Progress and Prospects in Insect Control

BCPC Monograph No. 43

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Contents

Preface

Invertebrates are probably the most numerous and diverse inhabitants of our planet and in many parts of the world they are a major competitor for the biological resources. Some invertebrates destroy substantial amounts of the world's agricultural production while others constitute a significant health hazard. There are over one million named species of insects alone and they have a considerable influence on the terrestrial, freshwater and marine environments.

The specific insects that present a threat to man are given particular attention by international organisations, governments, industry and academia; a major objective being to find acceptable control measures. Both the Agrochemical Industry and the Human Health Industry have sectors devoted to insect control.

In 1988 the end-user value of insecticides sold world-wide was about $7.5 billion and it is estimated that this investment in insect control prevented the destruction of about $165 billion worth of agricultural production. Even so substantial losses still occur and there is a continual search for new and better methods of insect control.

This year (1989) is a good time to review insect control, for several reasons

- Emerging sciences and technologies present new options for controlling insects.
- World knowledge has advanced to such a level that the rational design of insect control agents is approaching reality.
- The pressures on conventional materials have increased significantly in recent years and there is a drive for new products demonstrating improved performance and acceptability.
- 1989 is a significant year in insect control as it is the 50th Anniversary of the use of DDT [α,α-bis-(p-chlorophenyl)-ββ-tri-chloroethane] as an insecticide. This very important material has been of immense value, saving many lives, although it has also received considerable criticism. Putting DDT, and other materials, in perspective will allow us to benefit from the experience and continue the progression of product improvement.
- This Symposium continues the British Crop Protection Council theme of commemorating significant events in Crop Protection History. An earlier meeting (1985) in Bordeaux celebrated the centenary of the use of Bordeaux Mixture as a fungicide.

To do justice to the substantial amount of high quality R & D in progress is not possible in one monograph, however, the range of topics covered in this volume exemplifies many of the techniques that currently contribute to insect control or will be part of insect control in the future.

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NEIL R. McFARLANE

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This Conference was organised in association with the Agriculture Group of the Royal Society of Chemistry and the Pesticides Group of the Society of Chemical Industry. The assistance of the following organisations in promoting this meeting is gratefully acknowledged.

RSC Royal Society of Chemistry, Agriculture Group
SCI Society of Chemical Industry, Pesticides Group
GIFAP International Group of National Associations of Manufacturers of Agrochemical Products
FAO European Co-operative Research Network on Pesticides
BAA British Agrochemicals Association Limited

Additionally we wish to thank C.A.B. International Institute of Entomology for supplying material used in the production of the Monograph.

N. R. McFARLANE

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ABBREVIATIONS

Where abbreviations are necessary (in tables or figures) those below are permitted without definition. The full word, for example 'days', may be more suitable for use in text.

acid equivalent	a.e.	nuclear magnetic resonance	nmr
active ingredient	AI	number average diameter	n.a.d.
boiling point	b.p.	number median diameter	n.m.d.
British Standards Institution	BSI	organic matter	o.m.
centimetre(s)	cm	page	p.
concentration × time product	ct	pages	pp.
		parts per million by volume	mg/l
concentration required to kill 50% of test organisms	LC50	parts per million by weight	mg/kg
correlation coefficient	*r*	pascal	Pa
cultivar	cv.	percentage	%
cultivars	cvs	post-emergence	post-em.
day(s)	d	power take off	p.t.o.
days after treatment	DAT	pre-emergence	pre-em.
degrees Celsius (centigrade)	°C	probability (statistical)	*P*
		relative humidity	r.h.
dose required to kill 50% of test organisms	LD50	revolutions per minute	rev/min
dry matter	d.m.	second (time unit)	s
Edition	Edn	standard error	S.E.
Editor	Ed.	standard error of means	S.E.M.
Editors	Eds	soluble powder	SP
emulsifiable concentrate	e.c.	species (singular)	sp.
freezing point	f.p.	species (plural)	spp.
		square metre	m^2
gas chromatography–mass spectrometry	gc-ms	subspecies	ssp.
gas–liquid chromatography	g.l.c.	surface mean diameter	s.m.d.
gram(s)	g	suspension concentrate	SC
growth stage	GS	temperature	temp.
hectare(s)	ha	thin-layer chromatography	tlc
		tonne(s)	t
high performance (or pressure) liquid chromatography	h.p.l.c.	ultraviolet	u.v.
hour	h	vapour pressure	v.p.
infrared	i.r.	variety (wild plant use)	var.
		volume	*V*
International Standardisation Organisation	ISO	weight	*W*
Kelvin	K	weight by volume	*W/V*
kilogram(s)	kg	(mass by volume is more correct)	(m/*V*)
least significant difference	L.S.D.	weight by weight	*W/W*
litre(s)	litre	(mass by mass is more correct)	*(m/m)*
litres per hectare	l/ha	wettable powder	WP
mass	*m*		
mass per mass	*m/m*	approximately	*c.*
mass per volume	*m/V*	less than	<
mass spectrometry	m.s.	more than	>
maximum	max.	not less than	≮
melting point	m.p.	not more than	≯
metre(s)	m	Multiplying symbols—	Prefixes
milligram(s)	mg	mega ($\times 10^6$)	M
millilitre(s)	ml	kilo ($\times 10^3$)	k
millimetre(s)	mm	milli ($\times 10^{-3}$)	m
minimum	min.	micro ($\times 10^{-6}$)	μ
minute (time unit)	min	nano ($\times 10^{-9}$)	n
molar concentration	M	pico ($\times 10^{-12}$)	p

1.
The Invertebrate Nervous System as a Target

Chairman: DR J. A. PICKETT

DDT IN PERSPECTIVE

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INTRODUCTION

The discovery of the insecticidal properties of DDT fifty years ago was probably the most important development in the history of pest control that ever happened. It is therefore important that we should try to obtain an accurate picture of the way this discovery was made, and of the ways in which DDT was used, and misued, in the years that followed. There are so many myths and inaccurate stories regarding this substance that a balanced assessment is appropriate. Many people know that the discovery was made by Paul Müller in Switzerland, but the commonest myth suggests that this was the result of a lucky chance, rather than as the climax of a carefully planned scientific campaign. Then, particularly in the literature concerning wildlife conservation, and in the popular press, DDT is stigmatised as a major environmental pollutant rather than as the substance which should probably take first place among all chemicals for its contribution to saving human life.

THE PIONEER

Paul Müller was born at Olten, some 40 kilometres from Basle, in 1899. His school-days were interrupted for a period when he worked as a laboratory assistant, which probably contributed to his later skills as an experimental research worker. He attended the University of Basle, where he specialised in Chemistry and Physics. He took his doctorate, and in 1925 he joined the firm of J.R. Geigy at Basle as a research chemist.

At that time Geigy was mainly concerned with the manufacture of synthetic dyestuffs and tanning materials. Müller became involved in the latter field and succeeded in producing some useful research to discover more effective pesticides, particularly for use in agriculture. Those who are concerned with the organisation of research in Britain today should note that he was given free rein to work in this wide field using approaches which he himself thought would be most fruitful. There was no high powered committee to issue their instructions, no wordy "contract" setting out how he should spend his time. Also he was not a part of any highly organised team, though of course he had scientific colleagues with who to confer. But he was the essential "loner", responsible for his own programme - and therefore easily identified with his own successes and failures.

Müller was a chemist, not a biologist, but he insisted on making his own biological experiments, and he always stressed the idea that chemists should follow his own example, so they could themselves recognise the significance of their observations, and not leave the observations to others who might be less perceptive or have a different motivation.

Müller set himself the task of discovering an insecticide with very specific properties. These were as follows:

1. Strong toxicity to insects.
2. Rapid action with insects.
3. Relative harmlessness to warm-blooded animals and plants.
4. Non-irritant and virtually odourless.
5. Have a wide application.
6. Long-lasting effect through chemical stability.
7. Cheap and easy to produce in quantity.

No existing insecticide (in 1935) possessed all these properties. Those based on arsenic were lethal to warm-blooded animals. Some of vegetable origin, such as nicotine, were equally poisonous. Other vegetable products, such as pyrethrum or derris, rapidly lost their toxicity to insects when exposed to sunlight and to the atmosphere, and were difficult to produce in large quantities.

From the outset of his investigations, Müller had a mental picture of the sort of compound which would have the desired properties. There was not anything haphazard about his selection of chemicals - he certainly did not find a bottle of DDT, which had been synthesised in 1873 by O. Zeidler in Austria, collecting dust on a shelf and, by a stroke of good luck, try it out on his insects. He decided at the outset the sort of molecule which might be effective, and he produced a whole series of substances by combining chlorals with hydrocarbons and phenols. Several of these had insecticidal properties. In the autumn of 1939 his research yielded 4,4-dichlorodiphenyltrichloroethane, which proved to be the most effective of the substances he had synthesised.

Müller himself made the original experiments on DDT. He is said to have discovered its efficacy against houseflies when he confined these insects in a cage where DDT had been used, but which had been thoroughly - or apparently thoroughly - cleaned. The flies died, as the tiny traces of the chemical left after cleaning were sufficient to kill them. Incidentally less percipient workers who obtained similar results sometimes wrote them off as "unexplained accidents". The new insecticide was quickly tested by Müller and his colleagues against aphids, cockchafers, winter moths, Colorado potato beetles and others. At one stage Müller was disappointed to find that the chemical was somewhat slow acting, so it did not have the second of the properties he had stipulated when starting his researches. However, even when it was slow, it was sure, unlike rapidly acting substances like pyrethrum which gave a rapid "knock down" from which the insects sometimes recovered.

The further tests of DDT, its manufacture and marketing were the responsiblity of others within the Geigy organisation, and described later in this paper. Müller himself continued his research during his remaining years with the Company until 1961, and in fact right up to his death in 1965.

In 1948, Müller was awarded the Nobel Prize for Medicine and Physiology. All his friends say that he did not enjoy the limelight engendered by these ceremonies, nor did he appreciate the red-carpet treatment received when he visited the United States. At Stockholm he

spoke of the intensive work which was still needed to give a better understanding of the relationship between the constitution and the efficacy of insecticides. It is characteristic of the man that he gave the Nobel prize-money away to support young scientists - he himself was clearly happiest when working on his own, or at the most with one or two others, in the laboratory. He desired no honours for himself, and I think that, like Bernard Shaw's Captain Bluntschli, he might have said "My rank is the highest known in Switzerland : I am a free citizen".

EARLY APPLICATION OF DDT IN SWITZERLAND

Müller clearly showed that he had identified a substance, or rather a group of substances (in the earlier literature we find reference to the "DDT insecticides", and it was some time before the superiority of p.p.-dichlorodiphenyltrichlorethane was established) with potent insecticidal properties. At once more extensive tests and field trials were underway, conducted by such members of the Geigy organisation as R. Zinkernagel, F. Wille and R. Domenjoz. They studied the effects of the chemical, and of its toxicity to man. Their results more than confirmed Müller's earlier findings, and the work, in particular of R. Domenjoz, showed that, under the conditions of likely use, toxicity to man was minimal.

What may seem remarkable to us today is the speed with which DDT preparations were made available for general use. As early as August 1941 DDT containing dusts and wettable powders were marketed in Switzerland as M 1850 and M 1859, and in March 1942 they were given the trade name of Gesarol. This preparation - Gesarol - was sold for use in agriculture, and another preparation - Neocid - was made available for use in public hygiene, eg for use against human parasites such as body lice. In 1943, Trix powder, used to protect fabrics from insect attack, was also made available. It is salutory to realise that with the constraints which exist today, and which delay the marketing of any new insecticide, DDT would probably not have been available until the year 1949. Had that been the case, many millions of people throughout the world would have died of typhus and malaria, and food supplies in several countries would have been at risk.

As early as 1941 the new insecticide proved effective in checking the plague of the Colorado potato beetle, which threatened the Swiss potato crop at a time when the country was virtually isolated, and all food crops were of the utmost importance. Before the end of the year (1945) it had been successfully used against many agricultural pests, and in vineyards and forests. H. Storch had effectively deloused patients at the Zürich dermatological clinic. Flies of various species had been almost completely eliminated from many farms and agricultural buildings. All this had been successfully accomplished without any apparent harmful side effects.

WARTIME USE OF DDT

I shall deal more fully with the use made of DDT by Britain and our Allies during the 1939-45 war than with its use by other nations, as I saw something of this use at first hand. From the earliest recorded data, we have known that in war there are far more casualties from disease, including insect-borne disease, than from the weapons of the enemy. Thus

during the South African ("Boer") war of 1899 - 1902, 35 casualties occurred from disease to every one from enemy action. In the 1914-18 war, the figures were 9 to one. Even before 1939, various scientists had been studying the problem, with the hope of reducing or even eliminating casualties caused by insects if war ever broke out.

In Britain the man who made the greatest contribution to this work was the late Professor P.A. Buxton FRS, head of the Entomology Department at the London School of Hygiene and Tropical Medicine. He had seen military service as a medical officer in the Middle East and Palestine during the 1914 war and he continued his studies of relevant problems when he returned to London. I had the privilege of working in his department from 1930 to 1936. Part of my work was to study the physiology and ecology of the Body Louse, and I still bear the stigmata of Vagabonds Disease, ie patches of thickened and pigmented skin where the cultures of lice confined in pill boxes with gauze tops obtained their blood through my skin.

As soon as war was declared, Buxton rushed out his authoritative text book, "The Louse", and also published articles on Scabies and other arthropod parasites. He also stimulated extensive work on louse prevention and cure in his own and other departments as well as in several commercial firms. Various useful advances were made. The efficacy of pyrethrum was determined, though it was found to allow reinfestation very quickly. Work was done on derris (rotenone) and the preparation AL63 was found to have advantages even if it was sometimes irritating to the skin. The "Sherlice belt", a cotton corset with many small pockets attractive to lice was produced - if treated with a hot iron every night louse populations were reduced. Various other chemicals were tested with varying success. None proved ideal. Some, like pyrethrum, were in very short supply, and there were few possibilities of quickly improving the situation. Though we were much better prepared to deal with insect pests than in 1914, we were far from having solved the problem.

Then DDT arrived on the scene. Switzerland was neutral, and therefore unable directly to help any of the combatants separately. Information about DDT, samples of Gesarol and particulars of the work in Switzerland were sent to Geigy representatives in Britain, in America and in Germany. Work began in all these countries to see how the new chemical could be best used. The impression that the Allies were in some way more favourably treated than the Axis is not true, though they did indeed, greatly helped by local Geigy representatives, make the more effective use of the new chemicals. The Axis forces did make use of DDT in malaria control in South-east Europe, though in other fields their work was less significant.

As the first DDT product, Gesarol, was formulated for use in agriculture, the first tests were made by Dr. F. Tattersfield at Rothamsted Experimental Station, and by scientists at the Plant Pathology Laboratory at Harpenden, who alerted the Agricultural Research Council to what was happening. Then a report by H. Mooser, which extended the experiment of Domenjoz, on lice and fleas, arrived in January 1943 and was brought to the notice of Buxton and other medical entomologists. Work on its effects on lice then started in earnest. Official recognition of the problem included the setting up of a committee, the Insecticidal Development Panel of Experts, under the chairmanship of Sir Ian Heilbron FRS, Chemical Adviser to the Ministry of Production. For once a committee was effective, and not

an excuse for doing nothing. Widespread tests were made in many places, and the whole force of the Ministry of Production was mobilised to initiate and expedite the manufacture of the new insecticide.

I must confess that when I heard the first reports of the "wonder insecticide" I was somewhat sceptical, but I was soon convinced, perhaps by seeing whole cultures of insects in the insectary being wiped out because someone was working with DDT in another room along the same corridor! I was not myself involved in tests, except against the scabies mite. Here (as with some other arachnids) DDT was not nearly as effective as against lice, flies and mosquitoes.

The most spectacular use of DDT, and the most publicised, was when it controlled the incipient typhus epidemic in Naples, Italy, in 1944. In the 1914-18 war, it is likely that there were more than 40 million cases of typhus on all fronts in Europe, and that more than five million deaths occurred from this cause. In late 1943 typhus appeared among the civilian population of Naples. At first the Allied medical authorities got to work with their limited amounts of pyrethrum and derris, with reasonably successful results, though they realised that they had not controlled the louse population, and that those deloused were rapidly being reinfested. Early in January 1944 DDT dust became available. During that month 1,300,000 civilians were dusted at two delousing stations - 72,000 on the "peak" day. The great advantage of this method was that the patients did not have to remove their clothes. Only about half an ounce of powder containing 5 per cent active ingredient was puffed down the neck and via other suitable orifices to get under the clothes. This rendered those treated "louseproof" for several weeks. No serious side effects other than some very minor cases of skin irritation were detected. Within weeks typhus virtually disappeared. Equally important, the Allied forces were completely protected from typhus.

DDT had many other wartime uses. One which has received little publicity is the successful campaign against the plague epidemic in Dakar, in West Africa, in 1944. This was the first prophylactic use of the insecticide to control the fleas which carry plague.

More has been written about the use of DDT to control malarial mosquitoes in the Pacific, in South-East Asia and in Africa during the war. It was used to kill adult mosquitoes, by depositing a film of DDT on the walls of buildings where the mosquitoes rested. Larvae were also attacked by use of oil containing DDT on breeding grounds. These methods all proved successful, and greatly reduced mosquito numbers, but I am not sure whether they were always necessary from the military point of view. Most of the Allied troops were already fully protected from malaria by their daily prophylactic dose of mepacrine (syn: atebrine), the efficacy of which had been amply demonstrated by Brigadier Hamilton Fairley FRS in Australia, and by some of us working on similar lines in Europe.

The value of DDT was fully endorsed by the Prime Minister, Winston Churchill, in a broadcast on 28 September 1944:

> "We have discovered many preventatives against tropical diseases, and often against the onslaught of insects of all kinds, from lice to mosquitoes and back again. The excellent

> DDT powder, which has been fully experimented with and found to yield astonishing results, will henceforth be used on a great scale by the British forces in Burma, and the American and Australian forces in the Pacific and India in all theatres....."

THE MODE OF ACTION OF DDT

The way or ways in which DDT kills insects is still not fully understood. Müller himself determined that it affected the nervous system, but exactly how this happens, and its precise significance, is still somewhat of a mystery.

DDT may act as a contact insecticide or as a stomach poison. It is almost completely insoluble in water, but dissolves easily in lipoids. When applied to an insect's cuticle, which has a lipoid content, it is rapidly absorbed and enters the bloodstream. Work on the control of flies and mosquitoes has shown that lethal amounts may quickly be absorbed when a film of DDT is touched only by the insect's feet.

Normally the symptom of DDT intoxication is hyperactivity. This is soon followed by uncoordinated movement, which may be sufficiently violent for the insect's legs to be broken off. The primary site of action is thought to be the nerve axons. Later to the hyperactive stage, the insect becomes prostrated, and then dies. Few insects reaching the hyperactive state ever recover.

Though it is the nervous system which may be first affected, it is clear that death occurs because the normal control mechanisms of the insects have been seriously affected.

The fate of the DDT absorbed will be further discussed later in this paper, when resistance to the insecticide is discussed. Various metabolites of DDT, with different properties are produced within the insect body (and in the bodies of birds and mammals which may also pick up the chemical). Sometimes small sub-lethal doses may be excreted without doing permanent damage. In other cases they may build up to damaging levels in the tissues. Finally DDT is fat-soluble, and may be stored in fatty tissues, where it has few metabolic effects, but from which it may be released during starvation when the fat reserves are metabolised.

Different insects differ considerably in their susceptibility to DDT poisoning. In some of the earlier experiments, made during the war, J.R. Busvine found that bugs (Hemiptera) such as Rhodnius and the bed bug, Cimex, were only affected when placed on filter paper with 10 mg of DDT per square centimetre. For house flies only 1 mg was required to have a similar effect, while the yellow fever mosquito, Aedes aegypti, died on paper with only 0.001 mg per sq. cm. When used in agriculture, many pests were killed by applications which appeared to have no harmful effect on honeybees, or on beneficial insects like ladybirds (though higher doses did kill bees and ladybirds).

The most important property of DDT is its persistance, and the length of time for which applications in the field, and onto buildings, remain effective. This property of having a long-lasting effect was greatly

welcomed, for instance when it kept people free from lice for many weeks, or when it killed domestic pests like bedbugs and cockroaches over long periods. The trouble has been that persistance may have its disadvantages, because a chemical which is as stable as is DDT may become an environmental pollutant, which may have long term effects which are difficult to control. This subject is discussed below in the section on Ecological effects of DDT. It would seem impossible to discover any insecticide which remained potent for just the optimum length of time, and then broke down to some harmless residue.

AGRICULTURAL USES OF DDT SINCE 1945

Although there were several publications about DDT in Switzerland during the war, in Britain and America its use remained largely secret, and no DDT was available commercially , during the war. However, information about the chemical then became available, and there were gradually increasing amounts available for use in both agriculture and hygiene. The reaction of all those concerned was one of euphoria. We seemed to have the perfect weapon against harmful insects, and one which was harmless to man and his livestock. Many entomologists thought that they would soon be out of a job. I myself scrapped, before publication, the text of a book on economic entomology as being largely out of date due to the use of DDT.

In the volume "15 years of Geigy Pest Control", edited by A. Buxtorf and A. Spindler, and published in 1954 by J.R. Geigy S.A., Basle, Switzerland, there is a summary of the uses of the insecticide, in Switzerland and throughout the world, until that date. This is still very useful, as it covers a period before DDT began to receive severe criticism for its unwanted ecological effects. There is no doubt that during this period (and, in fact, in many cases for very much longer) DDT was a uniquely valuable tool to the agriculturalists.

This is not the place to try to give an exhaustive picture of all the agricultual pests which have been successfully controlled by DDT, but some of the most important crops which were protected must be mentioned. Mention must also be made, here and in other sections of this paper, of instances where DDT has failed to control insect pests.

As mentioned above, the first large scale use of DDT was in Switzerland, on pests of fruit trees, of vines and against the Colorado potato beetle. After the war these uses were adopted throughout the world, with continuing success. On fruit trees, the notorious pests such as codling moth, the Mediterranean fruit fly and the Mexican fruit fly were, at least for a time, practically eliminated. A high degree of protection was obtained for vines in most of the main wine producing countries. Being odourless and relatively non-toxic to man, and having a wide range of pests as victims, and good residual effects (so that one application could protect a crop throughout its growing season), these insecticides (particularly the formulation Gesarol) were widely used in gardens and in vegetable cultivation. Some of the pests successfully controlled in these situations were wireworms, cabbage butterflies, flea beetles, pea and bean thrips, pea midges, pea weevils, the greenhouse whitefly, asparagus beetles and very many more.

Special mention may be made of the continuing success against the

Colorado potato beetle. In Britain, where this pest has not yet established itself, invading individuals have been detected on potato crops from time to time. These were successfully prevented from colonising by the prompt use of DDT. In the USA it was calculated that the use of DDT had, overall, increased in potato crop by not less than 25 per cent. In India and the Far East, where the rice stem borer reduced the rice crop by more than one tenth,a loss sufficient to feed 15 million people, the impact of this pest was, at least in many areas, reduced nearly to zero.

Perhaps the greatest use of DDT in the tropics has been against the pests of cotton: the cotton bollworm, the corn earworm and pink bollworm. Here DDT has frequently been used in the form of "cotton dust" containing 10% DDT, 3% HCH and 40% sulphur. This and other preparations favoured in different parts of the world were for many years spectacularly effective, until unfortunately some of the pests became resistant to the insecticides (see section below on resistance).

Today the use of DDT is banned in many countries, for reasons discussed below. However it is still important in many parts of the world, where it is, for reasons of cost and efficacy, still the substance of choice. Carefully used in limited amounts it still has a part to play in safeguarding food supplies, particularly in developing countries.

DDT IN HYGIENE AND MEDICINE SINCE 1945

As has already been described, DDT came to public attention when the typhus epidemic in Naples in 1944 was controlled by the elimination of the body louse by the insecticide. After the war, DDT found many uses in controlling insect diseases and promoting hygienic conditions.

One success which today seems surprising was the control of filariasis, transmitted by the black fly, Simulium, in the Congo. The larvae of this vector live only in rapidly running water. Very small amounts of DDT, only one or two milligrammes per litre (ie one or two parts per million) were added to the running water to kill the larvae. Further to this, the vegetation along the river banks was treated from the air again with relatively small amounts of DDT. The area concerned was freed from an extremely troublesome pest. Somewhat surprisingly, the black fly appeared to be much more susceptible than other forms of life, and no serious ecological damage was reported.

Malaria is, however, the chief protozooal disease against which DDT has been used, and is still being used, on a large scale. The first work in this field was done by the Germans in the Balkans and Greece during the war, but this has had little publicity. Immediately after the war it was the insecticide of choice in the extensive anti-malarial campaigns in Italy. Here the larvae of the mosquitoes and their breeding places were ignored as far as treatment was concerned, though some wet sites which were breeding grounds were drained. The main emphasis was on treating the insides of all the buildings in which mosquitoes were likely to roost with one or two grams of DDT per square metre once in each year. Mosquitoes resting on such surfaces obtained a lethal dose of insecticide. With this technique, the slow action of DDT may be an advantage, for mosquitoes which rest, after feeding for only a short time, before symptoms appear, may fly off and die in the bush. Those which die within the house have, in a few

cases in some tropical countries, been eaten by geckos, who may in time build up a harmful concentration, which may be passed on with interest to domestic cats who have been reported as dying under these circumstances. No such side effects were ever reported in Italy. Here reports showed that within three years malaria was virtually eliminated.

Equally successful results were obtained in many other countries. To quote some examples: the disease was eliminated in Mauritius and Cyprus, and today tourists visiting these countries are no longer advised to take anti-malarial precautions. Malaria, once serious in Western Europe, has been eliminated from the Netherlands and Spain. In 1952 it was estimated that some 100,000,000 people in various countries were being protected by DDT.

One of the most successful was Ceylon (Sri Lanka as it is called today), but here, for reasons described below, treatment was stopped prematurely and there was a serious resurgence of the disease.

DDT has been less successful against the tsetse fly, the carrier of human sleeping sickness and nagana in cattle in Africa. Here the areas involved are huge, and the flies are present often in very small numbers. These may be as low as two flies per acre, so when spraying takes place, at least 99.99 per cent of the material must miss its target. DDT has proved effective in killing the fleas which carry bubonic plague - it is said that in some parts of India anti-malarial campaigns have also got rid of plague.

Lice, both head and body lice, were successfully treated with DDT for many years. The long-lasting effect of DDT made these treatments particularly successful, until, unfortunately, resistance in lice became too common. As I found in 1944, some arachnids are naturally resistant, and attempts to control both ticks and mites have generally proved disappointing. Fortunately other new insecticides, including HCH, proved in most cases to be more lethal.

I think that it will be generally agreed that, certainly up to 1955, DDT was the most important chemical ever used to combat human insect-borne disease. Since then its status has been somewhat equivocal, but I believe that it still has an important part to play, in medicine as well as in agriculture, in many parts of the world.

THE DEVELOPMENT OF RESISTANCE TO DDT BY INSECTS

J.R. Busvine, with whom I have had the privilege of discussing this phenomenon of resistance, says, in an article in the book "Pesticides and Human Welfare" edited by D.L. Gunn and H.G.R. Stevens and published in 1976:

> I can clearly remember hearing of DDT-resistant houseflies from the late Professor Missiroli in Rome in 1947. This was only five years after our initial tests of the new insecticide and, such was the reputation of DDT at that time, it was difficult to accept his story; but he kindly supplied me with some of the portentous flies, and we soon verified the fact.

This was not the first report of DDT resistance in flies. The first

that has been published describes the discovery by H. Speich, of the Geigy staff, of the failure to control flies in Northern Sweden in 1946. After that date, many other resistant houseflies were discovered in many parts of the world.

We should not have been surprised when we found that some pest insects became resistant to DDT. Thus as long ago as 1911 there was clear evidence that extensive fumigation of citrus trees in California had selected a strain of scale insects resistant to hydrogen cyanide gas; there were several other cases before the Second World War. But the growth of the incidence of resistance since 1946 has been much larger and faster, as the result of the introduction, on a vast scale, of new synthetic insecticides.

Resistance is not, as is often suggested, the result of some mutation which is induced by the application of a sub-lethal dose of the toxic pesticide. Most insect populations contain insects which vary greatly from one another, and one variation is in the susceptibility of different individuals to any pesticide. There are naturally resistant individuals, which survive the application of the pesticide. If only a small proportion of any population is killed, the resistant individuals will remain in a small minority and will not make their presence felt. Most insects, particularly pest insects, breed rapidly, and the proportion of resistant and non-resistant individuals is likely to be restored to normality. In most cases resistant individuals are not "super insects" - they often are among the less vigorous members of the population and so are not likely to predominate. However, when we attempt to eliminate all the pest insects in an area, then only the resistant individuals may remain. Under such circumstances they may increase without competition to pest dimensions. In some cases where heterozygous individuals in the natural population have some degree of resistence, in the depleted population homozygous individuals, with even greater resistance, may be bred.

The degree of resistance which is produced differs from species to species, and on the local conditions. In some cases insects which are not apparently affected by doses of DDT one thousand times greater than those which kill normal individuals have been detected. In such cases, any further use of the insecticide is obviously futile. In other cases where only slight resistance occurs, requiring only twice or three times the normal dose, DDT may continue to be used for some time, though it is likely that the situation will continue to deteriorate.

When the insecticidal pressure is removed, insect populations tend to become less resistant, and in some cases the same chemical can once more be used. However, unless great care is taken only to kill the minimum number of pests to give economic results (as discussed in the final section of this paper) resistance is likely to be quickly restored to the high level produced in the first instance. Generally the pest can only be controlled by using some chemically different insecticide. Even this may not always be successful, as in some cases resistance to one chemical induces resistance to another.

When the significance of increasing resistance has not been properly recognised, it has resulted in the greater and more excessive use of DDT or other pesticide, with the consequent greater contamination of the environment. This is one of the reasons for increasing concern about the

ecological effect, or rather side-effects, of pesticide use, as discussed in the following section.

The exact mechanism of resistance to DDT is not fully understood, notwithstanding a tremendous amount of work, and volumes of publications on the subject. J.R Busvine suggests that there are four possible mechanisms involved, and that these are as follows:

1. DDT detoxication to DDE, by the elimination, by suitable enzymes, of one chlorine atom.
2. "Knock-down" resistance caused by a change in the sensitive site.
3. Reduced penetration of the insect cuticle by DDT.
4. Microsomal detoxication.

The first of these processes is thought to be the most important. I do not find this suggestion completely convincing. There is no doubt that insects, and other animals, differ in the rate at which DDT is converted into DDE. It is DDE which we normally find stored in their tissues. DDE is also clearly less acutely toxic that DDT, but it is stil poisonous and has insecticidal properties. Even if what would otherwise be a fatal dose of DDT is turned, instantaneously on entering an insect's body, into DDE this might not be harmful in modest amounts, but where the resistant individual is one thousand times as resistant as the normal, it is surely probable that it would suffer some damage.

Though it is important that we should understand the mechanism of resistance so that we can devise ways of combating it, the important thing for pesticide users is that the phenomenon exists, and is one of the major reasons for worrying about the whole future of pest control. So far we have generally been able to find an alternative chemical to get over this difficulty. However, as it is now taking longer and longer to overcome the difficulties of introducing new substances as recognised and legally-accepted pesticides, the situation may get worse. Incidentally I find it rather nauseating, when I hear some of our so-called environmentalists expressing delight that so many more species of mosquito or other pest have become resistant to some insecticides, so that this chemical can no longer be used. They seem indifferent to the large number of people who may now perish from insect-borne disease or who may go even shorter of essential food.

There is one strange thing about the process of DDT detoxication. It is well established, as stated above, that the metabolite DDE is less toxic than DDT. However DDE does have its ecological effects, and is the substance which affects the endocrine system in birds and upsets the normal production of egg shells, something which has had its effect on the populations of several species, as described in the following section.

THE ECOLOGICAL EFFECTS OF DDT

There is widespread but quite erroneous opinion that until such writers as Rachel Carson erupted onto the scene, entomologists, other scientists, farmers and chemical manufacturers were splashing enormous amounts of DDT around the world without the slightest concern for any damage it might be doing to the environment. The real situation is quite

different. From the beginning, responsible entomologists, however much they welcomed the introduction of DDT for its control of pests causing human disease and crop losses, were fully aware of its potential dangers. When I came to study the literature, in preparing this paper, I was somewhat surprised to find that the first warning to be committed to print was given by me. At a meeting of the Royal Society of Tropical Medicine held in London on 15 February 1945, Professor Buxton gave a paper on "The use of the new insecticide DDT in relation to the problems of tropical medicine". I attended this function, and am recorded in the Transactions of the Society as contributing to the discussion as follows:

> Dr Kenneth Mellanby said that Professor Buxton had shown that the discovery of the properties of DDT was probably the greatest advance in insect control which had ever been made. But DDT was clearly no panacea, which could be broadcast indiscriminately to kill off all noxious pests, A greatly increased amount of field research was necessary whenever DDT was used. Fortunately, mosquitoes and muscid flies seemed particularly susceptible to this substance, but all arthropods were affected to a lesser or greater extent. MUCH WORK SHOULD BE DONE ON ITS EFFECT, IN THE FIELD, ON ALL MANNER OF APPARENTLY UNIMPORTANT INSECTS AND OTHER FORMS OF LIFE, TO ENSURE THAT THERE WAS NOT A SERIOUS UPSET OF THE "BALANCE OF NATURE" WITH SUBSEQUENT DISASTROUS EFFECTS.

I went on to report my finding that DDT was not a good acaracide, but that need not be dwelt upon at this time. I was of course not alone in making such statements. Shortly after this V.B. Wigglesworth, in the USA, spoke on "DDT and the balance of nature" and his thoughtful lecture is reproduced in full in the Atlantic Monthly magazine.

The responsible conservation bodies in Britain were soon becoming concerned about the ecological effects of various pesticides. Early in the nineteen fifties the Nature Conservancy complained that herbicides were affecting natural vegetation. and damaging the wildflowers particularly in roadside verges. The authorities responsible for spraying such areas willingly agreed to restrict sprays of herbicides to restricted lengths of verge where luxuriant growth of "weeds" was a traffic hazard. In general there is little complaint today about weedkillers, except that they are too efficient and make clean cultivation of cereals and other crops easier so that those birds, dependent upon weed seeds, are indirectly affected.

In the late nineteen fifties in Britain, in addition to the Nature Conservancy, the British Trust for Ornithology and the Royal Society for the Protection of Birds reported many unexplained deaths of song birds in the spring, and work was begun to find the cause and to remedy the situation. One reason why the Nature Conservancy established Monks Wood Experimental Station, of which I was the first director, was to house the Toxic Chemicals and Wildlife Section headed by N.W. Moore. This section had the task of studying the ecological effect of pesticides.

Work in Britain showed that the bird deaths were caused by the insecticides aldrin and dieldrin, organochlorines of the same group as DDT, used as seed dressings to protect the young plants from attack by the wheat bulb fly. This method had been adopted in an attempt to reduce the use of

these toxic insecticides - use as a seed dressing just where the larva attacked required only a tenth of the amount of chemical needed for use if it were broadcast. Unfortunately the seed-eating birds obtained a fatal dose, or, if slightly less was consumed, they fell as prey to sparrowhawks and other predators which were particularly susceptible and whose populations were devastated. In 1961 the farmers and manufacturers came to a voluntary agreement only to use these dressings in autumn sown wheat, when the birds had other sources of food. No dressings were used in spring-sown wheat. The result was striking. In 1962 and subsequent years the number of dead birds was reduced almost to zero. This was not quite all that happened, as a very few individuals who probably had been prevented by the weather from planting their dressed seed before Christmas, then planted it at a later date contrary to the agreement. This was generally detected because of a local effect, killing birds feeding on that farm. But in general the 'voluntary ban' was a great success.

At this time in Britain little evidence of actual bird deaths from DDT itself was found. Birds did have elevated levels of DDT in their tissues, and this was cause for worry. But it appeared that as a rule the chemical had been used conservatively with what, for the time being, seemed to be little widespread effect. A very different situation was found in parts of America, when DDT had been used much more liberally. There was good evidence that heavy use of DDT in an attempt to kill the bark beetles carrying Dutch elm disease had wiped out the American robin in many places, watercourses had been contaminated and many species of bird populations had been reduced.

In the autumn of 1962 Rachel Carson's "Silent Spring" appeared. As has already been pointed out, in Britain we had already taken effective action against the most important ecological effect of pesticides nearly a year earlier, so it is clear that British scientists, naturalists, farmers and manufacturers were already aware of possible dangers and were willing to act on good evidence. Nevertheless many people have praised Rachel Carson for doing so much to alert the public to the dangers of DDT and other pesticides. I am afraid I cannot agree. Although "Silent Spring" is generally accurate in its statement of facts, it is a very biased account of the situation, and one which gives a very partial picture of the situation. It did much to inflame public opinion, and to stimulate the rather hysterical anti-DDT campaign which culminated in legal actions in the USA in the nineteen seventies. More importantly, it did much to encourage various developing countries to stop using DDT at a time when malaria was still being successfully controlled. Those who were responsible for these premature bans on DDT must bear the responsibility for the thousands, perhaps millions, of deaths which might otherwise have been avoided.

Nevertheless, DDT has had harmful ecological effects. First, it has caused some pest species actually to increase, by eliminating the predators which previously controlled their numbers. One particularly striking instance has been that of the red spider mite on fruit trees. This mite had long been a pest in glasshouses but until DDT was widely used its numbers, on fruit trees grown in the open, never reached pest proportions. The situation changed when its slow-breeding predators were killed by DDT used to control various lepidopterous pests. The red spider mite itself was naturally resistant to DDT - in fact the chemical actually stimulated

the mite eggs to hatch.

Perhaps the most worrying long-term effect of DDT was the thinning of eggshells, particularly of rare predatory birds, which contributed to falls in the populations of these species. That this was happening was suspected by naturalists in the early nineteen sixties, but the evidence was largely circumstantial, and did not receive general acceptance. However, after a great deal of both field and laboratory study, the problem was solved. It is impossible for me to do justice to all the scientists who were concerned, and my summary must necessarily be selective. One of the first reports which contained hard evidence was that of D.A. Ratcliffe who compared the shells of peregrines' eggs in museum collections made since 1900 with those collected from 1959 onwards. The eggs before 1950 varied somewhat from egg to egg, but the mean thickness was unchanged. Then from 1950 the thickness decreased markedly. This change was correlated with the introduction of DDT.

This observation by itself did not convince everyone, but more field observations and laboratory feeding experiments followed. By about 1970 it was generally conceded that there was a strong correlation between the levels of DDT and its metabolites in such species as the peregrine, and that it was reasonable to assume that the chemical was the cause of the eggshell thinning. Laboratory tests showed that these chemicals affected the birds' thyroid and their calcium metabolism.

What was perhaps the most convincing observation was what happened next, when the use of DDT was stopped or very strictly controlled in both Europe and North America. Perhaps the best account is that given in the volume "Peregrine Falcon Populations, Their Management and Recovery" published by the American Peregrine Fund in 1988. This describes the effects of DDT residues in thinning eggshells, and also the way in which, as the chemical levels fell after 1970, the eggshells became thicker and eventually returned to normal. What is encouraging here is that the reduction in the use of DDT was so rapidly mirrored in the thickened eggshells. This contradicted the widely held view that it would be tens of years before the levels of DDT in the environment started to fall significantly.

One interesting finding in the eggshell studies was that it was not DDT itself, but its less toxic metabolite DDE, which was responsible for eggshell thinning. This shows that this metabolite, which was previously thought of as comparatively non-toxic, was sometimes of major environmental importance.

Much concern has been expressed because DDT and its residues have been found widespread in many species in many countries, even in penguins in the Antartic. In most cases the results of these analyses need not cause concern, because the DDT etc., is at levels far below those which have been shown to have biological effects. The public finds it difficult to distinguish between a toxic dose, and a trace whose discovery is mainly a tribute to the skill of the analytical chemist. But everyone should be less concerned today as even these very low levels are generally decreasing in parallel with those in the peregrines in Europe and the USA.

THE TOXICITY OF DDT

P.A. Buxton, in his paper delivered in London in February 1945, said, speaking of the toxicity of DDT: "My conclusion, given without reserve and in simple words is that DDT used as an insecticide is quite safe". This conclusion was based on the wartime use of the chemical, including the liberal application of the insecticide to millions of people in the anti-typhus campaigns. It echoed the results of work done by the Geigy scientist in Switzerland before DDT was put on the market, and extensive tests in the USA and Britain with the same results. DDT is of course a poison to all forms of life, but mammals and man seemed least affected. As an acute poison some ten grams is needed to affect a man seriously - a similar toxicity to aspirin. However DDT has one property which is quite different from aspirin, which can be taken daily in small doses without building up in the body. With DDT regular sub-lethal exposures can prove dangerous, for some of the DDT may be retained in the body and in time this can have harmful effects - as we have shown with wildlife.

However, particularly in the nineteen sixties and seventies, many reports contradicted these early findings. DDT was alleged to be a carcinogen, and to be much more toxic to man than had previously been accepted. These allegations were often repeated, particularly in the campaign against the use of DDT reported in the next section of this paper. Fortunately the toxicity of DDT has been thoroughly considered in many recent papers, the most outstanding being that by M. Spindler (1983) on "DDT: Health Aspects in Relation to Man and the Risk/benefit Assessment Based Thereon" and by F. Coulson (1985) entitled "Reconsideration of the Dilemma of DDT for the Establishment of an Acceptable Daily Intake".

With regard to the daily intake, Coulson found that, for a 50 kg man, a daily dose of 12.5 mg would have no toxicological effect, and, allowing a generous margin of safety, 1.0 mg was the acceptable daily intake. Looking at early results based on workers in processing factories, these findings may be considered conservative. What is important is that, even when DDT use in the USA was at its maximum, it was unlikely that anyone would exceed Coulson's acceptable dose from food purchased in the supermarket.

The present situation has been so well expressed by Spindler, that I now give his conclusions in full:

> After almost 40 years of worldwide experience in the use of DDT in agriculture and forestry and also in public health, the following conclusions can be drawn with regard to the health risk it constitutes for man and its undesirable impact on man's environment:
>
> (a) DDT is one of the safest and least hazardous insecticides in handling and application. The World Health Organization has stated: 'The safety record of DDT is phenomenally good', and quoted from another statement: 'The excellent safety record of DDT, never matched by any other insecticide used in antimalaria campaigns, other vector control programs and agriculture, is based mainly on its poor absorption through the skin'.
> (b) In the temperate zones of the Western World it is unlikely that the average daily intake of DDT from all contaminated sources (food, air, and drinking water) exceeds 0.05 mg/man. Based on

the body weight of a man weighing 70 kg this corresponds to 0.0007 mg/kg/day.

(c) Clinical studies in human beings as well as examinations of production workers and spraying personnel who had been exposed to DDT, for periods up to 25 years in the case of production workers, did not reveal any adverse health effects that could be attributed to DDT.

(d) DDT residues determined in human milk lie in the nontoxic range. This applies even to the extremely high residues that were detected in tropical countries where continued malaria campaigns with DDT were carried out, as for instance in Mareeba, Australia, or in Guatemala. This finding is even more reassuring with regard to safety, for the breast-fed infant may be considered as the terminal link of the human food chain (Shüpbach 1981). The fact that DDT residues in human milk exceed the ADI by a multiple factor is, therefore, irrelevant and has no consequences for the health of the child, since the intake is limited to the short lactation period and does not continue for a lifetime.

(e) Clinical studies in human beings mentioned above, as well as epidemiological investigations, have led to the conclusion that DDT is not carcinogenic in man.

(f) Relevant tests in mice, rats, and dogs showed no teratogenic effect of DDT and no adverse influence on reproduction.

(g) If the extremely low health risk of DDT for man is weighed against the enormous benefits resulting from its global use more particularly in the control of malaria, but also in agriculture, all the criticism alleging that DDT has had an alarming impact on the environment, including man, is completely unrealistic.

In 1979 the World Health Organisation summarized its view on the whole situation as follows:

> 'Finally, it should be noted that WHO has kept under review over the years any possible adverse effects of DDT, particularly in relation to the vector control program. The results of these reviews preceding the Critiera Document (WHO 1979a) have been published in 1971 (WHO 1971) and in 1973 (WHO 1973). The latter outlines the results of a study conducted by WHO on malaria spraymen exposed to DDT for more than 5 years. In practice, DDT has proved to be the safest pesticide used for residual spraying in vector control programs. If there had ever been any good evidence that workers, spraymen, or the general population were being adversely affected, it is inconceivable that the expert committees of the World Health Organization would have continued to recommend its use.'

THE CAMPAIGN AGAINST DDT

Few chemicals can have given rise to so much public concern as DDT. As the best known of the newer synthetic insecticides, it has sometimes been blamed for the effects of other chemicals, particularly the other organochlorines, aldrin, dieldrin and heptachlor, but it has also had its own particular supporters and opponents.

I like to think that in Britain we generally managed things in a reasonably rational way, basing our conclusions on scientific facts. Good

cooperation was generally found betwwen scientists employed by such bodies as the Nature Conservancy, the Government and the Chemical Industry. I do not pretend that all was sweetness and light, and that there were not acrimonious arguments between some of the protagonists. We were fortunate that it was the informed scientists and conservationists which were first to recognise the problem, so that slow but steady progress was made without too much emotion. When DDT was found to be having ecological effects, its use was restricted, particularly when other insecticides which could be substituted were available. As a comparatively wealthy country, more expensive substances could be used without bankrupting the users. The whole process is described by J. Sheail in his book "Pesticides and Nature Conservation. The British Experience 1950-1975" to which I hope those interested will refer.

In America the situation was very different. Here, where there are many more serious insect pests than exist in Britain, DDT had been used much more generously and the chemical industry was more aggresive than in Britain. As a result, those on the other side were equally violent in expressing their views. In fact we had what at best was a crusade, and at worst a vendetta, against DDT. The struggle is fully described, with some considerable anti-DDT bias, by T.R. Dunlap in his book "DDT, Scientists, Citizens and Public Policy". I will not try to summarise the issue. Sufficient to say that the lawyers had a field day, all the least well established "facts" about DDT's carcinogenicity were repeated ad nauseam, and the Environmental Protection Agency was sufficiently convinced to ban the chemical in 1972.

DDT has now been banned, or its use has been seriously restricted, in all the developed countries of the world. As one involved in wildlife conservation, I generally welcome this where alternatives are available and can be afforded. Personally my attitude can be ambiguous - I have not found an alternative which is as efficient in controlling the Greenhouse White Fly in my conservatory. But, as stated below, there are situations where DDT is still a lifesaver, and where the campaign, by affecting local public opinion, has done a great deal of damage.

DDT TODAY AND IN THE FUTURE

DDT is dead! Long live DDT!

DDT has unique advantages as an insecticide. It has six of the seven properties of the ideal insecticide, as formulated by P. Müller. Its relative slowness in action is not really a disadvantage, as although it may be minutes or hours before an affected insect dies, if it has picked up a lethal dose it is inevitably doomed.

Today, DDT has to contend with two disadvantages, that is with legal bans and with resistant pests. Fortunately as yet most of the developing countries with serious insect problems still allow DDT to be used, often with safeguards which need not impede its use. My worry is that extreme propaganda from some conservation bodies in developed countries will, as has already happened in some case, cause premature restrictions to be made.

With hindsight, except under the stress of wartime conditions, we should have been more selective in the use of DDT from the outset. The

onset of resistance could have been avoided, or at least delayed, if fewer attempts at eradication and more at pest reduction below the economic levels had predominated. Where DDT can be used in agriculture, if this is as part of an integrated pest control programme, long-term results are likely to be most satisfactory.

There is one major use for which there is, at present, no alternative chemical. That is in malaria control, by killing the adult mosquito by placing a residual deposit of DDT on the inside walls of buildings. This causes minimal environmental contamination with the maximum effect on the insect population. I hope that this use will be continued and extended.

There are still agricultural crops which are best protected by DDT, and this use should continue at present, but I do not see it having a very long-term future. The environmental disadvantages of extreme persistance outweigh the advantages, and more suitable chemicals are, or soon will be, available. So while I think that DDT should be used as much as possible in malaria control, I should expect it to have a limited future when used for other purposes.

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Addenda to 'DDT in perspective' K. MELLANBY

Page 4, 3rd paragraph, 3rd line from the bottom, after 'researches':-

Some of his colleagues told him that his insecticide had a slow action, but with a combination this shortcoming might be overcome. However, Müller remained persistent. It was indeed true, this action was slow, but it was sure, unlike rapidly acting substances like pyrethrum which gave a rapid "knock down" from which the insects sometimes recovered.

Page 4, last line from the bottom:-

.... when he visited Argentina. There, Dr. Müller was invited by Juan D. Péron, at that time President of Argentina, and his wife Evita Péron. Müller was granted an honorary professorship of the Eva Péron University and was offered a doctorate honoris causa of the University of Buenos Aires. Furthermore, he was invited to become Minister of Education in the Argentinian government. However, he refused this offer. At Stockholm ...

Page 17, 3rd paragraph

With regard to the daily intake, the following may be of interest:- based in part upon the data presented by Coulston as well as other considerations the Joint Meeting of the FAO Panel of Experts on Pesticide Residues in Food and the Environment and the WHO Expert Group on Pesticide Residues (JMPR) estimated 0.25 mg DDT per kg body weight/day to be an overall non-toxic level for humans. Applying a safety factor of approximately 10, an acceptable daily intake (ADI) of 0.02 mg/kg body weight per day was established for man. For a 50 kg man, a daily dose of 12.5 mg would have no toxicological effect.

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ACETYLCHOLINESTERASE INHIBITORS

K. Naumann

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SUMMARY A retrospective on the history of inventions in the field of organophosphates and carbamates is given together with an analysis of agricultural use and economic importance of these insecticides. Future developments and the possibility of modes of action different than the caption ones are briefly discussed.

Commemorating at this meeting the 50th aniversary of DDT as insecticide it is also necessary to point to other very recent jubilees of important groups of highly effective insecticides.

TABLE 1

Commemorating important insecticides/Reading conference 1989

53 years	Lindane
52 years	Organophosphates
50 years	DDT
45 years	Polycyclic chlorohydrocarbons
41 years	Carbamates
40 years	Industrial production of synthetic pyrethroids

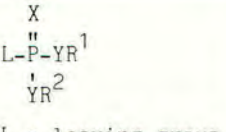
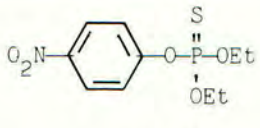
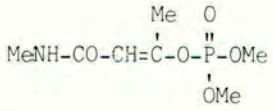
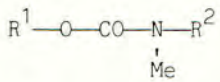
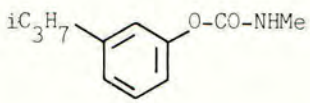
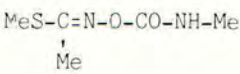
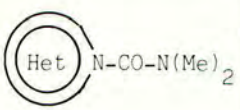
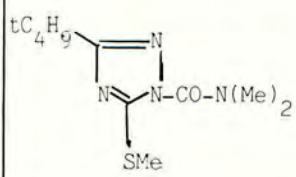
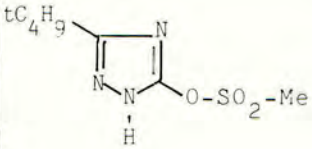
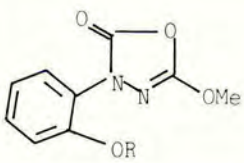
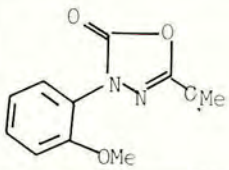
The most important group of insecticides, the organophosphates, actually had to be celebrated two years ago; the carbamates last year. It would be a good occasion to celebrate 40 years of industrial pyrethroid production. It is my pleasure, that Bayer, who opened up the field of organophosphates in 1937, was invited to give this lecture on acetylcholinesterase inhibiting-insecticides. Many thousands of pages in patents, scientific publications, in numerous journals, review articles and books have been published in the past 40 years on chemistry and scientific and toxicological investigations. Hundreds of chemists and biologists have been involved. It is not my intention to repeat, what in the meantime became part of textbooks. I rather would like to give a historical retrospective on what had happened in the past 52 years in terms of inventions, effort of institutions involved, economical importance and future chances for innovations as well as scientific controversies.

The title of this paper needs a classifying definition. It comprises some classes of insecticides which are capable of inhibiting the very important neuroenzyme AChE. This refers traditionally to warm blooded animals in vivo as found out by Gross in the Bayer Labs. 1938. Most of them are also more or less convincing inhibitors of fly head AChE, the long standing standard enzyme test for insect AChE.

Table 2 shows the classes of compounds of concern. These are organophosphates, carbamates, certain carbamoylazols, certain sulfonats and oxadiazolons.

TABLE 2

Acetylcholinesterase inhibiting insecticides

class	general formula	examples
organophosphates	 L = leaving group X = O, S Y = O, S, N, - R = alkyl, aryl	 
carbamates	 R^1 = aryl, imin, hetaryl R^2 = H, Me	 
carbamoylazoles		
sulfonates	$RO-SO_2-Me$ R = aryl, hetaryl	
oxadiazolons		

I restrict myself to organophosphates and carbamates; only these two classes are of great economic importance, because they cover about 2/3 of the whole insecticide world market, as shown in table 3 and table 4.

TABLE 3

Commercial importance of organophosphate insecticides

Number of organophosphates	%	Information
147	100	introduction into market since 1940
79	54	commercial active ingredients of significant economic value in 1986 ~ 100.000 annual tons = 45% of insecticide world market = 4 x 10^9DM annual turnover/2.2 x 10^9US$*
36	25	cover 90% of organophosphate market
24	16	cover 75% of organophosphate market
19	13	systemic products cover 45% of organophosphate market

* Wood Mackinsey 1988

TABLE 4

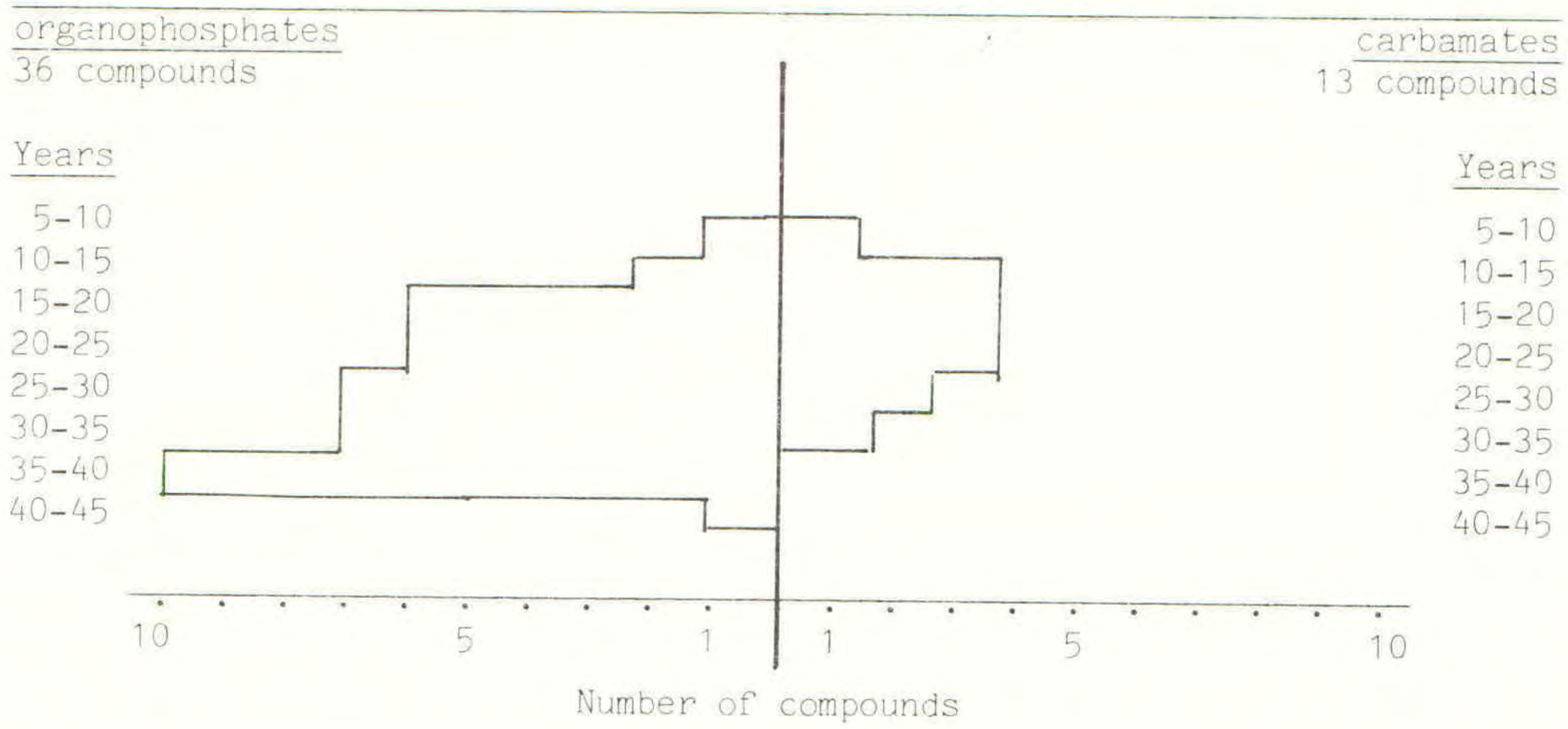
Commercial importance of carbamate insecticides

Number of carbamates	%	Information
45	100	introduction into market since 1948
29	64	commercial active ingredients of importance in 1986 ~ 30.000 annual tons = 17% of insecticide market = 1.5 x 10^9DM annual turnover
13	29	≙ 90% of carbamate market
6	13	≙ 75% of carbamate market
7	15	systemic products = 38% of carbamate market

The compounds of concern are very versatile. They still perform very well technically as insecticides, despite certain drawbacks. They are the indispensable backbone in the fight against voracious insects, which compete with us for cotton bolls, rice, corn, vegetables and fruits. But organophosphates and carbamates are in a certain way now commercially problematic. Compared to the innovative situation in most of other business sectors in life sciences they are oldtimers as shown in the figure 1, showing the age structure of the active ingredients covering 90% of their present respective market. The age is refered to the year of priority date. The top 10 organophosphates have an average age of 32 years.

FIGURE 1

Age structure of the more important (90% of the market) organophosphates and carbamates (1989)

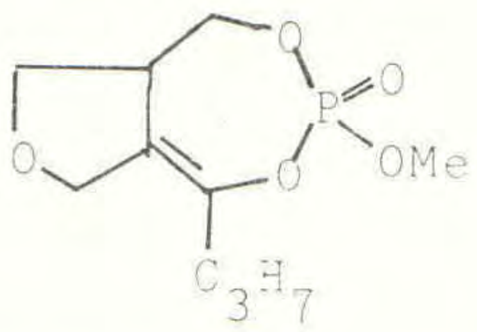


It is interesting to analyze the historical development of organophosphates and carbamates as important farming tools, which helped so much in the past to boost the yields of diverse crops to its present heights and still keep comparable low prices for them. Where did all these compounds originate from, what was the idea behind their discovery? No doubt, nature made them first.

FIGURE 2

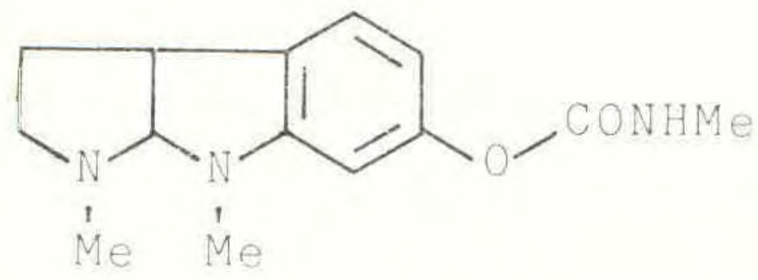
Natural Acetylcholinesterase Inhibitors

Neumann's phosphate



Streptomyces antibioticus

Physcstigmin



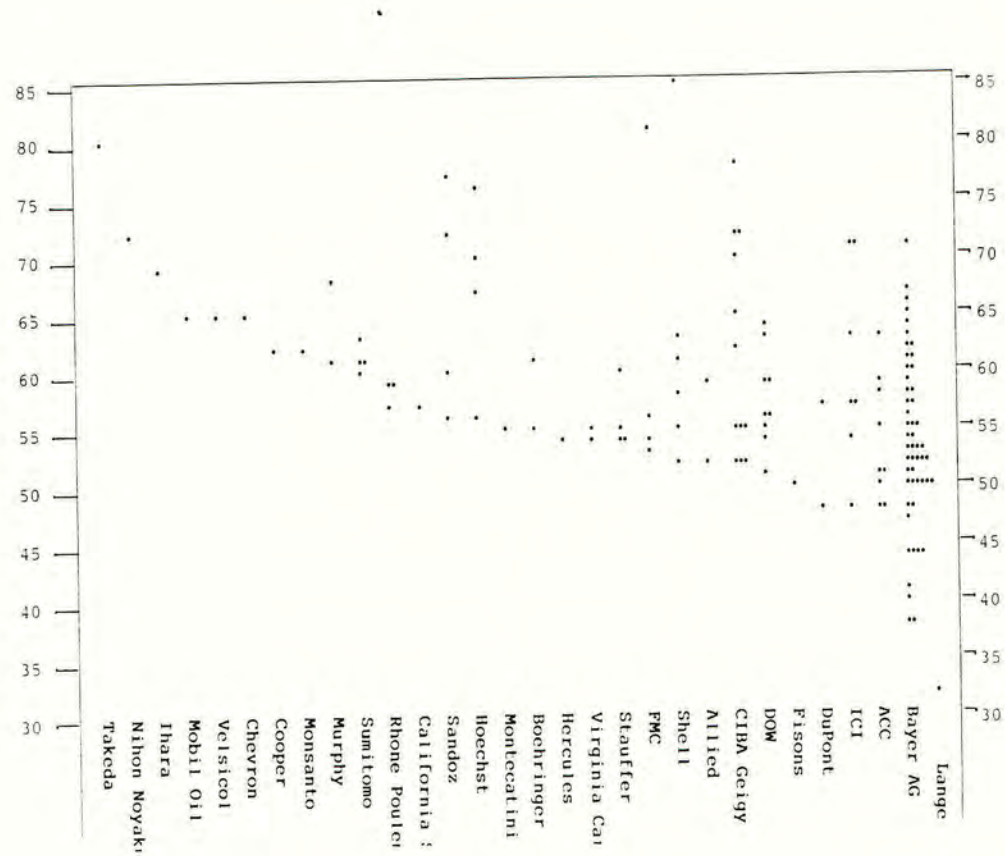
calabar beans

But this was not the story how the organophosphate and carbamate research came about. Organophosphate insecticides where discovered by Schrader in the Bayer Labs. in 1937, when he took up the leads he found with some sulfonylfluorids in connections with results of Lange in Berlin 1932. Lange synthesized the first phosphoryl fluorides, and his student Krüger made the acquaintance with their highly toxic properties. She recovered. This line was also followed up during wartime, especially in british and german laboratories for purposes which are not related to crop protection and which

still is the threatening and gloomy side of organophosphate chemistry. Schrader went on in looking for insecticides of lower toxicity, and soon found a general recipe for making insecticidally active organophosphates by his well known acyl-formula, which implies, that they are phosphorylating agents. The first of Schrader's organophosphates went to market in Germany in 1943 (TEPP). The progress in this field is shown in figure 3, which shows the bonanza like research activity of the successfull companies, after the german patents and biological properties of organophosphate insecticides became finally known to the world after the war through the american Fiat and Bios reports.

FIGURE 3

Years of priority of commercial organophosphates and inventing institutions

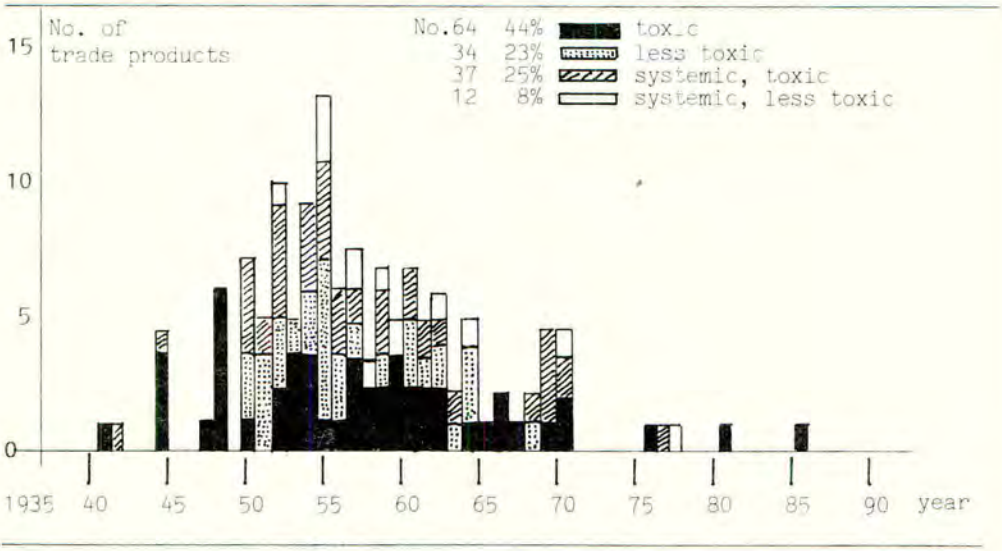


147 compounds were discovered and developed by 29 companies, 35% from Bayer. Developmental time in the fifties was 2-3 years, much shorter than nowadays with at least 6 years. And, of course, much cheaper.

Altogether 51 companies seriously were involved in R & D of this commercially very promising field of organophosphates in the last 50 years. This research has been carried out in 21 american, 13 japanese, 5 german, 3 british, 2 french, 1 italian companies and in russian institutes. The result of this tremendous effort is shown in figure 4.

FIGURE 4

Years of discovery of 147 organophosphate insecticides



It lists the annual number and years of priority of all organophosphate insecticides which became trade items. Additional information is provided by the classifications toxic, less toxic, systemic and toxic, systemic and less toxic. This 4 classifications determine together with the spectrum of insect-toxicity to a large extent the mode and field of application and hence the pest problem solved by the corresponding organophosphates and therefore the potential market size.

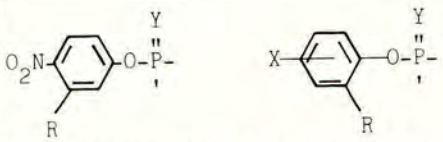
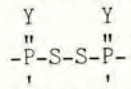
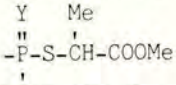
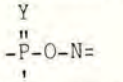
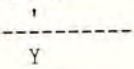
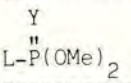
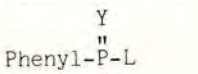
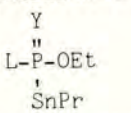
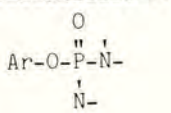
The biophysical property "systemic" means that the compound is taken up by the root or leaves of the plant and is distributed to other plant parts. By this means, newly grown leaves are protected as well as hidden pest, not accessible by direct contact with the poison, are controlled. Less toxic means compounds showing an oral acute toxicity to the rat, as LD50, of more then 100 mg/kg. The range of acute toxicity of organophosphates to rat is impressively large, going from LD50 less than 0.1 mg/kg up to >5000 mg/kg. But also the toxicity to different kind of insects may differ by a factor of 10,000. However, as less toxic organophosphates can be fed to the rat in larger doses (impossible to be taken up involuntarily under practical circumstances), there is a chance for long term toxicological effects, which may be prohibitive for registration. So we are faced in the field of organophosphates with the frequent paradox, that

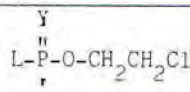
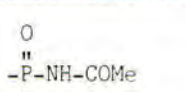
in terms of practical risk the toxic ones are considered toxicologically relatively safe and the nontoxic ones are considered toxicologically problematic, particularly for the treatment of organophosphate poisoning of humans by these compounds. This is also one of the darker sides of agricultural organophosphate history, particularly when safety demands for application in the field could not be met or in the cases of suicidal abuse.

Some structural principles (table 5) where elaborated in the course of the time which gave in most cases rise to acutely less toxic organophosphates. However, this is no guarantee for low toxicity.

TABLE 5

Important structural principles for acute less toxic organophosphates

partial structure	examples
ortho or meta substituent	
disulfide	
additional metabolizable group	
oxim	
thion	
dimethylphosphate	
phenylphosphone	
OEt/Snpropyl	
P-diamide	

partial structure	examples
chloroethoxy	
N-acyl prodrug	

Those principles take advantage of the more versatile metabolic pathways in rat compared to insects.

Some of the organophosphates caused delayed, but long lasting neurotoxicity due to effect on other nerve targets. Others were proven to exert mutagenic properties in the standard in vitro tests. Besides these toxicological problems insufficient performance or unacceptable residues in the edible agricultural product were reasons for withdrawals of some organophosphates from the market.

Behind the statistics there is hidden a very diverse chemistry. It turned out, that Schrader's general formula fits for many structural organophosphate classes, according to the host of several thousands patent applications in this field. Economically successfull proved to be to a very different extent only a limited number of structural principles, which are listed in Table 6 as discovered in the course of time. Some ideas of the chemists led to a very high annual turnover of money and chemicals and others led only to compounds of moderate demand.

On this occasion of a historical round up of meritorious insecticides it certainly is the place to give credit to the inventors and research institutions for the more important new structural principles starting from the Schrader rule. A structural principle - economic importance-relation of organophosphates is shown on Table 6.

TABLE 6

Relation of structural principles and economic importance of organopnosphate insecticides

discovery		partial structural principle	No. of R & D companies	No. of trade products			relative economic importance*
year	by			Inventor	1940-88	1986	
1937	Schrader/Bayer	P-CN	1	-	-	-	
1938	Schrader/Bayer	P-F	2	-	2	-	
1941	Schrader/Bayer	P-O-P		2	5	1	
1944	Schrader/Bayer	PO-C=CHCO-		-	8	5	
1944	Schrader/Bayer	POAryl	17	18	36	8	
1948	Ghosh/ICI	PS-CH_2CH_2N-	3	1	1	-	
	Hook/ACC	PS-CH_2S-	5	1	6	3	
	Cassady/ACC	PS-CH_2CON-	14	1	9	4	
	Cassady/ACC	PS-CH_2COO-					
	Jellineck/DuPont	C_6H_5P=	4	1	4	1	

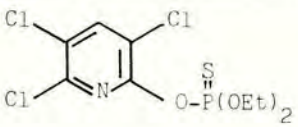
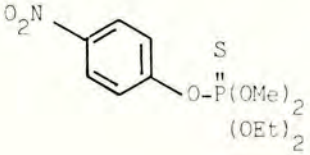
discovery		partial structural principle	No. of R & D companies	No. of trade products			relative economic importance*
year	by			Inventor	1940-88	1986	
1949	Schrader/Bayer	PS-CH_2CH_2S	7	6	13	5	
1951	Bryner/DOW	N-P-OAr	2	2	5	2	
	Sallmann/Ciba	PO-CH=CHCl	5	1	7	3	
	Lorenz/Bayer	P-CH(OH)CCl		1			
	Schrader/Bayer	PON=	2	3	4	1	
	Gysin/Ciba	PO-Hetar	12	3	12	6	
1953	Lorenz/Bayer	PS-CH_2-N-CO	6	2	7	4	
	Metivier/Rhone-P.	PS-CH_2-Hetar	4	1	2	-	
1957	Street/Cal.Res.Co.	P-O-C-Hal		1	3	2	
1960	Szabo/Stauffer	P-S-Ar	3	1	2	1	
1963	Addor/ACC	P-N=C-	1	1	1	-	
1964	Schrader/Bayer	N-P-O " S O	2	1	2	2	
	Lorenz/Bayer	CO-N-P-O " S O	2	-	1	1	
1965	Wilson/Mobil	O-P-S " S		1	2	1	
1968	Pianka/Murphy	PS-CH_2-Cl	4	1	1	1	
1971	Kishino/Bayer	EtO-P-SnPr "	3	2	4	4	

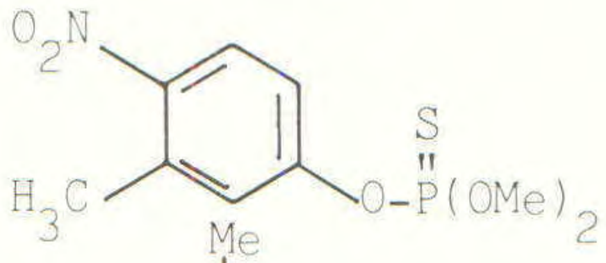
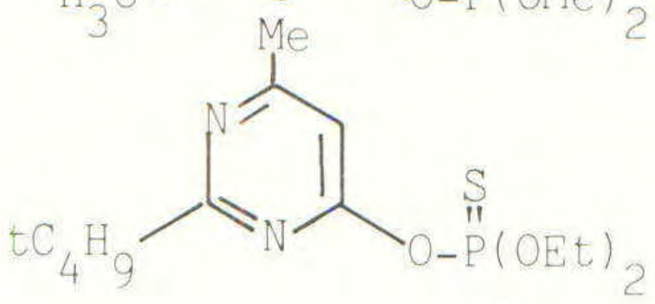
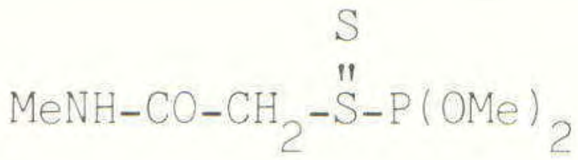
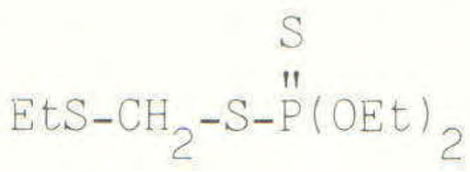
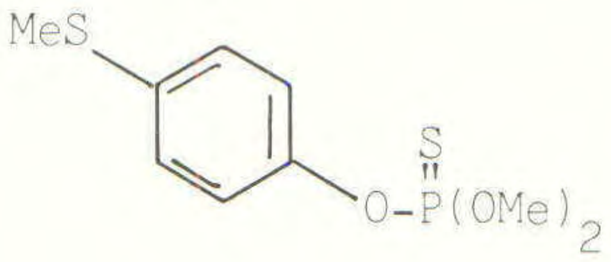
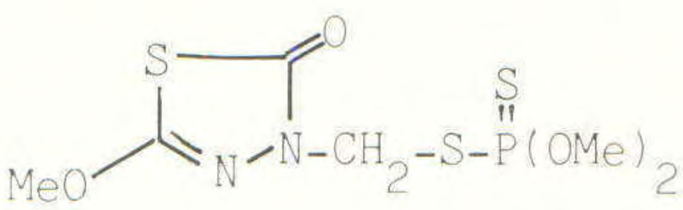
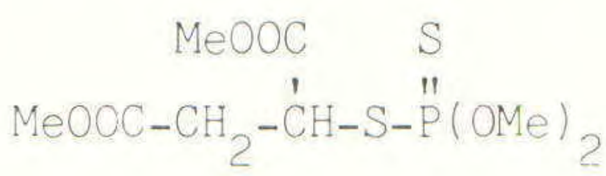
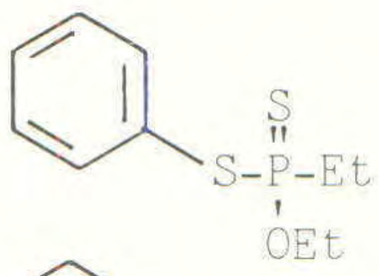
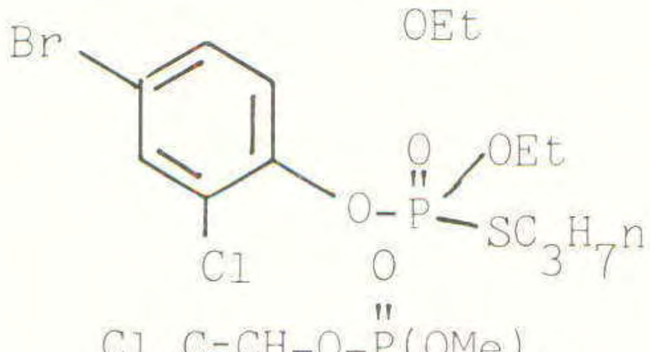
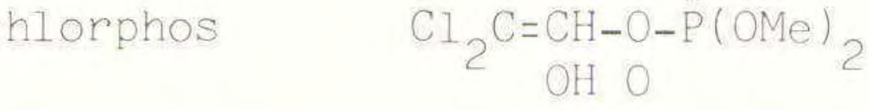
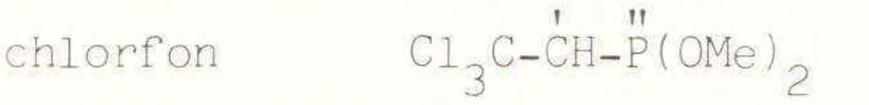
* area corresponds to the turnover

The most important organophosphates today are presented on figure 5.

FIGURE 5

18 Most important organophosphates 1986 (No. of producers/age/toxicity)

Chlorpyrifos		3	23	130
Monocrotophos	MeNHCO-CH=C(Me)-O-P(OMe)$_2$ " O	16	37	20
Terbufos	t-C_4H_9S-CH_2-S-P(OET)$_2$ " S	3	37	4
Parathiones		6 5	45 45	5 5

Methamidophos		13	23	30
Acephate		10	23	950
Fenitrothion		6	29	800
Diazinon		5	33	110
Dimethoate		10	39	220
Phorate		4	35	2
Fenthion		3	31	260
Methidathion		3	33	30
Malathion		11	38	1300
Fonophos		1	28	18
Propenophos		1	13	360
Dichlorphos		16	37	60
Trichlorfon		18	37	150
Disystones		2	37	2-84

X = O, S ;R = Me, Et

In the meantime besides the 29 american, western european and japanese companies engaged in R & D of organophosphates in the past, 47 more companies also in other countries are now producers of 28 different commodity-organophosphates (table 7).

TABLE 7

Commodity production of organophosphates for the world market companies* by not involved in R & D for agrochemicals.

Country	No. of producers	No. of products
S-Korea	1	6
Taiwan	6	11
India	8	13
Singapoor	1	5
Hongkong	1	6
Indonesia	1	1
Israel	1	9
Turkey	1	1
Italy	1	2
Spain	5	4
Danmark	1	6
Netherlands	1	6
Sweden	1	5
Argentine	3	9
Brasil	2	3
Mexico	1	4
Guatemala	1	1
USA	6	8

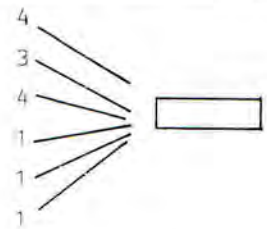
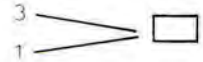
* Farm Chemical Handbook 1988

Synthetic organophosphate chemistry is a distinct field of organic chemistry and became now a mature technology for most cases, as shown on the previous table 7 of basic producers of commodities. The phosphorus chemical background is shown in table 8.

TABLE 8

Phosphorus chemical-basis for the more important organophosphate insecticides

Inorganic materia prima	consecutive intermediate	No. of OP	relative economical importance
P_4S_{10}	$(EtO)_2PS_2^{\ominus}$	7	
	$(MeO)_2PS_2^{\ominus}$	6	
$PSCl_3$	$EtOPSCl_2$	1	
	$(EtO)_2PSCl$	7	
	$(MeO)_2PSCl$	5	
	$(MeO)(NH_2)POS^{\ominus}$	2	

PCl_3	$(MeO)_2POS^{\ominus}$ $HPO(OMe)_2$ $P(OMe)_3$ $P(OEt)_3$ $EtPSCl_2$ $C_6H_5PSCl_2$	
$POCl_3$	$EtOPOCl_2$ $C_3H_7SPOCl_2$	

The organophosphates exert a host of physiological effects. The effects to humans go from burning taste (deClermont 1854), but plesent, strongly aromatic smell (Lange 1932), sometimes to very unpleasent feelings and even dangerous situtation due to their intrinsic toxic effects (Lange). Pharmacologically useful is their strong mitotic effect used in opthalmology. As mentioned before, organophosphates inhibit AChE in warm blooded animals. These animals die due to overexcitations of the cholinergic synapses and neuromuscular endplate by the endogenous acetylcholin neurotransmitter, which cannot be de activated by the blocked AChE. Ultimate cause of death is respiration failure due to dysfunction of the central nervous respiration center.

So far the group of chemicals of table 2 are connected by their ability to react with the standard AChE from human blood, pig, electric eal etc.. And this inhibition is nicely correlated with the rate of hydrolysis. However, the thionophosphates as such are unreactive at the enzyme, they need to be transformed by metabolic oxidation into the oxon form, known for a long time. But it has become increasingly difficult to explain in many cases the cause for the lethal action of organophosphates to insects. What do insects die of after treatment with organophosphates?

1. There is no nicotinic neuromuscular junction and no AChE to be found there.

2. An insect is unlikely to die from respiration failure, due to the different way of aquiring the air through the tracheae.

3. By far most of the numerous in vitro measurements for insects have been made with the readily available fly head AChE as a model for insects AChE. However, in many cases no correlation was found for inhibition of this enzym and toxicity to the whole animal. In other cases only certain ganglionic AChE of the fly was inhibited exclusively. The sensitivity to the AChE within one insect differs markedly, depending on the source from within the nervous system.

4. Moreover, depending on insect order the sensitivity of AChE-isoenzymes differ greatly toward a given organophosphate.

5. There are cases of complete inhibition of insect brain AChE but no toxicity to insects on one hand, and no inhibition of brain AChE in the deadly poisened insect in other cases.

6. In addition there are a number of examples of strong discrepancy of AChE inhibition and toxicity of enantiomers or diastereomers.

7. And even in cases, were the organophosphates needed a metabolic activation within the nerve fiber by MFO to become strongly inhibiting to AChE, in vitro, there was no correlation with toxicity to different insects.

8. Atropin and 2-PAM in contrast to rat, do not antagonize organophosphates poisoning in insects.

It was decided by an early majority, that organophosphates are toxic to insects because of AChE-inhibition, which in a number of cases has very convincingly been demonstrated. In view of the diverse results this decision needs further comments. Metabolic activation and of course, pharmacokinetics were called in for explanation, particularly in the case of organophosphate amides and organophosphate thiolesters.

In the meantime science came up with alternative mechanisms. These are effects of certain organophosphates on glutamat decarboxylase, glutamate receptor, glutamat-S-transferase and JH-esterase. The corresponding physiological effects are to be seen in the latter case. Moreover neurotransmitter mimetic activity was demonstrated for some organophosphates, such as antiproctolinergic action, action as ß-blocker and action directly on the nicotinic ACh receptor. A convincing general scientific explanation for the dead of insects due to organophosphate poisoning has yet to be presented after 50 years of application.

Economically more important seems to be the question of organophosphate resistance, which appeared since 1960, depending on the arthropod species. This resistance lead in some cases to complete control failure. Acarinae and fly have been shown to be particularly prone for developing organophosphate resistance. For example there is no organophosphate-market to combat ticks in Australia, Argentine, Brazil and South-Africa anymore and mites have now to be controlled by compounds from complete different groups of products. In the controll of other insect species however, organophosphates fortunately perform still convincingly, even in long lasting vector eradication programms. However, often application rates are elevated. Reason for resistance is as usual a combination of changes in insects: increase of enzymes for metabolic detoxification (e.g. glutathion-S-transferase, triesterase, MFO); and decrease of enzymes for oxidative metabolic toxification. The lowered AChE-sensitivity in a number of other cases of resistance is one of the strong arguments for the classification of compounds in table 2 as AChE inhibitors also for insects.

After this excursion into the story and some of the background of organophosphates the smaller sister group of the carbamates is to be considered. The historical development of commercial carbamates as developed to the market by the companies involved is shown in figure 6.

Four types of carbamates where discovered. Highly selectiv dimethylcarbamates against aphides, many more or less broad acting phenylcarbamates, some oximcarbamates and the less toxic N-sulfenylated derivatives. Starting with the discovery of dimethylcarbamates in the CibaGeigy laboratories by Gysin in 1947 45 carbamates where introduced to the market by 22 companies. 22% of the marketed compounds originate from the Bayer development. 37 companies where involved in R & D.

FIGURE 6

Commercial carbamates - Year of patent priority

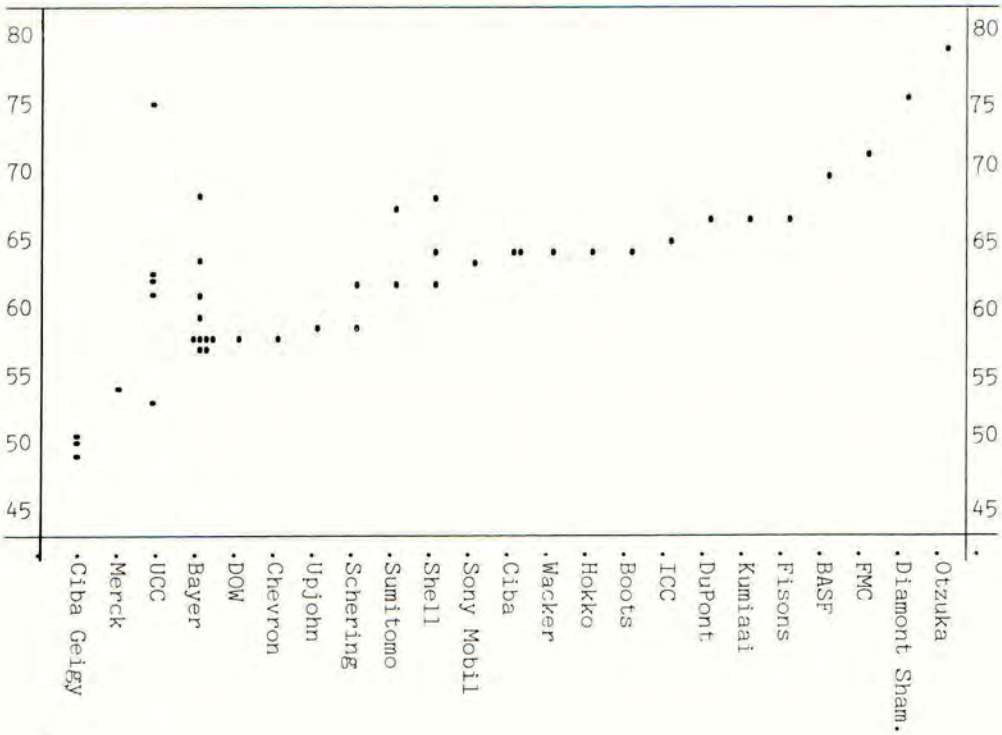


TABLE 9

Discovery of important structural principles of carbamates

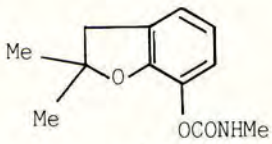
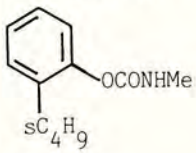
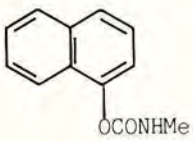
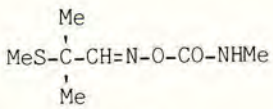
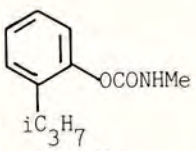
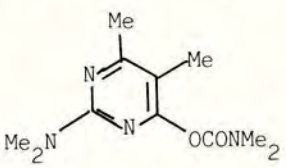
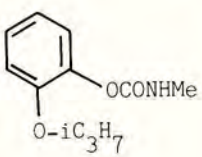
Year	Partial structure	Inventor	Company	economic success		
				*	**	***
1947	Dimethylcarbamates	Gysin	Geigy	+/4	+/1	ICI
1950	Monomethylcarbamates	Metcalf Fukuto	Uni of Calif.	-	-	
1952	Phenol-monomethylcarbamates	Jacobi Lust v. Schoor Zima	Merck	-	+++/21	UCC Bayer FMC
1962	Oximcarbamates	Addor	ACC	-	+++/7	DuPont/UCC
1962	Detoxification by N-acylation	Fraser	Boots	-	-	
1968	Detoxification by N-sulfenylation	Brown	Chevron	-	+/3	FMC/Ciba Otzuka

* for inventing institution/No. of trade products
** for others/No. of trade products
*** successfull companies

Table 9 outlines briefly the story of discovery of structural principles and gives credit to the inventors. The most important carbamates are shown in figure 7.

FIGURE 7

9 Most important carbamat insecticides

		(No. of producers/age/toxicity)		
Carbofuran		8	21	15
BPMC		2	27	400
Carbaryl		6	33	800
Methomyl		6	18	20
Aldicarb		1	23	0.9
Isoprocarb		8	19	450
Thiodicarb		1	14	66
Pirimicarb		1	19	140
Propoxur		4	26	100

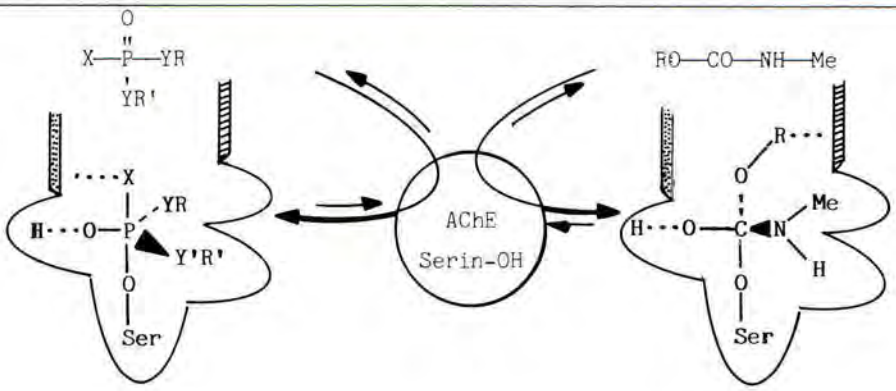
6 of the carbamtes are now produced for the world market by 19 companies in 11 countries, including former developing countries like Mexico, Taiwan, S-Korea, Indonesia and Singapoore. The reason for the wide aceptance of organophosphates and carbamates by agriculture is their broad spectrum of physical and biological properties as shown here:

- broad acting or (very) selective insecticides at reasonable application rates (0.5-1 kg/ha)
- some are selective nematicides, anthelmintics or acaricides highly toxic or of low toxicity to rats
- contact action and/or stomac action
- solubil or insolubil in water
- systemic, partially systemic or not-systemic in plants
- some have fumigant action
- very fast acting or slow acting
- very short or long lasting activity

Most of the organophosphates are cheap. Carbamates are about 1.5 times more expensive in the average. Organophosphates show a very broad structure-activity-range, defying any QSAR generalisation. They represent the most versatile class of insecticides. The properties of organophosphates are much more diverse than those of carbamates, which show a relatively moderate structure-activity variability. Different is also their mode of inhibiting AChE as shown in figure 8.

FIGURE 8

Differences in the mode of inhibition of AChE by organophosphates and carbamates



very labil
destabilisation of transient transition state by leaving group X

X = electron withdrawing aryloxy and other leaving groups

Inhibition of enzymfunction by cleavage of phosphate and irreversible blockage of catalytic serin-OH by phosphorylation

metastabil
stabilisation of metastabil intermediate by leaving group OR

OR = electron rich aryloxy or oximino

Inhibition of enzymfunction by sequestering of intact carbamates at the catalytic site

These properties alone or in combination make organophosphate's and carbamates to very effective agrochemicals for combatting biting caterpillars and beetles in cotton, rice and vegetables and succing pests in cotton, rice, vegetables and corn. Nematodes can be controlled in banana, potatoes and tobacco. This will certainly continue for more than only the next decade. There is no substitute in sight, particularly not for the valuable systemic action.

The main pest problems and their relative economic importance for each of the two classes of insecticides are shown in table 10.

TABLE 10

Main pest problems for organophosphates and carbamates

Species	commercial rel. importance	crop
lepidoptera	▨	rice, cotton, corn, fruits, vegetable
	▭	rice, cotton, corn, vegetable, soy, tobacco
aphids	▨	cereals, orchards
	▭	cereals, vegetable
plant hoppers	▨	rice, cotton
	▭	rice
coleopteres	▨	cotton, potatoe, rice
nematodes	▨	banana, potatoe, tobacco, citrus
	▭	
dipteres	▨	vegetable, citrus
	▭	
white fly	▨	vegetable, citrus
	▭	cotton
scales	▨	vegetable, citrus
mites	▨	cotton
	▭	citrus
household	▨	
public health	▭	

What will the future bring to acetylcholinesterase inhibitors, to organophosphates and carbamates insecticides? First of all, there will be an unchanged strong need for insecticides. The insects pests will have, as before, an acetylcholinesterase, which should be inhibitable also in organophosphates resistant insects with altered AChE binding properties. The spread of resistance will go on, crossing in certain cases the economical threashold. Nevertheless, these compounds will keep dominating the insecticide market also in the next two decades because of low prices,

relatively simple technology in the production, agricultural variability and a long term proofen ecological compatibility. Think of the most versatile metamidofos - the allmost ideal pesticide. Very simple in structure, it breaks down simply into the plant nutrients ammonia, phosphoric acid, and mercaptane, carriying the valuable sulfur. But there are a lot of problems ahead.

TABLE 11

Future developments for some organophosphates and carbamates

- more stringed toxicological/ecotoxicologial requirements for reregistration may not be met by certain compounds
- additional work for reregistration for economically marginal OP and CA not worthwhile
- economically marginal OP and CA will be cast off by larger R & D companies
- leaching problems into ground water table may become the final problem for certain OP and CA
- accelerated microbiological degradation in soil will render more OP/CA less usefull
- inorganic phosphate in the waste water of industrial production will become a critical factor for OP production in developed countries
- more market shares will go to pyrethroids
- OP will increase its share at the expense of carbamates
- highly toxic OP/CA increasingly under pressure by authorities
- innovative research in the field of OP/CA has stopped

Looking at the structural variability of organophosphates there are by no means all active compounds synthesized yet, as so in the field of carbamates. An interesting problem is the occurrence of chirality in about 20% of the tonnage of major organophosphates (about 12 compounds). Usually only one isomer is the main carrier of the desired properties. It still is technically impossible to tackle this academically fascinating area of stereochemistry. When will the first optically active single isomeric organophosphate appear on the market? A novel organophosphate will have a hard time to substitute the old cheap ones of the similar efficacy.

The figure 9 shows the state of innovation in the 5 classical insecticidal groups. Organochlorines, organophosphates, carbamates, pyrethroids and benzoyl ureas, which were so attractiv in the past for research and for their broad structure activity range, for high insecticidal activity. They are now faisly easy to synthesize and manufacture. Since other larger classes of insecticides of great variability are yet to be discovered

and insects in the fields have to be controlled, it is advisable, to apply a careful risk - benefit analysis, before discarding more of these meritorious agrochemicals. Research is sailing the wide open sea.

FIGURE 9

Rate of innovation in most important insecticidal classes

Published developmental products and market products (priority year)

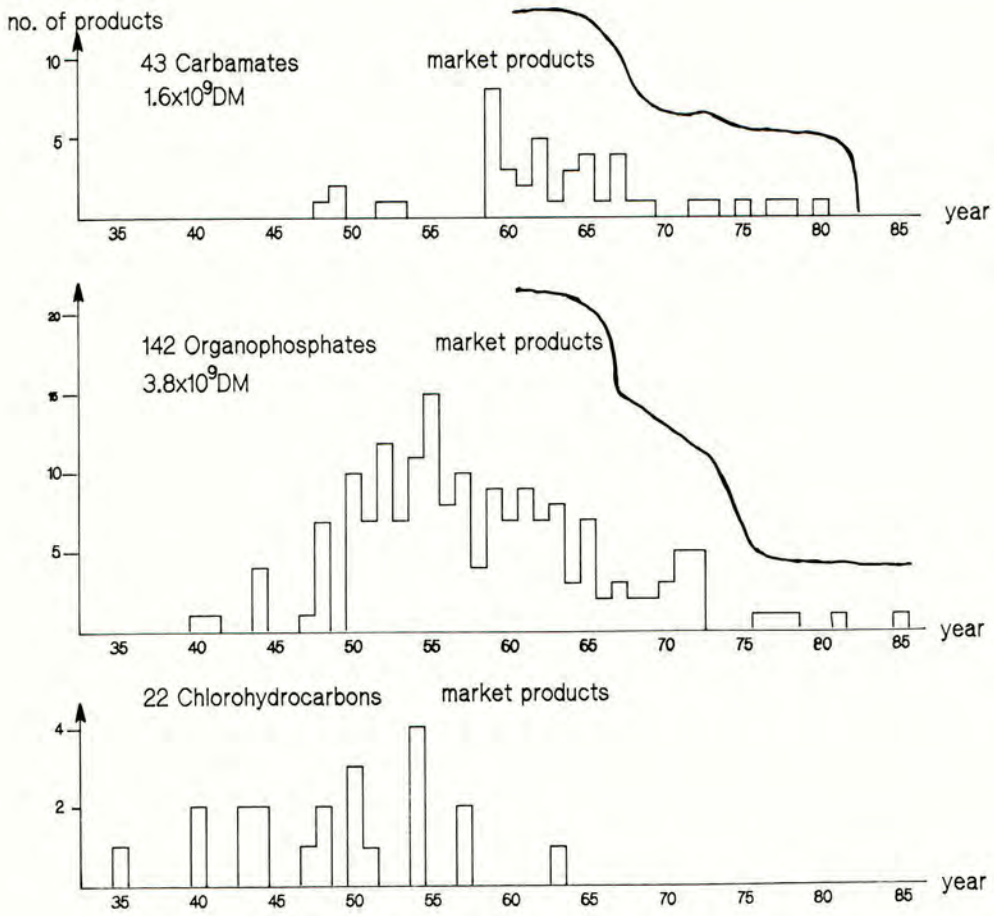
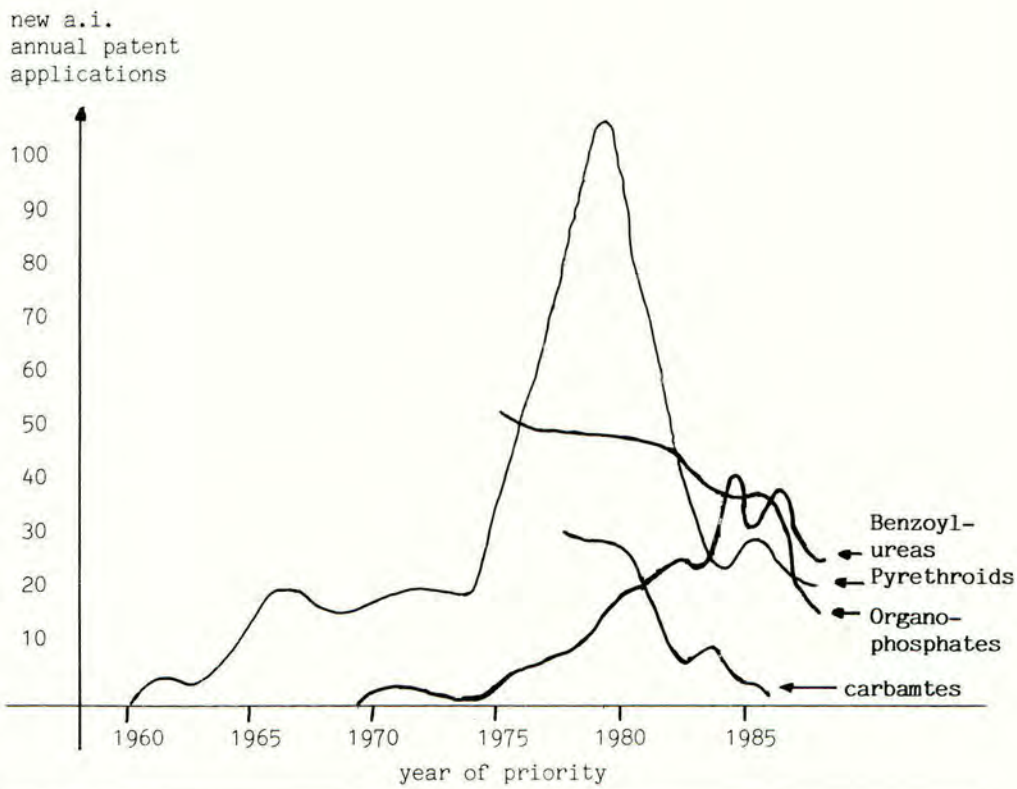


FIGURE 10

Rate of innovation in most important insecticial classes

Annual patent applications



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PYRETHROIDS - PAST, PRESENT AND FUTURE

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The natural pyrethrins are powerful insecticides which act rapidly against a wide range of insect species and which are harmless to mammals under all normal circumstances; however they are too unstable in light to control pests of agricultural crops and forests efficiently and economically. The first generation of commercial synthetic pyrethroids (allethrin, tetramethrin, resmethrin and bioresmethrin) did not greatly extend applications beyond those of the natural compounds but demonstrated the considerable scope for improvements in properties possible with structural variations, of which the first significant studies had been made as early as 1910. Continuing research identified the sites in the molecular structures of the natural and synthetic compounds which render them readily decomposed in light and showed that at selected positions alternative units could be introduced to give much greater overall stability whilst the features essential for their insecticidal activity and low mammalian toxicity could be retained. The economic importance of synthetic pyrethroids developed over the past decade (annual sales now ca. £900M) is consequently now comparable to that of longer established organochlorine, organophosphate and carbamate insecticides. Some of the new compounds are stable enough and have such high potency (e.g. deltamethrin, LD50 ca. 0.002 $mg.kg^{-1}$, insects) that they can protect crops at previously impracticable levels (10-50 $g.ha^{-1}$) and yet they possess unsurpassed selectivity between target and non-target organisms (e.g. deltamethrin, LD50 ca. 100 $mg.kg^{-1}$, mammals).

These favourable toxicological properties depend on their ability to penetrate rapidly to, and interact with, sites of action in insects; these are probably sodium channels of the nerve membrane, where compounds such as deltamethrin are active at concentrations as low as 10^{-12}M. In contrast, after external or oral administration to mammals, pyrethroids are largely converted by hydrolytic or oxidative attack to polar metabolites which are then eliminated in the faeces or urine, unchanged or as conjugates, before sensitive sites can be reached.

The family of synthetic pyrethroids is still expanding, but in all the members a relationship, albeit superficially tenuous, can be discerned with the prototype natural esters pyrethrin I or pyrethrin II. The first modifications of activity were achieved by substituting alternative unsaturated groups for the side chains in their alcoholic and acidic components, for example, benzyl for the *Z*-pentadienyl side chain in pyrethrolone and *Z*-butadienyl for isobutenyl in chrysanthemic acid. Next, it was shown that either or both rings holding the unsaturated side chains to which the ester function was attached could be modified with retention or considerable increase of insecticidal activity if the absolute (natural) configurations at the chiral centres were maintained. Typical of such components were 5-benzyl-3-furylmethyl alcohol, *S*-α-cyano-3-phenoxybenzyl alcohol and *S*-4-chlorophenyl-α-isopropylacetic acid. Subsequently, significantly active compounds with the dimethyl groups in the acid and/or the ester function itself replaced (for example in oxime and trifluoromethyl

substituted ethers and in alkenes) have indicated that the essential features for potency may be achieved in relatively simple structures. A formidable array of related, active compounds with a range of physical and chemical properties is therefore now available; in these, subtle structural variations influence potency and speed of action generally or variously against particular species of insects, mites, aphids, rice and soil pests and bees and fish, etc. Like the natural esters and photolabile pyrethroids, these compounds are readily degraded by metabolising systems such as those of mammals and soil micro-organisms but remain effective for long enough to ensure adequate control.

As well as being applied to agricultural crops (over 70 million ha. were estimated to have been treated in 1987) especially cotton, selected pyrethroids are now used in the animal sector as ectoparasiticides and in the health and household insecticide market. Knowledge of how structure affects potency for important practical uses (activity against economically important pest species, acaricidal activity, selectivity between pests and beneficial species, persistence on crops and in the environment, rapidity of action, antifeedant or repellent properties, toxicity to mammals, birds and fish, etc.) is being rapidly accumulated. Strategies to guard against resistance development and to diminish adverse effects to biological systems by appropriate choices of compounds, timing and sites of application are increasingly recognized as of outstanding importance.

The synthetic pyrethroids therefore now constitute a valuable group of pest control agents which do not leave residues that might contaminate biological systems or the environment.

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AMINO ACID SYNAPSES AND RECEPTORS

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ABSTRACT

Receptors for γ-aminobutyric acid and L-glutamic acid serve important roles in neurotransmission in insects where they are found in the central nervous system (CNS) and on muscle. The pharmacological properties of these membrane macromolecules will be described with particular emphasis given to the antagonism of L-glutamate receptors by polyamine amides.

INTRODUCTION

In any discussion of insecticides and their neurotoxic effects the importance to the pesticide industry of cholinergic synapses and nicotinic acetylcholine receptors (nAChR) as major targets in insect CNS becomes readily apparent. However, insect central and peripheral nervous systems contain many other types of synapses and membrane receptors which may figure in the future as target sites for insecticides. Receptors for dopamine, 5-hydroxytryptamine, octopamine and peptides have already been identified in insects and some insecticides interact with one or more of these membrane macromolecules, yet our knowledge of their basic properties remains somewhat fragmentary. Another class of signalling molecules, the amino acid receptors are much better understood and are widely distributed in insect CNS and peripheral nervous systems, but they have not yet been successfully exploited. There are two major classes of amino acid receptor, those sensitive to γ-aminobutyric acid (Gaba) and those sensitive to L-glutamic acid (Glu).

RECEPTORS FOR GABA

Our understanding of Gaba receptors in insects and other animals has advanced considerably over the past two decades and the properties of these signalling molecules were extensively considered at Neurotox '88 (e.g. Lunt et al, 1988). Therefore, this review of their properties will be succinct.

Gaba is a major inhibitory transmitter in insects, with Gabaergic synapses located in the central nervous system and on muscle. The ligand binding properties of both central and peripheral Gaba receptors are similar to those of vertebrate CNS $Gaba_A$ receptors except that insect Gaba receptors are insensitive to bicuculline. The pharmacological properties of insect

Table 1 - Antagonists of insect amino acid receptors

Receptor	Ligand	Mode of Action	References
Gaba	(lindane endrin)	non-competitive	1
	picrotoxin	non-competitive antagonism	2,3,4,5
	dihydroavermectin B_{1a}	non-competitive antagonism	6
	trioxabicyclo-octanes	non-competitive antagonism	7
	(benzodiazepine flunitrazepam barbiturate)	allosteric potentiator	8
	bicuculline	nil (CNS) nil (PNS)	9 3
	baclofen	nil	4
Glutamate (excitatory)	δ-philantho-toxin	non-competitive antagonism	9, 10
	argiotoxins	non-competitive antagonism	11, 12, 13
("inhibitory")	picrotoxin	non-competitive antagonism	14

CNS - central nervous system; PNS - peripheral nervous system.

References: 1, Eldefrawi & Eldefrawi (1988); 2, Usherwood & Grundfest (1965); 3, Scott & Duce (1987); 4, Benson (1988); 5, Lunt et al (1988); 6, Duce & Scott (1985); 7, Casida et al (1988); 8, Lees et al (1987); 9, Eldefrawi et al (1988); 10, Piek et al (1988); 11, Bateman et al (1985); 12, Volkova et al (1986); 13, Adams et al (1987); 14, Lea & Usherwood (1973).

Gaba receptors are complex (Table 1). In common with the $Gaba_A$ receptors of vertebrate CNS they have a benzodiazapine binding site which can be allosterically modulated by Gaba and pentobarbitol (Lunt *et al*, 1988), and a convulsant binding site to which picrotoxin binds and which is especially sensitive to the cage convulsants, the trioxabicyclooctanes (Casida *et al*, 1988). Given the growing industrial interest in insect Gaba receptors it might be timely to herald a slight note of caution of the dangers that the pesticide industry may face as it develops its biorational approach to exploit these and other neurotransmitter receptors. These arise from its tendency to focus down too much both conceptually and systematically and, thereby, become trapped within the contemporary time frame. To illustrate this point we will refer to the current controversy on the bicuculline sensitivity of insect Gaba receptors. Bicuculline is a potent inhibitor of Gaba binding to vertebrate Gaba receptors, but both electrophysiological and biochemical studies of insect central and peripheral Gaba receptors have claimed that this compound is inactive. However, a recent report by Waldrop *et al* (1988) has claimed that 10^{-4}M bicucculine reversibily blocks Gaba receptors in the deutocerebrum of *Manduca sexta*. Hildebrand (1988) suggests that one interpretation of these conflicting data is that bicuculline blocks postsynaptic Gaba receptors (presumably located exclusively in the neuropile), but is inactive on extrasynaptic Gaba receptors. The recent work of Fraser *et al* (1989) on *Xenopus* oocytes injected with poly(A)$^+$ mRNA challenges this idea. They found that when mRNA extracted from locust muscle and CNS is injected into toad oocytes, Gaba receptors are expressed which are insensitive to bicuculline. This suggests that, at least in locust, Gaba receptors are bicuculline-insensitive, although there remains the unlikely possibility that the *Xenopus* oocytes fail to translate bicuculline-sensitive receptors. It is worthwhile remembering that Gaba receptors are present peripherally on muscle as well as in the CNS of insects. In fact studies by Usherwood & Grundfest (1964, 1965) on locust leg muscle led to the initial discovery of insect Gaba receptors and the non-competitive action of picrotoxin on these membrane macromolecules. The reason for raising this point is that the muscle Gaba receptors are more readily accessible than their CNS counterparts and, with appropriate electrophysiological techniques, they can be identified unequivocally as either synaptic or extrasynaptic. Despite this, the recent studies by Scott & Duce (1987) which show that locust muscle Gaba receptors are insensitive to bicuculline remain largely ignored. Biorational research in the pharmaceutical industry has exceptionally strong foundations derived from a large, worldwide academic community of pharmacologists. Although the pesticide industry is less favourably placed and at times is forced to pursue its own basic research from which to launch biorational programmes of pesticide discovery and development, because of the lack of consensus views amongst academic scientists, it would be advised to maintain a broad outlook.

GLUTAMATE RECEPTORS

Although insect muscle (Lea & Usherwood, 1973) and insect neurones (Giles & Usherwood, 1985) express receptors for Glu which gate chloride channels and could, therefore, be reasonably classed as inhibitory, there is no evidence that this type of GluR is associated with inhibitory synapses either centrally or peripherally. The chloride channel gated by these receptors is blocked by picrotoxin, but it remains to be established whether

it has other pharmacological properties in common with the chloride channel gated by Gaba receptors. Given the apparent ubiquity of GluR linked to chloride channels in insect excitable systems, perhaps it would be worth determining whether the trioxabicyclooctanes and other Gaba receptor antagonists affect their function.

To appreciate fully the role of excitatory GluR in synaptic function in insects it is necessary to contemplate the wealth of information that is available on the glutamatergic excitatory synapse of insect skeletal muscle, which has recently been comprehensively reviewed by Duce (1988). As one might anticipate from their excitatory function, these GluR gate cation-selective ion channels causing muscle membrane depolarization. There is growing evidence of similarities between insect excitatory GluR and some types of mammalian CNS GluR, but the presence of three main classes of GluR in mammalian CNS, the quisqualate-sensitive (QUIS), the N-methyl-D-aspartate sensitive (NMDA) and the kainate-sensitive (KAIN) GluR is not matched completely in insect nervous systems which seem, at this time, to lack the NMDA class. However, KAIN-GluR have been reported for insect CNS and the QUIS-GluR is the type of receptor present postjunctionally at insect excitatory nerve-muscle junctions. Recent studies of mammalian CNS GluR have shown that at least some QUIS-GluR have a single channel conductance approaching that of locust muscle QUIS-GluR i.e. 100-150pS (Patlak et al, 1979), and exhibit desensitization properties which are remarkably reminiscent of those of the locust muscle QUIS-GluR, even with respect to their inhibition by concanavalin A (Sansom & Usherwood, 1989). Thus, the QUIS-GluR may have been well-conserved during evolution, in which case the pesticide industry should not be too optimistic about discovering insect-specific poisons targeted at this receptor type. However, one major difference between locust muscle and mammalian CNS QUIS-GluR is the insensitivity to DL-α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) of the insect receptor.

Pharmacological studies of the locust muscle QUIS-GluR led Lea and Usherwood (1973) to conclude that L-glutamic acid binds to its site or sites on this receptor in a partly extended conformation. Their suggestion that a distance of between 2.6-4.6Å between N^+ and C_3 of L-glutamic acid is optimal for binding of this ligand has been confirmed by subsequent computer graphic studies allied to more detailed, quantitative pharmacology of this receptor. The results of these studies are referred to by Bycroft & Jackson (1988) who concluded that a distance of 4.54Å between N^+ and C_3 of L-glutamic acid is optimal for binding. This clearer definition of the binding conformation of L-glutamic acid will provide further insight into those structural changes to the binding molecule which may be permitted and which may, perhaps, lead to perturbations of GluR function.

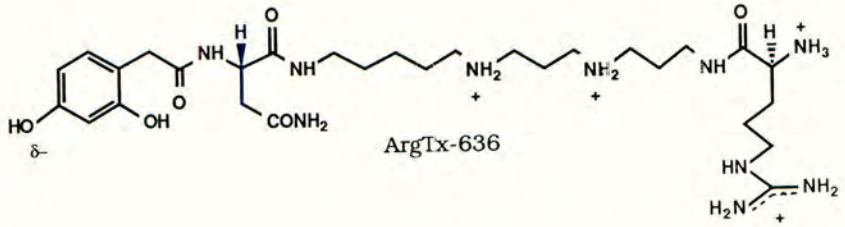
One approach to pesticide design might be to determine the structural characteristics of the QUIS-GluR binding site(s) together with an account of its chemical properties. To this end a number of laboratories have embarked upon procedures designed to isolate and structurally to characterise QUIS-GluR of insect muscle and CNS using a combination of molecular biological and electrophysiological techniques (Fraser et al, 1989). $Poly(A)^+$ mRNA isolated from leg muscles of adult and nymphal locusts and injected into Xenopus laevis oocytes leads to the translation of GluR and the appearance

of QUIS-GluR in the surface membrane of the oocytes. Although Xenopus oocytes endogenously express several types of neurotransmitter receptor GluR does not rank among their number. Appearance of GluR occurs within 5-10 days post-injection, but the success rate is low in terms of the percentage of oocytes injected which respond to Glu, i.e. 10-30%. More recent studies undertaken in our laboratory show that expression of locust QUIS-GluR can be obtained within 24h postinjection of mRNA if the latter is extracted from 9-day-old locust embryos (where mRNA production is at a peak), but the response to Glu is probably complicated by the presence of CNS mRNA in addition to muscle message. Furthermore, the use of embryos as an mRNA source does not increase the rate of successful expression. Further work needs to be done to establish whether the low success rate is associated with attempts to translate and express invertebrate message in a vertebrate vehicle. A more logical approach might be to use locust oocytes to translate and express locust mRNA. We are currently attempting this alternative strategy in Nottingham, as well as attempting to translate locust message in cell-free preparations. In the longer term it may be possible to fractionate poly(A)$^+$ mRNA obtained from locust to identify the specific message for QUIS-GluR. The success of this approach will rest largely on the QUIS-GluR being composed of homogenous sub-units. Although single channel studies of locust muscle QUIS-GluR suggest that this is likely (Kerry et al, 1988), there is no structural evidence to support such optimism.

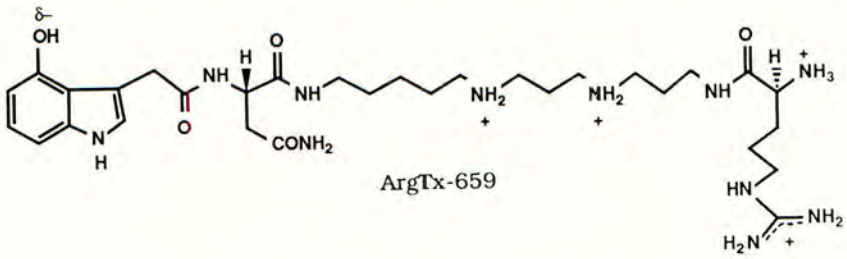
There are other reasons, of course, for using mRNA techniques for pesticide discovery. Insect CNS contain receptor macromolecules which have not yet been fully evaluated and probably receptors which have yet to be discovered. The Xenopus oocyte technique, or an analogous approach, might provide a more rapid route towards identifying and pharmacologically and structurally characterizing such molecules and, thereby, towards their exploitation by the pesticide industry. It may also provide opportunities to study receptors from nervous systems of pest insects which are too small to be investigated easily using contemporary electrophysiological approaches.

ANTAGONISTS

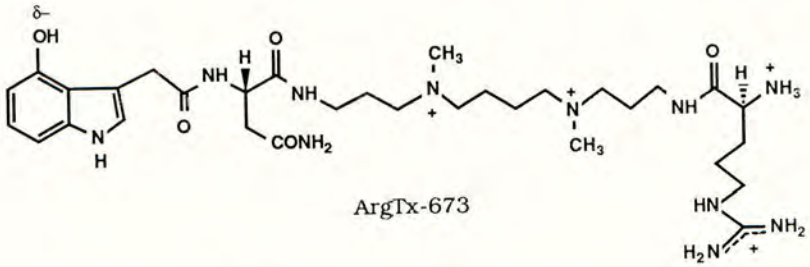
Since the discoveries of low molecular weight toxins, the polyamine amides, in the venoms of certain spiders (Usherwood et al, 1984; Bateman et al, 1985; Volkova et al, 1986; Aramaki et al, 1986; Adams et al, 1987) and parasitic wasps (Piek & Spanjer, 1986; Eldefrawi et al, 1988; Piek et al, 1988) and the demonstrations that these compounds are GluR antagonists, there have been many reviews of the pharmacological properties of this new class of compounds, particularly with respect to their use as leads for pharmaceutical and pesticide agents (e.g. Jackson & Usherwood, 1988). There is no point in retracing the ground covered in these reviews, given that little additional experimental information has been published on these compounds during the past 12 months. However, a number of issues concerning the site and mode of action of the polyamine amides remain unresolved and there are conflicting reports on their structure-activity properties.



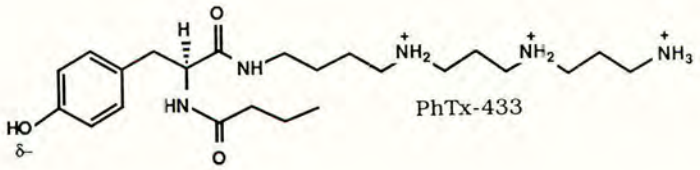
ArgTx-636



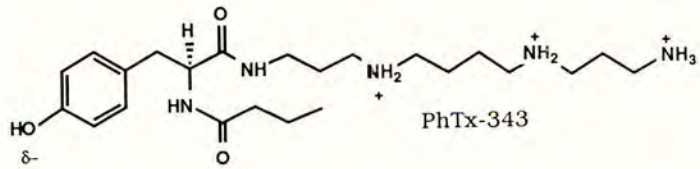
ArgTx-659



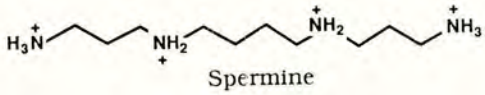
ArgTx-673



PhTx-433



PhTx-343



Spermine

Figure 1

Which components of the polyamine amides are essential for GluR antagonism?

Figure 1 illustrates the structures of naturally occurring polyamine amides and their analogues. N-Butanoyl-L-tyrosylspermine (philanthotoxin-433, PhTx-433; the numbers refer to the number of carbon atoms separating the amine groups of the polyamine moiety in the toxin) is an antagonist of insect QUIS-GluR (Eldefrawi et al, 1988; Blagbrough et al, 1989b). Pharmacological studies suggest that this antagonism is non-competitive. The analogue PhTx-343 is almost as potent as the natural product, the polyamine spermine replacing the thermospermine in the toxin. Activity is enhanced in the thermospermine analogue PhTx-334, but only by 30%. PhTx-33, an analogue with a shorter chain, and which is only capable of protonation at two nitrogen centres, is inactive at 10^{-5}M. Although thermospermine has not been tested, the isomeric polyamine spermine interacts with sheepfly larval muscle GluR (Robinson, 1980), and other results from our laboratory confirm this non-competitive antagonism, but only at mM concentrations. The polyamine amide argiotoxin-636 (ArgTx-636; molecular weight 636), together with the spider venom toxins ArgTx-659 and ArgTx-673 (Adams et al, 1987; Budd et al 1988) constitute a new class of toxins, the argiotoxins (ArgTxs). They may best be viewed as dipoles, containing a phenolic and a guanidine group at each end of the molecule; these functional groups will be charged (hydrogen bonding and ionization respectively) at physiological pH. In our structure-activity studies (Blagbrough et al, 1989a), we have prepared the first two residues of the six residue peptide-like toxin. 2,4-Dihydroxyphenylacetic acid, and the readily available 3,4- and 2,5-analogues, are not antagonists of QUIS-GluR of locust muscle. These findings contrast with those of Pan-Hou & Suda (1987) who reported that 2,4-dihydroxyphenylacetic acid inhibits L-glutamate binding at a similar concentration to that observed with a Japanese spider toxin (JSTx). When L-asparagine was acylated with this acid, the product was not an antagonist in our insect muscle preparations at 10^{-5}M. Pan-Hou et al (1987) have shown that this product is almost as active as JSTx-3 in the inhibition of glutamate binding to rat brain synaptic membranes. They propose that the acid is the functional part of the molecule, with additional binding from the L-asparagine residue (Hashimoto et al, 1987).

Competitive or non-competitive antagonism?

It has been claimed by Miwa et al (1987) that JSTx- and NSTx- are competitive antagonists of both vertebrate and invertebrate GluR, exhibiting a mode of action analogous to that of α-bungarotoxin on the nAChR of vertebrate peripheral nervous system. Michaelis et al (1984) had previously shown that venoms obtained from three spider species belonging to the Argiope family inhibit L-glutamate binding to rat brain synaptic membranes and to the glutamate binding protein that Michaelis (1975) had isolated from this tissue. It was assumed initially that the venoms contained competitive antagonists, but, like Usherwood (1985) they found high concentrations of L-glutamic and L-aspartic acids in the venoms. Therefore, at least some of the inhibition of glutamate binding was due to competition between labelled L-glutamate and the non-radioactive amino acid. More recent studies (Michaelis et al, 1988) suggest that the competition is not entirely due to these amino acids. Some other components in the venoms must be present which prevent activation of GluR of rat brain by L-glutamic acid. However, it was not established whether the action of these components is competitive as claimed by Miwa et al (1987), nor has it been established that the

components are polyamine amides. In a recent publication Yoshioka et al (1988) have developed their proposal that JSTx is a competitive antagonist of GluR by suggesting that the toxin acts as a metal chelator in removing the metal of the proposed metalloproteinaceous GluR. In contrast to this claim, there is evidence from other laboratories that both spider (G. Fagg cited in Usherwood, 1987) and wasp (K. Nakanishi, personal comm.) polyamine amides do not inhibit binding of L-glutamate to mammalian brain membranes, so unless the toxins studied by the Japanese group exhibit markedly different properties from other polyamine amides it is difficult to account for the conflicting results. It is also claimed from electrophysiological experiments on lobster skeletal muscle and a variety of other invertebrate preparations that JSTx and NSTx are competitive antagonists of GluR. However, a number of laboratories working with insect (Usherwood et al, 1984; Bateman et al, 1985; Volkova et al, 1986; Usherwood, 1985, 1987; Adams et al, 1987; Piek et al, 1988; Kerry et al, 1988), crustacean (Antonov et al, 1989) and mammalian (Priestley et al, 1989) preparations have concluded that ArgTxs are non-competitive antagonists of GluR and that PhTxs behave in a similar fashion.

Reversible or irreversible antagonism?

Despite continuing claims from Kawai and colleagues (e.g. Nakajima et al, 1988) that the polyamine amide toxins obtained from spider venoms are irreversible antagonists of GluR all other electrophysiological and biochemical studies of these compounds suggest that their action is reversible. Reports on the irreversibility of NSTx and JSTx action are based mainly on studies of GluR of lobster nerve-muscle preparations, which may interact with the polyamine amides in a manner which is quite different from that with other GluR (e.g. of insect muscle). This is an unlikely possibility since Antonov et al (1989) have shown that ArgTx-636 is a reversible antagonist of crayfish muscle QUIS-GluR. The results of the Japanese group might suggest that JSTx and NSTx differ from other polyamine amides, but JSTx and NSTx also reversibly antagonise insect muscle QUIS-GluR (Piek et al, 1988). How then can one account for the results of the Japanese group vis-a-vis those of other laboratories? A clue lies in the first report of the action of spider venom on lobster muscle GluR (Abe et al, 1983) where it was found that the action of the active component (presumably JSTx) was partly reversible after application of low 'concentrations' of toxin. The rate of recovery from treatment of locust leg muscle with ArgTx-636 is directly proportional to toxin concentration and this is also the case for rat cortical neurones (Priestley et al, 1989). The report of conversion of JSTx from an irreversible blocker into a reversible blocker after storage in the freezer (Miwa et al, 1987) supports the view that concentration may hold the key to resolving this controversy, since we know that the polyamine amides do not store well in an aqueous environment.

Open channel block?

When ArgTxs are applied to insect nerve-muscle preparations and to rat cortical neurones they antagonize GluR in a manner suggestive of open channel block, since the toxins have no effect in the absence of agonist. This was demonstrated by first equilibrating the preparations with toxin and then repeatedly applying agonist (e.g. L-glutamate). The first response to the agonist was usually the same amplitude as a control response recorded in

the absence of toxin. If the toxin were either a competitive antagonist or blocked GluR function by binding to the closed channel conformation of the unliganded receptor then one would not expect to observe this use-dependent antagonism.

Kerry et al (1988) employed single channel recordings to study the influence of ArgTx-636 on locust muscle QUIS-GluR and confirmed that the antagonism exhibited in this system could be explained by binding of the toxin to the open channel conformation of this receptor. However, Magazanik et al (1986) proposed that part of the non-competitive antagonism exhibited by ArgTx-636 is due to binding of the toxin to the closed GluR channel. This idea has been elaborated in a recent study by Anotov et al (1989) on single QUIS-GluR of crayfish muscle. One mechanism, which is potential-dependent, can be relieved by raising the membrane potential above -100mV. The second type of block was termed "flickering block" in which channel openings were interrupted by high frequency brief closing events. However, this is not classical open channel block, where the blocking and unblocking rates of the non-competitive antagonist are high (Neher & Steinbach, 1978), since against expectations there was a clear shortening of the total open time per channel opening. This "flickering block" was voltage-independent, which contrasts with the open channel block by ArgTx-636 of blowfly muscle GluR recorded by the same workers. Although the crayfish muscle studies challenge the notion that block of GluR by ArgTx-636 is not only at the level of the open channel, Antonov et al (1989) agree that for both types of antagonism agonist is necessary for the development of block. Their suggestion that the toxin binds to the, presumed short-lived, agonist-GluR closed channel state, and, thereby, prevents the channel from opening, deserves consideration, although having argued that agonist is essential for block they then propose that the toxin may also bind to GluR in the absence of agonist. Clearly more studies are required to determine if, and to what extent, the binding of toxin to the closed channel conformation of GluR contributes to antagonism by the polyamine amides.

Specific antagonists of GluR?

Since ArgTx-636 is an open channel blocker of GluR some caution should be exercised over claims that this and other polyamine amides are specific antagonists of GluR (Usherwood, 1987). The relatively non-selective channels gated by GluR, nAChR etc. are likely to share many pharmacological properties in common and there is already a wealth of scientific literature attesting to this (Usherwood & Blagbrough, 1989). It comes as no surprise, therefore, to discover that ArgTx-636 blocks the channel gated by nAChR of frog skeletal muscle (Magazanik et al, 1986) and that the related polyamine amide PhTx-343 (Eldefrawi et al, 1988) blocks transmission postsynaptically at the cholinergic synapses of frog skeletal muscle, inhibits the response of the cockroach coxal depressor neurone to acetylcholine and competes with the channel blocker perhistrionicotoxin (a non-competitive antagonist of nAChR) for binding sites in membranes isolated from Torpedo electroplax and honeybee brain (Rozental et al, 1989).

Specific antagonists of non-N-methyl-D-aspartate GluR?

It is understandable that the discovery of antagonism of arthropod QUIS-GluR by the polyamine amides should initiate studies to determine whether these toxins specifically antagonize this class of GluR. JSTx has been reported to block responses to kainate and quisqualate in rat hippocampal neurones (Akaike et al, 1987). Antonov et al (1987) showed that ArgTx-636 blocks dorsal-root potentials and the action of L-glutamate in the isolated frog spinal cord, both effects presumed to be largely mediated by non-NMDA GluR. A further indication of the interactions of polyamine amides with non-NMDA GluR was obtained by Sugiyama et al (1987) who showed block of kainate currents by JSTx in Xenopus oocytes injected with rat brain mRNA. These data suggest that the polyamine amides may be specific antagonists of non-NMDA receptors. However, the ability of non-competitive antagonists to bind to ligand-gated membrane receptor channels does not depend upon the agonist sensitivity of the receptor. The strength of this argument is made clear by the recent work of Priestley et al (1989) who showed that ArgTx-636 is an open channel blocker of the NMDA receptor in rat cortical neurones, with at least a 30-fold selectivity over QUIS- and KAIN-GluR. One possibility is that the ion channels gated by the three classes of GluR in vertebrate CNS vary from site to site within the nervous system.

Glutamate re-uptake block and possible presynaptic effects?

Van Marle et al (1983) and Piek et al (1988) have shown that relatively high concentrations of PhTx-433 inhibit uptake of L-glutamic acid into glia and axons terminals at locust glutamatergic nerve-muscle junctions. JSTx and NSTx increased uptake of L-glutamic acid at locust nerve-muscle junctions, but according to Pan-Hou et al (1989) JSTx inhibits L-glutamate uptake by rat brain synaptosomes. Differences in the actions of spider and wasp polyamine amides are seen when testing these compounds on isolated locust nerve-muscle preparations and using reduction in amplitude of the neurally-evoked twitch contraction as the measured parameter (Bateman et al, 1985). ArgTx-636 causes a use-dependent reduction in twitch amplitudes, whereas addition of PhTx-343 produces complex changes including a reduction in twitch amplitude and an enhancement of activity with repetitive responses to single neural stimuli following removal of the toxin (Eldefrawi et al, 1988). The complex responses to wasp toxin may be due to the effects of PhTx-433 on glutamate uptake leading to prolongation of the excitatory postsynaptic current and perhaps repetitive firing of the muscle fibre electrically-excitable membrane. However, in evaluating the effects of polyamine amides on insect glutamatergic synapses one needs to take into account the presence of GluR in the axon terminals of locust excitatory motoneurones (Dowson & Usherwood, 1972), which may lead to additional depolarization of the axon terminal if the rate of transmitter removal from the synaptic cleft is depressed by polyamine amides.

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INSECT NICOTINIC ACETYLCHOLINE RECEPTORS AS TARGETS FOR INSECTICIDES.

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ABSTRACT

The voltage- and ligand binding-activated ion channel complexes of the nervous system are important targets for bioactive compounds both in pharmaceuticals and pest control. The continuing definition of pharmacologically distinct subtypes raises the prospect of species-specificity in the effects of compounds active at these receptors. Such receptors are central to the normal activity of the nervous system, they have structures and functions that can be studied quantitatively, and they are accessible to molecular genetic and CAMM analysis, all properties that should facilitate the target-directed synthesis and lead optimisation comprising "biorational" insecticide development. The cholinergic synapse is a proven site of action for successful insecticides, and the nicotinic cholinergic receptor thus epitomises the kind of target suited to biorational research. The nitromethylene heterocycle insecticides evoke the form of activity aimed for in receptor-active leads. They are selective and potent nicotinic antagonists with pronounced agonistic effects at higher concentrations. Compounds, such as these, that are highly potent, target-specific and display a complex mode of action, likely to cause maladaptive changes in the activity of the insect nervous system, exhibit the optimal characteristics to be sought in the most promising leads.

INTRODUCTION

The site of action of most modern insecticides is the insect nervous system and, indeed, there are good reasons why the nervous system should remain a major focus in the search for new insecticides. From the insecticide development angle, the activity of the nervous system is accessible to *quantitative* monitoring by well-tried methods (extra- and intracellular electrophysiology, patch clamping, binding assays). Furthermore, many potential leads are available from research in the pharmaceutical field where the properties that make a compound uninteresting as a therapeutic agent can be the very characteristics called for in a pesticide. From the viewpoint of being a likely target, the nervous system has the virtue of being central to the normal functioning of the insect on a short time scale. In contrast to compounds that affect reproduction or development, neurally active insecticides can provide rapid knockdown, in some cases by affecting only a small proportion of the molecular target sites. For example, doses of pyrethroids that elicit repetitive firing and symptoms of poisoning probably alter the

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gating mechanism of less than 1 % of the voltage-gated sodium channels in the axons (Lund & Narahashi, 1982).

MOLECULAR TARGETS FOR PESTICIDES IN THE INSECT NERVOUS SYSTEM

The molecular targets within the nervous system are the voltage- and ligand-activated ion channels and the associated ligand recognition sites. For simplicity, these complexes are referred to below as "receptors". These molecules exhibit several useful properties. They are discrete in structure and function so that structure/activity correlations can be investigated in quantitative detail. This means that the mode of action as well as the absolute potency of a lead at its actual site of action can be determined. This is essential for biorational lead optimisation, since any non target-associated influences affecting the efficacy of the lead and its analogues (e.g. metabolism, blood brain barrier) nullify the value of quantitative comparison. Some of these channels and receptors play a major rôle in the correct functioning of the nervous system, so that interference with them results in significantly maladaptive changes in neural activity. Perhaps most importantly of all, from the perspective of current industrial and social requirements, some of them offer the possibility of designing insecticides specific to pest insects. The remainder of this paper is an attempt to show how this is might be achieved.

Receptor diversity and classification

Over the past three to five years, a virtual revolution has begun in our conception of transmitter receptors and their structural, pharmacological and evolutionary relationships with one another. The cause has been two-fold. Firstly, as recent advances in molecular genetic cloning techniques have been applied to the receptor proteins in the nervous systems of both vertebrates and invertebrates, comparison of their genes and primary amino acid sequences has revealed that receptors are encoded by multigene superfamilies. The members of these superfamilies have apparently diverged during evolution resulting in a hitherto unsuspected diversity of subtypes. Secondly, there has been and continues to be a dramatic increase in the number of highly specific, receptor-binding drugs and consequently a proliferation of newly characterised receptor subtypes that has stretched the traditional pharmacological classification to its limits. It is likely that a new classificatory scheme for receptors will emerge in the near future to reflect presumed evolutionary relationships or at least the degrees of sequence homology, as well as to rationalise the growing chaos of pharmacologically defined subtypes (Barnard, 1988; Benson, 1988a,b; Hartig, 1988). For our purposes, these developments provide an even more significant message: there are likely to be differences between closely related receptors occurring in different phyla and perhaps even different species. The implication of this is that there is a real possibility to discover or design compounds that act on receptors in pests but which are inactive on the related receptors in other species.

Modelling receptor structure and function

The nicotinic acetylcholine receptor from the *Torpedo* electric organ, a modified muscle, was the first receptor to be purified, and in 1982 the primary structures of two of its subunits were deduced from their cDNA sequences (Ballivet *et al.*, 1982; Noda *et al.*, 1982). Since that time, the entire pentameric structure has been elucidated and the quaternary structures and amino acid sequences of several other receptors and ion channels have been

reported. Modelling the three-dimensional structures of receptors depended for many years on inferences derived from changes in function induced by biochemical, immunological and photoaffinity probes, as well as from the characteristics of natural toxins and other ligands and channels blockers. Now, as the list of known primary structures grows, efforts are being made to identify the amino acid sequences forming the ligand recognition site itself, as well as other important functional domains of the molecule such as the ionophore. The nicotinic receptor has been studied with particular intensity (reviewed by Lindstrom *et al.*, 1987).

For the purposes of the insecticide scientist, these recent research trends point towards the concept of biorational design of compounds that will bind in a predictable manner to a pre-selected site on the chosen molecular target, evoking a predictable and desirable effect (Geissbühler *et al.*, 1983). How can this be done? A structural understanding of the functionally important parts of a receptor or an ion channel at the atomic level has yet to be achieved, but progress in this direction is accelerating as several important techniques improve and come into wider use. These techniques include CAMM (computer-aided molecular modelling) and heterologous expression of the *m*RNA for receptor subunits and even smaller fragments, in expression systems such as yeasts, bacteria and the *Xenopus* frog oocyte.

The application of CAMM methodology to the problem of molecular target-directed insecticide design falls, in principle, into two broad fields. On the one hand, there is modelling by conformational search through the structures of compounds known to act at the target, the search for a common "pharmacophore" that could be a basis for synthesis of novel ligands (e.g. Sheridan *et al.*, 1986). One potential limitation of this approach is that it is currently difficult to predict changes in the conformation of the ligand that might take place during the binding reaction. Odell (1988) comprehensively discusses other limitations and possibilities in relation to a pharmacophore CAMM study of the cholinergic nitromethylene heterocycle insecticides described below. It cannot be over-emphasised that a synthetic programme oriented around CAMM-based optimisation of a receptor-directed lead is crucially dependent on quantitative feedback from assays carried out directly at the molecular target site.

The second broad field involves modelling from the known structure of the target itself. Of the two CAMM fields, only modelling from known active compounds is currently practicable. The currently available combination of CAMM software and databases cannot unambiguously or even fully model receptor functional domains to the atomic level, especially the form of the molecule existing *in situ* (aqueous medium, lipid and other associated material *etc.*). However, data are rapidly accumulating to indicate the identities and approximate conformations of the primary sequences contributing to the nicotinic ion channel selectivity and channel blocker binding (the hydrophobic segment M2 and its vicinity; Imoto *et al.*, 1988, Leonard *et al.*, 1988), and the acetylcholine recognition site (the α-subunit peptide 188-201; e.g. Gotti *et al.*, 1988). It seems to be generally agreed that for the foreseeable future improvement in CAMM approaches to receptor structure will depend on the accumulation of empirical data correlating amino acid sequences with experimentally determined three-dimensional structures. Working from basic principles upwards appears to be both theoretically and practically impossible on this scale. It is unclear whether advances in the CAMM technique will be sporadic, depending on quantal improvements in concepts, or slow and continuous as the fund of empirical data increases and already existing but scattered information becomes more readily accessible. Structural information from x-ray crystallography awaits production of high quality crystals of receptor protein.

THE INSECT NICOTINIC RECEPTOR

Purification, molecular genetics and heterologous expression

Despite the allocation of comparatively fewer resources to the isolation and molecular genetics of insect nicotinic receptors, considerable progress is be ng made. An α-bungarotoxin-binding *Locusta* neuronal nicotinic receptor has been purified and it is hypothesized to consist of four similar or identical subunits (Breer *et al.*, 1985). The same receptor was subsequently reconstituted in an artificial lipid bilayer and single receptors that behave similarly to functional receptors *in situ* could be activated with nicotinic agonists (Hanke & Breer, 1986). The expression of functional *Locusta* nicotinic receptors in *Xenopus* oocytes injected with locust neural *m*RNA has also been demonstrated (Breer & Benke, 1985). These essentially classical biochemical experiments are now being supplemented by gene cloning. Hermans-Borgmeyer *et al.* (1986) isolated two overlapping cDNA clones encoding a non-ligand binding subunit from a *Drosophila* nicotinic receptor, and the putative ligand binding subunit gene has also been isolated by Bossy *et al.* (1988). The gene for an unidentified, putatively-nicotinic subunit was isolated by Wadsworth *et al.* (1988) and shown to be expressed in the *Drosophila* CNS. There is considerable conservation in the organisation of nicotinic receptor genes between vertebrates and insects (40 to 45% of the amino acids have been conserved since their divergence), but they are by no means identical (Sawruk *et al.*, 1988; Bossy *et al.*, 1988; Wadsworth *et al.*, 1988). In both these insect neuronal cDNA clones, the degree of homology in the deduced amino acid sequences is greater with the vertebrate *neuronal* nicotinic receptor which, unlike its muscular counterpart, does not bind α-bungarotoxin. Analysis of landmark residues within the ligand-binding subunit sequence and expression of the relevant polypeptide sequence suggested to Bossy *et al.* (1988) that their *Drosophila* receptor is not of the α-bungarotoxin binding type. Functional α-bungarotoxin-insensitive nicotinic receptors are rare in *Locusta* (where easier neuronal electrophysiology has provided more information on functional receptors than in *Drosophila*), but they do occur on certain identified *Locusta* neurones (Goodman & Spitzer, 1979) and have been located in cultures of embryonic cockroach brain neurones (Lees *et al.*, 1983). It is to be hoped that the cDNA for the more commonly observed α-bungarotoxin-sensitive insect nicotinic receptor will be isolated soon. The observations made so far are intriguing in themselves and promise advances in the near future that will facilitate the development of the kind of CAMM-based biorational synthesis described above.

Recently, Lester (1988) has brought together the arguments in favour of utilising the *Xenopus* oocyte and mammalian cell line expression systems as a way to test pharmaceutical leads on specific receptor subtypes. This method, called heterologous expression by Lester, also has applications in insecticide research. As noted above, insect nicotinic receptors are readily expressed when the appropriate *m*RNA is injected into *Xenopus* oocytes, and glutamate receptors as well as A-type voltage-sensitive potassium channels from insects have been expressed by the same method (Saito *et al.*, 1987; Iverson *et al.*, 1988; Timpe *et al.*, 1988; MacKinnon *et al.*, 1988). The advantage of this approach is that *Xenopus* oocytes are ideal for electrophysiology. This means that the receptors from pest insects that are too small for electrophysiology, as most of them are, become available for the detailed structure-activity studies required for biorational design. In addition, by this means more attention could be given to synaptic receptors which might differ from the somal receptors that are the object of most current insect electrophysiology.

Another advantage of heterologous expression is that the appropriate system can produce the large quantities of receptor protein required for crystalisation as a step towards obtaining structural information by X-ray crystallography. Individual injection of oocytes is not a practical approach to obtaining the quantities of protein required. As Lester has pointed out, so far there have been no reports of successful expression of receptor proteins using yeasts and bacteria, but he sees transfected mammalian cell lines as a feasible alternative (Lester, 1988).

Pharmacology

The pharmacology of vertebrate nicotinic receptors and to a lesser extent those of the insects is well-known and will not be discussed in detail here (reviewed most recently by Eldefrawi & Eldefrawi, 1988). Broad similarities between the insect synaptic and somal nicotinic receptors and both muscular and neuronal nicotinic receptors in vertebrates are sufficient to show that these receptors are all products of the same gene family. There are, however, many differences in pharmacological detail (Benson & Neumann, 1987; Benson, 1988a,b,c). For practical purposes (binding assays, probing cDNA expression libraries), one of the most important differences is that many insect neuronal nicotinic receptors bind α-bungarotoxin, as described above, and this toxin is readily radiolabelled. Unlike at the vertebrate muscular nicotinic receptor, antagonism by α-bungarotoxin binding at the insect nicotinic receptor is reversible (Benson & Neumann, 1987; Benson, 1988b).

Specificity

Is there any evidence that the differences in pharmacological profile between nicotinic receptors in different species could be of practical significance with regard to pesticide specificity? Not much quantitative comparative electrophysiological information has been published, but a good example is provided by the anthelmintics Morantel, Pyrantel and Levamisole. These compounds are used for the treatment of both larval and adult nematode infections in domestic animals. Harrow and Gration (1985) showed that they act as agonists at the acetylcholine receptor on the muscle cells of the nematode *Ascaris*, and that relatively high concentrations ($> 10^{-5}$ M) of Morantel blocked the effects of acetylcholine. The order of potency was Morantel = Pyrantel > Levamisole > acetylcholine, with the agonist threshold for Morantel and Pyrantel being between 10^{-8} and 10^{-7} M. In contrast, when tested on the locust neuronal soma using the methods described below, these compounds were low potency blockers of the nicotinic cholinergic response and were nicotinic agonists at concentrations above 3×10^{-5} M. All were less potent than acetylcholine itself on this system. Not surprisingly, they possess only weak insecticidal and acaricidal activities. These observations corroborate the idea that the differences among receptors belonging to the same family but occurring in different phyla, if not species, are sufficient to provide an opportunity for exploitation by pesticide chemists.

Cholinergic insecticides

Nicotine itself is a naturally occurring insecticide that has evolved in tobacco plants presumably as a defence against insects. It has been a commercial insecticide, reaching a peak use in the USA of 1.2 million pounds in 1944 (Metcalf, 1948), and its mode of action is certainly as a potent agonist at nicotinic receptors. Cartap, (4-*N*,*N*-dimethylamino-1,2-dithiolane),

appears to break down *in vivo* to form a compound identical to a toxic component, nereistoxin, found in the venom of the polychaete worm, *Lumbriconereis*. The nereistoxin acts both as an agonist and a blocker at the nicotinic receptor (Eldefrawi *et al.*, 1980), a combination that we shall see again in relation to the nitromethylene heterocycle insecticides. Of far greater economic significance are the organophosphates and carbamates which are anticholinesterases. Their primary mode of action is to decrease the rate of hydrolysis of acetylcholine in the vicinity of the post-synaptic nicotinic receptors at cholinergic synapses. This prolongation of the synaptic event is assumed to be the basis of their insecticidal action. These compounds are well known and they need not be considered further here. The important conclusion is that very successful insecticides exist that act via cholinergic synapses, and this is in itself sufficient evidence to establish the importance of this target in the life of the insects. In addition, it is worth noticing again that the target molecule should be an important functional component of the nervous system: "subtle effects", especially if they are inhibitory or mediated by uncommon receptor types, are not likely to provide a mode of action for a successful insecticide. A negative aspect of focusing on a target such as the nicotinic receptor is that single-gene-coded molecular targets are, at least in principle, more accessible than "dispersed" targets to the selective pressures leading to resistance. On the other hand, they are less likely to be by-passed or to tolerate selection-induced modifications if they are fine-tuned to highly specific and essential functions.

Nitromethylene heterocycle insecticides

An attempt has been made above to show how, in principle anyway, one might set about designing an insecticide. But what kind of action is called for at the receptor? What type of activity is displayed by likely leads? Soloway *et al.* (1979) reported that certain nitromethylene heterocycles (NMH) are extraordinarily active towards insects, especially lepidopterous larvae, but affect vertebrates only weakly. The NMHs are active by both contact and oral ingestion. In addition, they are relatively non-persistent in the environment, a combination of properties rather attractive to insecticide chemists. Schroeder & Flattum (1984) deduced that one site of action of these compounds in the cockroach is the cholinergic synapse in the sixth abdominal ganglion. They observed that micromolar concentrations elicited an increase in giant axon activity followed by inhibition, but that the giant axon membrane itself was unaffected. Furthermore, 5×10^{-3}M d-tubocurarine (a nicotinic blocker) and a very high dose of atropine (a muscarinic blocker) suppressed the giant axon discharges induced by the NMH, although 10^{-6}M α-bungarotoxin did not. They tentatively concluded that the NMHs act on the post-synaptic nicotinic receptors at this synapse. This was confirmed by Harris *et al.* (1986) who showed in addition that these compounds can act as cholinergic agonists at the nicotinic receptors on insect neuronal somata. See Odell (1988) for a CAMM-based comparison of the conformations of acetylcholine and the NMHs.

To determine the mode of action of the NMHs more precisely and to resolve some of the pharmacological paradoxes, 1-(pyridin-3-yl-methyl)-2-nitromethylene-imidazolidine (PMNI) (Shiokawa *et al.*, 1984) was tested against the somal cholinergic receptors on isolated neuronal somata from the thoracic ganglia of the locust. The method used was as described previously (Lees *et al.*, 1987). These isolated somata can be readily voltage-clamped in order to characterise the membrane currents activated by various transmitters (Neumann *et al.*, 1987; Benson, 1988a,c). It has been shown that the somal membrane is endowed with both nicotinic (ACh1) and muscarinic (ACh2) receptors (Benson & Neumann, 1987; Benson, 1988a) so that the effects of test compounds on the two receptor types can be monitored simultaneously using

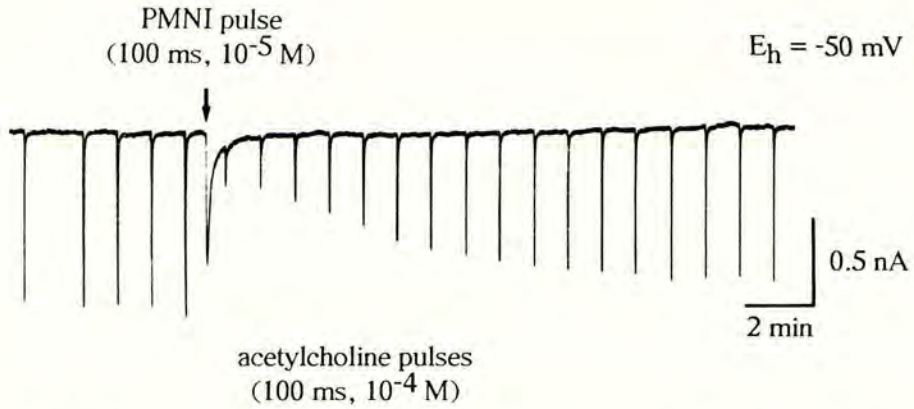

Fig. 1. Chart record showing the membrane current recorded from an isolated locust neuronal soma voltage-clamped at a holding potential (E_h) of -50 mV. Pulses (100 ms) of acetylcholine (10^{-4} M) were applied to the soma via a pressure micropipette at regular intervals resulting in transient inward current (downward deflections). A single pulse (100 ms) of 1-(pyridinyl-3-yl-methyl)-2-nitromethylene-imidazol (PMNI) (10^{-5} M) was similarly applied (arrow), evoking a slower inward current and a decrease in the amplitude of the responses to acetylcholine.

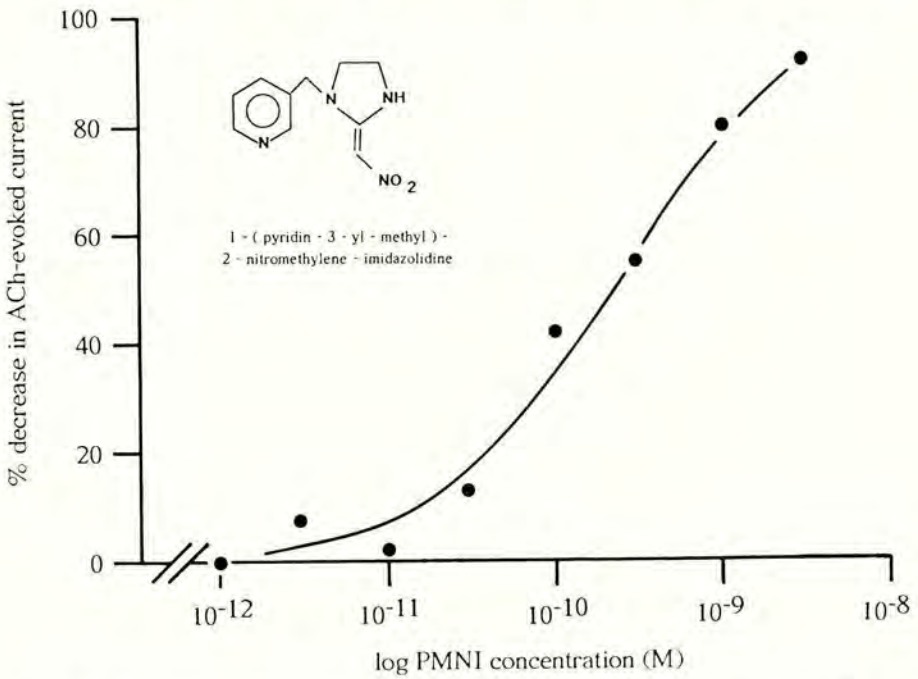

Fig. 2. Dose response curve showing the concentration-dependence of the antagonistic effect of bath-applied PMNI on the amplitude of the response of an isolated voltage clamped neuronal soma to pulse-applied acetylcholine (100 ms, 10^{-4} M, E_h = -50 mV).

the same neurone. The ACh1 receptors are apparently about 100-fold more numerous than the ACh2 receptors. The former are blocked by low doses of α-bungarotoxin and are closely related, if not identical, to the synaptic nicotinic receptors in insects (Benson, 1988b), while the latter exhibit pharmacological resemblances to vertebrate m_1 muscarinic receptors (Benson, 1989).

Pressure application of a 100 ms pulse of 10^{-5} M PMNI onto a voltage-clamped locust thoracic neuronal soma evoked a fast, transient inward current, following which the responses to pulses of acetylcholine were reversibly reduced in amplitude (Fig. 1). Bath application of increasing doses of PMNI induced a dose-dependent decrease in acetylcholine-evoked inward current and an inward shift in the base-line current at higher concentrations. The EC_{50} for the antagonistic effect was $1.0 \pm 0.8 \times 10^{-10}$ M (m ± SD, n = 3) (sample dose response curve in Fig. 2), while the threshold for the inward shift in base-line (agonism) was about 3×10^{-8} M. Further experiments showed that the current activated by higher doses of PMNI had the same voltage-dependence as the ACh1 response and was blocked by 10^{-6} M α-bungarotoxin, 10^{-6} M mecamylamine and 10^{-5} M d-tubocurarine but not by 10^{-5} M atropine. In addition, 10^{-8} M PMNI had no effect on the ACh2 (muscarinic) response of these neurones. These results demonstrate that PMNI is a highly specific, nicotinic antagonist that probably has its synaptic action by causing total blockade of the nicotinic receptors. It is only at comparatively high doses that the compound acts as an agonist, and application at these higher doses can evoke abnormal excitation which is followed by blockade. These effects are most likely the basis of its insecticidal activity. The high doses used, in combination with the very high potency of PMNI and other NMHs, even in comparison with mecamylamine and α-bungarotoxin, account for the failure of Schroeder & Flattum to observe blockade of the NMH response by α-bungarotoxin. Similarly, atropine, although it is a selective muscarinic antagonist, will block ACh1 (nicotinic) receptors if applied at sufficiently high concentrations (J.A. Benson, unpublished observations).

The NMHs exhibit the characteristics to be sought in the most promising leads. They are highly potent; indeed, they are active at concentrations lower than the three compounds most active to date at the ACh1 receptors in locust (α-bungarotoxin, lobeline and mecamylamine; J.A. Benson, unpublished observations). In addition, they are receptor-specific and display a complex mode of action likely to cause maladaptive changes in the activity of the insect nervous system.

CONCLUSIONS

The cholinergic synapse and the Na channel of the insect nervous system are the sites of action of important insecticides. The nicotinic receptor continues to provide an ideal target for biorational design of insecticides. Like the Na channel, it combines with a central rôle in neural activity the discrete structure and function, and accessibility to molecular genetics and CAMM, common to all the ion channel and receptor proteins. However, in apparent contrast to the Na channel, the nicotinic receptor shows a variation among species and different parts of the nervous system that might provide a basis for species specificity in nicotinic insecticides. Potent leads active at this receptor are known and their activity can be monitored quantitatively. They seem to be characterised by a complex mode of action involving both agonism and antagonism.

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2.
Non-nervous System Targets for Chemicals

Chairman: DR J. P. FISHER

THE INTEGUMENT AS A TARGET FOR INSECTICIDES: DISRUPTION OF CUTICLE CHEMISTRY, STRUCTURE, AND FUNCTION

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ABSTRACT

The insect cuticle presents a potentially vulnerable and specific target for the lethal disruption of its chemistry, structure and function by insecticides. Such chemicals can interfere directly with the synthesis of cuticle macromolecules or their post-depositional modification. Two successful commercial insecticide classes are discussed: acylureas, known to inhibit chitin synthesis, and substituted melamines, which appear to interfere directly with the cuticle's mechanical properties.

THE INTEGUMENT AS A TARGET

The importance of the cuticle

Insects and other Arthropods differ from vertebrates and most other animal taxa in their reliance on a stiff exoskeleton or cuticle made of chitin and protein and impregnated with lipids, phenolics and other small molecules. The cuticle is a layered structure, laid down by an underlying sheet of epidermal cells. The layers of the cuticle constitute a record in space of the changing secretory actvities of the epidermis through time. The whole tissue, cellular epidermis plus extracellular cuticle, is referred to as the integument.

The integument is the insect's interface with the environment. Not only does the cuticle serve to support the insect's body shape and provide the necessary leverage for locomotion, but it also serves as a waterproofing layer; body armour that defends against predators, parasites and pathogens; and the raw material from which offensive and defensive structures, mouthparts, and even sense organs are constructed. Many authors have commented that the evolutionary success of the insects is based largely on the extraordinary variety of form that the possession of a cuticle has permitted. The importance of the cuticle in the insect's life must make it a vulnerable target for insecticides.

Moulting

Furthermore, because it covers the external surface of the body completely, the possession of a cuticle has profound implications for the way in which an insect grows and develops. The relative stiffness and inextensibility of the cuticle means that growth in size can only be accommodated by its periodic replacement.

Moulting in insects is a dramatic affair that involves the whole body. Although the actual shedding of the old cuticle is often accomplished in minutes (Reynolds, 1980), the requirement that this event is preceded by the laying down of a new cuticle means that the whole moulting process can take anything from days to weeks. The synthesis of the materials of a new cuticle and the digestion and recycling of the old one lead to massive fluxes of raw materials through the insect's metabolic pathways (Ziegler, 1985). At the same time, the process of renewing cuticular structures associated with feeding imposes a state of temporary starvation during moulting. The temporary loss of waterproofing

is accompanied by increased water loss (Wigglesworth and Gillett,1936). The use of a hydrostatic skeleton during ecdysis requires an increased blood volume, so that insects retain water prior to the moult and actively lose it thereafter (Reynolds, 1980). The vulnerability of the moulting insect to predation and accidental damage leads to the seeking out of safe places. The difficulty of escaping the old cuticle leads to the adoption of special ecdysial behaviour patterns (Reynolds, 1980). The soft and extensible mechanical properties of the new cuticle, essential to allow successful moulting, are inappropriate to normal behaviour, and so moulting is accompanied by the localised incorporation into the cuticle of reactive sclerotising chemicals that cross-link cuticle macromolecules (Suguruman, 1988). The correct sequence of all these processes is assured by the orderly succession of endocrine timing events involving several hormones (Reynolds,1987a).

From all this it is evident that disruption of moulting must be very damaging to the affected insect. A sure way to derange moulting will be to interfere with the structure and/or chemistry of the cuticle. Because so much of the integument's synthetic and metabolic activity is compressed into the moult, it will not be surprising to find that insects with damaged integuments commonly succumb at the time of ecdysis.

Why the integument is a selective target.

The Arthropod integument is a characteristic feature that is not shared with any other major Phylum (Barnes, 1987).

Vertebrates do not have an exoskeleton. Most Molluscs have a shell, but this does not completely cover the body surface. Although several Invertebrate Phyla have complete cuticles, including Trematodes, Cestodes, Pseudocoelomates (including Nematodes), and Annelids, these do not resemble the Arthropod cuticle in structure. Only some minor Phyla (eg Onychophorans, Tardigrades), thought in any case to be related to Arthropods, have a cuticle somewhat resembling that of the Arthropod type (Neville, 1975).

The Arthropod cuticle has a remarkably similar structure throughout the Phylum (Figure 1). In all cases, an external epicuticle bounds a thicker procuticle. The procuticle is a fibre-composite type material, with a helicoidal array of very long, thin, crystalline rods of chitin embedded in an apparently formless matrix of impregnated protein. Wax and cement layers on the outside of the epicuticle are more variable. The cuticle of Crustacea is calcified, but even here, it is easy to see in decalcified specimens that the underlying structure of the organic matrix is of the typical Arthropodan type.

The characteristic structural macromolecule of the Arthropod cuticle is chitin (see below). Chitin is completely absent from animals of the Deuterostome branch (Echinoderms, Vertebrates), and occurs only sporadically in animals other than Arthropods (Rudall and Kenchington, 1973; Muzzarelli, 1977). In the majority of cases where it does occur, this non-Arthropod chitin is of a different crystallographic form (beta or gamma rather than alpha). Chitin occurs in some algae, but is absent from higher plants. Only in Fungi is chitin relatively common, and even there shares its structural role in the cell wall with several other polysaccharides. As we shall see, there is reason to think that the process of chitin synthesis is substantially different in Fungi from that in Arthropods, and it may be that Fungal chitin is structurally dissimilar too.

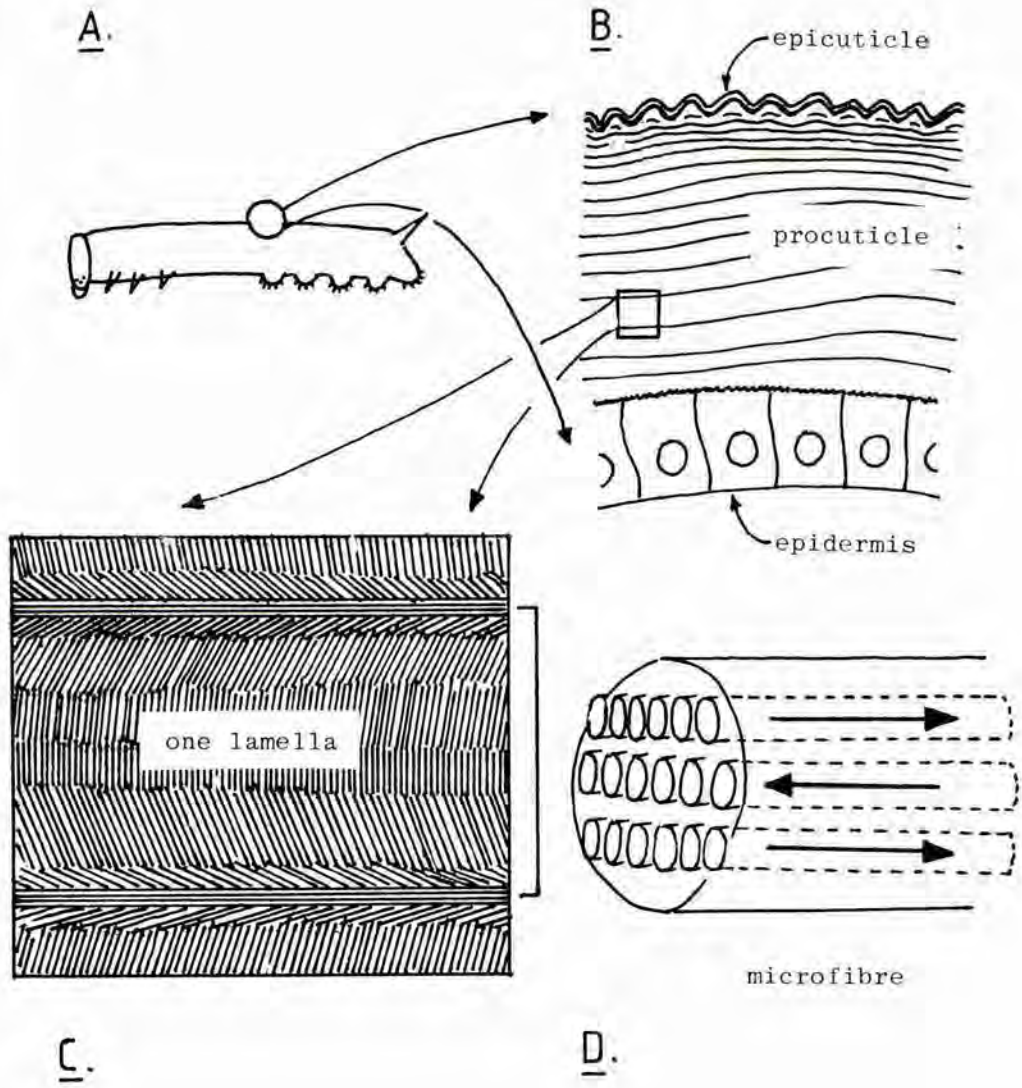

FIGURE 1

The structure of insect cuticle.
A The cuticle is an exoskeleton that completely covers the body surface; B In cross-section the cuticle is revealed as a layered extracellular matrix attached to an underlying sheet of epidermal cells; C The layers within the procuticle correspond to the rotation through 180° of a helicoidal array of chitin rods; D Each alpha-chitin microfibre is 2.8 nm in diameter and contains 18 individual poly(GlcNAc) chains held in a crystalline array by interchain H-bonds. Nearest neighbour poly(GlcNAc) chains are antiparallel.

Most insect cuticle proteins so far identified (eg Snyder et al, 1982; Henzel et al, 1985; Hojrup et al,1986; Rebers and Riddiford, 1988) are not homologous with any other known proteins, from Vertebrates or anywhere else. There is some similarity between the N-terminal sequences of one family of proteins isolated from rigid regions of cuticle in the Lepidopteran Hyalophora cecropia and of some kinds of intermediate filament proteins (Willis, 1989). However, there is so far no evidence that the insect cuticle contains proteins closely related to collagen or keratin, proteins which are the principal components of epidermally secreted structures in other animals.

Thus we may say that there are good reasons to think that the Arthropod integument will present potential targets for novel insecticidal chemicals that are not shared by other organisms. The opportunity for such selectivity seems like a golden opportunity. Of course, there will be many occasions when even this degree of selectivity is inadequate. Arthropods other than insects are important in both terrestrial and aquatic ecosystems, and may be affected by integument-specific insecticides.

HOW TO DISRUPT THE INTEGUMENT

In what follows I shall only deal with the disruption of cuticle at the level of synthesis and post-secretory modification. I shall exclude the disruption of developmental hormone systems that indirectly leads to disruption of the timing of moulting, malformed cuticle morphology etc, since this is dealt with elsewhere in this monograph.

Potential and actual targets within the integument

The rich complexity of cuticle chemistry, structure and function, only roughly sketched in the treatment above, affords several potential targets (Chen and Mayer, 1985; Reynolds, 1987b). We may group these together according to the integumental functions that we would hope to disrupt.

One category of damage that would be lethal would to impair the function of the cuticle as a barrier to water loss. As small terrestrial animals, insects are frequently subject to dehydrating conditions (Edney, 1977). Inert dusts can achieve this kind of damage, either by abrasion or sorption of cuticle lipids (Chen and Mayer, 1985). Another approach to this end would be to interfere with the synthesis and secretion of cuticle lipids. To my knowledge, no commercial insecticide works in this way, although some compounds have been synthesised that inhibit sterol metabolism in insects, and which have growth regulator (IGR)-like effects (Svoboda et al,1972). This has been attributed to their effects on moulting hormone (ecdysteroid) levels, but effects on cuticle lipids are possible too.

Another lesion that we could hope to inflict on the integument would be to impair the usefulness of the cuticle as a mechanical support. This might be achieved by decreasing or increasing its stiffness. Damage of either kind would prevent the insect behaving normally. Movement would be impaired, also the ability to feed. We might expect that reproduction would be secondarily affected, because the developing embryos would be inviable. This kind of damage to the cuticle's mechanical function will have particularly serious consequences at the times of hatching and ecdysis, when the new cuticle must be flexible enough to allow extrication from the confines of the old, but yet is under considerable stress.

Compounds inhibiting chitin synthesis

Since chitin is so characteristic of Arthropods, a particularly promising potential target for an integument-specific insecticide would appear to be some aspect of chitin metabolism. This might include biosynthesis or degradation.

Many compounds have been reported to disrupt chitin synthesis. Full reviews of chitin synthesis inhibition that list such chemicals have been published (eg Marks et al, 1982; Cohen, 1987). It must be said that many of the compounds reported to inhibit chitin synthesis are rather ineffective and only act at high concentrations; I will not attempt to be exhaustive in discussing them here. However, at least two classes of commercially useful insecticidal compounds appear to act primarily by inhibiting the deposition of chitin in the cuticle. These are acylureas (eg diflubenzuron: Grosscurt and Jongsma, 1987), of which a large number are now contending for commercial viability; and the thiadiazine compound buprofezin (Izawa et al, 1985). Whereas the latter compound is apparently rather selective for Homoptera, the acylureas are useful in controlling a wide variety of insect pests (Retnakaran and Wright, 1987). The success of these compounds demonstrates the commercial potential of the integument as a target.

Acylureas

There is wide agreement that these compounds kill insects by inhibiting chitin synthesis. This is worth stating, because it will not necessarily be the case that every compound having a detrimental effect on chitin deposition has the inhibition of chitin synthesis as its primary effect. Starvation and arrest of growth in general, for instance, are likely to inhibit the deposition of chitin. Similarly, compounds that inhibit chitin synthesis in vitro may not do so in vivo. Some such in vitro systems appear to be very easily disrupted. For example, Marks et al (1982) found that DDT inhibited chitin synthesis in cultures of regenerating cockroach legs. No-one would imagine that this was relevant to DDT's action as an insecticide.

The evidence that the prototype acylurea, diflubenzuron (DFB), acts primarily by inhibiting chitin synthesis is overwhelming. It includes the observations that the incorporation of [^{14}C]-GlcNAc into chitin is rapidly suppressed by DFB both in whole insects (eg Deul et al, 1978) and in isolated tissues in vitro (eg Hajjar and Casida, 1979); that this inhibition is achieved at low doses reflecting toxicity (eg Mitsui et al, 1980); and that the efficacy of structural analogues in preventing chitin synthesis corresponds well with their toxicity (Hajjar and Casida, 1979). So called "second-generation" acylurea insecticides are also potent inhibitors of chitin deposition (Figure 2). I regard the fact that some authors have reported that treatment with acylureas can lead to other effects (eg on epidermal proliferation - Meola and Mayer, 1980) as evidence of secondary effects consequent on the primary inhibition of chitin deposition.

It is a puzzle that after more than 10 years of research on the biochemical mechanism of action of acylurea insecticides, we still do not know how they work (Cohen, 1987; Reynolds, 1987b). It is clear that the step in chitin biosynthesis that is inhibited is a very late one, since a number of studies (eg Van Eck, 1979) have shown that treatment with DFB leads to a build up in the epidermis of UDP-GlcNAc, usually regarded as the ultimate precursor for the final polycondensation step in chitin assembly (Candy and Kilby, 1962). Initially it was proposed that it must be chitin synthase itself that was inhibited, Gijswijt et al (1979) noting that the effects of DFB poisoning were

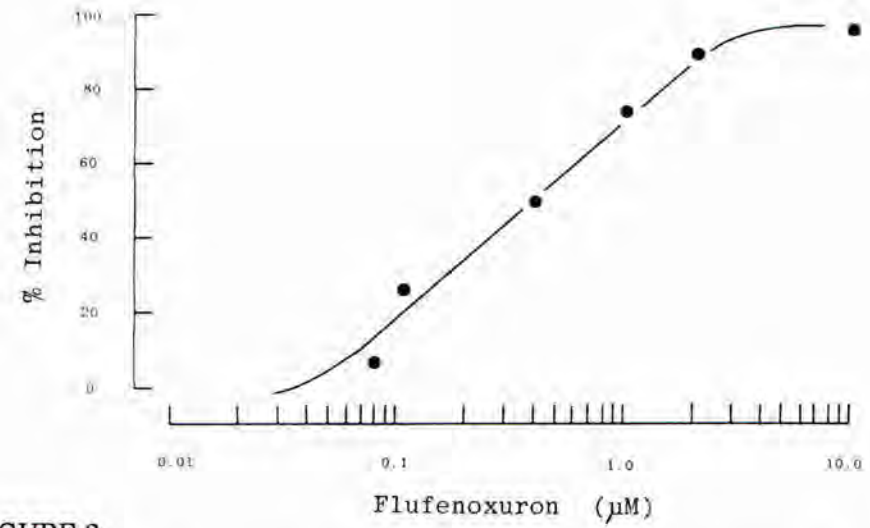

FIGURE 2

Inhibition by flufenoxuron of incorporation of [^{14}C]-GlcNAc into chitin by M.sexta proleg epidermis in vitro (Chandler and Reynolds, unpublished). Prolegs were from day 0, fifth instar caterpillars. Tissue was preincubated in insecticide for 15 min and then incubated with insecticide and labelled substrate for 1h. Incorporation of radioactivity into alkali-insoluble, chitinase soluble material was measured. IC_{50} value is 0.40 μM. Means of 5 determinations

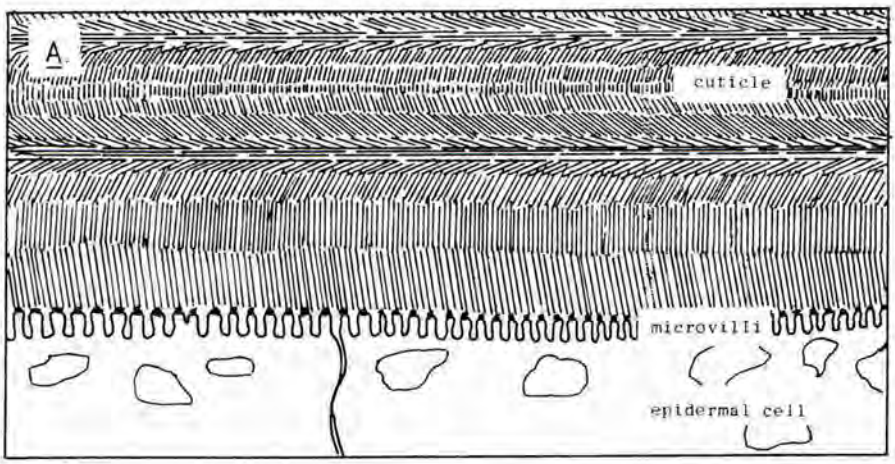

FIGURE 3

A Chitin synthesis is generally agreed to take place at the apical membrane of epidermal cells. Dense plaques associated with the tips of epidermal microvilli may be sites of chitin microfibre assembly. Microfibres are long, and appear in electron micrographs to extend right to the cell surface membrane implying that chitin crystallites are assembled directly at the time of poly(GlcNAc) synthesis.

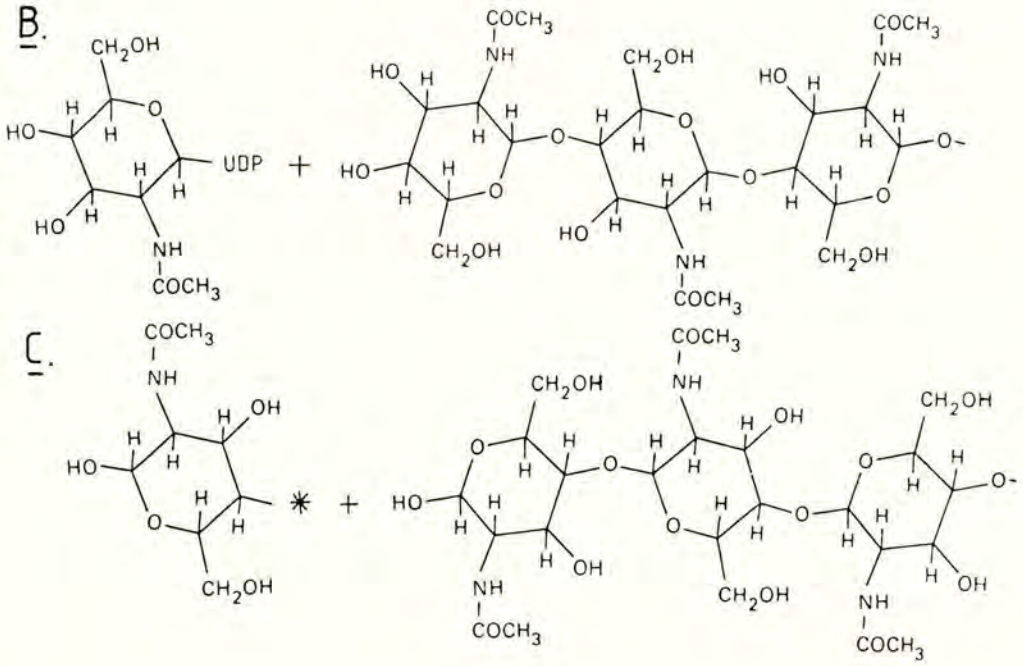

FIGURE 3 (continued)

B The conventionally accepted chitin biosynthetic pathway supposes that GlcNAc monomers are added only to the 4'end of the growing poly(GlcNAc) chain, the 1-phosphorylated monomer being first activated to UDP-GlcNAc. The inhibition of chitin deposition by the UDP-GlcNAc analogue peptidylnuceoside antibiotics supports the existence of this reaction.
C If chitin microfibres are assembled directly at the time of poly(GlcNAc) synthesis, however, half of the existing poly(GlcNAc) chains have the wrong orientation for this reaction, having no available free 4' end.In this case, addition to the 1' end of the nascent poly(GlcNAc) chain must occur, activation of the GlcNAc monomers occurring at the 4' end by the addition of an unknown group (*). Is it possible that acylureas act at this step?

identical to those produced by the peptidylnucleoside Polyoxin D. This compound is well known to be a specific inhibitor of chitin synthase in fungi. However, it is clear that there are differences in the actions of these two groups of compounds.

First, although the peptidylnucleosides (which probably act as competitors of UDP-GlcNAc) inhibit chitin synthesis in both fungi and insects, acylureas are completely ineffective in fungi (Marks et al, 1982). Second, several studies have found that broken-cell preparations of insect chitin synthase are not inhibited by acylureas, although they are inhibited by Polyoxins and Nikkomycins (see Cohen, 1987, for a review of this).

If acylureas do not inhibit chitin synthase, then how do they prevent the deposition of chitin? It seems clear that the problem here is that we simply do not understand the biochemistry of chitin synthesis in insects well enough.

First, the analogy between chitin synthesis in insects and fungi may not be a good one. It is not certain that the crystalline form of chitin in fungi is the same as that in insects and other Arthropods (Muzzarelli, 1977). Chitin in Arthropod cuticle always occurs as alpha-chitin, in which neighbouring poly(GlcNAc) chains are antiparallel. If the crystalline form of chitin in insect cuticle differs from that in fungi then the enzymes making chitin are unlikely to be the same.

A point which does not seem to have been considered previously is that if alpha-chitin really is antiparallel in the native state, as has been convincingly argued by Neville (1975), then it is hard to see how chitin microfibres can be assembled solely by the addition of UDP-GlcNAc monomers to the free 4' end of the growing poly-GlcNAc chains within the microfibre - the mechanism usually supposed to operate (Neville, 1975; Kramer, 1985). This problem is illustrated in Figure 3. Since chitin microfibres can be seen in electron micrographs to be very long, and since the molecular weight of chitin is known to be in excess of 1MDa (implying a degree of polymerisation >5000 - Hackman and Goldberg, 1974), one of the following scenarios must be true.

Either, chitin microfibres arise by self assembly of relatively short oligomers assembled at some distance from the microfibre, (which would require an additional enzyme to cement the incoming oligomers to the existing polymer chains), or the microfibre is assembled directly by a membrane chitin synthase complex. In this case, exactly half of the existing poly(GlcNAc) chains will be in the wrong orientation for the conventional polycondensation reaction to occur, and thus half of the GlcNAc monomers must be added not to the 4' end of the growing poly(glcNAc) chain, but to the 1' end. In this case an additional enzyme and a novel 4'-activated GlcNAc monomer would be required. Electron microscopic evidence would appear to favour the latter option, since chitin microfibres can be seen to run right up to the surface membrane of the epidermal cells. Since to date all published cell-free assays of chitin synthase activity have measured only the incorporation of UDP-GlcNAc into 'chitin', any differential effects of acylureas on the addition of 4'-activated monomers to the 1'-end of the chitin chain would have been missed.

Second, it is not certain that in the studies which have failed to find any acylurea inhibition the 'chitin' product synthesised in vitro was in fact identical to the chitin that is made in vivo. Although this product was in many cases not only identified as an alkali insoluble polysaccharide (the minimal criterion for

chitin) but also confirmed to be chitinase soluble, this still does not prove that it was alpha-chitin constituted in microfibrils of the appropriate dimensions. Such proof would be very hard to obtain.

It thus remains possible that previous studies of chitin synthase activity using broken cell preparations did not in fact achieve their aim of an in vitro system that is a true model of that operating in vivo.

The parallel with cellulose biosynthesis

We ought not to be surprised if this mismatch between in vivo and in vitro proves to be the case. It is instructive to examine the problems which have beset biochemists studying the biosynthesis by plant cells of cellulose, an analogue of chitin in which the monomer is glucose instead of N-acetylglucosamine. These are nicely reviewed by Delmer (1987). Clearly much of the interest in cellulose comes from the fact that it is the major polysaccharide of most plant cell walls. Nevertheless, despite much effort, unambiguous success in the cell-free synthesis of this apparently simple biopolymer has only been achieved with simpler organisms such as bacteria. The suspicion has grown that in plant cell membranes, the enzyme that would normally synthesise cellulose (a beta-1,4-glucan), switches to synthesising a different polysaccharide, callose (a beta-1,3-glucan) under in vitro conditions.

A clear lesson from the work on cellulose is that soluble regulatory factors may prove to be very important in studying the function of the membrane bound enzymes that synthesise the polysaccharide polymer. Particularly intriguing is the observation (Delmer et al, 1987) that a photaffinity label analogue of the specific cellulose synthesis inhibitor 2,6-dichlorobenzonitrile (DCB) binds to a soluble 18 kDa protein, which can under some circumstances become associated with membranes. It is speculated that this protein is a regulatory factor that specifies the type of linkage of the glucosyltransferase reaction catalysed by a membrane bound cellulose synthase. The ability of DCB to inhibit cellulose biosynthesis would then be a consequence of its action on this regulation. Is it possible that acylureas bind not to chitin synthase itself, but to a regulatory protein? I think that it will be approaches analagous to this one, probably involving the use of affinity labelled insecticides, that will finally reveal the mechanism of action of acylureas.

Compounds affecting chitinolysis

Insects use several enzymes to break down chitin in the old cuticle during moulting (Kramer, 1985). One or more endochitinases function to cleave the poly(GlcNAc) chains into oligomers, with several exochitinases (beta-N-acetylglucosaminidases) following on to reduce these products to GlcNAc monomers. Inhibition of either class of enzyme would effectively prevent chitinolysis, since the two enzymes are synergistic, with the endochitinases providing substrate for the exochitinases, and the latter preventing end-product inhibition of the former (Fukamizo and Kramer, 1985).

So far as I know, only one compound inhibiting chitin breakdown has been reported. Allosamidine is a highly effective inhibitor of Bombyx chitinase (Sakuda et al, 1987). As a modified beta-(1-4) dimer of allosamine (an isomer of glucosamine), it probably acts as a competitive inhibitor of the enzyme. It is toxic to insects, although probably not sufficiently so to be a useful insecticide. Nevertheless, it demonstrates the potential for compounds acting in this way.

Sclerotisation as a target

Another way of affecting the mechanical properties of the cuticle would be to interfere with sclerotisation. This is a post-deposition process that stiffens and hardens the cuticle by the incorporation of phenolic compounds derived from tyrosine (Kramer and Hopkins, 1987). The chemistry of tanning precursors is somewhat variable among insects, but it is clear that N-acetyldopamine and N-betaalanyldopamine are widely used. DOPA decarboxylase is an essential enzyme in the synthesis of these compounds. It is clear that at least some of these sclerotising compounds stiffen the cuticle by reacting with cuticle macromolecules to form chemical cross-links between them.

It is difficult to study these sclerotising reactions, because the products are insoluble; nevertheless, considerable progress has been made recently, although there is some disagreement about the importance of the various pathways proposed (Suguruman, 1988; Andersen, 1989). Three principal types of reaction have been suggested to occur : all oxidise the sclerotising agents to reactive intermediates which then attack cuticle macromolecules. The pathway longest recognised involves the formation of a quinone from the sclerotising agent. This is probably catalysed by a laccase-type enzyme (Barrett, 1987). A second pathway requires the existence in cuticle of a desaturase enzyme that produces a side-chain unsaturated derivative. This dehydro- compound can either add to cuticle macromolecules directly, or can be further attacked by the laccase to produce a quinone (Andersen, 1985). A third pathway postulates a cuticular phenoloxidase that results in the formation of a quinone methide from the sclerotising agent. This then attacks macromolecules to form cross-links (Suguruman, 1988). Different types of cuticle probably employ these various pathways to different extents.

There is no doubt that the formation of cross links actually occurs. The use of solid state NMR spectroscopy has confirmed their existence in sclerotised cuticle, and suggests that in Manduca pupal cuticle the prime target of these cross-links is the secondary amino group of histidine residues in matrix proteins (Kramer et al, 1987). Some of the stiffening effect of the incorporation of sclerotising agents may however be due to the increased hydrophobicity of the phenolic impregnated cuticle matrix, which leads to a reduction in water content, and consequent decreased extensibility (Hillerton and Vincent, 1979).

All insects employ some form of sclerotisation to modify cuticle properties, but the extent of sclerotisation varies greatly. Some insects have extensively sclerotised cuticles (eg adult beetles), while others are almost entirely unsclerotised (eg Dipteran larvae). Even maggots have sclerotised mouthparts, however. The time at which sclerotisation occurs varies too. Some insects (beetles again) are extensively sclerotised prior to ecdysis, while others (eg cockroaches) restrict sclerotisation to a brief period immediately after the old cuticle is shed (see Reynolds, 1980).

It would seem that sclerotisation ought to be a good target for a novel insecticide. The process is vital to the insect's well-being, and involves distinctive chemistry. Possible approaches would be to interfere with the production and storage of sclerotising agents, their transport into the integument, and their enzymic conversion to reactive intermediates. It is possible to envisage the prevention of sclerotisation, which would produce a soft and extensible cuticle, or the inappropriate stimulation of sclerotisation, producing a cuticle that would be too stiff. As yet however, little success has been achieved in either direction. Carbidopa, an inhibitor of DOPA

decarboxylase, and therefore of the synthesis of sclerotising agents, has been reported to be insecticidal to blowfly maggots (Turnbull et al, 1980), probably because it interferes with the stabilisation of the epicuticle.

Another target

An insecticide that has for some time been recognised as affecting the cuticle is the substituted melamine, cyromazine (2-cyclopropylamino-4,6-diamino-s-triazine). This compound has IGR-like effects, causing reduced growth, and eventually death from integumental lesions (eg Price and Stubbs, 1984). A closely related compound, furyldiaminotriazine, has similar effects and may act in the same way (Pessah et al, 1985). An early suggestion that cyromazine might inhibit chitin synthesis (Miller et al, 1981) has not been confirmed, several studies agreeing that chitin deposition is not affected (eg Turnbull and Howells, 1982).

We have recently discovered (Reynolds and Blakey, 1989; Kotze and Reynolds, this monograph) that cyromazine has the specific effect of rapidly causing the cuticle of Manduca sexta caterpillars to become much less extensible than normal. This is evident whether the cuticle is subjected to creep tests under constant load, or to Instron tests in which tension is measured during extension at a constant rate (see Table 1). This decreased cuticle stiffness has the effect of elevating internal pressure within the body, restricting movement, and hampering feeding. Not only is stiffness increased, but also cuticle strength. This means that the force required to break the cuticle is greater than normal. Interestingly, the extent to which the cuticle must be strained (increased in length) before it breaks is actually decreased. We believe that these changes are responsible for the decreased growth shown by poisoned caterpillars, and ultimately cause the development of fatal integumental lesions.

TABLE 1

Cyromazine effects on cuticle mechanical properties (Kotze and Reynolds, unpublished).

	Control	Cyromazine
Stiffness (N mm^{-1})	0.106 ± 0.008	0.622 ± 0.031
Strength - Force at yield point (N)	0.677 ± 0.038	1.624 ± 0.108
- Strain at yield point	0.481 ± 0.024	0.367 ± 0.018

Insects (fifth instar Manduca sexta) were given 50 ppm cyromazine in diet on day 0 and tested after 24h. Testing utilised loops of abdominal body wall stretched at 1 mm min-1 in an Instron floor-standing mechanical testing machine (used courtesy of the School of Materials Science, Bath University). Means ± SE (N=10).

These effects on the mechanical properties of the cuticle are more rapid than any previously described, and other symptoms can readily be explained as

consequences of them. The effects on cuticle stiffness occur at doses sufficiently low to explain the compound's toxicity. As yet, the mechanism whereby cyromazine increases cuticle stiffness is unknown. It seems not to involve effects on the synthesis of cuticle macromolecules, and so may involve the post-secretory modification of cuticle components. The effects of the compound on cuticle mechanical properties would be explained by an increase in the extent of chemical cross linking between cuticle proteins. It may be significant that Hughes et al (1989) have recently observed that [^{14}C]-labelled cyromazine accumulates within the integument of treated insects. Cyromazine could be involved in the formation of cross-links itself, or might affect the activity of sclerotising enzymes and /or transport systems for sclerotising agent precursors.

PROSPECTS

The Arthropod-specific chemistry and structure of the cuticle, combined with its extreme importance to the insect, must make the integument an important target for the development of new insecticides.

While I have tried to show in this article that the cuticle also contains many other potential targets, it is to be expected that the existence of relatively straightforward assays for chitin synthesis in organ cultures or cell lines will lead to much screening of potential chitin synthesis inhibitors in insecticide discovery programmes. It is already evident that chemical classes other than acylureas will prove to be inhibitory. It is likely that the complexity of the chitin synthase system will provide multiple biochemical targets that all have the same final effect of inhibiting the assembly of chitin microfibres. As yet, with only very meagre knowledge of chitin synthase, traditional methods of discovery are likely to be most important - we are a long way off the rational design of an effective chitin synthesis inhibitor. The recognition of the cuticle as the target of cyromazine may lead to renewed interest in substituted melamines as insecticides.

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JUVENOIDS AND NEUROPEPTIDES AS INSECT CONTROL AGENTS: RETROSPECT AND PROSPECTS.

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ABSTRACT

This paper presents a review of the development of juvenoids, and examines the prospects for future use of such compounds as pest control agents. In addition, we review current developments in insect neuropeptide research. The search for selective insect control agents based on biorational principles has focused research attention on insect hormones and neuropeptides. It appears now that all life processes in insects are regulated by neurohormonal systems. Presently over 40 insect neuropeptides have been isolated and sequenced. Among these, those regulating diuretic, antidiuretic, eclosion, juvenile and anti-juvenile hormone and behaviour activity (PBAN) appear to be the more promising neurohormones with potential for exploitation as model systems for insect control. It is likely that neuropeptides will be exploited through the development of processing enzyme agonists/ antagonists, peptidomimetics and through cloning of peptide genes into baculoviruses that will express a peptide gene in the host insect cells.

JUVENOIDS

Following many pioneering studies in basic insect endocrinology during the 1930s and 40s, the discovery of the chemical structure of naturally-occuring juvenile hormone (Roller *et al.* 1967) provided the impetus for the development of related compounds for insect control.

Currently, at least 4 naturally-occuring hormones have been identified, and all are closely related chemically. It appears that multiple homologues (JH0, JH1, JHII and JHIII) occur in the Lepidoptera, while most other orders utilise only JHIII (Schooley *et al* . 1984). Some exceptions appear to be the higher Diptera where another hormone (JHB3) has recently been identified (F.C. Baker pers. commun), and some hemipterans where the hormone remains to be identified (Baker *et al.* 1988). Interestingly, the juvenile hormone precursor methyl farnesoate has been found in a Crustacean (Laufer *et al.* 1987) where it is believed to have a gonadotropic role (Borst *et al.* 1987). Indeed it is likely that juvenile hormone evolved initially as a gonadotropic hormone, and was later adapted by insects, and perhaps other arthropods, as a regulator of metamorphosis.

Classically, the role of juvenile hormone in insects has been associated with the control of the process of metamorphosis (i.e. maintenance of the larval condition), and with the regulation of

reproduction in adult insects, especially females. Thus, in larval insects it is believed that the hormone is present (in varying titers) more or less continuously until the final larval instar. In the presence of juvenile hormone, metamorphosis is inhibited, and the insect remains juvenile. The absence of the hormone in the final larval instar permits development to the adult stage, either directly as in the Hemimetabola, or via the pupal stage in holometabolous insects. Following metamorphosis, the hormone may reappear in the adult insect as a regulator of reproductive physiology. The gonadotropic role of juvenile hormone is most obvious in those insects producing cyclic batches of eggs (e.g. cockroaches) where peaks of endogenous juvenile hormone coincide with the regular production of oothecae (Weaver *et al.* 1975). In addition to the control of metamorphosis and regulation of reproduction, juvenile hormones have been implicated in a variety of other physiological processes in insects including phase and caste determination, diapause, pigmentation and colour polymorphism, pheromone production, and some aspects of behaviour (Edwards & Menn, 1981). Thus it is clear that these molecules influence a wide range of important physiological processes in both the developing and the mature insect.

The juvenile hormones are produced by a pair of endocrine glands (the corpora allata) which are linked by nerves to the brain and to the suboesophageal ganglion. Within the corpora allata, the hormones are biosynthesised from simple precursor molecules (e.g. acetate and propionate) via pathways which are similar to those involved in the early steps of steroid and isoprenoid biosynthesis in vertebrates and other organisms. Thus, the steps in the synthesis of JHIII proceed via acetyl-CoA, HMG-CoA, mevalonate, isopentenyl pyrophosphate, farnesyl pyrophosphate and farnesoic acid. The latter molecule is converted to methyl farnesoate (the methyl moiety being transferred from methionine) before being epoxidised to JHIII. Once synthesised, the hormone is secreted from the glands into the haemolymph where it is transported to the sites of action bound to a carrier protein. The carrier protein may serve to protect the molecule from enzymic degradation, and to facilitate the transport of an essentially non-polar compound through an aqueous medium.

Effects of juvenoids on insects.

Application of juvenile hormones, their synthetic mimics and analogues (collectively termed juvenoids) to insects results in a variety of effects depending on the type of insect, stage of development, time of application etc. In general, application to larval insects prior to the final larval instar produces no marked effects. By contrast, application during the final larval instar (and during the early part of the pupal stage in holometabolous insects) can produce delay or disruption of metamorphosis. At its most obvious, this is manifest as the production of further larval stages that are often the result of several supernumerary moults, and the resulting individuals are frequently substantially larger than normal fully-grown larvae. Eventually such "giant" larvae may undergo successful metamorphosis to large adults. The production of extra large insects has always been quoted as one of the major drawbacks to the use of juvenoids in pest control, although it is seldom appreciated that such insects are produced only at doses that are generally much higher than those necessary to disrupt metamorphosis and reproduction. Moreover, the production of large pupae following juvenoid treatment (Nihmura *et al.* 1972) has been

usefully exploited in sericulture, where cocoon size and therefore silk production has been increased by rearing silkworms on juvenoid treated diets.

Notwithstanding the formation of "permanent" or supernumerary larvae described above, the most typical effect of juvenoid application during late larval or early pupal development is the marked disruption of metamorphosis. This frequently occurs after the larval-pupal transformation in holometabolous insects, although occasionally disruption occurs at the larval-pupal moult resulting in the formation of larval-pupal intermediates. Disruption of metamorphosis following the formation of the pupal stage can vary from the production of "permanent" pupae, through the production of pupal-adult intermediates, to the production of adultoids with varying degrees of deformity. In the majority of cases, such insects are unable to complete metamorphosis to the adult stage, and are thus inviable. In such insects death is often associated with rupture of the cuticle during an attempted moult. In some cases, especially in hemimetabolous insects, juvenoid treatment may be followed by successful metamorphosis to the adult stage. However these "adults" often exhibit minor external deformities of wings, legs and genital ia. Such insects are frequently unable to reproduce - probably because of morphological derangement of the reproductive system, rather than a direct action of juvenoids on the mature adult reproductive organs. This is certainly the case in some Coleoptera (e.g. *Tribolium* spp., DeVries & Brown, 1977) and in some cockroaches (e.g. *Blatta orientalis*, Edwards & Short, 1988). The various manifestations of development-disruption caused by juvenoids has been termed the morphogenetic action of these chemicals and it is this action which is the basis of their use as novel pest control agents.

There is considerable evidence that juvenile hormones act as gonadotropins in the adult stage of many insect species, so it is slightly surprising that in only relatively few species are adult insects affected by juvenoid treatment. The most notable example is the juvenoid induced sterilisation of queens in certain ant species (Edwards 1975; Vinson & Robeau, 1974). In other insects, low doses of juvenoids may actually stimulate egg production (e.g. the increased rate of production of oothecae by cockroaches). Recently the stimulatory action of a juvenoid on egg production has also been reported in the flour mite *Acarus siro* (Thind & Edwards, 1989).

However, in most cases juvenoid treatment of adult insects results in neither increase nor decrease in reproductive capacity. Juvenoids have been shown to have ovicidal effects in several species, although sometimes these effects may have been due to the toxic or suffocating action of the application solvent or of the oily juvenoid itself (Critchley & Almeida, 1973). True ovicidal activity is manifest by disruption of embryogenesis occuring at or before blastokinesis. For this reason, in those insects that are sensitive to the ovicidal action of juvenoids, susceptibility is often limited to the very early stages in embryonic development i.e. soon after oviposition, or even earlier when the eggs are still within the body of the gravid female.

The development of juvenoids.

The realisation that insect juvenile hormones were both unique and vital to the life of insects, prompted the synthesis of literally hundreds of chemical analogues. Some of these exhibited substantial increases in biological activity by comparison with their naturally occuring relatives, and a few have shown promise as insect control agents (Edwards & Menn, 1981). Natural juvenile hormones are unstable in ultra-violet light, and are rapidly metabolised by insects and by other organisms. In insects, metabolic degradation of natural juvenile hormone to primary (inactive) metabolites occurs by enzymic attack on the ester moiety by JH esterase (Weirich *et al.* 1973) or at the 10,11 epoxide ring by JH epoxide hydratase (Brooks, 1973). For these reasons, the synthesis of analogues was directed towards the stabilisation of the molecule by protection or substitution of epoxide and ester groups, and by various attempts to improve UV stability through alteration or substitution of the 2,6 diene double bonds. Predominant among the early molecules showing promise as pest control agents were the substituted alkyl 2,4, dienoates - especially methoprene and hydroprene. Subsequently, both compounds were developed commercially, and methoprene was first registered for use as a mosquito larvicide in the USA in 1973 and, in the UK for the control of pharaoh's ants, in 1980. Following the commercialisation of methoprene, efforts continued to be directed towards improving UV stability of juvenoids. A significant advance came with the synthesis of epofenonane. Unlike methoprene, this compound showed considerable UV stability, so much so that several trials against field crop pests were undertaken (Schmid *et al.* 1978). Strangely, epofenonane was never commercialised, but it did provide the basis for the more recent development of juvenoids like fenoxycarb and pyriproxyfen. In these compounds, isoprene groups have been substituted by linked phenoxy groups giving increased stability and substantial improvement in biological activity.

Practical use of juvenoids - Advantages and Limitations.

The main advantages of juvenoids over conventional insecticides are linked directly to their low toxicity to vertebrates, and their specificity of action which appears to be confined to insects and other arthropods. The combination of such attributes has undoubtedly fostered the widespread interest in these compounds as a new generation of pesticides. The negligible acute toxicity of such molecules is illustrated by methoprene which has an acute oral LD50 (rat) of >34,600 mg/kg, and is therefore essentially without measurable acute toxicity. Moreover, compounds like methoprene and hydroprene do not persist or accumulate in the environment, and are degraded ultimately to simple molecules (e.g. carbon dioxide and acetic acid) that are unlikely to cause problems of environmental contamination. Obviously, the more stable molecules like fenoxycarb will show greater persistence, but even so, are most unlikely to cause problems in the environment. Balanced against these advantages are several potential disadvantages associated with the practical use of juvenoids. Some of these have been over emphasised (e.g. the production of "giant" larvae), but there is one that represents a real problem, namely that juvenoids do not have a rapid toxic action, and thus are not useful in situations where immediate control is demanded. For this reason, and because juvenoids are ineffective against early larval stages, their use has been limited mainly to two pest situations - either against pests where

only adult stages are pests (e.g. mosquitoes, ants and fleas), or where it is efficacious to prevent small pest populations, causing negligible damage, from increasing to levels causing economic loss (e.g. the protection of stored products, or the long-term control of cockroach populations).

Insect control with juvenoids - target pest groups.

As a consequence of the constraints described above, the main target pests groups for juvenoids are found largely outside the major insect pests of field crops. However, juvenoids have shown remarkable promise for use against a variety of different pest types. The early development of methoprene was largely a result of good activity against flood-water mosquitoes, with minimal impact on other aquatic organisms (Miura & Takahashi, 1973). Improved stability of methoprene in water was obtained by microencapsulation. Subsequently, this compound and several other juvenoids have been shown to be effective against a variety of water-breeding Diptera, including *Aedes*, *Culex* and chironomid species (Schaefer & Wilder, 1973; Mulla *et al.* 1974).

Juvenoids have also shown promise for the control of manure-breeding flies, either by direct application to the breeding sites or by feed-through treatment of the manure producing animals. Good results have been obtained against stable flies (*Stomoxys* spp.) and the horn fly (*Haematobia irritans*), culminating in the development of a sustained-release bolus for horn fly control in cattle (Miller *et al.* 1977).

Several early attempts to control housefly larvae in manure in intensive poultry rearing units were frustrated by the instability of juvenoids in the avian digestive tract. However, the more recent development of more stable molecules like piriproxyfen has led to the development of effective fly control measures by direct application to manure. Thus, in recent years there has been considerable progress made in the utilisation of juvenoids for the control of both water-breeding and manure-breeding flies. This group of target pests illustrates well the efficacy of juvenoid-based control measures in those instances where larval stages are not pests.

The protection of stored products is another area in which juvenoids have been shown to be effective. Methoprene has been used to control cigarette beetle (*Lasioderma serricorne*) and tobacco moth (*Ephestia elutella*) in hogsheads of stored tobacco, and this juvenoid is also used for the control of flour beetles (*Tribolium* spp.) and the lesser grain borer (*Rhyzopertha dominica*) in stored wheat. However, methoprene is relatively ineffective against grain weevils (*Sitophilus* spp.) and more active compounds like fenoxycarb show greater promise for protection of bulk stored grain against attack by stored products insects (Edwards *et al.* 1987).

Against household and domestic pests, juvenoids have demonstrated remarkable promise. Methoprene and fenoxycarb are highly active against cat and dog fleas, and the former compound has been effectively used to eradicate infestations of pharaoh's ant in large buildings (Edwards & Clarke, 1978). In addition, outstanding results have been obtained with juvenoids against cockroach populations. Hydroprene has been developed for use against the German cockroach (*Blattella germanica*) and is under

development for use against other cockroach species (e.g. *Blatta orientalis*, Edwards & Short, 1988). Juvenoids are especially suited for use in domestic situations where absence of toxicity and environmental hazard are of paramount importance.

Future prospects for the use of juvenoids in pest control.

There are undoubtedly many applications for the effective use of juvenoids against pest populations that remain to be explored. In particular, the advent of UV-stable compounds has opened the way for the development of juvenoids as main-stream crop protection agents. The good toxicological and environmental properties of these chemicals will become increasingly important as the older, broad-spectrum poisons are withdrawn from use. As with any new development, changes may need to be made in terms of application technology, formulation and, not least, the attitude of the end-user towards population control of pests as opposed to instant kill. In summary, juvenoids cannot provide the answer to all insect pests problems, but they do have definite advantages over conventional compounds, and where applicable, often provide a level of efficacy and safety that has hitherto been lacking.

JUVENILE HORMONE ANTAGONISTS.

The absence of substantial effects of juvenoids on insect larvae prior to the final instar, stimulated the search for ways of reducing or antagonising endogenous hormone levels in larvae. Bowers (1976) discovered that extracts from the plant *Ageratum houstonianum* caused premature metamorphosis in some hemipterans. Subsequently, the active molecules (precocenes) were shown to destroy the corpora allata after being converted to highly-reactive metabolites by tissue-specific enzymes within these glands (Brooks *et al.* 1979). Since the discovery of the precocenes, a number of other active anti-juvenile hormone agents have been reported. These include inhibitors of hormone biosynthesis like fluoromevalonate (Quistad *et al.* 1981) and compactin (Monger & Law, 1982), and compounds with less obvious modes of action like ETB (Staal, 1977; Edwards *et al.* 1983) and a variety of substituted imidazoles (Kuwano *et al.* 1983). In addition, recent studies (Wing *et al.* 1988) have shown that a non-steroidal ecdysone agonist produces premature moulting in *Manduca sexta*. However, although many of these chemicals have become useful research tools, none have, so far, shown sufficient promise to be developed as practical insect control agents (see Staal, 1986). Nevertheless, the concept remains valid, and the search for ways of manipulating the activity of the corpora allata has moved towards the characterisation of neuropeptides responsible for the internal regulation of endocrine gland activity.

INSECT NEUROPEPTIDES.

It is generally accepted that the field of insect neuropeptides had its genesis in the pioneering research of the Polish neurobiologist Stefan Kopec over seventy years ago (Kopec, 1917, 1922). These papers advanced the concept for the first time that nervous tissue produced hormones, and the brain of the gypsy moth (*Lymantria dispar*) is the source of chemical secretions including a pupation hormone or, as we know it now to be, the prothoracicotropic hormone (PTTH) that stimulates ecdysone production. Only recently, Nagasawa *et al.*(1986) elucidated the amino acid sequence of the 4KD PTTH II of *Bombyx mori*. They also showed its

sequence homology to human insulin. The conservation of amino acid sequences among insects, other invertebrates and vertebrates was noted in several identified neuropeptides and suggested a common genetic origin (Scharrer, 1987) and conservation of ancestral genes in the course of evolution.

The pentapeptide proctolin was the first insect neuropeptide to be discovered and fully characterised by Starratt & Brown (1975). The significance of their discovery is even greater considering the difficulties in isolating and characterising an oligopeptide present in vanishing amounts by less than optimal analytical procedures. They obtained 180 μg of peptide from 125 kg of *Periplaneta americana*. Proctolin occurs widely in insects, and functions as a neurotransmitter in visceral and somatic muscles (O'Shea & Adams, 1986). Whether it functions as a neurohormone has not been clearly established as yet.

The advent of new analytical methodology, largely developed in isolation schemes from the mammalian neuropeptide field, has resulted in the elucidation of over 40 insect neuropeptide sequences (Holman *et al.* 1990). These sequence identifications were facilitated by reverse-phase, high-performance liquid chromatography (RP-HPLC), fast-atom bombardment mass spectrometry (FAB-MS), gas phase sequencing, and advanced nuclear magnetic resonance (NMR) procedures including circular magnetic dichroism for tertiary structure conformation. Undoubtedly, many more neuropeptides will be discovered in the next few years. These discoveries will also be aided by immunocytochemical methods (Sternberger, 1986), improved bioassays, and the availability of receptor preparations to facilitate isolation of neuropeptides.

The organisation of cerebral neurosecretion in insects was recently reviewed by Scharrer (1987). Paired neurosecretory cells in the protocerebrum extend axon bundles into the corpus cardiacum (CC). Neuropeptides are subsequently released from the CC into the haemolymph and transported to the target organ or gland. In some instances nerve projections extend from the CC into the glandular corpus allatum (CA), and subsequently release the material into the circulatory system. The CC-CA complex of insects is the major neurohaemal organ in these organisms (Meola, 1983). Insect neuropeptides may also be elaborated in somatic elements (Scharrer, 1974), and in the accessory gland (Meola, 1988). The sources, structure and function of the currently known insect neuropeptides were described in several recent reviews including: Cook & Holman (1985); O'Shea (1986); Grimmelikhuijzen *et al.* (1987); Keeley & Hayes (1987); Thorpe & Duve (1988); Menn & Borkovec (1989); and Holman *et al.* (1990).

PROCESSES CONTROLLED BY NEUROPEPTIDES

Virtually all life processes in insects are regulated by neural and endocrine systems. As early as 1928, Ernst Scharrer provided evidence that neurosecretory neurons in the hypothalamohypophysial tract in the preoptic nucleus of a teleost fish, *Phoximus laevis*, produced circulating proteinaceous messenger substances which communicated with the endocrine system. These early findings were confirmed also in insects in subsequent studies by Berta Scharrer and other investigators (cf. Scharrer, 1987). Most neurons are presently considered to be peptidergic, secreting neuropeptides from secretory granules (Fujita, 1985).

Menn and Borkovec (1989) grouped the known neuropeptides into four categories, those regulating:- 1) development and reproduction; 2) behaviour; 3) homeostasis and metabolism; and 4) muscle function. These functions were discussed in detail by Cook and Holman (1985). The diversity of regulated functions attests to the versatility and importance of these molecules as biochemical messengers in insects.

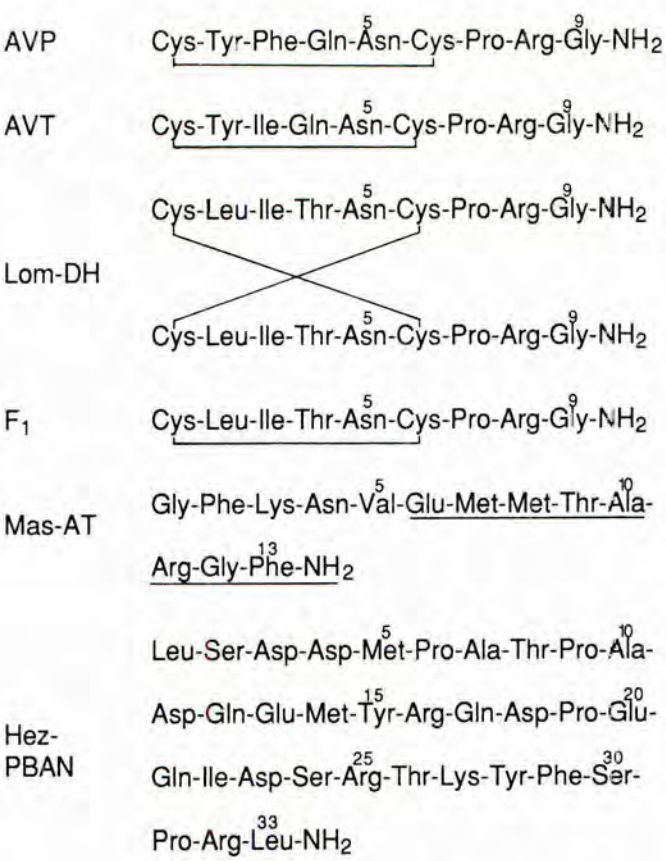

Fig. 1. Amino acid sequences of arginine vasopressin (AVP); arginine vasotocin (AVT); locust diuretic hormone (Lom-DH); and its naturally occurring monomer (F_1); *Manduca sexta* allatotropin (Mas-AT); and *Heliothis zea* pheromone biosynthesis activating neuropeptide (Hez-PBAN).

Of the fully sequenced insect neuropeptides, a few appear to us to be useful models for further research toward potential practical application, these include; diuretic and antidiuretic neurohormone, eclosion neurohormone (EH), allatotropins (AT) and allatostatins, PTTH and pheromone biosynthesis activating neuropeptide (PBAN). These neuropeptides, based on our current knowledge exert critical control over key developmental,

reproductive, metabolic, and behavioural functions in insects. Disruption in biosynthesis, availability and removal (metabolism) of these neuropeptides would lead either directly or indirectly to death and elimination of affected populations.

Diuretic and Antidiuretic Neuropeptides

Maintenance of internal water balance in insects is most critical for their survival. Water loss (dehydration) or retention (toximeia) would result in death. Proux *et al* (1987) identified the locust, *Locusta migratoria*, diuretic neuropeptide (Lom-DH) from suboesophageal and thoracic ganglia, as the antiparallel dimer of the inactive naturally occurring monomer (F1). Lom-DH and F1 were isolated based on their immunological similarity to arginine vasopressin (AVP) (Figure 1). Significantly, F1 and Lom-DH show 78% sequence homology with arginine vasotocin (AVT) and 67% with AVP. Lom-DH stimulates water transport by increasing cAMP levels in Malpighian tubules (Proux & Herault, 1988).

Most recently Kataoka *et al* (1989a) reported the isolation and sequence of a 41-residue diuretic peptide from pharade adult heads of the tobacco hornworm, *M. sexta* (Mas-DH) that stimulated fluid excretion *in vivo* upon injection into larval *M. sexta*, with an ED_{50} of 0.1 ng/larva; and in newly emerged adult *Pieris rapae*.

Spring and co-workers (1988), isolated from CC extracts of the house cricket, *Acheta domesticus*, an antidiuretic hormone (ADH) that inhibits fluid secretion by Malpighian tubules. This hormone is detected in haemolymph as a result of dehydration. The ADH has no effect on reabsorption of water by the isolated cricket rectum.

Sequence identification of ADH, determination of processing and degradation steps and minimal structural requirements for function are requisites for further exploitation of control of diuresis in insects. It is also to be noted that confirmation of activity of exogenously applied DH and ADH in the intact insect is only now emerging (Kataoka 1989a). *In vivo* bioassays are most essential for further developmental work in this area of research.

Eclosion Hormones

The EHs are attractive for further exploitation due to their wide range of activity in the developing insect. They are synthesised in the brain and released from the CC, triggering ecdysis behaviour in the larval, pupal and adult stages (Truman, 1985). According to Morton and Truman (1988), EH acts directly on the central nervous system (CNS) to release the stereotyped motor program involved in ecdysis. Truman and coworkers have also established that the response of the CNS to EH involves pre-sensitisation of the CNS by exposure to 20-hydroxy ecdysone (HE) (Truman *et al*. 1983). The hormone isolated from pharate adult brain tissue of *M. sexta* (Mas-EH) is a relatively large neuropeptide, consisting of 62 amino acid residues (Kataoka *et al*. 1987; Marti *et al*. 1987).

Two forms of EH were determined in *B. mori* by Kono *et al*. (1987). The Mas-EH has 80% homology to Bom-EH, with only 12 residues differing. Since PTTH stimulates the synthesis of ecdysone it is obvious

that further chemical, biochemical and molecular studies on the inter-relationship of EH, PTTH and ecdysteroids may lead to discovery of potent sites for interference with these interactive neuropeptides as targets for selective insect control agents.

Allatotropin and Allatostatin

The synthesis and release of JH by the CA is under the control of the stimulatory neurohormone allatotropin (AT) and inhibitory neurohormone allatostatin (AS),(Rankin and Stay 1987). The recent report by Kataoka *et al.* (1989) describing the purification, sequence analysis, and *de novo* synthesis of AT from brain-CC-CA complex of adult *M. sexta* (Figure 1) provides an exciting step forward in our knowledge of the regulatory events involved in JH release by the CA. Approximately 150% increase over control in *in vitro* secretion of JH from CA was obtained at a dose of $2x10^{-15}M$. Most significantly, bioactivity was fully elicited with the truncated analog from the amino terminus AT(6-13) (Figure 1). Activity was specific to the adult stages in *M. sexta* and *Heliothis virescens*, but not in a beetle and a grasshopper species. No information is as yet available on the identity of an AS. The emerging possibility to harness the regulatory processes and events which regulate production, release and cessation of JH biosynthesis are most tantalising prospects for totally novel approaches to biorational control of insects. The search for viable JH antagonists (AJHs) has been long and arduous (Menn, 1985; Staal, 1986). Possibly a new avenue of research is opening based on these new discoveries.

Pheromone Biosynthesis Activating Neuropeptide (PBAN)

Sex pheromones are used by most species of moths for mate finding. Pheromones are produced and released at discrete times to coincide with the period of reproductive activity. Raina and Klun (1984) showed for the first time that pheromone production in the corn earworm, *Heliothis zea*, was controlled by a factor originating in the head of the female. This was demonstrated through a simple experiment which also provided an excellent bioassay for the putative hormone (Figure 2). Subsequently it was shown that the hormone is a neuropeptide produced in the suboesophageal ganglion (SOG) and released via the corpora cardiaca to induce pheromone biosynthesis (Raina *et al.* 1987; Raina and Menn, 1987). PBAN was isolated from brain-SOG complexes of both male and female *H. zea* by HPLC. Amino acid analysis and gas phase sequencing revealed the primary structure of this peptide (Hez-PBAN) (Raina *et al.* 1989) (Figure 1). The peptide was synthesised and after controlled oxidation of the two methionine residues was shown to match the natural hormone in both chemical and biological characteristics. Synthetic PBAN when injected at $2x10^{-12}M$ stimulated significant production of the sex pheromone in ligated *H. zea* females.

It was previously reported (Raina & Klun, 1984; Raina *et al.* 1987) that crude brain-SOG homogenates from a number of moth species and a cockroach species caused pheromone production when assayed in ligated *H. zea* females. Recently it has been shown that the synthetic Hez-PBAN caused pheromone production in six other species of moths, in each case the chemistry of the sex pheromone was different from that of *H. zea* (Raina *et al.* 1989). In three of these species the titer of pheromone produced was 6-7 times greater when compared to that of control females.

Based on these observations, it is suggested that PBAN in different species of moths must be similar if not identical. A confirmation of that speculation came from a partial sequence of PBAN from the silkworm *B. mori*, (Nagasawa *et al.* 1988) in which 8 of the 9 amino acids sequenced from the N-terminus are the same as in Hez-PBAN. Because of the diversity in pheromone chemistry of various species, it is further speculated that PBAN activates an early step in the biosynthetic pathway of these pheromones. It is also proposed that PBAN actually causes the release of a secondary substance such as a neurotransmitter from the terminal abdominal ganglion that in turn activates pheromone production (Teal *et al.* 1989). However, the intermediary steps between the release of Hez-PBAN and stimulation of production and release of the sex pheromone have not been elucidated as yet. Matsumoto *et al.* (1984, 1985) reported the partial N-terminal amino acid sequence of a peptide (isolated from the heads of the armyworm, *Leucania sparata*, and *B. mori*) and named it the melanization and reddish coloration hormone (MRCH). Subsequently, a partial sequence of PBAN isolated from *B. mori* heads was reported to be identical to MRCH (Nagasawa *et al.* 1988). When Hez-PBAN was injected into 3rd instar *H. zea* larvae, they developed intense black colour immediately after their moult to the 4th

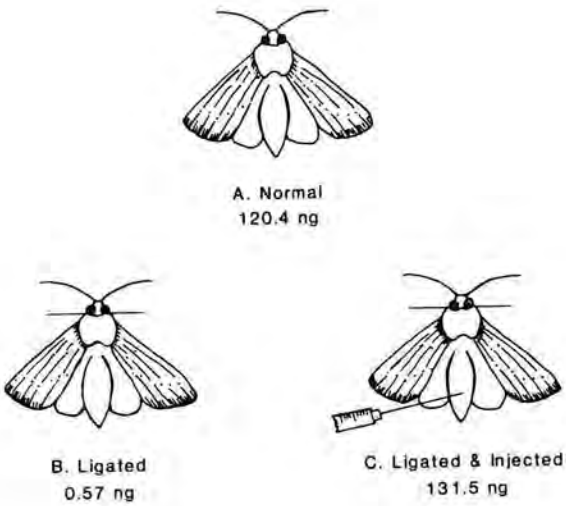

Fig. 2. Discovery of the neurohormonal control of pheromone production in *Heliothis zea* females and development of a bioassay. A virgin female during 3rd scotophase has about 120 ng of (Z)-11-hexadecenal (major component of its pheromone) A. When ligated between head and the thorax, the pheromone titer drops to 1 ng within three hours, B. If a ligated female is injected with a homogenate of brain suboesophageal ganglion or HPLC fractions thereof, and the pheromone extracted after three hours, the titer goes up to normal level, C. Ovipositor containing the pheromone gland is excised, extracted in heptane containing a known quantity of an internal standard, and quantified by capillary gas chromatography.

instar (Raina, unpublished results). Depressed levels of JH (but higher than the level that would cause premature pupation) have been reported to cause an intense black pigmentation in 3rd and subsequent instar larvae of *M. sexta* (Safranek & Riddiford, 1975; Staal, 1976). Nagasawa *et al.* (1988) have also reported that PBAN isolated from *B. mori* showed MRCH activity in that insect. The exact nature of a possible interaction between PBAN and JH while not yet clear offers intriguing possibilities for the involvement of JH regulation via other neuropeptides in the larval stages.

Recently, Cusson & McNeil (1989) reported that in the true armyworm, *Pseudaletia unipunctata*, JH acts on the central nervous system to allow the release of PBAN for initiation of pheromone biosynthesis. They further speculated that JH also acts to allow the release of neural signals that mediate calling.

EXPLOITATION OF NEUROPEPTIDES IN INSECT CONTROL.

Most commercial insecticides can be classified broadly as nerve poisons that, qualitatively at least, exert similar action in vertebrates and invertebrates. Selective toxicity is usually a function of differential uptake, transport and metabolism. In contrast, insect neuropeptides, as already noted previously, while showing sequence homology to vertebrate neuropeptides, in several instances show specific action in insects. Limited sequence variation in the peptide chain confers a high degree of selectivity on these neuroregulators. The extraordinary sensitivity of target organs and tissues to the action of neuropeptides provides most attractive inducements for further search for avenues leading to successful exploitation.

To illustrate the latter point, Katakoa and coworkers (1989) showed that native allatotropin (AT) strongly stimulated the secretion of JH from CA *in vitro* at concentration of 2×10^{-15}M. At this concentration the biological activity was increased almost 200-fold over the control.

Such levels of activity are common for neuropeptides, and justify intensified efforts to research the enzymology involved in biosynthesis and metabolism of these oligopeptides. In due course appropriate processing enzyme inhibitors could be developed as possible models for selective insect control agents. Several successful examples of this approach are already available from the human pharmacology field (Menn & Borkovec, 1989).

Speculations and proposed approaches to exploit neuropeptide technology for selective insect control have been discussed in several recent articles including: Keeley & Hayes (1987) Menn & Borkovec (1989), and Holman *et al.* (1990). Broadly, these can be divided into two categories:-

1/ Design of peptidomimetics and processing enzyme antagonists/agonists for direct application to insects. Such compounds would mimic, block or overstimulate action of neuropeptides, respectively.
2/ Insertion of neuropeptide genes into the genome of insects through recombinant DNA technology using baculoviruses as a cloning expression vector.

We have chosen PBAN as a model neuropeptide to elaborate further the foregoing strategies.

Design of Peptidomimetics and Processing Enzyme Antagonists/Agonists

Most likely, as is the case with vertebrate neuropeptides and already shown by Hekimi & O'Shea (1987) who isolated two adipokinetic neuropeptide (AKH-1) proneurohormones from *Schistocerca* CC cultured with radiolabeled amino acids, PBAN may be processed from a proneurohormone to an active form. At the present time, it is unknown whether the 33-residue Hez-PBAN binds to a receptor or is further truncated to a final active form. Processing of the proneurohormone would involve truncating enzymes. Interference with such enzymes with nonpetidic inhibitors (antagonists) should effectively block the action of the neuropeptide (Menn & Borkovec, 1989). In the process of this research, receptor ligands that block PBAN-receptor binding may also be discovered. Based on such leads, potent synthetic chemicals may be developed to disrupt pheromone production. Further biochemical research on absolute structural requirements of PBAN and tertiary structure requirements could lead to the discovery of stable antagonistic and/or agonistic peptidomimetic compounds. Such compounds would bind to the PBAN receptor and render the native neuropeptide ineffective.

Genetic Engineering - Recombinant DNA Technology

It is unlikely that native neuropeptides could ever be used as insect control agents due to their lability, to degradation, hydrophilicity, inability to penetrate the cuticle, susceptibility to UV radiation and high cost (Menn & Borkovec, 1989). A potentially promising approach to circumvent these obstacles would be to clone the gene controlling the biosynthesis of PBAN into an expression vector such as a baculovirus (Keeley & Hayes, 1987). Such a gene placed next to a viral promoter could turn an infected cell into a production site for the neuropeptide in the living insect. However, it is important that the neuropeptide expressed in the larval cell exert a strong regulatory role, since baculoviruses are known to multiply primarily in the larvae and not the adult moth. If successful, *H. zea* larvae infected with an engineered baculovirus, would express production of PBAN through intense melanisation, a symptom with yet unknown consequences. Successful expression in the adult female would result in disruption of normal periodicity of pheromone production and in turn reproduction, an ultimate target to be achieved via behavioural deregulation.

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OTHER HORMONAL AGENTS: ECDYSONE AGONISTS

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ABSTRACT

The novel insect growth regulator RH 5849 (1,2-dibenzoyl, 1-t-butyl hydrazine) is a compound which shows the rich potential for an as-yet unexploited biochemical target site in insects. RH 5849 is the first nonsteroidal ecdysone mimic as determined by data from Drosophila melanogaster and Plodia interpunctella tissue culture cells, ecdysone receptor extracts therefrom, and Manduca sexta larvae; this seems to be its primary mode of action in Lepidoptera. It also has chemosterilant activity on female Diptera, Coleoptera and Lepidoptera. RH 5849 thus represents a prototype ligand for a novel, invertebrate-specific target site.

INTRODUCTION

It is clear to those in the field of insecticide toxicology that the majority of agriculturally important insecticides are neurotoxins; of these, the primary targets are acetylcholinesterase, the GABA-gated chloride channel, the voltage-regulated sodium channel and the postsyntaptic acetylcholine receptor (for review see Lund 1985). The pesticide industry's inability to identify and exploit hormonally-associated lesions in agriculturally important insects may be due in part to a lack of fundamental understanding of these systems, and also in part to industrial screening mechanisms which select for rapidly-acting toxins. However, the ability to optimize cost-effective compounds acting on more insect-specific biochemical sites will become increasingly important, due to enhanced safety to nontargets, the potential for combatting resistance, and the likelihood for more favorable public perception.

Invertebrate moulting has been oft-considered as an excellent general target; as of now the commercial juvenoids act to alter the outcome of a normal moult (maintain a larval or nymphal state) while the benzoylphenylureas disrupt cuticular deposition after a normal moult has been initiated by endogenous hormonal signals (Retnakaran et al. 1985; Menn & Edwards, and Reynolds, this volume). While useful in specific circumstances, neither compound class has reached the agricultural importance of the conventional neurotoxins. In short, there has been a paucity of insect growth regulator (IGR) targets to serve as alternatives to juvenoid or benzoylphenylurea action; for instance a viable chemical lead which acts to mimic or disrupt levels of the steroid molting hormone 20-hydroxyecdysone (20-OH E) had previously been elusive (Bergamasco & Horn 1980; Galbraith et al. 1981). RH 5849 is a newly discovered representative of just such a class of IGRs, and a discussion of its biological effects and potential for insecticidal use (Hsu & Aller 1987, Aller & Ramsay 1988) will be the topic of this chapter.

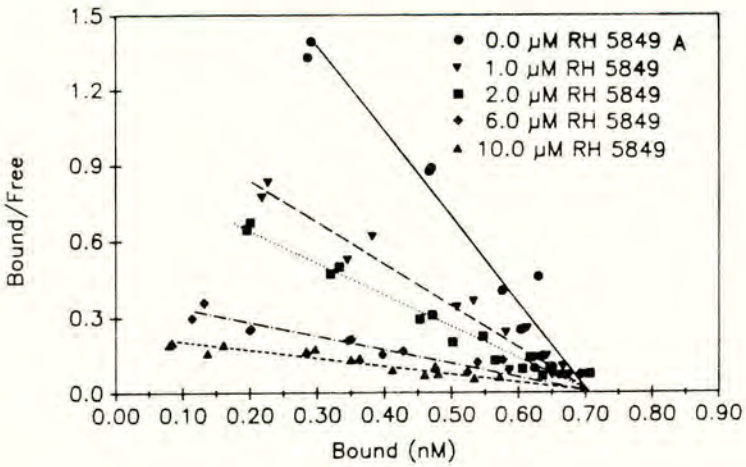
As is the case with any steroid hormone, the molting hormone axis in insects is composed of a biosynthetic gland (the prothoracic gland or ring glands), a blood medium which carries the hormone (the haemolymph), target tissues (practically all cells including those of the nerves, muscles, gut and epidermis) and mechanisms for degradation (the gut and fat body) (for a review on the physiological actions of ecdysones see Kerkut & Gilbert 1985). During larval development in a lepidopteran such as the tobacco hornworm Manduca sexta, increases in a prothoracicotropic hormone from the brain, which occur after some obligate feeding period, stimulate surges of ecdysteroid synthesis in the prothoracic glands (Bollenbacher & Granger 1985). The steroidal prothoracic gland products are metabolically activated by other tissues to the active molting hormone 20-OH E (Warren et al. 1988). Once released into the haemolymph, the hormone enters the target cell via some poorly understood mechanism, binds to specific receptors located in the nucleus, and the hormone-receptor complex binds to DNA sequences where it initiates stage-specific gene expression, such as cuticle synthesis in the epidermal cells (Riddiford 1984). At peak titers in Lepidoptera, these haemolymph ecdysteroid surges are generally 250-1000 fold over basal levels and typically reach 1 - 3 µg/ml haemolymph. However, it is also critical that this surge falls rapidly; this is necessary for successful molting fluid activation, ecdysis (shedding of the old cuticle by the underlying pharate larva), and post-ecdystial tanning (Truman 1984). This entire process is highly regulated temporally; a persistent ecdysteroid agonist which appeared in the bloodstream during feeding, forced a molt well before the normal time and then failed to drop to low levels would clearly have devastating developmental consequences. Thus insect ecdysteroid receptors are inherently sensitive and attractive targets for insecticides; unfortunately the void in our knowledge of ecdysteroid receptors at the cellu-

lar or biochemical level is such that only a few Dipteran cells and tissues have been studied (Yund and Osterbur 1985).

The Drosophila K_c cell line, first described by Echalier and Ohanessian (1969), has been widely used by developmental and molecular biologists to study mechanisms of steroid-dependent gene expression and is also a source of ecdysteroid receptors (Cherbas et al. 1984). Multiple Scatchard plots show that RH 5849 lowers the equilibrium dissociation constant (K_d) of the binding of ^{3}H-ponasterone A (a potent ecdysteroid) to the K_c cell receptor in a dose-dependent manner, while the total ligand bound (B_{max}) remains the same (Figure 1). In addition, kinetic analyses of the binding reaction shows that while RH 5849 lowers the on-rate k_a of ^{3}H-ponasterone A binding to the receptor, the off-rate k_d is unaffected (Wing 1989). These data indicate that RH 5849 is probably sharing some common binding domain in the ecdysteroid receptor with ponasterone A.

Figure 1

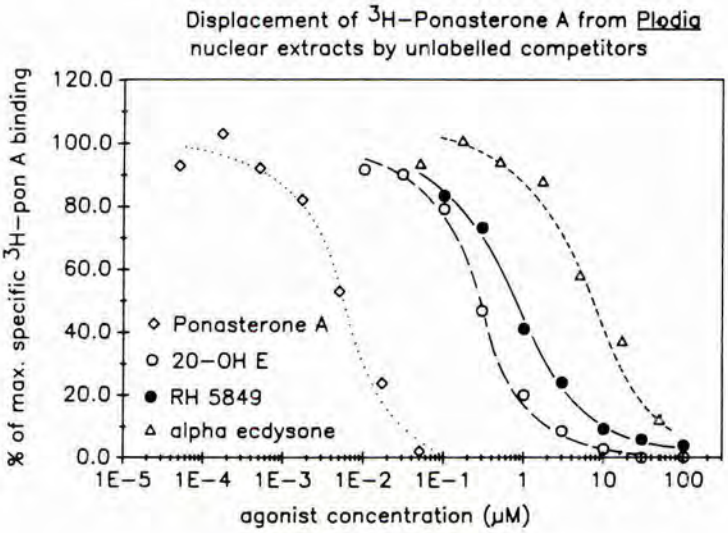
Scatchard analysis of the dose-dependent effect of RH 5849 on ^{3}H-ponasterone A binding to the Drosophila K_c cell ecdysone receptor (Wing 1988).



Similar data are accumulating for a putative ecdysteroid receptor in IAL-PID2 imaginal disc cells of the Indian meal moth, Plodia interpunctella (Wing 1989). While this cell line had been shown previously to be responsive to 20-OH E (Lynn & Oberlander 1983; Porcheron et al. 1988), biochemical evidence for a receptor in this or any other lepidopteran tissue had not been previously described. Figure 2 shows that a ^{3}H-ponasterone A receptor from nuclear extracts of these cells is saturable, of high affinity, heat-sensitive, and shows the appropriate structural selectivity for the cold competitors ponasterone A, 20-OH E, RH 5849 and alpha ecdysone. RH 5849 binds to the lepidopteran ^{3}H-ponasterone A receptor in a competitive manner, as had been observed with the Drosophila K_c cell receptor (Wing 1989).

Figure 2

Competition of nonradiolabelled competitors for specific ^{3}H-ponasterone A binding sites in nuclear extracts of P. interpunctella imaginal disc cells (Wing 1989).



Intact ecdysone-responsive K_c cells undergo dramatic morphological differentiation after treatment with active molting hormones by undergoing a reduction in cloning efficiency, halting their proliferation, elaborating long, branched processes and inducing an increased specific activity of acetylcholinesterase, beta-galactosidase and dopa-decarboxylase (for review see Cherbas et al. 1984). Of the responses in whole cells

examined thus far, RH 5849 mimicks the action of the natural molting hormone 20-OH E precisely, though it is over one hundred-fold less potent; in addition RH 5849 is 30-fold less potent at displacing 0.5 nM ^{3}H-ponasterone A from the K_c cell receptor (Table 1).

Table 1

Relative potency of 20-OH E and RH 5849 in ecdysone-dependent assays on Drosophila K_c cells in vitro (Wing 1989).

Assay	EG_{50} (μM) 20-OH E	RH 5849
Intact K_c cell process elaboration, proliferative arrest	0.035	4.8
Intact K_c cell acetylcholinesterase induction	0.007	1.05
^{3}H-ponasterone A displacement from receptor	0.10	3.0

In addition, K_c cells which are reared in the continuous presence of 20-OH E eventually become resistant to hormone treatment (Stevens & O'Connor 1982). I have found that cells raised in the continuous presence of either 1 μM 20-OH E or 100 μM RH 5849 become nonresponsive to both compounds as monitored by process elaboration and inhibition of proliferation; they also contain measurably lower levels of ecdysteroid receptor (Wing 1988). Taken together, these data indicate that RH 5849 may be a useful ligand with which to study ecdysteroid-responsive cells and provide evidence at the cellular and biochemical levels that it is capable of mimicking the moulting hormone.

However, of greater relevance to insect control, RH 5849 has highly potent ecdysonergic properties in whole M. sexta larvae or their isolated abdomens. Manduca and other lepidoptera signal the onset of larval apolysis (the separation of the old exocuticle from the underlying epidermal cells) by slipping the head capsule forward over their developing pharate mouthparts; this normally occurs at peak haemolymph levels of 20-OH E. However, administration of RH 5849 either orally or by injection causes an immediate initiation of head capsule slippage (within 24 hours), when given to M. sexta larvae at any instar, and at any day within an instar (Wing et al. 1988). Because these moulting effects can occur without an increase in endogenous hormone titers, RH 5849 is not acting as a trophic hormone for endogenous ecdysone production. In addition, other quanti-

fiable moulting responses occur in isolated abdomens which no longer have a glandular source for endogenous hormone; these would include indicators of larval moulting in fourth instar, day 1 animals such as the formation of new proleg crochets and spiracle cuticle, and indicators of pupal development in fifth instar, day 3 such as dorsal vessel exposure and prepupal contraction and cuticle formation. In these and especially in assays on intact larvae, RH 5849 is far more potent than is the natural hormone (Table 2).

Table 2

Relative potency of 20-OH E and RH 5849 in ecdysone-dependent assays on M. sexta ligated abdomens and intact larvae (Wing et al.1988).

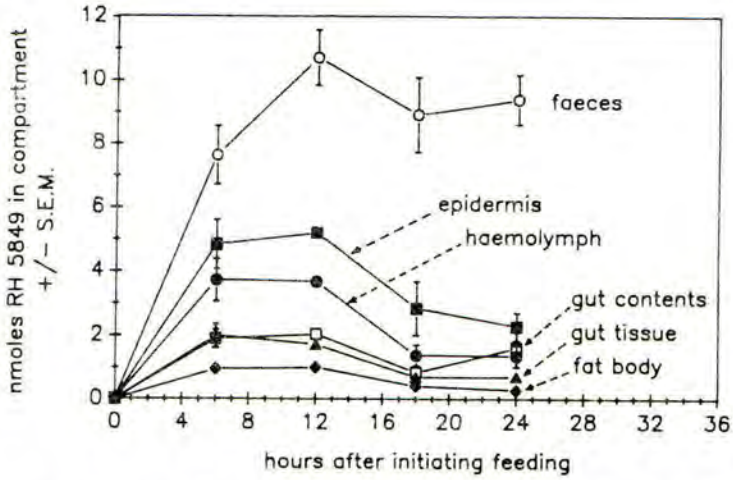
Assay (units)	EC_{50} 20-OH E	RH 5849
L4D1 Abdomens (µg/abdomen)[a]	35.2	1.3
L4D3 Abdomens (µg/abdomen)[b]	30.7	1.1
L5D0 Larvae, injected (µg/gr body wt.)[c]	181.0	3.4
L5D0 Larvae, oral (ppm in diet)[c]	>2000.0	3.0

[a] bioassayed for larval moulting
[b] bioassayed for pupal development
[c] bioassayed for head capsule apolysis

Thus, although RH 5849 seems to be a poor agonist at the ecdysteroid receptor relative to the hormone in assays in vitro, it is remarkably potent in lepidopteran tissues and whole larvae. This is apparently due to its facile absorption from the gut into the haemolymph and epidermis; Figure 3 shows the distribution of parent ^{14}C-RH 5849 in different compartments after feeding the label to newly ecdysed fifth instar Manduca larvae, dissecting the tissues at appropriate time intervals, and extracting and chromatographing the residues on TLC (with RP-HPLC verification). Note that while the majority of the 13 ug of compound is excreted unchanged, that which is present in the insect's body is located mostly in the haemolymph and epidermis and is largely parent compound (other metabolites have been identified such as ring-hydroxyllated RH 5849 and hippuric acid). This is a remarkable contrast to oral administration of 20-OH E, where initial reports indicate the hormone is probably efficiently metabolized and excreted (Robinson et al. 1987).

Figure 3

Distribution of ^{14}C-RH 5849 in different compartments in L5D0 M. sexta larvae, after feeding on 10 µg compound/gr body weight in artificial diet (Wing 1989).



Corroborative evidence for the ecdysonergic actions of RH 5849 in Lepidoptera are accumulating for P. interpunctella whole IAL-PID2 imaginal disc cells and imaginal disc tissues, as well as imaginal disc cells of the cabbage looper Trichoplusia ni (IAL-TND1) (Silhacek and Oberlander, personal communication). Ecdysone-agonist effects are also being seen in the eastern spruce budworm Choristoneura fumiferana and the forest tent caterpillar Malacosoma disstria, both at the whole organism and cuticular ultrastructural level, and in tissue culture cells from the latter (Retnakaran and Sohi, personal communication). In our hands, we have observed symptoms consistent with a rapid moult induction in several lepidopteran larvae of the families Arctiidae, Gelechiidae, Geometridae, Hesperiidae, Lasiocampidae, Lymantriidae, Lyontiidae, Oleuthreutidae, Noctuidae, Pyralidae, Sphingidae, Tortricidae and Zygaenidae; thus, this phenomenon seems to be widespread across the Lepidoptera.

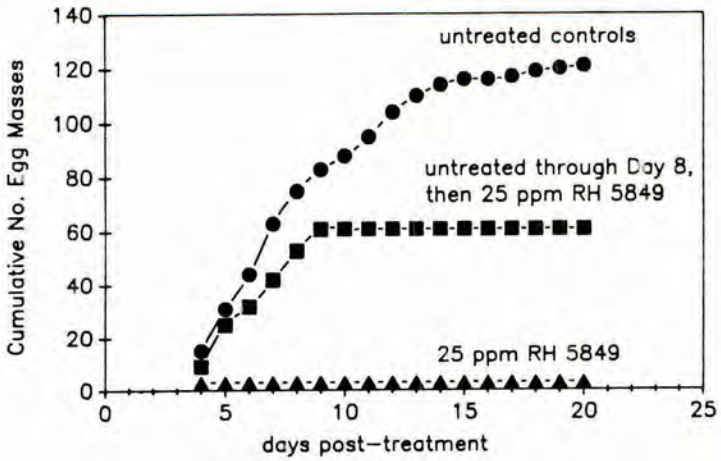
Although we have observed inhibition of ecdysis in Coleoptera such as the Mexican bean beetle Epilachna varivestis and the Colorado potato beetle Leptinotarsa decemlineata, as well as in larval Diptera such as the housefly Musca domestica and the yellow fever mosquito Aedes aegypti, it is unclear whether this is due to actual ecdysonergic effects or to some other biochemical lesion such as neurotoxicity. It is important to note that ecdysis failure can be induced by any of a number of compound classes and environmental stresses (Reynolds 1975), and may or

may not be caused by an ecdysone-like action. This is an important area worthy of further investigation, especially at the ultrastructural level.

RH 5849 also has unique chemosterilizing properties in Diptera, Coleoptera and Lepidoptera. This is exemplified by the data shown in Figure 4, where it is clear that RH 5849 rapidly halts egg laying in gravid L. decemlineata females, and inhibits the formation of new ovarioles. Similar results have also been observed in female M. domestica; in this case administration of the compound at 0.01 to 1.0% in the diet of adult female flies leads to a dose-dependent inhibition of ovarian development; however, all eggs which are laid are viable. This is in marked contrast to the benzoylphenylurea Dimilin, which has strong ovicidal effects. The role of ecdysteroids in insect reproduction is not clearly defined, except to state that the ovaries of many insects synthesize ecdysteroids, that ecdysteroids and their conjugates are found in insect embryos, and that ecdysones seem to stimulate vitellogenesis in Diptera (Hoffmann et al. 1986). Thus, while it has previously been shown that ecdyteroids can be effective insect chemosterilants (Robbins et al. 1968, Robbins et al. 1970), it is at present uncertain whether the reproductive effects observed with RH 5849 are truly due to a hormone agonist effect.

Figure 4

Rapid inhibition of oviposition and chemosterilization of adult female L. decemlineata, after foliar spraying of tomato plants (Lycopersicon esculentum) with RH 5849.



Certain natural products are also known to affect moulting in insects, or at least to disrupt ecdysis. Examples would include the limonoid antifeedant and growth regulator azadirachtin (Jacobson 1988), the naphthoquinone plumbagin (Kubo et al. 1983), the antineoplastic and cytotoxic quassinoids (Klocke et al. 1985, Lidert et al. 1987), and the Streptomyces broth product alanosine (Matsumoto et al. 1984). The precise endocrine effects these various compounds have on insects have not been fully elucidated; however it is known that azadirachtin at least is capable of severely lowering levels of both juvenile hormone III (Rembold et al. 1983) and ecdysones (Sieber & Rembold 1983), possibly by acting on the corpora cardiaca, the neural organs for the release of various neuropeptides (Rembold et al. 1989). These natural product "leads" may eventually prove to be useful probes into other commercially exploitable hormonal lesions.

In closing, it seems apparent that our ability to utilize insights into insect endocrinology for the development of novel pesticides is primitive at best, and often limited to a few hormone/integument systems. However, it is hoped that the discovery of the 1,2-dibenzoyl, 1-alkyl hydrazines will serve as an example of how aspects of both "basic" and "applied" research can help uncover agriculturally useful new IGRs acting on novel biochemical targets.

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SEMIOCHEMICALS FOR THE CONTROL OF INSECT PESTS

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ABSTRACT

The use of commercially available slow release pheromone formulations for the control of economically important insect pests by mating disruption is discussed. Other pheromonal control strategies using mass-trapping, lure and kill and bioirritation are described. The difficulties of implementing a commercially acceptable control programme are illustrated by reference to the adoption of pheromones for the control of the pink bollworm Pectinophora gossypiella (Saund.) in Egypt.

INTRODUCTION

Semiochemicals are employed for both intraspecific and interspecific communication in insects. Compounds which convey information between members of the same species are known as pheromones. Since the isolation of the first pheromone, that of the silk worm moth Bombyx mori L. by Butenandt et al. (1959), progress in the identification and use of pheromones and other behaviour modifying chemicals has been uneven. Most work has been on sex pheromones of Lepidoptera and aggregation pheromones of Coleoptera, and to a lesser extent on epideictic pheromones which influence oviposition behaviour in Coleoptera, Diptera, Homoptera, Hymenoptera, Lepidoptera and Orthoptera and alarm pheromones which stimulate escape and other defensive behaviour in Dictyoptera, Hemiptera and eusocial Hymenoptera.

The chemical components range from blends of aliphatic alcohols, aldehydes, esters and epoxides as found in lepidopterous insects to alkenoic acids and aldehydes, branched alkenones, esters, monoterpene alcohols and aldehydes as employed by Coleoptera.

SLOW RELEASE PHEROMONE FORMULATIONS

Slow release formulation is essential for pheromones used in control strategies in order to prolong the release and efficacy of compounds which are otherwise highly volatile and to provide stabilization of remaining material under field conditions. Several formulations have so far been used commercially.

1) Hollow fibres, developed by Albany International (subsequently Scentry and now Yellowstone International) in the USA and now sold under licence by Sandoz in Switzerland. Polyether or polyester fibres, measuring 1.5 cm in length with an inner diameter of 0.2 mm, are sealed at one end and contain about 250-275 ug of pheromone (Svenson and Weatherstone, 1989).

2) Laminate flakes, developed by the Hercon Division of the Health-Chem Corporation in the USA and now sold under licence by BASF in Germany. The flakes, consisting of two layers of plastic laminate in between which is sandwiched a porous layer impregnated with pheromone, can be varied in size to give different rates of release or numbers of point sources per unit area to suit different types of ground or aerial application equipment (Quisumbing & Kydonius, 1982).

3) Microcapsules, developed jointly by the Overseas Development Natural Resources Institute (ODNRI) and ICI Agrochemicals. The microcapsules, made of polyamide and polyurea, consist of tiny spheres with a median diameter of 2-3 um. They are miscible in water and contain light-stable additives to prevent degradation of the capsule wall and the enclosed pheromone particularly in the presence of sunlight (Hall et al., 1982).

4) Twist-tie dispensers, developed by the Shin Etsu Company in Japan and marketed by Mitsubishi Corporation of Japan. This formulation consists of a polyethylene tube, 10-20 cm long, containing the pheromone and a soft-wire stiffener.

5) A polyvinyl chloride resin formulation developed jointly by ODNRI and Biological Control Systems Ltd which has provided good protection for more labile pheromones containing both aldehydic and conjugated diene functionality. (Cork et al 1989).

Of the five formulations listed, the hollow fibres and laminate flakes have to be applied with a glue to ensure adherence to the plant foliage; the microcapsules, being a water-based suspension, can be sprayed with conventional applicators without the need of any special adhesives, while the twist-tie and PVC dispensers can as yet only be applied by hand, tied or attached around the stem of the plant.

The relative merits of sprayable and hand applied pheromone formulations must depend on circumstances. Relatively stable pheromones such as for the pink bollworm, a 1:1 mixture of (ZZ) and (ZE)-7,11-hexadecadienyl acetate applied in hollow fibre or microencapsulated formulations have a field persistence of two to three weeks. Much greater persistence of up to 100 days can be achieved in the field using the twist-tie or PVC resin formulations.

Pheromone formulations need not be excessively expensive and recent studies conducted in Egypt suggest that the pink bollworm pheromone formulated in hollow fibres, microcapsules and twist-ties offer cost savings of up to 20% compared with conventional insecticides. Additional savings are possible using the twist-tie formulation which is applied by hand only once during the cotton season compared with three or four applications of the hollow fibre or microencapsulated pheromone formulations.

CONTROL POSSIBILITIES USING PHEROMONES

Control of lepidopterous pest species is possible by means of mating disruption whereby chemical communication between the sexes

for the purposes of mating is prevented in the presence of excess amounts of the synthesised pheromone (Campion et al. 1989).

An example of the successful use of this technique is for the pheromonal control of oriental fruit moth Cydia molesta (L.), a world-wide pest of peaches and nectarines which was first achieved on a commercial scale in Australia in 1985. An area of 160 ha was treated season long with the pheromone blend of (Z)-8-dodecenol and (Z)-8-dodecenyl acetate dispensed in twist-ties at a rate of 75g/ha. The levels of control achieved were comparable to those in a conventional programme of four applications of azinphosmethyl and malathion. However there was an upsurge of two-spotted mites in the insecticide treated plots which was not found in those treated with pheromone. (Davidson 1985). The technique was subsequently also adopted in California and France and by 1989 3200 ha of peach orchards had been successfully treated. (Casagrande et al. 1987 K. Ogawa private communication). Successful control of C. molesta was also reported from France and Switzerland using a laminate flake formulation and commercial use registration achieved (see review by Campion et al. 1989).

The European grape berry moth Eupoecilia ambiquella (Hubner) has been successfully controlled using a laminate flake formulation of the main pheromone component (Z)-9-dodecenyl acetate and commercial registration granted for use in wine growing areas of Germany (H. Wolgast private communication).

Hollow fibre, laminate flake and twist-tie formulations of the pheromone of the artichoke plume moth Platyptilia carduidactyla have all been used successfully and have achieved commercial registration for use in the United States.

Mass trapping trials for a whole range of Lepidopteran and Coleopteran pest species have generally not been successful. The technique would appear more appropriate using the aggregation pheromones for controlling beetle pests since both sexes are attracted, rather than for Lepidoptera where in most cases only the males are caught and therefore a very high proportion of the population needs to be trapped which creates practical difficulties.

Large scale mass-trapping of the Spruce bark beetle Ips typographus (L.) was initiated in Scandinavia in 1979 when 2.9 billion beetles were trapped using a lure mixture of cis-Verbenol, methylbutenol and Ipsdienol. Appropriate trap technology was developed as part of an IPM programme which also included the prohibition of storage of unbarked logs in the forest during the summer and a general clean-up after storm damage and logging as well as the felling and removal of beetle infested trees. (Bakke and Lie 1989).

The combined use of semiochemicals and insecticides whereby insects are attracted to pheromone sources spiked with insecticides is receiving some attention for the control of certain lepidopterous pest species but no example has reached the stage of wide scale commercial adoption (Campion et al. 1989).

Bioirritation techniques by semiochemicals to enhance the contact effectiveness of insecticides is well exemplified by the use of alarm pheromones in the control of aphids (Pickett 1988).

CONSTRAINTS TO THE IMPLEMENTATION OF PHEROMONES FOR THE CONTROL OF INSECT PESTS

In view of the large amount of research devoted to the identification of by now, several hundreds of semiochemicals and their subsequent field evaluation it is perhaps surprising that so few have yet reached the stage of commercial utilisation. There are however several constraints which have to be overcome in order to implement a pheromonal control programme. These are illustrated by reference to the commercial adoption of pheromones for the control of the pink bollworm Pectinophora gossypiella (Saund.) in Egypt.

There has been for some years a collaboration between ODNRI and the Egyptian Ministry of Agriculture. It was evident that to introduce a new control approach into a conservative farming community and for the outcome to be successful the method should be:

1. Technically possible
2. Practically feasible
3. Economically desirable
4. Environmentally acceptable
5. Politically advantageous.

Pectinophora is considered to be the most important cotton pest in Egypt. Most of the larval stage occurs within the cotton boll and thus protected it is difficult to control with conventional insecticides. An alternative control method aimed at the adult stage is therefore advantageous.

Technical feasibility

Control of insect populations by mating disruption is achieved by applying pheromone throughout the crop. Slow release pheromone formulations which persist in the crop for several weeks after application are initially applied early in the season when insect populations are low and successive applications made at regular intervals to prevent population build-up.

Hollow fibre, laminate flake and microencapsulated pheromone formulations were first evaluated in Egypt in a series of large scale trials using cotton blocks of 50 or 100 ha. as the sole means of controlling the pest insect. Pheromone treatments were compared with conventional insecticide spray treatments in other blocks of cotton of similar size sited in the same localities. Comparisons of numbers of infested flowers and green bolls, open boll counts, crop development and yields of seed cotton in pheromone and insecticide treated blocks showed that adequate levels of control were achieved using the pheromone formulations which were equal in effect to the insecticide sprays (Critchley et al. 1983, 1985, El-Adl et al. 1987).

Practical feasibility

Cotton in Egypt is increasingly grown in large blocks as the result of cooperation between a number of small farmers. Larger scale commercial trials were therefore undertaken and to ensure area-wide application and aerial spraying of the pheromone formulations was undertaken using helicopters or fixed-wing aircraft (Campion et al. 1989). Since the aerial application of insecticides for controlling cotton pests is widely used in Egypt, sufficient aircraft and experienced pilots were available to apply the pheromone formulations. The only disadvantage was that since pheromone applications begin several weeks before the scheduled insecticide sprays, there is sometimes a shortage of aircraft at the time of the first application which has caused delays. Nevertheless it has generally been accepted that the method is practically feasible.

Economically desirable

Pheromones are often thought to be too expensive to be of practical value. Large-scale synthesis of Pectinophora pheromone has however resulted in a relatively cheap product with the result that pheromone formulations supplied by ICI and Sandoz Agrochemical Companies are now on average 20% cheaper than conventional insecticides. However to achieve competitiveness with insecticides the number of pheromone applications made during the season has been reduced from six to only three or four and the relative loss of pheromone could pose problems under conditions of high insect pressure.

More recently the 'twist-tie' pheromone formulation marketed by the Mitsubishi Corporation requiring only one application for season-long control has been successfully evaluated in large-scale trials and is now being used commercially in Egypt. Only hand application is possible but this may be of particular relevance in developing country situations in rural areas.

All the pheromone formulations at present available thus appear to have economic advantages.

Environmentally acceptable

The pheromone formulations have been shown to be completely non-toxic both to man and higher animals and since selective in action for only one insect species, beneficial insects have been conserved in greater numbers in pheromone treated areas compared with those found in areas treated with conventional insecticides (Critchley et al. 1985, El-Adl et al. 1987).

It has also been shown that bee hives sited next to cotton fields treated with pheromones contained substantially more honey than those next to insecticide treated fields (Moawad et al. 1989). Thus in comparison with current practices pheromonal control is environmentally acceptable. This has been recognised by the Environmental Protection Agency in the United States, since it has agreed to consider pheromones

as biorational agents and as such require less rigorous evaluation procedures for usage registration (Punja 1989).

Politically advantageous

The Egyptian Ministry of Agriculture through its Pesticide Recommendation Committee selects which insecticides are to be applied to the cotton crop each year and also organises their application. Once convinced of the effectiveness of the pheromone formulations it is thus able to implement newer control strategies over wide areas. However, full responsibility for controlling the cotton pest complex rests with the Ministry of Agriculture and although an environmentally acceptable control strategy is politically desirable, possible risks of failure with a relatively new technique has resulted in a policy of caution and the need for further annual demonstrations of efficiency in cotton growing areas at present in the region of 20,000 ha.

Remaining constraints to pheromone usage for the control of cotton pests in Egypt and their resolution

It is suggested that the main hurdles to the implementation of pheromonal control for the control of pink bollworm over a wide scale have been overcome but some doubts still exist about their effectiveness; which are:-

1. The risks of using selective control methods
2. The availability of broad spectrum insecticides as an easy alternative
3. Misconceptions about pheromone control strategies.

It has been argued that in the absence of conventional pesticides in the pheromone treated areas there could be an upsurge of the Egyptian cotton leafworm Spodoptera littoralis (Boisd.) early in the season or the spiny bollworm Earias insulana (Boisd.) late in the season.

At present Spodoptera infestations early in the season are generally contained throughout the country by the hand collection of egg masses using teams of children (Hosny, 1980), supplemented later in the season by application of insect growth regulators. In other words two other insect species specific control techniques are already being used and regarded as acceptable. It was suggested that late season control of Earias and possibly Pectinophora could be achieved by substituting the last pheromone application for a broad spectrum insecticide. (Campion and Hosny, 1987). Such an integration of pheromone usage coupled with other selective control methods early in the season and insecticides late in the season had been shown to be effective in Egypt in a series of 40 ha blocks of cotton by Critchley, et al. (1984).

During 1987 a similar strategy was adopted by the Egyptian Ministry of Agriculture in a total area of 8,000 ha where after hand collection of egg masses for Spodoptera control two or three Pectinophora pheromone applications were followed by one to two sprays of conventional insecticides and successful control of the cotton pest complex was achieved (Moawad et al. 1989).

Work is also in progress to develop an effective pheromone formulation for the control of Earias spp. Recent trials conducted in Pakistan suggest that a joint application system for both Earias and Pectinophora pheromones using a Mitsubishi twist-tie formulation is possible (Critchley et al 1987).

CONCLUSIONS

Critics of those advocating selective control strategies have argued that such allegedly complicated and possibly risky procedures are not worthwhile at a time when new and more powerful pyrethroid insecticides are available. Indeed in some countries scheduled spraying of four or five successive pyrethoid insecticides is used for season-long control of the cotton pest complex. There is sufficient documentation throughout the world of the dangers of such an approach which eventually leads to an upsurge of secondary pests including American bollworm, whitefly and mites. Restricting the use of pyrethroid insecticides to later in the season must help to avoid such complictions while the consequent destruction of the beneficial insect fauna would be at a time of a natural decline in numbers.

It has taken about 25 years to reach the point where the practical commercial feasibility of using behaviour modifying chemicals in the control of economically important pest species has been achieved. There are still relatively few examples that demonstrate this achievement and these in particularly favourable circumstances. It has been the aim of this paper to emphasise that there are many problems both biological, chemical, technological, environmental and social that have had to be overcome. It is anticipated that the lessons learnt from the success so far achieved will help to advance the technique in less favourable circumstances that are also acceptably convincing.

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