct approach is naturally required to test this hypothesis. Due to asynchronous character of the tissues, higher plants pose some experimental difficulty. *Chlorella*, which can be grown synchronously with relative ease, would provide a useful model system for biochemical study of this particular aspect of cellular life.

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Activity and Mode of Action of Benzylidenemalononitriles

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An attempt was made to develop a new pesticide by modification by butylated hydroxytoluene (BHT) which is used as an antioxidant. Rationale for this attempt was partial analogy in structure of BHT to 3, 5-diiodo-4-hydroxybenzonitrile which is a commercial herbicide. During the course of investigation, the authors came upon a compound, 3, 5-di-tert-butyl-4-hydroxybenzylidenemalononitrile (coded SF-6847) having pesticidal efficacy against fungi and mites (1). Due to its newness in structure as a pesticide, influence of structural variation on the biological activity was studied. In vitro studies were also performed which showed that SF-6847 had remarkable potency as an uncoupler of oxidative phosphorylation in mitochondria isolated from rat liver. The results reported here had been published by Horiuchi et al (1) and Muraoka and Terada (2).

The forthcoming compounds will be arbitrarily classified into five groups, A through E (Table 1). In group A methyl group at 1-position was oxidized to various degrees. None of them gave detectable biocidal activity. When the structure was further modified into a benzylidenemalononitrile, remarkable biocidal activity appeared. Among the analogues in which alkyl group at 3- and 5-positions were varied, di-tert-butyl derivative gave the highest activity. The activity increased with increase in the van der Waals radii of the alkyl groups. When 3- and 5-positions of the aromatic ring were substituted with electron-withdrawing groups such as nitro groups and chlorine atoms, the activity was almost completely lost. Edwards and Pianka (3) studied fungicidal properties of nitro- and chloro-substituted benzylidenemalononitriles, and they were shown to be inactive. Replacement of one of the cyano groups in the malononitrile moiety by carboalkoxy or related group reduced the activity (group C). Modification of both the cyano groups reduced the activity more drastically (group D). The free hydroxy group at 4-position seemed necessary for the highest activity since the acetylated derivative possessed lower activity (group E).

In order to evaluate these compounds as agricultural fungi-

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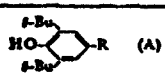
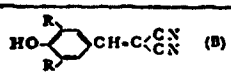
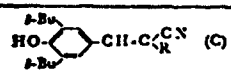
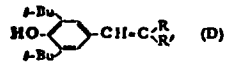
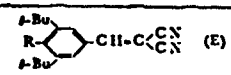
Table 1 Fungicidal and Acaricidal Activities

No.	Structure	P. oryzae Inhibition % of spore germination (conc. μM)				T. telarius LC_{50} in μM
		400	40	4	0.4	
	 (A)					
I	R=CH_2OH	0	0	0	0	>500
II	=CHO	0	0	0	0	>500
III	=COOH	0	0	0	0	>500
	 (B)					
IV	R=Me R=Me	100	100	0	0	>500
V	=Isopr =Isopr	100	100	98	76	>500
VI	=sec-Bu =sec-Bu	100	98	79	47	>500
VII	=tert-Bu =tert-Bu	100	100	100	100	14
VIII	=tert-Am =tert-Am	100	100	100	66	13
IX	=Me =tert-Bu	0	0	0	0	>500
X	=Cl =Cl	100	0	0	0	>500
XI	=NO_2 =NO_2	100	0	0	0	>500
	 (C)					
XII	R=H	0	0	0	0	>500
XIII	=COOH	100	70	0	0	>500
XIV	=COOMe	100	100	97	76	198
XV	=COOEt	100	100	0	0	>500
XVI	=$CONH_2$	0	0	0	0	>500
	 (D)					
XVII	R=COOH R'=H	0	0	0	0	>500
XVIII	=COOEt =COOEt	0	0	0	0	>500
XIX	=COOEt =COMe	100	97	10	0	>500
XX	=COMe =COMe	0	0	0	0	>500
	 (E)					
XXI	R=H	0	0	0	0	>500
XXII	=OAc	100	100	0	0	>500

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cides, they were subjected to various biological tests (1). Results of pot tests showed that SF-6847 completely inhibited rice blast (Table 2). SF-6847 exhibited high toxicity to mammals. The acute oral LD_{50} to mice was 29 mg/kg. Symptoms caused by SF-6847 suggested that respiratory function might possibly be severely impaired. Consequently, effects of this series of compounds on mitochondrial respiration *in vitro* were studied.

Table 3 shows the effects of these compounds on ATPase and state 4 respiration in rat liver mitochondria. Analogues possessing electron-withdrawing groups at 1-position displayed high activity. Among them, SF-6847 showed the most potent uncoupling activity in parallel to the results of *in vivo* tests. It was found that 3×10^{-9} M SF-6847 caused approximatly 75% uncoupling (taking the effect of 3×10^{-5} M dinitrophenol as 100%). The amount of SF-6847 giving half-maximal uncoupling (S_{50}) is less than 1.2 n moles/g protein and potency of this compound is more than 8300 times that of dinitrophenol (S_{50}: 10 μmoles/g protein). Slight modifications of the malononitrile moiety or hydroxyl group caused decreases in the uncoupling activity. The two tertiary butyl groups seem to play an important role for the uncoupling activity. Introduction of electron-withdrawing group at 1-position caused remarkable increase in the activities of these compounds. A similar fact was reported by Williamson and Metcalf (4) for powerful uncouplers, such as 5-chloro-3-*tert*-butyl-2'-chloro-4'-nitro-salicylanilide (S-13), 4, 5, 6, 7-tetrachloro-2-trifluoromethyl benzimidazole (TTFB) and carbonyl cyanide-*m*-chlorophenyl hydrazone (CCCP), in which the strong electron-withdrawing centers are located within a certain spatial distance of a halogenated ring. The strong electron-withdrawing property of the malononitrile group in SF-6847 seemed confirmed by solvent effect experiments; a large shift of the absorption peak was observed when the absorption spectrum of SF-6847 was measured in various non-polar solvents with different refractive indices. However, the shift was small in the derivatives with less activity and was negligible in some known uncouplers such as dinitrophenol and flufenamic acid. A wide range of spectral changes of malononitrile compounds in mixtures of alcohols and water were also described by Lauerer *et al*, who proposed the formation of charge transfer complexes between the compounds and solvent molecules (5).

It is possible that the electronwithdrawing group interacts with the energy conservation site in a direct manner such as by nucleophilic attack or formation of a charge-transfer complexes. This may easily be explained by a chemical (6, 7) or conformational hypothesis, but scarcely by chemiosmotic hypothesis (8). The hydroxyl group might act as an electron donor to the electron-withdrawing group as illustrated in Fig. 1. The figure also illustrates that bulky hydrophobic groups might bind to the hydrophobic region located in the vicinity of the energy conservation site. The distance between these groups may be about 5 Å. It is likely that the scheme visualizes a topographical aspect of the

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TABLE 2 RESULTS OF POT TEST ON RICE BLAST

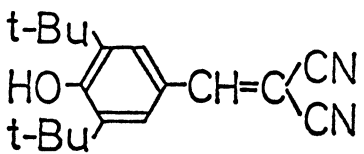
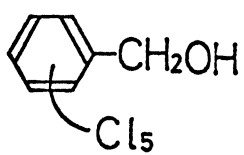
COMPOUND	CONC. (PPM)	INFECTED LEAVES (%)	PREVENTIVE VALUE (%)
	100	0	100
	20	0	100
	10	17	97
	100	0	100
	20	69	91
	10	100	76
NON-TREATMENT		100	0

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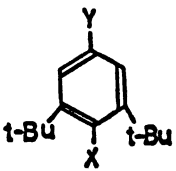
TABLE 3

UNCOUPLING ACTIVITIES OF 3,5-Di-tert-BUTYL-4-HYDROXYBENZYLIDENEMALONONITRILE AND ITS DERIVATIVES



No.	X	Y	Uncoupling Activity			
			ATPase (M)	R.A.	Respiration (M)	R.A.
	-OH	-H	3.2×10^{-4}	0.31	7.0×10^{-5}	0.69
	-OH	-COOH	1.3×10^{-3}	0.08	1.8×10^{-3}	0.03
	-OH	$-CH_2OH$	9.8×10^{-4}	0.10	9.0×10^{-4}	0.05
	-OH	-CH=C(CN)COOH	1.2×10^{-4}	0.83	1.1×10^{-4}	0.44
	-OH	-CH=NOH	2.0×10^{-4}	0.50	8.5×10^{-5}	0.56
	-OH	$-CH=NN(CH_3)_2$	2.7×10^{-4}	0.37	7.5×10^{-5}	0.64
	-OH	-CH=CHCN	1.2×10^{-4}	0.83	3.6×10^{-5}	1.33
	-OH	$-CH=C(COCH_3)COOC_2H_5$	1.2×10^{-4}	0.83	2.4×10^{-5}	2.00
	-OH	$-CH=C(CN)CONH_2$	2.3×10^{-5}	4.30	7.0×10^{-6}	6.86
	-OH	$-CH=C(CN)COOCH_3$	2.3×10^{-6}	43.0	5.0×10^{-7}	96.0
	-OH	$-NO_2$	2.1×10^{-6}	47.1	7.3×10^{-7}	65.8
	-OH	$-CH=C(CN)_2$	8.6×10^{-8}	1151	2.9×10^{-8}	1655
	$-CO_2CH_3$	$-CH=C(CN)_2$	1.4×10^{-6}	70.7	7.2×10^{-7}	66.7
	2,4-dinitrophenol		9.9×10^{-5}	1.0	4.8×10^{-5}	1.0

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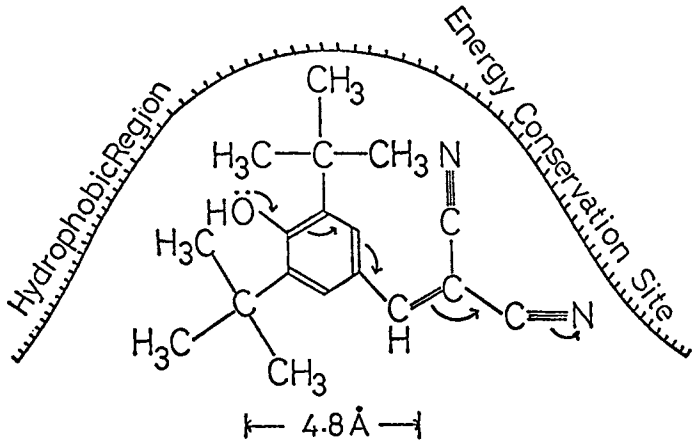
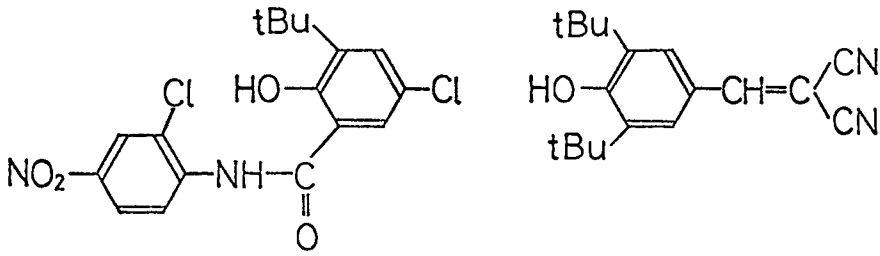


Figure 1



S—13
1.7×10^{-9} M(50%)

SF—6847
6×10^{-9} M(100%)

Figure 2

In Mechanism of Pesticide Action; Kohn, G.;
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coupling site in the mitochondrial membrane.

SF-6847 reported here (2), and S-13 reported by Williamson and Metcalf (4), are, to our knowledge, among the most powerful uncouplers ever reported (Fig. 2). Because of their utmost potency, biochemists use them as tools to study mechanism of oxidative phosphorylation which occupies one of the central positions of fundamental biochemistry. It is particularly interesting to note that those compounds which were originally produced through investigation in search of new pesticides for practical purposes now make contribution also to the advancement of pure science.

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