SESSION 7A

RECENT ADVANCES IN BIOPESTICIDES

CHAIRMAN DR B. KERRY

SESSION ORGANISER DR A. T. GILLESPIE

INVITED PAPERS 7A-1 to 7A-3

VIRAL INSECTICIDES: POTENTIAL, PROBLEMS AND PROSPECTS.

J., HUBER

Biologische Bundesanstalt für Land- und Forstwirtschaft, Institut für biologische Schädlingsbekämpfung, D-6100 Darmstadt, Germany F.R.

ABSTRACT

From more than 1100 insect viruses known today, it is mainly the baculoviruses which are used in pest management. A major advantage of these viruses is that they are highly specific and can be used to suppress pests without harming beneficial arthropods, man or the environment. However, this selectivity is also one of the major obstacles to the commercialisation of viral insecticides as it restricts the market potential.

The paper discusses possible solutions to this dilemma.

WHY DO WE NEED VIRAL INSECTICIDES ?

One of the main principles in integrated pest management is the use of specific pesticides for a directed control of the few pest species that surpass the economic damage threshold in a given crop. The intention is to leave the ecosystem as undisturbed as possible, in order to protect the natural enemies present in a intact fauna and integrate them into the pest control strategies. For economic reasons, most chemical insecticides are broad spectrum pesticides and therefore fit very poorly into such schemes. To make things even worse, a single application of a broad spectrum pesticide can annihilate the effect of a series of selective control measures. The lack of selective insecticides is therefore a major problem in integrated control programmes in agriculture and forestry.

In addition to being fatal to the natural antagonists of pests, many chemical pesticides have been identified as major pollutants of our environment. Therefore, the use of chemicals in plant protection is becoming increasingly restricted by the very rigid tolerance levels for residues of chemicals in water, soil and air.

Agriculture without pest control measures, on the other hand, is not economically feasible, either for the producer, or for the consumer. Fortunately, there are many methods of biological control which can give relief in this situations. Through the insect viruses, for instance, nature itself is providing us with highly specific insecticides.

WHAT ARE INSECT VIRUSES ?

Viruses pathogenic for insects do not form a taxonomic entity. We can find them in such different virus families as Iridoviridae, Parvoviridae, Poxviridae, Reoviridae, and Baculoviridae. Most of these families also have representatives which infect not only insects but also vertebrates or even plants. There is one exception to this: the members of the family of the Baculoviridae, the baculoviruses, which infect arthropods only. Until today no baculovirus has ever been isolated from a non-arthropod host. They have been found in crustacea and mites, but mostly in insects. This absence from organisms other then arthropods is strong indirect evidence that baculoviruses are safe for man and the environment. This is one of the main reasons why it is mostly these viruses that are being used in pest control.

Baculoviruses are characterized by double stranded circular DNA which is included in rod shaped capsids. They are formed mostly in the nucleus of the host cells. In common with many insect viruses from other virus families, the virions of most baculoviruses are contained within proteinaceous particles, the so called occlusion bodies, which often have a polyhedral shape (therefore the name polyhedrosis virus). The thick layers of protein provide protection against adverse physical and chemical factors in the environment, allow survival outside the host cell, and enable the viruses to kill their host rapidly without jeopardizing their own existence. It is obvious that the good protection of the virus particles by the occlusion bodies also is of a great advantage for the use of these viruses as biological insecticides. Since they are so resistant, in general, they can be used like chemical pesticides.

WHAT IS THEIR POTENTIAL IN PEST CONTROL ?

Baculoviruses have several properties which make them ideally suited for use in integrated plant protection programmes. Their most important attribute in this regard is their extremely high host specificity. Though they are found in several families outside the arthropoda, a given virus in most cases only infects a few insects species usually belonging to the same family or even the same genus. In many cases, just a single susceptible host is known. This means, when used in plant protection against a given pest species, only the target pest is affected and all the beneficial or even neutral arthropod species in the same ecosystem are left unharmed. Therefore, the whole potential of the natural antagonists of the pest, present in an intact ecosystem, can be exploited. Many secondary pests are kept below economic damage levels and the necessity for additional plant protection measures is greatly reduced. As a consequence, a treadmill situation as often results from the use of broad spectrum pesticides, can be avoided. At the same time, selectivity means that baculoviruses are harmless for man, environmentally and ecologically safe, and do not present residue problems.

In contrast to chemicals, viruses have the ability to multiply in their host. After dissemination for pest control, they may persist or even spread in the population of the target pest. They can even initiate real epizootics which may keep the pest at a low level for several years and make further control measures unnecessary. So far we have no indication that resistance in the pest population will become a major problem, as it is for many chemical pesticides. In nature, insect populations have been exposed to viruses for thousands of years without ever becoming resistant.

From the more than 1100 insect viruses known today, about 60% belong to the baculoviruses. There are estimates that baculoviruses can be used against nearly 30% of all the major pests of food and fibre crops. In Central America, by replacing chemical insecticides with insect viruses, pesticide consumption could be reduced by nearly 80%. This vast potential for pest control so far has hardly been exploited. World-wide, little more that a dozen baculoviruses are registered for use as biological insecticides in forestry and agriculture. Whereas in the past most registrations were held by government agencies in the United States of America and in Canada, recently several virus preparations were being produced and sold by private companies, particularly in Europe (see Table 1).

WHAT ARE THEIR LIMITATIONS ?

It can not be denied that the wider use of insect viruses in pest control is confronted with some reluctance. Insect viruses have also some features which are negative, particularly with regard to their economic use in plant protection. As profitable and desirable specificity is from a ecological viewpoint, it causes problems with regard to the economics of the commercialisation of viral pesticides. In most countries, insect viruses have to be officially registered for use as insecticides and are subjected to the same regulations as chemical pesticides. Therefore, the expenses for their commercialisation are in the same order of magnitude as for conventional insecticides. But, due to their selectivity, their market size and their sales potential is very limited - an aspect which is not very attractive for a potential producer, especially not for big companies which have the necessary experience for handling registration, but which need a big market to make production profitable. As a consequence, microbial pesticides, if they ever make it to the market, find it difficult to compete in price with chemical pesticides.

Furthermore, the use of selective pesticides in the framework of integrated pest management programmes requires good knowledge of the biology of the pest species and their antagonists in the crop, and is therefore not as simple as the use of broad spectrum chemicals. Since insect viruses do not act on contact and are less persistent than most chemical pesticides, much more attention has to be given to the correct timing and application of the sprays. All this makes the use of selective pesticides more cumbersome and more costly for the farmer. So, whereas the main advantage of using a selective insecticide is on an environmental and social level, there is usually hardly any immediate economic benefit for the farmer as the direct user of the product. The basic constraints for a wider use of viral pesticides arise therefore from the fact that the negative and the positive aspects of the use of selective pesticides do not afflict the same group of people. Whereas it is mostly the general public who has the direct benefit from the use of ecologically non-disruptive pesticides in the form of an intact environment, the farmer has mostly to deal with the negative side of selectivity, since he has to pay for the high price. Nobody can blame the farmer for not being particularly keen to pay for somebody else's advantage. Therefore, a way has to be found that the public pays its share in the costs, so that the use of environmentally safe products becomes profitable to the farmer.

WHAT ARE THE PROSPECTS FOR THE FUTURE ?

A change in this controversial situation can not come from the farmer or from the plant protection industry. They both are subjected to the law of commerce. Only the consumers or the government can alter the situation. If the consumer prefers agricultural products which are produced with the help of environmentally save pesticides, the farmer is induced to abandon his traditional pest management practices. A change in the habit of the consumer can easily alter the whole market. The government on the other hand, has the possibility to change the market from the outside through regulations, taxes and subsidies. It could, for instance, raise taxes on environmentally disruptive preparations or pay subsidies for less harmful products. The government could even ban the use of a given broad spectrum chemical, if another environmentally safe product is available for the same purpose. It is obvious that in any case we have to be prepared to pay higher prices for the agricultural products.

Recent advantages in genetic engineering have risen hopes that this novel technique would be capable of improving some of the negative pesticide qualities of insect viruses. It has been suggested for instance to create viruses which a) exhibit a broader host range, b) produce a toxin for faster kill, c) show increased virulence, or d) have a better environmental stability than the natural occurring parent viruses. Technically this way is feasible and some very promising results have already been achieved, but in view of the public's general apprehension of genetic engineering, release of such recombinant viruses into the environment probably will meet great resistance. Since, as we have seen above, the main hindrance for wider use of insect viruses is not so much lack of efficacy as economical constraints, this would not solve the problem anyhow.

Whereas insect viruses, for reasons outlined above, face many problems in industrialized countries, they look very promising for use in third world nations. Their production technique is simple and they can be produced in a "cottage type" industry, using local resources and manpower. Viral pesticides offer the possibility for developing nations to produce their own insecticides within the country. By this they become less dependent on the industrialized nations, from which they had to buy the chemicals before. This fact has been recognized, and the potential of insect viruses is already being exploited in countries like Brazil, Guatemala, Thailand, Columbia, Zimbabwe, Malawi and so on.

To summarize, only if we accept that the preservation of nature has its price and if we are willing to pay it, then, and only then, will viral pesticides be as widely esteemed and used in the future as they should be with regard to our environment.

Tab.1: Virus preparations registered in Europe

target pest	virus type[1]	product trade name	registered country	registered date
Agrotis segetum	GV	AGROVIR	DK	01/90[2]
Cydia pomonella	GV	MADEX	CH	12/87
		GRANUPOM	D	03/89
Neodiprion sertifer	NPV	Monisärmiövirus (Kemira Sertifvirus)	SF	05/83
		VIROX	GB	1984
Adoxophyes orana	GV	CAPEX	CH	02/89[3]
Mamestra brassicae	NPV	MAMESTRIN	F	12/88[3]

[1] NPV = nuclear polyhedrosis virus, GV = granulosis virus
[2] notification only, no registration needed
[3] experimental use permit

BIOLOGICAL CONTROL OF SOIL-BORNE DISEASES

R. CAMPBELL

Department of Botany, University of Bristol, Woodland Road, Bristol, BS8 1UG

ABSTRACT

The general principles of biocontrol have been known for many years, but there are at present few field scale commercial products available. There are many reasons for this, but one of the main ones is that present control agents, and those in development, do not give consistent results. Some of the causes of this are examined and the future prospects assessed.

INTRODUCTION

Biological control can be broadly defined as any method of control of plant diseases which uses organisms, other than man, to reduce disease (Campbell, 1989a). This can include plant breeding and host defence mechanisms (Hornby, 1990), but this paper will consider especially the use of selected microorganisms inoculated onto the crop or the soil to control disease (Campbell, 1989b). There is some overlap with deleterious bacteria and plant growth promoting rhizobacteria. The former cause reductions in plant growth without obvious disease symptoms and the latter may improve plant growth by various mechanisms including affecting hormone balance and plant nutrition, and by controlling deleterious bacteria (Schippers *et al.*, 1987). These bacteria, and some fungi, exist in the rhizosphere and any isolation and screening programme will produce microorganisms that benefit the plant and those which harm it, as well as a lot of apparently neutral organisms.

REASONS FOR BIOCONTROL

The general principles of biocontrol, and many laboratory demonstrations, have now been well documented (Cook & Baker, 1983; Lynch, 1988; Campbell, 1989a, b; Hornby, 1990). Attention has centred on the control of root diseases, largely because there are adequate chemical or plant resistance controls for foliar pathogens, and root diseases are now seen as the main limitation on crop production (Cook, 1986). Working with root diseases is more difficult, more time consuming and therefore more expensive, than work with foliar pathogens. Biological control is being considered because the soil is a place where introduced microorganisms might survive, as opposed to the harsher environment of the leaf surface for example, though investigations of both sites are hampered by the rather poor knowledge of microbial ecology. The advantage of biological control is that the commercial development and registration costs are now considerably

less than for chemicals (Lethbridge, 1989). The number of compounds that are screened to produce a chemical pesticide is rising. This is partly a reflection of the ever more stringent registration requirements, but it may also reflect a fear that most of the 'good' chemical groupings have been discovered over the past 50 years of intensive searching by the world's chemical companies.

One reason for the current interest in biological control is that there is pressure from the general public and bodies concerned about 'the environment' for a reduction in the use of chemical pesticides; whether this is a valid fear need not concern us here, if it exists then it will affect the way in which the control of plant diseases is viewed by the industry, as well as by people at large. Biological control is perceived as being environmentally less damaging than chemical pesticicdes. There is no *a priori* reason why a culture of a microorganism, and possibly some of its metabolic products, should be inherently safer than a chemical, provided both are adequately tested and registered prior to release. There is the added complication with microorganisms that some may be genetically engineered (see below).

There are very few inoculants commercially available at present for soil borne diseases (Lynch, 1988). There is *Agrobacterium* against crown gall and *Bacillus subtilis* (Quantum 4000) against *Cercospora* on peanuts. A pseudomonad (Dagger G) for the control of *Pythium*, which was available (Lynch 1988) has now been withdrawn. Those control agents that there are, apparently work by the production of siderophores, antibiotics or possibly lytic enzymes, or by competition with the pathogen for nutrient requirements or for attachment sites, and these modes of action have been descibed in many publications (Lynch, 1988; Campbell, 1989a; Hornby, 1990).

There is much potential for biocontrol that does not use commercially viable inocula, and never will do, though it is none the less important for that. This includes particularly the manipulation of crop systems to favour antagonistic organisms (Cook, 1986; Hornby, 1990), and various organic farming systems which reduce the effects of root pathogens (Hoitink & Fahy, 1986; Lennartsson, 1988). These systems will be particularly important to people in the Third World who may not be able to afford commercial inoculants.

PROBLEMS WITH BIOCONTROL

If biocontrol agents have so many advantages, real or imagined, why do we not have more of them in commercial use ? The main reason is that there has until recently, been no serious research efforts to find, commercially develop and exploit biocontrol agents. This has now changed, and most agrochemical companies around the world, and many small biotechnology companies, have programmes (Lynch, 1988), because of the lower costs amongst other more altruistic reasons.

It is not anticipated that there will be any major problems with protecting commercial property by patents on the microorganisms

themselves, on the production process, or on the formulation and use (Crespi, 1989). There is beginning to be a case law for biotechnology in general, but there have been few tests of these patents in the courts.

Though most of the present commercially available biocontrol agents are inoculants derived from natural populations by selection, it is likely that in the future more of the organisms will be genetically engineered. Natural organisms selected by traditional laboratory strain improvement do not, at present, require special permission for release in the country of origin, though there are of course safety regulations for the production and distribution of commercial quantities. The position for the release of microbial pest control agents, engineered or not, is further advanced:there are established protocols in most countries (Laird *et al.*, 1990), and it is likely that these will form the legal basis for biocontrol agents in general. Release of engineered organisms does require special permission and safety protocols (Klingmuller, 1988; Sussman *et al.*, 1988; Royal Commission, 1989) and releases are being dealt with on a case by case basis at the moment. General laws are in existance in some countries or are in the process of being enacted. These regulations may delay release of biocontrol agents and increase the cost of registration, but are unlikely to prevent the release of 'reasonably' manipulated organisms. There is reported to be one genetically engineered control agent already cleared for release, a strain of *Agrobacterium* for the control of crown gall (Jones *et al.*, 1988; Wright, 1989; Ryder & Jones, 1990), but most potential organisms are still in development.

The main problem at present is that biocontrol agents do not usually give such good control as chemicals, and the results are not so reproducible. There are many reasons for this unreliability, and it has been known about for many years (Suslow, 1982). We should bear in mind that it is not unknown for chemical control and host plant resistance to vary: indeed host plant resistance may not exist for some races of the pathogen. This variation is usually rather small, and if it were not the chemical would not be used. The problem with biological control may therefore be that the selection of the organisms has not been rigourous enough. The variation in biocontrol agents can be caused by changes in the agent itself. It may also take the form of different results from experiments in different years, suggesting perhaps a climatic effect (Becker *et al.*, 1990). Alternatively there may be different results from different sites in the same year and this may be caused by soil variation, differences in pathogen race or inoculum potential, or differences in host species or cultivars. I will now examine these possible problems in more detail.

Since the biocontrol agent is alive it may mutate or change its physiology in storage, production or during use. The mutant may not be an effective biocontrol agent. This requires stringent quality control in development and manufacture, and genetically stable strains, giving repeatable results in trials, should be selected.

Biocontrol agents are expected to grow, or at least survive, in the environment and they are therefore subject to environmental factors. This may be a reason why some work only in certain soil types or in particular seasons when the soil is wet. Some biocontrol agents may survive better in soils with a higher silt or clay content (Wessendorf & Lingens, 1989). Other strains, even though they survive, do not work so well in soils high in clay (Campbell & Ephgrave, 1983), and it was postulated that this was because of adsorption of the antibiotic produced by the bacterial control agent on the clay. This is not a problem unique to biocontrol, it may be one of the reasons why some chemicals do not work well as soil drenches. In horticulture, rather than arable agriculture, a similar problem exists in that prepared potting and seeding composts vary in their effects on the pathogen and the introduced biocontrol agents (Hoitink & Fahy, 1986). The key factor seems to be the degree of decomposition of the organic matter in the compost, high microbial activity leading to suppressive growing media and lower microbial activity allowing disease development (Chen *et al.*, 1988). Similar effects are thought to be responsible for the varying suppressiveness of different sorts of peat used in horticultural composts.

Water availability is another major determinant of microbial activity, and this is, to some extent, affected by the clay content of the soil. However, taking water availability alone, there should be no problem with biocontrol if the pathogen and the proposed control agent react in the same way. If the pathogen can grow at either higher or lower water levels than the control agent, then control will break down. Thus *G. graminis* can grow over a wide range of water potentials, but proposed antagonists may not grow in even moderately dry soil so control of the pathogen can break down at low water potentials when the antagonist was inactive while the pathogen could still grow (Campbell & Clor, 1985). This is reflected in field trials data where proposed antagonists survived many frosts and extreme weather conditions in the winter, but fell in numbers in the soil during the spring drought (Campbell & Renwick, unpublished). Similarly the pathogen and the control agent will respond to temperature and nutrient availability and if they respond differently control can be either enhanced or reduced.

There are clearly problems with using live biocontrol agents, and the only solution(s) is to run the sceening systems to select the control agents at realistic soil moisture levels and temperatures and with conditions as close as possible to the field.

The final problem, or it could be an advantage, with biocontrol agents is their specificity for host and/or pathogen. If the agent is very specific it may control one race of the pathogen on one cultivar of the host. This may be ecologically desirable, but it would be a commercial disaster if a separate control agent was needed for each race/cultivar combination. Particular strains of biocontrol agents may colonize one plant host better than others, and this may be linked with a specific characteristic such as motility, or it may be some unknown strain characteristic(s). More difficult to explain is the

fact that biocontrol or plant growth promoting bacteria may be beneficial to one host species or cultivar, but deleterious on another (Astrom & Gerhardson, 1988; Becker *et al.*, 1990).

A development of host specificity is that biocontrol agents are generally selected by looking for microorganisms on the host plant of the target disease, so it is considered that isolates from wheat might work best on wheat plants and wheat diseases. This is to be expected, for it has long been known that the host genome controls the rhizosphere population and therefore particular species or cultivars of plants have their own unique rhizosphere. Biocontrol agents are not, however, always host specific. In a study of the control of wheat root diseases, isolates were obtained from long term wheat monoculture and from grass pastures and rotation crops. The majority of the more promising isolates for the control of wheat root disease came from non-wheat systems, even though the sampling was heavily biased against this (Campbell, Renwick, & Coe, unpublished). Furthermore, isolates from this screening have been shown to be effective against other pathogens on other plant hosts. Some fungal biocontrol agents are claimed to have a very wide host range, colonizing and giving protection to every plant species so far examined (Wood, 1990), but this report awaits detailed evidence and confirmation.

Biocontrol agents may operate best against particular strains of the pathogen they are controlling, and this might be expected, especially for those that operate by the use of antibiotics, for the pathogen could well develop resistance to a particular chemical. This has been shown for both fungal and bacterial agents against *Gaeumannomyces graminis* and *Rhizoctonia solani* (Campbell, Lewis & Gurol, unpublished). This will complicate screening procedures if the potential control agents have to be tested against several strains of each pathogen.

Chemical control agents also show pathogen race specificity, or at least some races of the pathogens are resistant to some chemicals, and there can be cultivar specificity (resistance) to some herbicides. So, for both chemicals and biocontrol agents, host and pathogen specificity has good and bad points.

All the problems described have only become apparent once serious programmes have been developed to look for biocontrol agents. With hindsight some of them seem obvious. They will not prevent the eventual development of control agents, but they may well necessitate more complex screening systems. Chemicals have some of the same problems, and these have been partly overcome, but the extra disadvantage which biocontrol agents have is that they are alive and therefore more sensitive to the environmental variables than are most chemicals.

GENETIC ENGINEERING IN BIOCONTROL

There are various levels of involvement of genetic engineering in biological control. The first, and most widespread, is to use it as a research tool to introduce or delete genetic material so that a mode of action can be studied. Secondly introduced genetic material is used as a marker for tracking studies, either probing the nucleic acid directly or depending on the formation of some gene product or new capability to identify the engineered strain. Thirdly, and of more long term importance, is the possible construction of new strains of organisms such as the *Agrobacterium* mentioned above. There is also the well know case of the transfer of the genes for *Bacillus thuringiensis* toxin production to *Pseudomonas* and now to crop plants (Beringer *et al*, 1989). In the case of root diseases the main interest has been to transfer genes for various characteristics, thought to be important in biocontrol, into potential agents or carriers of the gene which might subsequently be used to pass it on to a root colonizing microorganism. Thus cloned DNA for chitinase production, which might enable the organism to lyse fungal hyphae, has been transferred to *Escherichia coli* which was then shown to control *Sclerotium rolfsii* in laboratory experiments (Shapira *et al.*, 1989). As regards the fungi, there are technical difficulties in transferring the DNA, and the additions and deletions may not be so precise, but it is possible to combine genetic information from two strains into one to improve the biocontrol activity (Pe'er & Chet, 1990).

The main restraint on biocontrol agents does not however seem to be whether they can produce this or that enzyme or antibiotic that might be added or 'improved' upon; making superproducers of antibiotics, for example, does not necessarily improve their biocontrol performance, even though the antibiotic is known to be the mode of action (Beringer *et al.*, 1989).

The main problem is the one of repeatability, and the ability to colonize the roots and survive in the soil. Many of the factors which control these characteristics are not known, or are different for different organisms. It is not therefore possible to genetically engineer an organism to be a good survivor, or a good colonizer. Until this is known genetic engineering will be a very useful experimental tool, but will have little commercial use in biological control of root pathogens.

FUTURE OF BIOCONTROL

Are we then wasting time looking for biological control agents; are they just too complicated and unpredictable to be worth the bother ? It is certain that they do have a future, and there will be good reliable control agents that are commercially viable. The long term research priority is to understand microbe/microbe interactions, and the interactions with the host plant roots (Hornby, 1990), but we could wait a long while for the day when you can sit down and

logically select or design an organism for a particular host or disease. We do not do this for chemicals, so why should we wait to be able to do it for biologicals ? Chemicals have been selected on a 'try it and see' basis, and there have been some very successful, commercially available chemical controls for plant diseases. In view of some of the specificity characteristics of biocontrol agents discussed above, we may need to think again on where or how we look for potential control agents, and the key is in the development of realistic screening systems that will only select genetically stable microorganisms that are not particularly sensitive to environmental variables and that will survive and grow in the proposed site of action. This means selecting them under soil conditions with the disease and live plants, not using *in vitro* screens in petri dishes, however tempting the cheapness and high throughput of the latter systems might be.

It takes a lot of money to develop, register and market a chemical. One of the advantages of biocontrol agents noted above, is that the research and development of them is cheaper than for chemicals (Lethbridge, 1989). Maybe this is the problem: if as much time and effort went into screening and developing biologicals as has gone into chemicals, more progress might have been made.

ACKNOWLEDGEMENT

I thank Dr Annabel Renwick and Dr Keith Powell for helpful discussion and comment on the original manuscript.

REFERENCES

Astrom, B.; Gerhardson, B. (1988) Differential reactions of wheat and pea genotypes to root inoculation with growth affecting rhizosphere bacteria. *Plant and Soil*, **109**, 263-269.

Becker, J.O., Hepfer, C.A., Yuen, G.Y., Van Gundy, S.D., Schroth, M.N., Hancock, J.G., Weinhold, A.R.; Bowman, T. (1990) Effect of rhizobacteria and metham-sodium on growth and root microflora of celery cultivars. *Phytopathology*, **80**, 206-211.

Beringer, J.E.; Powell, K.A.; Poole, N. (1989) The use of engineered and genetically distinct inocula on plants. In: *Microbial Inoculation of Crop Plants*, R. Campbell and R.M. Macdonald (Eds), Oxford, IRL Press, pp. 101-108.

Campbell, R. (1989a) *Biological Control of Microbial Plant Pathogens*, Cambridge, Cambridge University Press, 218 pp.

Campbell, R. (1989b) The use of microbial inoculants in the biological control of plant diseases. In: *Microbial Inoculation of Crop Plants*, R. Campbell and R.M. Macdonald (Eds), Oxford, IRL Press, pp. 67-77.

Campbell, R.; Clor, A. (1985) Soil moisture affects the interaction between *Gaeumannomyces graminis* var. *tritici* and antagonistic bacteria. *Soil Biology and Biochemistry*, **17**, 441-446.

Campbell, R.; Ephgrave, J.M. (1983) Effect of bentonite clay on the growth of *Gaeumannomyces graminis* var. *tritici* and on its

interactions with antagonistic bacteria. *Journal of General Microbiology*, **129**, 771-777.

Chen, W.; Hoitink, H.A.J.; Schmitthenner, A.F.; Tuovinen, O. (1988) The role of microbial activity in suppression of damping-off caused by *Pythium ultimum*. *Phytopathology*, **78**, 314-322.

Cook, R.J. (1986) Plant health and the sustainability of agriculture, with special reference to disease control by beneficial microorganisms. *Biological Agriculture and Horticulture*, 3, 211-232.

Cook, R.J.; Baker, K.F. (1983) *The Nature and Practice of Biological Control of Plant Pathogens*, St. Paul, Minn., American Phytopathological Soc., 539 pp.

Crespi, R.S. (1989) Microbiological inventions and the patent law - international developments. *Biotechnology and Genetic Engineering Review*, **7**, 221-258.

Hoitink, H.A.J.; Fahy, P.C. (1986) Basis for the control of soil-borne plant pathogens with composts. *Annual Review of Phytopathology*, **24**, 93-114.

Hornby, D. (1990) Root diseases. In: *The Rhizoshere*, J.M. Lynch (Ed), Chichester, England, John Wiley, pp. 233-258.

Jones, D.A.; Ryder, M.A.; Clare, B.G.; Farrand, S.K.; Kerr, A. (1988) Construction of *tra*⁻ deletion mutant of pAGk84 to safeguard biological control of crown gall. *Molecular and General Genetics*, **212**, 207-214.

Klingmuller,W. (Ed) (1988) *Risk assessment for deliberate release*, Berlin, Springer-verlag, 193 pp.

Laird, M.; Lacey, L.A.; Davidson, E.W. (1990) *Safety of Microbial Insecticides*, Boca Raton, Florida, CRC Press, 259 pp.

Lennartsson, M. (1988) Effects of organic soil ammendments and mixed species cropping on take-all disease of wheat. In: *Global Perspectives on Agroecology and Sustainable Agricultural Systems*, P. Allen and D. Van Dusen (Eds), Santa Cruz, University of California, pp. 575-580a.

Lethbridge, G. (1989) An industrial view of microbial inoculants for crop plants. In: *Microbial Inoculation of Crop Plants*, R. Campbell and R.M. Macdonald (Eds), Oxford, IRL Press, pp. 11-28.

Lynch, J.M. (1988) Biological control of plant diseases: achievement and prospects. Brighton Crop Protection Conference - Pests and Diseases 1988, 2, 587-596.

Pe'er, S.; Chet, I. (1990) *Trichoderma* protoplast fusion; a tool for improving biocontrol agents. *Canadian Journal of Microbiology*, **36**, 6-9.

Royal Commission on Environmental Pollution, 13th Report. (1989) *Release of Genetically Engineered Organisms to the Environment*, London, Her Majesty's Stationary Office, pp. 144

Ryder, M.A.; Jones, D.A. (1990) Biological control of crown gall. In: *Biological Control of Soil-borne Plant Pathogens*, D. Hornby (Ed.), Wallingford, England, CAB International.

Schippers, B.; Lugtenberg, B.; Weisbeek, P.J. (1987) Plant growth control by fluorescent pseudomonads. In: *Innovative Approaches to Plant Disease Control*, I. Chet (Ed), New York, John Wiley, pp. 19-39.

Shapira, R.; Ordentlich, A.; Chet, I.; Oppenheim, A.B. (1989) Control of plant disease by chitinase expressed from cloned DNA in *Escherichia coli*. *Phytopathology*, **79**, 1246-1249.

Suslow, T. V. (1982) The role of root-colonizing bacteria in plant growth. In: *Phytopathogenic Prokaryotes*, Vol. 1, M.S. Mount and G.H. Lacey (Eds), New York, Academic Press. pp. 187-224.

Sussman, M.; Collins, C.H.; Skinner, F.A.; Stewart-Tull, D.E. (1988) *The Release of Genetically Engineered Microorganisms*, London, Academic Press, pp. 306.

Wessendorf, J.; Lingens, F. (1989) Effect of culture and soil conditions on survival of *Pseudomonas fluorescens* R1 in soil. *Applied Microbiology and Biotechnology*, **31**, 97-102.

Wood, I. 1990. Plant fungus keeps its host in good health. *New Scientist*, **125** (no. 1709), 27.

Wright, B. (1989) Gene spliced pesticide uncorked in Australia. *New Scientist*, **121** (No. 1654), 23.

BACILLUS THURINGIENSIS, BIOENGINEERING AND THE FUTURE OF BIOINSECTICIDES

WENDY D. GELERNTER

Mycogen Corporation, 5451 Oberlin Drive, San Diego, CA 92121. USA.

ABSTRACT

Naturally occurring protein biotoxins produced by *Bacillus thuringiensis* offer great potential for new, ecologically sound crop protection and animal health products. Renewed interest in the development of bioinsecticides has spurred the discovery of novel activities against insect and nematode pests. Recombinant DNA technology provides the means for improving, stabilizing and delivering *Bt* proteins to a wide variety of agricultural environments.

INTRODUCTION

During the past decade, growing public awareness of the impact of pesticides on food and environmental safety has been the most significant issue affecting agriculture and the pest control industry. Scientific organizations, consumer and environmental groups and government regulatory agencies have called for more severe restrictions on the use of toxic chemicals in agriculture, and for increased funding for the development of alternative pest control methods. By the year 2,000, the arsenal of pest control products available to growers will be significantly altered.

The insect pathogenic bacterium, *Bacillus thuringiensis* (*Bt*), was first developed during the 1950's as a biological insecticide for control of caterpillar pests in agriculture and forestry (Rowe and Margaritis, 1987). Since that time, a wide variety of products based on naturally occurring isolates of *Bt* have been commercialized for control of caterpillar, mosquito, black fly and beetle pests. Sales of *Bt* based products have increased to approximately $100 million per year, and are expected to reach close to $300 million by the year 2000 (McKemy, 1990), making *Bt*s the most successfully commercialized group of biopesticides developed to date. Increased public concern regarding the use of toxic pesticides, coupled with the application of techniques in genetic engineering to agriculture have escalated research and industrial efforts with *Bt* in recent years. In this paper, the scientific and commercial development of *Bt* based insecticides will be reviewed, and the outlook for new products, especially those based on bioengineered *Bacillus thuringiensis* will be discussed.

BIOLOGY OF *Bacillus thuringiensis*

The delta endotoxin

Bacillus thuringiensis is a soil dwelling, Gram positive, spore-forming, rod-shaped bacterium that is distinguished from other members of the large *Bacillus* genus by the production in each mature cell of a proteinaceous crystal. When *Bt* is commercially produced in large scale fermentation tanks, the mature *Bt* cells break open, or lyse, at the completion of their growth cycle, releasing delta endotoxin crystals and spores into the liquid medium. These naked crystals and spores constitute the active ingredient of conventional *Bt* products.

For most isolates of *Bt* thus far described, the protein or proteins (known as delta endotoxins) making up the crystal have toxic activity for specific insects or other invertebrates. The composition of the delta endotoxin crystal, which is usually coded for by genes located on bacterial plasmids, determines the host range activity for the *Bt* which produces it (Rowe and

Margaritis, 1987). While *Bt* delta endotoxins can be quite toxic to target organisms (LC_{50} values of 5 - 20 ug delta endotoxin/ml are common for susceptible beetle, mosquito and caterpillar larvae [Mycogen Corporation unpublished data]), results of toxicology tests with *Bt* varieties *kurstaki, aizawai, israelensis , tenebrionis* and *san diego* have indicated a consistent lack of toxicity against non-target organisms: mammals, birds, fish, ducks, aquatic invertebrates, beneficial insects and plants (Faust, 1982). In addition, the *Bt* active ingredient -- a protein -- breaks down in the environment, usually within 1 - 4 days after application, which confers the advantage of biodegradability to *Bt* products. Although *Bt* delta endotoxins are regarded as the primary active ingredient in *Bt* based products, it should be noted that *Bt* spores appear to play an as yet uncharacterized role in certain *Bt*/host interactions (Heimpel and Angus, 1959).

Host range

The thousands of *Bt* isolates thus far discovered are currently classified into over 30 varieties or subspecies in a taxonomic system which relies on serotyping *Bt* flagellar antigens, microbial biochemistry and insect host range (Dulmage, 1982). As illustrated in Table 1, the majority of *Bt* varieties are active against specific caterpillar, or lepidopterous insects, although activities have been reported on mosquitoes and black flies (Order Diptera), beetle larvae and adults (Order Coleoptera) (Hofte and Whiteley, 1990) and nematodes (Edwards et. al., 1990). There are also several *Bt* varieties reported with no apparent toxic activity, although this is probably a function of incomplete host range testing, rather than true lack of biological activity.

Although highly toxic to susceptible species, the host range for each *Bt* isolate is usually restricted to a small number of related organisms. For example, *Bt* variety *san diego* is active against chrysomelid insects including the Colorado potato beetle (*Leptinotarsa decemlineata*) and the elm leaf beetle (*Xanthogaleruca luteola*), but this *Bt* has no effect on the closely related chrysomelid, the corn rootworm (*Diabrotica longicornis*). *Bt* products may be age-specific as well, as for *Bt* variety *san diego* and *Bt* variety *tenebrionis*, which are effective for early instar Colorado potato beetle larvae, but have little or no effect on older larvae and on adults (Zehnder and Gelernter, 1989).

TABLE 1. Biological activities of *Bacillus thuringiensis* varieties (subspecies)

Target Pests	**Active *Bt* varieties**
Lepidoptera (caterpillars)	*aizawai, alesti, canadensis, darmstadiensis, dendrolimus, entomocidus, fukuokaensis, galleriae, kenyae, kurstaki, kyushuensis, morrisoni, ostriniae, pondicheriensis, shandogiensis, sotto, subtoxicus, thompsoni, thuringiensis, tohokuensis, tolworthi, wuhanesis, yunnanensis*
Diptera (mosquitoes, blackflies)	*aizawai, fukuokaensis, israelensis, kenyae, kyushuensis, morrisoni, thuringiensis, tolworthi*
Coleoptera (beetles)	*morrisoni, tenebrionis, san diego,* unclassified *Diabrotica* and scarab-active isolates.
Nematoda (nematodes)	five unclassified isolates

Mode of action

Bacillus thuringiensis is characterized as a stomach poison. When susceptible organisms ingest *Bt* protein crystals, the first gross symptom observed is the cessation of feeding, usually within one hour. This is followed by a slow, apparent poisoning of the insect, resulting in death 1 - 7 days after ingestion (Heimpel and Angus, 1959). The basis of the feeding inhibition and toxic response is primarily due to the delta endotoxin crystals which are rapidly digested and activated within the insect gut by proteolytic enzymes. The activated toxin molecules then appear to attach to the microvillar membrane of midgut epithelial cells, a specific binding interaction which occurs only if the correct protein or glycoprotein is present on the microvillar surface. Different *Bt* toxins appear to require the presence of different binding proteins, which partially explains the very specific host range activities observed for different *Bt* toxins. Following the toxin binding step, the midgut epithelial cells begin to break down, usually within minutes after the crystals are ingested. Ultimately, the microvillar membrane disintegrates and the epithelial cells break down, resulting in destruction of the midgut. Once the mid-gut ceases to function as an effective barrier between the hemocoel (body cavity) and the gut, the hemocoel and gut contents mix, resulting in poisonous changes in pH and ion balance. This, coupled with the effects of starvation caused by feeding inhibition, results in death of the target organism (Hofmann, 1988).

COMMERCIALIZATION

There are more *Bt* based products commercially available today than at any other time in history. Of these, the majority of products is based on *Bt* variety *kurstaki* (Table 2). Until 1977, it was generally believed that *Bts* were active exclusively on lepidopteran larvae. At that time, however, researchers in Israel discovered an unusual isolate of *Bt* (later named *Bt* variety *israelensis*) with high levels of activity for mosquito and black fly larvae, but no activity for caterpillar pests. Since its discovery, *Bt israelensis* has served as the basis for several commercially available products that control biting flies (Rowe and Margaritis, 1987). The discovery, in 1983 and 1985 of beetle active isolates, *Bt* variety *tenebrionis* and *Bt* variety *san diego*, respectively, further expanded the commercial potential of *Bts* (Krieg et. al., 1983; Herrnstadt et. al., 1986). Finally, in 1990, regulatory approval was received for genetically manipulated (Carlton et. al., 1990) and dead, bioengineered *Bt* endotoxin based products (Gelernter, 1990) with activity against caterpillar and beetle pests.

Benefits of conventional *Bt* based insecticides.

Conventional *Bt* based insecticides -- those based on the spores and crystals of naturally occurring *Bts* -- are currently enjoying the widest usage in their history. A summary of the benefits of *Bt* products includes:

- **environmentally friendly features:** lack of toxicity to non-target organisms (mammals, birds, fish, beneficial insects) and biodegradability
- **highly toxic delta endotoxin protein and unique mode of action:** makes *Bts* good pest management tools for insects resistant to chemical insecticides, and for growers striving to reduce their use of more toxic products.
- **economical production methods:** based on successful fermentation methods utilized for production of pharmaceuticals, *Bt* production methods can be quickly and economically developed.
- **streamlined regulatory review:** toxicology testing costs are generally less than $500,000 (U.S.) and regulatory review may take less than one year, as opposed to the multimillion dollar costs and 7 - 10 year review period associated with more toxic products.

TABLE 2. Commercial products based on *Bacillus thuringiensis*

Target organisms	Target markets	*Bt* varieties	Products	Companies
Lepidoptera (i.e., cabbage looper, imported cabbage worm, diamondback moth, gypsy moth, Indian meal moth, European corn borer)	vegetable and fruit crops, forestry, stored products, corn	*kurstaki*	Bactospeine Biobit Condor* Cutlass* Dipel Javelin Larvo Bt	Duphar Novo Labs Ecogen Ecogen Abbott Labs Sandoz, Inc. Knoll Labs
"	"	encapsulated *kurstaki* endotoxin	MVP^	Mycogen
Lepidoptera (greater wax moth)	bee keepers	*aizawai*	Certan	Sandoz, Inc.
Diptera (mosquitoes and black flies)	vector management agencies, mosquito abatement districts	*israelensis*	Skeetal Teknar Vectobac	Novo Labs Sandoz, Inc. Abbott Labs
Coleoptera (Colorado potato beetle, elm leaf beetle)	potato and vegetable crops, tree care	*san diego, tenebrionis tenebrionis/kurstaki*	M-One Trident Foil**	Mycogen Sandoz, Inc. Ecogen
"	"	encapsulated *san diego* endotoxin	M-One Plus^	Mycogen

* genetically manipulated transconjugant
** genetically manipulated transconjugant with beetle and caterpillar activity
^ genetically engineered, killed recombinants. Experimental Use Permit granted 1990; full registration pending.

Limitations of conventional *Bt* based insecticides

While conventional *Bt* based products will undoubtedly enjoy increased recognition and use in agriculture over the next decade, there are several features, which if they remain unaddressed, will tend to limit the widespread adoption of *Bt* in agriculture. These include:

- **insect host range specificity:** while viewed as an environmental attribute, the inability of *Bt* products to control the majority of insect pests present on a given crop makes the product more difficult to use, and will therefore decrease rapid adoption of the product.
- **lack of delivery to cryptic and sap feeding insects:** because *Bts* are stomach poisons, insects that feed within the plant are difficult, if not impossible to target. Examples include codling moth larvae, *Cydia pomonella,* which are highly susceptible to *Bt* variety *kurstaki* when it is administered in a diet incorporation assay, but in the field feed only briefly on the surface of the apple before boring into the fruit and completing their life cycle protected from foliar *Bt* sprays. Similarly, sap feeding heteropteran and homopteran insects such as aphids, whiteflies or leafhoppers insert their stylets into the plant vascular system, and are never exposed to products sprayed onto the plant surface.
- **short residual activity:** this is perhaps the most important factor contributing to inconsistent field performance and lack of adoption of conventional *Bt* products. Because the *Bt* active ingredient is an unprotected protein crystal, it is rapidly degraded on the foliage by plant and microbial enzymes, secondary plant compounds such as tannins, extremes in pH, ultraviolet light, wind and rain (Dulmage and Aizawa, 1982). Typical degradation

curves illustrate that *Bt* insecticidal activity degrades rapidly within the first two days after application, while by four days post-application, insecticidal activity has virtually disappeared (Gelernter, 1990).

NEW DEVELOPMENTS IN *Bt* BASED INSECTICIDES

Screening programs

To expand the number of pest organisms that may be controlled by *Bt*, massive screening programs have been implemented by industry, university and government research laboratories. These efforts have in the past three years identified thousands of new *Bt* isolates from diverse environments and geographies. Unique activities have so far been identified against insect pests such as scarab beetles (Family Scarabaeidae) (Wigley and Chilcott, 1990), the corn rootworm (*Diabrotica undecimpunctata*) (Gawron-Burke et. al., 1990) and leaf feeding beetles such as the cottonwood leaf beetle (*Chrysomela scripta*) (Bauer, 1990) as well as against non-insect pests such as nematodes parasites of mammals and plants (Edwards et. al., 1990). Given the level of ongoing effort in the discovery and characterization of novel *Bt* isolates, it is reasonable to expect that biological activities against additional pests will soon be discovered. In addition to serving as the basis for new products based on naturally occurring *Bts*, unique isolates identified in *Bt* screening programs may also be utilized by molecular geneticists as the raw materials for creating improved recombinant microbial pesticides.

Molecular genetics and improved *Bt* based products

Since Schnepf and Whiteley first sequenced and cloned the delta endotoxin gene from *Bt* variety *kurstaki* in 1981, the nucleotide sequences for over 40 *Bt* genes have been published (Hofte and Whiteley, 1989). Researchers have based their efforts on the thesis that techniques in genetic engineering can be utilized to improve the potency, stability and delivery of *Bt* delta endotoxins, while still maintaining the features of environmental compatibility that had made this organism so attractive as a pest control tool . To date, the most successful efforts have been made in the area of increasing *Bt* endotoxin performance through development of improved delivery systems-- in plants and in microorganisms.

Development of transgenic plants

Delta endotoxin genes from *Bt* varieties *kurstaki* and *tenebrionis* have been successfully transferred from *Bt* cells to crop plants such as tomatoes, potatoes, tobacco and cotton (Vaeck et. al., 1987). The transformed plants have been shown to produce the *Bt* delta endotoxin in sufficient quantity to kill target insect pests, and have been tested in greenhouse and restricted field trials. These tests have shown that cryptic insects such as the tomato pinworm (*Keiferia lycopersicella*) which feed inside the tomato were adequately controlled without the application of any insecticide products. The advantages of this approach are numerous. Because insecticide applications are no longer necessary, the cost of scouting fields to determine appropriate timing and frequency of sprays, and the cost of applying the product can be subtracted from the cost of production. The imprecision associated with foliar insecticide applications, from the effect of rain, wind or high temperatures on spray coverage and on foliar persistence is no longer a factor. And the ability to target insects which feed inside the plant -- an impossibility in the past -- can now be easily achieved. The market potential for transgenic crop plants has been recognized by many companies including Monsanto, Plant Genetic Systems, Biotechnica and Rohm and Haas, all of whom have active research and development programs in this area.

As for all new technologies, however, there are also disadvantages associated with the development of transgenic plants. First and foremost, the regulatory framework for approving the use of transgenic plants, or any living recombinant organism, currently demands submission of expensive multi-year studies, as registrants attempt to answer questions

regarding the spread and mutation potential of recombinant genes in the environment. For this reason, the current regulatory procedure for approving even small plot, crop destruct field trials may take several years. Public perceptions of living recombinant organisms tend to be sceptical, if not negative, a factor which could inhibit the development of transgenic seed, even if regulatory approval were secured. Secondly, there are many technical issues to overcome before transgenic seeds can be marketed to the public -- primarily a confirmation that the new, *Bt* endotoxin producing plants still express the other desirable agronomic features (taste, nutritional value, yield, etc) for which they were originally bred. Finally, there is concern among some scientists that the continuous production of *Bt* endotoxin in the tissues of transgenic plants would create the ideal conditions for development of pest resistance to *Bt*. While none of these problems is insurmountable, they make the commercial availability of transgenic seed unlikely before the year 2000.

Development of recombinant, plant colonizing bacteria

Bacteria which are natural colonizers of crop plants have been isolated and engineered to produce various *Bt* delta endotoxins. When these recombinant organisms are applied to the plant, they produce *Bt* toxin as they multiply and spread over the plant surface. Monsanto has created endotoxin- producing root colonizing bacteria to protect crop plants from soil dwelling insects, while Crop Genetics has demonstrated that corn borer larvae (*O. nubilalis*) feeding on the vascular system of corn plants can be targeted by the endophytic bacterium, *Clavibacter xyli*, which grows within the plant's vascular system and has been genetically engineered to produce the *Bt* variety *kurstaki* toxin. Similarly, several companies have successfully transformed leaf colonizing bacteria to produce *Bt* endotoxins. Advantages of these potential products are similar to those for transgenic plants; ease of use, and the ability to target cryptic insects, such as those living beneath the soil, or those feeding within the plant vascular system. Disadvantages include regulatory and public perception hurdles, which again make commercialization within the next 10 years unlikely.

Development of dead, recombinant bacteria

In attempt to improve the foliar persistence of *Bt* toxins, Mycogen Corporation has developed the CellCap™ encapsulation system. This system is based on a non-pathogenic bacterium, *Pseudomonas fluorescens,* which has been genetically engineered to produce *Bt* delta endotoxin, and has been killed prior to field release (Barnes and Cummings, 1987). In this patented system, the dead bacterial cell wall serves as a biological microcapsule or biopackage which protects fragile *Bt* protein biotoxins from environmental degradation. Products based on the CellCap system are developed by selecting a naturally occurring *Bt* isolate to serve as the source of the insecticidal biotoxin. Once identified, the *Bt* gene responsible for production of the desired biotoxin can be transferred to *Pseudomonas fluorescens (Pf)* cells. When the *Pf* cells are grown in flasks or fermentors, the biotoxin is produced and forms a crystal within the cell. At the end of the growth cycle, while still in the fermentation tank, the *Pf* cells are killed via chemical treatment. This process also fixes the *Pf* cell wall, causing it to become thicker and more rigid through cross linking of cell wall components. In contrast to the unprotected toxin crystals present in conventional *Bt* products, the rigid wall of the dead *Pf* cell serves as a protective biological microcapsule for the enclosed biotoxin, protecting it from environmental degradation.

Mycogen is developing several different products using the CellCap delivery system including MVP™ Bioinsecticide, based on a delta endotoxin from *Bt* variety *kurstaki* and targeted for caterpillar larvae on vegetable, fruit and grain crops, and M-One® Plus Bioinsecticide, based on the *Bt* variety *san diego* delta endotoxin and targeted for control of the Colorado potato beetle (*L. decemlineata*) and elm leaf beetle (*X. luteola*). Because these products are based on dead organisms, concerns regarding spread and mutation of recombinant genes have not been raised by regulatory agencies or environmental groups, and registration has proceeded similarly to that for naturally occurring *Bt* products. For this reason, Mycogen received permission to conduct small plot field tests with MVP in 1985, and was the first company to receive permission to test recombinant organisms in large scale, on-farm trials,

when two 5,000 acre Experimental Use Permits were granted for MVP and M-One Plus in 1990. Based on this streamlined review process, full registration of both CellCap products is expected in 1991.

Results from field trials demonstrate that CellCap products have two to three times the residual activity of conventional *Bt* products. While insecticidal activity of conventional *Bts* typically declines by 96 hours post-application, an equal dose of the biotoxin delivered in MVP or M-One Plus demonstrates high levels of insecticidal activity 5 - 7 days post-application. This increase in persistence results in better insect control and higher yields of marketable produce for the CellCap products (Gelernter, 1990). It is important to note that although the CellCap system enhances foliar persistence of *Bt* biotoxins, environmental degradation does occur 7 - 10 days after application. A summary of the advantages of CellCap based products includes:

- **increased residual activity:** results in improved insect control and higher crop yields, with less effort necessary to strictly time applications, as compared to conventional *Bt* products.
- **flexibility:** new products can be rapidly created based on a "cassette" concept, where the delta endotoxin(s) of interest can be readily transferred into the *Pseudomonas* system to target the appropriate insect pest(s).
- **non-target and environmental safety:** because CellCap products are based on *Bt* delta endotoxins, they have the same environmentally friendly attributes as conventional *Bt* products.
- **streamlined regulatory review**
- **increased shelf life:** because there are no living organisms in formulations of CellCap products, microbial growth and production of endotoxin-degrading enzymes within the product jug -- problems which have plagued spore-containing *Bt* products -- are eliminated.
- **proprietary system:** patents issued in 1987 (Barnes and Cummings) protect Mycogen's dead microbial cell/encapsulation technology.

Development of genetically manipulated *Bt* based products

In an effort to expand the host range activity of naturally occurring *Bt* isolates while still avoiding the regulatory issues associated with the development of recombinant microbes, Ecogen Inc. has developed bioinsecticides which are based on genetic manipulation, rather than genetic engineering, techniques. Relying on the naturally occurring process of conjugal transfer, modified *Bts* are constructed to produce two delta endotoxins -- one coded for by a plasmid from the original, or parental *Bt*, and a different toxin coded for by a plasmid which has been transferred, via conjugation, from a second *Bt* isolate. Thus, Ecogen's product Foil® is based on a caterpillar-active isolate of *Bt* variety *kurstaki*, which acquired a beetle-active *Bt* variety *morrisoni* plasmid through conjugation. The end-product, a *Bt* cell which produces two distinct crystals, is active against caterpillar pests such as the European corn borer (*O. nubilalis*) and beetle pests such as the Colorado potato beetle (*L. decemlineata*) (Carlton et. al., 1990).

SUMMARY: THE NEED FOR EDUCATION

Products based on *Bacillus thuringiensis* offer great potential in beginning to fulfill the growing demand for effective and safe insecticides, and will provide a prototype for the development of other biological pesticides in the future. The application of techniques in genetic engineering has already produced improved *Bt* products, but it is important to recognize that without public support for these innovations, commercialization and even research efforts may be seriously hampered in the near future. Invention and development of new and improved products is only a portion of the effort that will be required to bring bioengineered products to the market. Education -- of the grower/end-user, so that expectations for product performance are reasonable, and so that new technologies are used correctly; and education of the public --environmental groups, legislators and communities -- is critical. Without the support of these groups, the prospects for development of new technologies in agriculture will be difficult, if not impossible to achieve. Thus, simultaneous with the development of

biological pesticides should be development of educational programs designed to illustrate the characteristics and benefits (and perhaps even an honest appraisal of the limitations) of new products. Our challenges over the next 10 years are therefore not only technical, but also social and political as we strive to achieve this year's Brighton Crop Protection Conference theme of "crop protection in sympathy with the environment by the year 2,000".

REFERENCES

Bauer, L.S. (1990) Response of the cottonwood leaf beetle to *Bacillus thuringiensis* var. *san diego* . *Environmental Entomology,* **19** (2), 428-431.

Barnes, A.; Cummings, S.E. (1987) U.S. Patent No. 4,695,455

Carlton, B.C.; Gowran-Burke, C.; Johnson, T.B. (1990). Exploiting the genetic diversity of *Bacillus thuringiensis* for the creation of new bioinsecticides. *Proceedings of the Vth International Colloquium on Invertebrate Pathology and Microbial Control, August, 1990.* D.E. Pinnock (ed.), Adelaide, Australia: Society for Invertebrate Pathology, pp. 18 - 22.

Dulmage, H.T.; Aizawa, K. (1982) Distribution of *Bacillus thuringiensis* in nature. *Microbial and Viral Pesticides*. E. Kurstak (ed), New York, Marcel Dekker, Inc., pp. 209-237.

Edwards, D.L.; Payne, J.; Soares, G.G. (1990), U.S. Patent No. 4,948,734.

Gawron-Burke, C.; Chambers, J.; Jelen, A.; Donovan, W.; Rupar, M.; Jany, C.; Slaney, A.; Baum, J.; English, L.; Johnson, T. (1990) Molecular biology and genetics of *Bacillus thuringiensis. Proceedings of the Vth International Colloquium on Invertebrate Pathology and Microbial Control, August, 1990.* D.E. Pinnock (ed.), Adelaide, Australia: Society for Invertebrate Pathology, pp. 456-460.

Gelernter, W.D. (1990) Targeting insecticide-resistant markets: new developments in microbial based products. *Managing Resistance to Agrochemicals: From Fundamental Research to Practical Strategies*. M.B. Green, W.K. Moberg and H. LeBaron (eds), Series 421, Washington, D.C., American Chemical Society, pp. 105-117.

Heimpel, A.M.; Angus, T.A. (1959) The site of action of crystalliferous bacteria in Lepidoptera larvae. *Journal of Insect Pathology,* **1,** 152-170.

Herrnstadt, C.; Soares, G.G.; Wilcox, E.R.; Edwards, D.L.; (1986) A new strain of *Bacillus thuringiensis* with activity against coleopteran insects. *Bio/Technology* **4**, 305-308.

Hofmann, C. (1988) The binding of *Bacillus thuringiensis* delta endotoxin to cultured cells and brush border membrane vesicles. Ph.D. thesis, Swiss Federal Institute of Technology, Zurich, Switzerland. 129 pp.

Hofte, H.; Whiteley, H.R. (1989) Insecticidal crystal proteins of *Bacillus thuringiensis*. *Microbiological Review,* **53** (2), 242-255.

Krieg, A.; Huger, A.M.; Langenbruch, G.A.; Schnetter, W. (1983) *Bacillus thuringiensis* variety *tenebrionis*, a new pathotype effective against larvae of coleoptera. *Journal of Applied Entomology* **96,** 500-508.

McKemy, C. (1990). Bt: keeps bugs from bouncing back. *The Grower*. August, 1990. pp. 22 - 24.

Rowe, G.E.; Margaritis, A. (1987) Bioprocess developments in the production of bioinsecticides by *Bacillus thuringiensis*. *CRC Critical Reviews in Biotechnology,* **6**(1), 87-127.

Vaeck, M.; Reynaerts, A.; Hofte, H.; Jansens, S.; DeCeuckeleer, M.; Dean, C.; Zabeau, M; Van Montagu, M.; Leemans, J. (1987) Transgenic plants protected from insect attack. *Nature*, **328**, 33-37.

Wigley, P.; Chilcott, C. (1990) *Bacillus thuringiensis* isolates active against the New Zealand pasture pest, *Costelytra zealandica. Proceedings of the Vth International Colloquium on Invertebrate Pathology and Microbial Control, August, 1990.* D.E. Pinnock (ed.), Adelaide, Australia: Society for Invertebrate Pathology, p. 344.

Zehnder, G.W.; Gelernter, W.D. (1989) Activity of the M-One formulation of a new strain of *Bacillus thuringiensis* against the Colorado potato beetle: relationship between susceptibility and insect life stage. *Journal of Economic Entomology*, **82** (3), 756-761.

SESSION 7B

PROSPECTIVE AND RETROSPECTIVE SAFETY ASSESSMENT OF PESTICIDES: GOALS FOR THE 1990s

CHAIRMAN DR B. BUCKLEY

SESSION ORGANISER DR A. COCKBURN

REPLACEMENT OF *IN VIVO* STUDIES BY *IN VITRO* STUDIES

M. BALLS

FRAME (Fund for the Replacement of Animals in Medical Experiments), 34 Stoney Street, Nottingham NG1 1NB

ABSTRACT

Scientific, ethical and legal reasons for recognition and promotion of the Three Rs (*reduction, refinement, replacement*) concept of alternatives to animal procedures are considered, and proposals for the orderly development, validation and regulatory acceptance of *in vitro* methods are discussed, with emphasis on the LD50 test and the Draize eye irritancy test. It is emphasised that the limited value of animal models must be more readily recognised, and that the genuine replacement of *in vivo* tests will remain the goal of the moderate animal welfare movement.

INTRODUCTION

A number of introductory points need to be made before the possibility of replacing *in vivo* studies by *in vitro* studies in the context of the safety of new and existing pesticides can be adequately discussed.

1. As is the case with many other types of chemicals and products, the toxicity of pesticide ingredients and formulations must be investigated, as an essential component of risk assessment, risk limitation, and risk avoidance and/or management.

2. Pesticides represent a special case, for a variety of obvious reasons: they are designed to kill pests; in general, their target species specificity is imperfect (as is even their target *kingdom* specificity); they are distributed widely in the environment on a large scale; they can be hazardous to human beings, to domestic animals and plants, and to wildlife; they are potentially hazardous at many levels – e.g. in their concentrated form during manufacture, transport and use, and as residues accumulating in food and water.

3. Since, by contrast with drugs and cosmetics, human studies are not regularly carried out on pesticides, only accidental poisoning and epidemiological studies can provide direct evidence on the actual hazard they represent to man.

4. Great reliance is currently placed on toxicological studies in animals, many of which are performed to meet national and international regulatory requirements. Sometimes, as in the case of rodenticides and insecticides, the target species itself can be used in the toxicological studies, but standard laboratory animal species are usually used, as "models" for man and other animal species. However, like all models, they are imperfect, and species differences represent an insurmountable problem.

5. *In vitro* systems are increasingly used in many branches of the biological sciences, including pharmacology and toxicology, and they include:

a. the use of certain physico-chemical techniques;

b. the use of "lower" organisms not protected by legislation controlling animal experiments, including invertebrates, plants and microorganisms;

c. the use of the early developmental stages of vertebrates not protected as "animals" – e.g. under the terms of the British *Animals Scientific Procedures Act 1986* (Anon, 1986a), before half-way through gestation (mammals), incubation (birds and reptiles), or the stage when independent feeding occurs (amphibians and fish);

d. the use of sub-cellular fractions; short-term maintenance of tissue slices, cell suspensions and perfused organs; and tissue culture proper (cell and organ culture).

This is a rather liberal interpretation of the term "*in vitro*", but such methods, together with improved storage, exchange and use of information, the use of mathematical models and computer programs, and human studies, make up the *replacement* component of the Three Rs (*reduction, refinement, replacement*) concept of alternatives to animal experiments (Balls, 1983).

6. There are scientific, ethical and legal reasons for accepting the introduction of relevant and reliable, properly validated and independently assessed and recommended, animal toxicity tests and testing strategies into toxicological and regulatory practice.

THE LIMITED VALUE OF ANIMAL MODELS

Greater realism in attitudes toward animal models must precede consideration of the potential for their replacement by other systems – there is little point in seeking to duplicate or replace what is itself unnecessary or scientifically unsound. The value of animal toxicity tests is accepted too readily and too uncritically. From a survey on post-marketing experience with drugs, Heywood (1990) has recently concluded that many adverse reactions in man are unpredictable in animal models, that correlations between target system toxicity in the rat and a non-rodent species are around 30%, that most *in vivo* toxicological data cannot be interpreted, and that a best guess for the correlation of adverse reactions in man and animal toxicity data is somewhere between 5% and 25%.

ETHICAL AND LEGAL CONSIDERATIONS

Since the mid-1970s, there has been mounting public pressure against the use of animals in laboratory procedures – particularly in relation to certain kinds of products (especially cosmetics), and in relation to certain kinds of procedures (notably the LD50 test and the Draize eye and skin tests). This is reflected in new legislation, such as the British 1986 Act, which includes the following clauses:

> The Secretary of State shall not grant a project licence unless he is satisfied that the applicant has given adequate consideration to the feasibility of achieving the purpose of the programme to be specified in the licence by means not involving the use of protected animals.

> In determining whether and on what terms to grant a project licence the Secretary of State shall weigh the likely adverse effects on the animals concerned against the benefit likely to accrue as a result of the programme to be specified in the licence.

We are also subject to Directive 86/609/EEC (Anon, 1986b), which states, *inter alia*, that:

> An experiment shall not be performed if another scientifically satisfactory method of obtaining the result sought, not entailing the use of an animal, is reasonably and practicably available.
>
> All experiments shall be designed to avoid distress and unnecessary pain and suffering to the experimental animals.

Gerhard Zbinden (1988) has emphasised that these legal requirements represent a major challenge to current practice in regulatory toxicology:

> Many of the toxicological test procedures inflict substantial pain and anxiety on the laboratory animals. This is particularly true for animals used in acute toxicity studies, and those included in the high dose groups of repeated-dose experiments. Considerable suffering must be assumed in animals bearing large tumors or afflicted with organ damage, e.g. perforated gastrointestinal ulcers, myocardial infarctions, liver necrosis and muscle wasting. Functional disturbances such as paralysis, excessive central nervous system stimulation, diarrhea, polyuria, hypotension and sensory organ disfunction cause stress and anxiety. Repeated injections often induce considerable local pain, and animals sometimes struggle desperately to avoid another injection. Topical administration of irritant and corrosive substances to the skin and nucous membranes is a painful procedure that has come under particularly heavy criticism by animal welfare advocates.
>
> A common feature of many new animal protection laws is the requirement to demonstrate the advisability, in some countries even the unconditional necessity, of all proposed animal experiments. It is probable that those reviewing applications for toxicological studies will expect more justification for an animal experiment than the simple statement that the proposed test is necessary because it is required by a regulatory guideline. In particular, permission to conduct a toxicological experiment will not easily be obtained if the country in which the study will be conducted does not require the proposed test, or is satisfied with an experiment involving fewer animals or a shorter duration of treatment. Thus, the easy way out described above, i.e. to conduct toxicity studies always according to the most demanding national guidelines, will, in future, often not be possible.

It is particularly important that controls on animal experimentation are not regarded as subordinate to regulatory requirements. Thus, one of FRAME's aims in the 1990s will be to see that demands for *specific* justification of the use of animal tests in particular circumstances are constantly made.

THE ORDERLY EVOLUTION OF *IN VITRO* METHODOLOGY

The use of animal procedures in new drug discovery has fallen dramatically during the last two decades, particularly since progress in understanding of events at the cellular and molecular level has led to more emphasis on drug design rather than empirical screening of large numbers of molecules for pharmacological activity. However, the impact of non-animal methods in toxicology in general, and in toxicity testing in particular, has been less dramatic.

This situation is now changing, at least with respect to fundamental toxicology, where non-animal methods are now more and more employed in studies on molecular toxicology, target organ toxicity (including hepatotoxicity and nephrotoxicity), and systemic toxicity (e.g. neurotoxicity and reproductive toxicity). New journals have appeared, such as *Toxicology In Vitro* and *In Vitro Toxicology*.

Toxicity testing is a different matter, for two principal reasons. Firstly, the use of a whole organism, with all its integrated body systems in operation, can provide information on the expected effects, whereas, as currently used, the non-animal methods are more suited to answering specific questions. Secondly, and more importantly, because of the monolithic block to progress represented by toxicity testing guidelines (which are, in effect, requirements for conventional live animal tests) and attitudes toward them within the regulatory agencies themselves and among some industrial toxicologists.

It is generally agreed that, if non-animal tests are to replace any of the currently-accepted animal procedures, they must be no less relevant, no less reproducible, and no less useful for identifying the toxic potentials of chemicals, their toxic potencies and the hazards they might represent under certain conditions of exposure – as a basis for risk assessment, risk limitation and/or risk management. If non-animal test procedures are to meet these requirements, they must be properly developed, proceed through a final validation process, be independently assessed and recommended for acceptance by regulatory authorities. Until recently, these questions had not been adequately addressed (Balls & Clothier, 1989), but, through its involvement with ERGATT (European Research Group for Alternatives in Toxicity Testing), FRAME has been involved in two important workshops, held earlier this year, to discuss validation and the promotion of the regulatory acceptance of validated non-animal test procedures.

Validation

The validation workshop was organised in collaboration with the Center for Alternatives to Animal Testing at Johns Hopkins University, and was held at Amden in Switzerland. The report of the workshop contains 15 recommendations and an idealised scheme for validation, the aim of which is to make available reliable and relevant methods for use for specific purposes in toxicology research and testing (Balls *et al.*, 1990a).

Validation consists of four main steps, namely, *intra-laboratory assessment, interlaboratory assessment, test database development,* and *evaluation*, and is preceded by *test development* (the steps involved in establishing and defining a new procedure) and *acceptance* (the steps involved in taking the decision to use a particular procedure for a particular purpose).

One crucial problem with validation is that it is frequently, perhaps inevitably, based on the retrospective comparison of non-animal test data with historical *in vivo* test data.

However, this gives the animal data a credibility which they do not necessarily deserve, and an alternative test which does not duplicate them is said to give "false positive" or "false negative" results. The animal data, which are themselves of limited relevance and reliability, are thus made "true"! Thus, wherever possible when evaluation of potential hazard to man is the principal objective of the testing exercise, human experience data should be used as the *in vivo* component of the *in vivo/in vitro* comparison. It is also likely that much useful information on pesticide effects is not made readily available or sufficiently taken into account. As the Amden report puts it:

> As in the case of human toxicology, veterinary toxicology involves the collection of data from a variety of sources and for a variety of purposes, which are not always compatible with each other. For example, data may be received on the acute effects of household products on domestic pets following accidential exposure, or on acute and chronic data on the poisoning of farm animals and wild animals by industrial chemicals and agrochemicals. It is highly desirable that more use be made of such information. For example, experience with pets and wildlife could be used, more frequently than at present, to warn of hazard within the home or in the environment in general.

Regulatory Acceptance

The final recommendation of the Amden workshop was that *"The regulatory and legislative authorities should be encouraged to welcome scientifically-validated methods and to accept their incorporation into toxicity testing practices"*. How such acceptance should be promoted and achieved was the subject of the second workshop, held at Vouliagmeni, near Athens, and financially supported by the EC. The participants included a number of members of European regulatory agencies, as well as *in vitro* toxicologists. The report of the workshop contains 14 main recommendations, including the following (Balls *et al.*, 1990b):

a. The replacement of some current animal procedures by non-animal methods should be achievable in the foreseeable future.

b. Before formal acceptance and incorporation of new methods into regulatory toxicology is considered, the results of a validation study should be considered by at least one independent assessment panel.

c. Assessment panels should assess the value of adequately-validated tests or batteries of tests in competition with other methods already validated or in the course of development and validation, and the need for the method and the practicability of its use as part of the regulatory process.

d. When it has been independently concluded that a test or battery of tests could be considered relevant, reproducible and needed for use for regulatory purposes, a recommendation of this effect, and a summary of the reasons for it, should be published and should be brought to the attention of an appropriate national authority. Ideally, the national authority should take the validated test or battery of tests to appropriate other national, supra-national and/or international agencies for consideration for acceptance and incorporation into regulatory practice.

e. Some agencies, notably the OECD and the EC, already have procedures for the notification, consideration, concensus agreement and adoption of new test methods. Such procedures need to be harmonised and rationalised, and the principle of mutual acceptance of data should be promoted and extended to

include the acceptance of well validated and independently assessed alternative tests or stratgegies.

f. The acceptance and incorporation process should not be rigid. Flexibility is essential, in view of a number of other considerations. For example, tests which have been only partially validated or which address restricted toxicological endpoints should be used within the regulatory context under certain conditions. Also, the conduct of selected non–animal tests in parallel with the required animal tests should be accepted as an aspect of the validation process, and industry should be encouraged to obtain in vitro data alongside the required in vivo data and to submit both data sets to the regulatory authority.

g. There are already circumstances in which it should be accepted that the labelling of chemicals (e.g. as severe eye irritants) should be permitted on the basis of tests conducted only with non–animal methods. Classification of other chemicals as of lower toxicity, or confirmation of lack of effect, might still require further testing in animals.

REPLACING THE LD50 TEST

The proposals put forward by these two workshops are eminently reasonable and objective. It is to be hoped that they will contribute to the still–awaited revolution in thinking in relation to toxicity testing, sought by the FRAME Toxicity Committee in 1982, leading to the introduction of genuine replacement alternative toxicity tests in the near future. However, there is likely to be resistance to the very idea of replacement, since, while no toxicologists or regulatory authorities would be likely to want to oppose the use of *in vitro* methods in fundamental research (especially in the elucidation of toxic mechanisms or as a basis for establishing structure–activity relationships), in screening tests (carried out before animal tests) or in adjunct/complementary tests (carried out alongside animal tests), giving up an animal procedure with which they are familiar is another matter.

Sadly, we have an example currently before us. The British Toxicological Society (Van den Heuvel *et al.*, 1987) proposed a workable scheme to replace the LD50 test with a procedure which would cause less animal suffering, the Fixed Dose Procedure (FDP). A national validation trial was undertaken, followed by an extensive initial trial, largely funded by the EC. The results of the EC trial were announced at a seminar on the LD50, held in Brussels in September 1989 (Van den Heuvel, 1990).

It transpired that many companies still carry out the LD50 test according to 1982 OECD guidelines, because some regulators refuse to accept tests according to the 1987 OECD guidelines (which require fewer animals), even though they are based in OECD member countries! It further emerged that, even if the USA, Canada and Japan could be persuaded to follow the lead of the EC in accepting the FDP as a basis for the classification and labelling of chemicals, companies would still have to do the classical LD50, because of transport regulations laid down by the United Nations. If it is not possible to obtain universal acceptance of modifications to the LD50 test itself, let alone an animal method, which does not require death to be the endpoint, what prospect can there be of replacing animal procedures by non–animal methods?

The participants in the EC seminar did accept that total replacement of the use of animals was a desirable long-term objective – but how is that objective to be achieved? The following eight-point plan was put forward at the seminar, on behalf of FRAME (Balls, 1990):

1. A ban on performance of the classical LD50 test or any test more rigorous than that specified in the 1987 OECD Guideline.
2. An early end to the use of death as an endpoint in acute toxicity tests.
3. Acceptance of the Fixed Dose Procedure.
4. Willingness by regulatory authorities to accept any well-designed and appropriate acute toxicity study.
5. The rationalisation and harmonisation of regulatory guidelines and classifcation schemes.
6. The greater encouragement and financial support of the development, validation and use of computer models and *in vitro* systems in toxicity testing.
7. The establishment of a scheme for recording, assessing, expressing and making available data from past acute toxicity tests in animals and acute exposure in humans, as a basis for the validation and evaluation of alternative methods and strategies.
8. The development of effective procedures for the more-rapid international evaluation and regulatory and legal acceptance of new and scientifically-validated testing strategies, including those not involving animal tests.

Good correlations between animal LD50 data and results in *in vitro* cytotoxicity tests have been obtained in a number of studies (e.g. Clothier *et al.*, 1989; Hulme *et al.*, 1989). The MEIC (multicentre evaluation of *in vitro* cytotoxicity) programme of the Scandanivian Society for Cell Toxicology is especially important, since it involves comparison of *in vitro* and animal data with human toxicity (Ekwall *et al.*, 1989). Preliminary results for the first ten MEIC chemicals indicate that some *in vitro* tests predict human lethal dosage at least as well as rodent LD50 tests.

REPLACING THE DRAIZE EYE TEST

A number of potential alternatives to the Draize eye irritancy test are in the course of development and validation in Europe and North America, including the use of:

1. isolated, enucleated rabbit eyes;
2. isolated bovine corneas;
3. isolated sections of the rabbit intestine;
4. the extra-embryonic membranes of the chick embryo;
5. physicochemical tests (e.g. the EYTEX™ method, based on the breakdown of proteins in solution).
6. cytotoxicity tests (to assess effects on cell viability, cell morphology, cell adherence and/or detachment, cell membrane integrity, and cell proliferation).

Some of these methods (1–3) involve killing animals, albeit animals already used for other purposes or being slaughtered for meat. The chorio–allantoic method (4) involves the use of chicken eggs more than half–way through the incubation period, so it is classed as an animal experiment in the UK, under the terms of the 1986 Act. FRAME is therefore concentrating its efforts on the physicochemical (5) and cytotoxicity (6) testing strategies. A evaluation of the EYTEX™ method is being undertaken in collaboration with its developers. The results obtained will then be compared with published Draize eye test data and with human experience.

Our research group in the Department of Human Morphology at the University of Nottingham have recently developed the FRAME *neutral red release* (NRR) method (Reader *et al.*, 1989). In order to make the cell culture approach more relevant to what happens when chemicals are splashed into the eye, we decided to use a confluent layer of cells (instead of rapidly growing cells), and exposure to a small volume of concentrated test material for a brief period, namely, one minute – instead of exposure to diluted materials for 24 or 72 hours, as in the FRAME *kenacid blue* (KB) method for general or intrinsic cytotoxicity (Clothier *et al.*, 1988). Cells are preloaded with neutral red (a vital dye used for staining living cells), then release of the dye after a one–minute exposure to a range of concentrations of the test sample is determined.

More than 150 chemicals and formulations have to date been tested by the NRR method, and it is already clear that it provides information different from that obtained when cytotoxic effects on growing cells are measured by the KB method. The NRR method has performed very well with samples used in the first stages of the multi–centre validation schemes of the US Cosmetic, Toiletry & Fragrance Association (CTFA) and of the European Commission, the results of which are to be published shortly. Samples have also been tested for a number of cosmetics companies, including the ingredients of new formulations (to provide information on comparative cytotoxicities), new finished product ranges about to go to the market (to provide further assurance of safety), and products which have had to be withdrawn because of adverse reports from consumers (to provide explanations of unexpected irritancy).

Another method has recently been added to the emerging battery of FRAME tests, the fluorescein leakage (FL) method (Shaw *et al.*, 1991). Madin–Darby canine kidney (MDCK) cells, grown on porous filters, develop tight junctions and desmosomes to form an impermeable barrier analogous to those found in many epithelia in the body. Chemically–induced loss of trans–epithelial impermeability is measured by determining fluorescein leakage through the cell layer. Moreover, repetition of this, measured after further incubation of the damaged cells can provide information on the degree and rate of reconstitution of the cellular barrier.

Our current feeling is that the intelligent use of the KB, NRR, FL and EXTEX™ methods as components of an adaptable battery of tests would provide much valuable information on eye irritation and its consequences. The extent to which this or any other battery could partly or totally replace the Draize eye irritation tests should be decided in the near future, ideally, as a result of implementation of the principles established at Amden and Vouliagmeni.

FUTURE PROSPECTS

The increasing contribution being made by non-animal methods in toxicology and toxicity testing is very welcome. As a result of toxicological screening, the most favourable candidate compounds can be selected for further development. This contributes not only to a reduction in the total number of animals used, but often also to a reduction in the suffering caused to those that are used in subsequent *in vivo* tests.

However, there are signs that some toxicologists, even some *in vitro* toxicologists, are prepared to settle for such *reduction* and *refinement* contributions and see genuine *replacement* as an unachievable goal. This is *not* acceptable to members of the moderate animal welfare movement – nor is it likely to be acceptable to those responsible for the Three Rs legislation which now governs us.

REFERENCES

Anon. (1986a) *Animals (Scientific Procedures) Act 1986*, London: HMSO, 24pp.

Anon. (1986b) Council Directive of 24 November 1986 on the approximation of laws, regulations and administrative provisions of the Member States regarding the protection of animals used for experimental and other scientific purposes. *Official Journal of the European Communities*, **29(L358)**, 1–29.

Balls, M. (1983) Alternatives to animal experimentation. *ATLA*, **11**, 56–72.

Balls, M. (1990) Alternative approaches. In: *LD50 Testing and Classification Schemes – the Possibilities for Change*, Brussels: CEC, pp. 58–64.

Balls, M.; Clothier, R.H. (1989) Validation of alternative toxicity test systems: lessons learned and to be learned. *Molecular Toxicology*, **1**, 547–559.

Balls, M.; Blaauboer, B.; Brusik, D.; Frazier, J.; Lamb, D.; Pemberton, M.; Reinhardt, C.; Roberfroid, M.; Rosenkranz, H.; Schmid, B.; Spielmann, H.; Stammati, A–L.; Walum, E. (1990) Report and recommendations of the CAAT/ERGATT workshop on the validation of toxicity test procedures. *ATLA*, 18, in press.

Balls, M.; Botham, P.; Cordier, A.; Fumero, S.; Kayser, D.; Koeter, H.; Koundakjian, P.; Gunnar Lindquist, N.; Meyer, O.; Pioda, L.; Reinhardt, C.; Rozemond, H.; Smyrniotis, T.; Spielmann, H.; Van Looy, H.; Van der Venne, M–T.; Walum, E. (1990b) Report and recommendations of an international workshop on promotion of the regulatory acceptance of validated non-animal toxicity test procedures. *ATLA*, **18**, in press.

Clothier, R.H.; Hulme, L.M.; Ahmed, A.R.; Reeves, H.L.; Smith, M.; Balls, M. (1988) *In vitro* cytotoxicity of 150 chemicals to 3T3–L1 cells, assessed by the FRAME kenacid blue method. *ATLA*, **16**, 84–95.

Clothier, R.H.; Hulme, L.M.; Smith, M.; Balls, M. (1989) A comparison of the *in vitro* cytotoxicities and acute *in vivo* toxicities of 59 chemicals. *Molecular Toxicology,* **1**, 571–577.

Ekwall, B.; Bondesson, I.; Castell, J.V.; Gomez–Lechon, M.J.; Hellberg, S.; Högberg, J.; Jover, R.; Ponsoda, X.; Romert, L.; Stenberg, K.; Walum, E. (1989) Cytotoxicity evaluation of the first ten MEIC chemicals: acute lethal toxicity in man predicted by cytotoxicity in five cellular assays and by oral LD50 tests in rodents. *ATLA*, **17**, 83–100.

Heywood, R. (1990). Clinical toxicity – could it have been predicted? Post–marketing experience. In: *Animal Toxicity Studies: Their Relevance for Man*, C.E. Lumley & S.R. Walker (Eds.), Lancaster: Quay Publishing, pp. 57–67.

Hulme, L.M.; Reeves, H.L.; Clothier, R.H.; Smith, M.; Balls, M. (1989) Assessment of two alternative methods for predicting the *in vivo* toxication of metallic compounds. *Molecular Toxicology*, **1**, 589–596.

Reader, S.J.; Blackwell, V.; O'Hara, R.; Clothier, R.H.; Griffin, G.; Balls, M. (1989) A vital dye release method for assessing the short-term cytotoxic effects of chemicals and formulations. *ATLA*, **17**, 28–33.

Shaw, A.J.; Clothier, R.H.; Balls, M. (1991) Loss of trans-epithelial impermeability of Madin-Derby canine kidney (MDCK) cells in the assessment of ocular irritancy potential and of cellular recovery from chemical damage. *ATLA*, **19**, in press.

Van den Heuvel, M.J.; Dayan, A.D.; Shillaker, R.O. (1987) Evaluation of the BTS approach to the testing of substances and preparations of their acute toxicity. *Human Toxicology*, **6**, 279–291.

Van den Heuvel, M.J.; (1990) A fixed-dose procedure as a replacement for the classical LD50 test: the UK initiative. In: *LD50 Testing and Classification Schemes: the Possibilities for Change*, Brussels; CEC, pp. 71–82.

Zbinden, G. (1988) A look behind drug regulatory guidelines. In: *National and International Drug Safety Guidelines*, S. Alder & G. Zbinden (Eds.), Zollikon: M.T.C. Verlag, pp. 7–19.

WHY IN VIVO STUDIES ARE STILL REQUIRED

A.D. DAYAN

DH Department of Toxicology, St. Bartholomew's Hospital Medical College, University of London, Dominion House, 59 Bartholomew Close, London, EC1 7ED

ABSTRACT

Determining the safety of a chemical requires a fine balance between the risk of its toxicity and the benefit of its use. Studies in animals are essential in determining toxicity because only with in vivo studies can the full range of direct and indirect toxic effects and disturbed physiological mechanisms be studied in a way that will support extrapolation to man and other species. Methods may be improved, and some studies will be done in man, but the need for extensive experiments in animals will remain for the foreseeable future.

"Neither the voice of authority nor the weight of reason and argument are as significant as experiment, for thence comes quiet to the mind".

Bacon

INTRODUCTION

This essay does not attempt to cover the use of any living organism in any type of scientific investigation, but rather it is focused on 'Safety', which usually implies studies in vertebrates, and especially mammals. It is important, however, to realise that the ethical, scientific and practical issues are common throughout basic and applied biology. The arguments for and against work in vivo apply as much in toxicology as to basic investigative research. Put at its starkest "Why and for what reasons do we use animals in experiments?"

To respond to the specific question requires clear understanding of what is meant by "safety", and of the nature of animal experimentation, before analysing its place and limitations.

WHAT IS "SAFETY"?

"Safety", as a lack of harm, can only be demonstrated retrospectively by continued health or well-being despite exposure to some circumstance or preparation. To be useful, however, protection against harm, which is predicting 'Safety', requires forecasting absence (or minimisation) of risk in the future on the basis of present evidence. 'Safety', too, is not an absolute, because every action may have good and bad consequences, i.e. some likelihood of risk, but its essential feature is deciding what is acceptable.

The elements behind "safety" are knowledge first of the details of effects:

- systems and organisms at risk

- harmful effects produced

- circumstances of their occurrence
  - causal factor(s)
  - dose
  - duration of exposure
  - any interactions (genotype, co-administered substance etc.)

- nature of the effects
  - frequency
  - type
  - severity
  - reversibility
  - consequences

Second, there must be some means of predicting the extent and type of harm that the entire system or population of organisms may experience under given conditions of exposure.

And third, and with the most difficulty, a judgement is required whether any conceivable harm is acceptable in relation to the anticipated benefit of the exposure. Safety is more than just a prediction of damage, it is as much a decision that something is acceptable, and therefore it is to be considered safe because it is worth more overall than the harm it may cause (Moore, 1983; BMA, 1987).

"Safety", then, means both knowing and balancing benefit and harm. For comparison the latter pair should be assessed on a common scale, although in practice that is very difficult and is almost never done, because most judgements, being rooted in empiricism, have a subjective basis, which is politely termed 'wisdom and experience', or more acerbically 'complaisance'.

The ultimate decision is that something is safe, because the risk that it carries is acceptable; "risk" being a combination of the likelihood that a harmful event will occur, of its mature and magnitude, and of its perception by those directly involved, and increasingly by the general public, too.

Such a hybrid measure, combining probability, individual and group psychology, and monetary value is an unsatisfactory basis for rigorous analysis, but it has rightly been the subject of vigorous debate about definition, evaluation and communication in science psychology and insurance (NRC 1984, 1989; Royal Society, 1983; Moore, 1983; BMA, 1987).

There is general acceptance that the concept of risk must somehow combine both probability of occurrence and the severity of the event caused in the individual and the population. This remains a largely unexplored field, except in a few simpler instances, where cause and consequence can be clearly linked, for example, exposure to ionising radiation and the frequency and onset and the lethality of various diseases. The concept of overall 'health detriment' combining all these as one presentation of risk to man has been devised by radiobiologists (ICRP, 1985, 1988), and it might be worth developing it as a more general index of harm.

The judgement of safety is also clouded by our asymmetrical perception of risk and our common inability to measure simplistically most benefits, such as economic gain or the value of prolongation of human life. We are far more scared of unfamiliar and involuntary risks than of everyday occurrences, or of those we willingly accept, as in travel and sport. Some current risks to man are shown in Table 1, and it must be presumed that they represent "safety", because they appear to be accepted.

TABLE 1. Accepted risks which presumably are regarded as "safe" because they appear to be accepted.

Activity	Risk of dying in any year
Smoking 10 cigarettes/day	1 in 200
Violence (GB)	1 in 3,300
"Influenza"	1 in 5,000
Accident on road	1 in 8,000
Accident at home	1 in 26,000
Accident at work	1 in 43,000
Murder	1 in 100,000
Lightning	1 in 10,000,000
Radiation from power station	1 in 10,000,000

Attempts have been made to devise financial scales to assess the value of non-fatal effects on human health of disease and treatment, but such approaches as 'Quality of Life Years' etc. carry too many uncertainties for general application (Walker and Rosser, 1988).

This discussion has been concentrated on man and his safety and the corresponding risks, but there is need for as much concern about nature and the environment at large. Unfortunately, there are no adequate tools yet to quantify harm to such a broad system, rather than to individual populations or small sub-systems. The imprecision here has led to baffling confusion in debates over acid rain, the greenhouse gases and other broad environmental issues. These are risks of a nature and scale that makes them extremely important, but judging safety in such contexts so far has shown more intent than achievement (e.g. Otway and Peltu, 1985).

Risk covers both harm and the probability of its occurrence under given circumstances, and is quite distinct from the innate harmful properties of a substance, which is its hazard. Practical realisation of a hazard requires exposure and a responsive system that results in the harm (risk). The experimenter, however, basically assesses hazard in animal and other experiments, before considering the circumstances of exposure that lead to risk.

From the viewpoint of use of any chemical, be it medicine, food additive or crop protectant, and whether developer-user, exposed public or regulator balancing in the middle, the need then is to define the hazard in experiments and to predict the risk under relevant circumstances. Each of us, as polled

and taxed citizens, should take part in the debate about acceptability of the risk, noting the difficulty of defining the harm and the benefit. For our narrower purpose here, these steps can now be assumed, and attention focused on hazard and its demonstration and prediction by animal experiments.

The appropriate questions are :

i) What can and cannot be studied in animal experiments?

ii) How successful have such studies been and in what ways may they fail?

iii) Are there imminent developments affecting the scope or need for animal experimentation?

My purpose specifically excludes the study of useful activities and confines me to safety and its entailed risk, although the separation would be unacceptable in the real world of manufacturing practice and regulatory restraint.

ANIMAL EXPERIMENTATION

Hazards that are studied

A range of in vivo toxicity experiments is done in the development of a pesticide, which has grown empirically over the years. It generally comprises :

(a) Mammals

Acute toxicity
Aspects of general pharmacology
Local irritancy
Sensitising potential
Toxicokinetics and metabolism
Subacute - chronic toxicity
Genotoxicity
Carcinogenicity
Reproduction and teratology

The substance of interest will be examined and possibly residues or metabolites found in the diet or drinking water.

b) Wildlife (as appropriate to use)

Subacute toxicity	(birds
Accumulation	(fishes
and/or metabolism	(crustacea
	(insects
	(ruminants (or other target species if not already covered)

This will be done in the laboratory under controlled conditions, and to some extent as field investigation of model ecosystems exposed under near-use conditions.

Inevitably, exposure will be to the test substance, its formulations, and its natural breakdown products.

Man is likely to be investigated by measurement of exposure in field use, possibly in deliberate trials to explore toxicokinetics and metabolism in the field or in the laboratory, and via occupational health records, and perhaps epidemiological inquisition for post-use detection of associated illnesses.

Experiments will also be done into adsorption, migration and breakdown in soils and plants.

Well done studies in mammals of these types should reveal major functional and morphological effects on target organs, their relation to dose and duration of treatment, and the importance of kinetics and metabolism in determining their occurrence. Similarly, the work in wild species or surrogates, and in the field or in experimental plots, ought to demonstrate direct harmful effects or actions via other mechanisms in ecosystems.

In principle, therefore, many types of hazard should be identified and measured in a manner suitable for the prediction of risk in workers, the public and animals (and in vertebrates) in the environment, provided that their exposure can be determined and the assumptions supporting the predictions can be verified.

WEAKNESSES AND DEFICIENCIES

The well-publicised inadequacies in animal experiments arise for fundamental and practical reasons.

i) Animals usable in experiments may have physiological and metabolic responses that diverge from their wild counterparts and man. The differences may arise because prediction jumps from one species to another, because laboratory conditions make their physiology and behaviour diverge from natural circumstances, or because special strains of animals have inevitably been selected for the laboratory, which are unrepresentative of the general population.

ii) The statistical power of experiments in the necessarily limited groups in the laboratory means that smaller or weaker effects, or unusual or idiosyncratic actions, cannot be detected.

iii) And, have the appropriate effects been sought by adequate methods?

Toxicity testing is a complex, costly and all-consuming purpose and there is often reluctance to add further experiments to explore further endpoints without very clear evidence of their value. This inertia must always risk inattention to exploration of a novel action, or failure to do a confirmatory study to link a pattern of effects, possibly suggesting an underlying toxic mechanism; for example, should anything extra be done to examine a behavioural abnormality seen in a standard test? Or, are there changes in, say, the pituitary, thyroid, ovary, breast and pancreas all due to a common action on prolactin via the hypothalamus, or are they distinct forms of toxicity, a question that might require a further experiment to assay prolactin levels etc.

All these daily worries of the toxicologist are discussed in standard works, e.g. Calabrese (1983), Tardiff and Rodrick (1988), Dayan (1990).

In addition, in evaluating a practical risk from a laboratory-determined hazard there is the well known problem of determining exposure in the field, be it of the spray operative, say, or, with more difficulty, the consumer of residue-containing food (especially if exposure is indirect, e.g. the eater of meat from cattle fed on treated fodder).

The determinants of individual susceptibility are not well known, except for the limited instances of genetic polymorphism of xenobiotic metabolism (e.g. slow acetylators and fast oxidisers). They may account for a small proportion of the population in the tail of a distribution, but the main sources of person-to-person variation remain to be uncovered and their importance decided, as extensively discussed in an analogous area of the human response to pollutant gases (Brain et al., 1988).

COUNTERVAILING STRENGTHS

Again, there is a mixture of basic and practical aspects.

i) Animal life as an individual or in a community depends on a complex web of integrated responses and a toxic action may occur at one level but only be manifested at another. Only in animals can important second order interactions be demonstrated, for example, increased pituitary prolactin secretion via an action in the hypothalamus may ultimately cause pancreatic and mammary tumours in the rat. To reveal such a chain means to identify its links, which is essential if the hazard is to be evaluated, and it can only be done by work in animals in whom all the components can operate, so revealing the action and displaying each step for study.

Similarly, activation to a toxic metabolite may occur in one organ, with the end effect occurring at a remote site, e.g. the metabolism of aromatic amines to proximate carcinogens in liver that only act in the bladder. Vital body systems have evolved to respond homeostatically to adverse stimuli, e.g. the controls over water and electrolyte metabolism via the kidneys and endocrine glands, the inter-related neural and paracrine regulators of cardiac function and blood pressure, and the entire humoral, cellular and innate immune systems. Toxic effects on these multilevel systems can only be investigated at first by in vivo studies, because otherwise there would be no indication of which target or mechanism to examine.

ii) Social and other physiological interactions between animals are very important, especially amongst sub-human species, and they form the very basis of ecotoxicology. If investigations are not done in vivo it is impossible to explore important actions, say, on breeding performance, social behaviour, etc.

iii) High level physiological mechanisms, too, can only be studied as integrated responses in vivo, notably the cognitive functions of the nervous system.

iv) Experience gained over several decades of toxicity testing of substances ranging from natural products to industrial chemicals, pesticides and pharmaceuticals should usually show when extrapolation can be done with

confidence and when it may be less certain because of the novelty of the actions. It is very important that the toxicologist be able to refer the latest problem to accumulated experience, as a guide to assessment and as a lead to any additional experiments.

PRACTICALITIES, ETHICS AND ECONOMICS

These factors point in several directions.

There is a large investment in the skills and facilities for animal experimentation, but it is not immutable, as shown in related areas by the gradual introduction of *in vitro* pharmacopoeial assays and genotoxicity testing, albeit with growing knowledge of how *in vitro* enthusiasm must not outrun empirical reality (COM 1989). The intellectual and financial costs of other than *in vivo* experiments must always be considered, e.g. the availability of skills and resources and the degree of confidence it may be reasonable to have in a prediction.

The question of ethics is at the core of this debate. It is not an absolute matter, except to a few extremists, but rather it represents individual societal opinions based on diverse value systems (Paton, 1984; Dunstan, 1979; Warnock, 1985). Is it justified to use some animals to protect many others and man? I believe it is - with safeguards such as we have under current UK legislation.

VALUE OF ANIMAL EXPERIMENTS

At first, it would seem appropriate to list a series of decisions based on *in vivo* work, where safety was attained or harmful effects unexpectedly occurred. This is impossible because of the common practices of not developing or withdrawing substances that appear too risky, and of conducting exhaustive investigations to defend marketed substances whose apparent safety has been impugned by new findings.

Most publicly available data reflect past errors and tragedies, and accidental or deliberate poisoning today. They are all biased and are grossly unsuitable for assessment.

At one level published statistics on poisonings, accidents and even pensions for industrial injuries suggest that harm to man is rare, unless there is a breach of current safety standards (see Annual Reports of Health and Safety Commission) but they are equally selected data. Similar and equally inadequate surveys have been used to suggest widespread harm from exposure to pesticides or contaminants, e.g. Young and Reggiani (1988) - dioxin on its own and in 'Yellow Rain' in Vietnam. Even the most rigorous sources have been troubled by the inadequacies and obfuscation of competing claims, e.g. the attempts by IARC and others to judge competing claims about the carcinogenicity or non-carcinogenicity of the phenoxyherbicides (Alderson, 1985; IARC).

Statistical support for the value of toxicity testing from related areas, such as pharmaceuticals, has also been limited for the same reasons. In general, toxicity tests have been capable of predicting most risks and even of over predicting some, when it has been possible to compare findings in man and animals (Lumley and Walker, 1987).

DEVELOPMENTS IN ANIMAL EXPERIMENTATION?

Scientific, regulatory and practical considerations may all affect the usage of animals in different ways.

i) Newly discovered diseases and their causes, and more importantly advances in understanding that reveal new cellular targets, would all tend to increase the extent of animal experimentation or the species or numbers required.

If there were agreement, for example, that more detailed studies of behavioural or immunotoxicity were desirable, because of a perceived toxic threat, that would cause a sharp increase in animal usage.

Similarly, if a close parallel between toxicokinetics in man and an animal species were desired, there might be a trend to greater use of non-human primates, as has sometimes happened in other instances. The advent of genetically engineered products, which often are active only in a narrow range of susceptible species, is an area where such a trend can already be seen.

ii) More precise or more focused studies during current in vivo tests might lead to a reduction in the numbers used whilst maintaining adequate discriminative power.

iii) Our accelerating ability to do studies in man, thanks to much more sensitive techniques, and the availability of guidelines to the ethical and professional problems they cause, may lead to some reduction in the need for animal experiments.

iv) Similarly, improved epidemiological surveillance, and perhaps detailed employment records in 30 years time, may eventually demonstrate the true value of some in vivo tests and encourage others to be discarded.

A toxicologist might wonder about the importance of mixed exposures and interactions in causing low level effects, and he could even ponder if they should be explored in even more experiments?

v) At a regulated level within Britain, the increased surveillance and control over animal experimentation afforded by the 1986 Animals (Scientific Procedures) Act, and the development of clear and comprehensive guidelines by the Home Office, Universities' Federation for Animal Welfare and Laboratory Animal Science Association will support a high standard of experimentation, but they have little concern with amount of work in the area of risk. Indirectly, like GLP and the broader European Community and other international initiatives for regularisation of toxicity testing, they will encourage the interchangeability of results, thus reducing even near duplication of experiments. As most of this work is already concentrated in relatively few centres, it is likely to have little overall impact.

vi) What about the replacement of *in vivo* by non-animal or *in vitro* techniques?

In the field of toxicity testing for risk assessment, it seems unlikely to have any major quantitative effect for many years for the reasons discussed above. Even when the likely absence or presence of given effects is forecast with some assurance on the basis of QSAR or other *in vitro* procedures, ethics and the practical need for personal and legal protection will still require animal tests to confirm the prediction, especially to reveal the unexpected and the novel that can only be unpredictable, because of the limited range of reactivity inherent in *in vitro* methods relative to studies in animals.

In selected tests, the numbers of animals might be slightly reduced, because of refined and better directed observations, but without a major jump in basic knowledge (e.g. in the mechanisms of carcinogenesis or the control of development), there seems no good reason to anticipate a reduction in the general scale of toxicity testing.

Better selection of compounds may reduce overall numbers of tests, but that, too, may not make much difference overall. *In vitro* and other methods for prior screening may help to reduce numbers of animals a little in certain areas, e.g. in acute toxicity and irritancy testing.

CONCLUSIONS

The "Safety" in my title is a *post hoc* judgement, and depends on controlling exposure to a risk limit set by extrapolation from hazard demonstrated in various tests.

Essential information about direct and indirect effects on integrated and regulatory systems in the body can only come from toxicity studies in animals, and there seems little prospect of reducing the need for them in the near future. Some refinement of techniques may be possible if more useful information could be obtained from other procedures and even from man.

It would be timely for industry to collate its experience to show the value of toxicity testing, as is being done for pharmaceuticals. The best defence would be to show how much we depend on what is now done. As Goethe more succinctly wrote, "There is nothing so terrifying as ignorance in action." That itself is too harsh a judgement on safety studies but it does appear to me that they have reached a state where accretion of new procedures based on proven methods affords the best protection against present concerns. To change the foundation of that knowledge will require a powerful leap in understanding.

"Next to being right in the world, the best of all things is to be clear and definitely wrong." T.H. Huxley

Toxicity testing based on animal experimentation is still right, I believe.

REFERENCES

Alderson, M. (1985) Occupational Cancer. Butterworth, London.
BMA (1987) Living with Risk. Wiley, Chichester.
Brain, J.D.; Beck, B.D.; Warren, A.J.; Shaikh, R.A. (1988) Variations in Susceptibility to Inhaled Pollutants. Johns Hopkins, Baltimore.
Calabrese, E.J. (1983) Principles of Animal Extrapolation. Wiley, New York.
COM (1989) Guidelines for the Testing of Chemicals for Mutagenicity. HMSO, London.
Dayan, A.D. (1990) Toxicity Testing. In: Principles of Drug Development, O'Grady, J.G.; Kolar, O. (Eds). MacMillan, London.
Dunstan, G.R. (1979) A Limited Dominion. Conquest, 169, 1.
IARC (1987) Chlorophenoxy herbicides. IARC Monographs, Suppl. 8, 156.
ICRP (1985) Quantitative bases for developing a unified index of harm. Ann. ICRP (15) no. 3.
ICRP (1991) Recommendations of the Commission. Ann. ICRP in press.
Lumley, C.E.; Walker, S.R. (1987) Predicting the Safety of Medicines from Animal Tests. Arch. Tox., S11, 295-304.
Moore, P.G. (1983) The Business of Risk. CUP, Cambridge.
NRC (1983) Risk Assessment in the Federal Government. National Academy Press, Washington.
NRC (1989) Improving Risk Communication. National Academy Press, Washington.
Otway, H.; Peltu, R. (1985) Regulating Industrial Risks. Butterworth, London.
Paton, W. (1984) Of Mouse and Man. Clarendon Press, Oxford.
Royal Society (1983) Risk Assessment. London.
Tardiff, R.G.; Rodricks, J.V. (1987) Toxic Substances and Human Risk: Principles of Data Extrapolation. Plenum, New York.
Walker, S.R.; Rosser, R.M. (1988) Quality of Life: Assessment and Application. MTP, Lancaster.
Warnock, M. (1985) Law and the Pursuit of Knowledge. Conquest, 175, 1.
Young, A.L.; Reggiani, G.M. (1988) Agent Orange and its Associated Dioxin: Assessment of a Controversy. Elsevier, London.

"THE DEVELOPMENT AND UTILITY OF *IN VITRO* TECHNIQUES - AN INDUSTRY VIEWPOINT"

G J A OLIVER

ICI Central Toxicology Laboratory, ICI plc, Macclesfield, Cheshire, SK10 4TJ

ABSTRACT

Toxic hazard is usually determined by established test methods, the majority of which use animals, and the results of which are credited internationally. The toxic profile of a chemical may have an impact on its initial selection and subsequent successful development as a product. The timing of toxicity studies and their predictiveness can therefore contribute significantly to commercial decisions.

In vitro methods are only of value in this process if they allow reliable decisions on toxic potential to be made. Thus the utility of *in-vitro* techniques will be governed ultimately by their performance against accepted standard test methods or known human response.

The potential of *in vitro* techniques, how they may be applied in industry to aid product selection and development and used in hazard and risk assessment is exemplified by two models - for assessing absorption through and effects on skin following dermal exposure.

INTRODUCTION

The principal role of a toxicologist working within industry is to define the toxic hazard to man of 'substances' be they raw materials, intermediates or final products. When toxic hazard is linked to an understanding of potential exposure, then risk to health can be assessed and, if necessary, protective measures introduced. The importance of this process is reflected in the close regulation of the introduction and use of industrial chemicals and particularly pesticides into the community (EEC 1984; EPA, 1984;). The initial definition of toxic potential -dose related adverse effects - is therefore critical to the above process and the toxicologist in industry and the regulator in government must have confidence in the methods used.

For ethical reasons, it is usually impossible to assess toxicity directly in man. Over a number of years numerous surrogate test methods have been developed, the majority of which use live animals, to determine adverse effects which may range from skin irritancy to cancer. Protocols for these methods are international and in many cases harmonised, enabling mutual acceptance of data between different countries (OECD, 1981).

Against this established background there has been a substanial understandable, and generally well-supported move to reduce the use of live animals for toxicity evaluation (Balls et al, 1983; Paton, 1984; Reinhardt et al, 1985). *In vitro* methods have been used in biological sciences for decades and in toxicology, mainly for the purposes of

screening or studying mechanisms of toxicity. Undoutedly, whilst in vitro methods offer potentially many advantages there are corresponding pitfalls. Essentially, the industrial toxicologist has to make decisions regarding health effects on the results of his studies; the consequences of a poor decision may influence product development or introduce an unnecessary risk to an exposed population. The utility of any methods including in vitro tests, must be judged in this context.

HAZARD ASSESSMENT AND PRODUCT DEVELOPMENT

The development of a pesticide from it's initial synthesis and efficacy screening to sales may take 5-10 years and cost more than £15m. Regulatory toxicology tends to be commissioned in the development schedule after favourable efficacy and other commercial profiles have been sufficiently well established and a final route of synthesis is known. The regulatory toxicology requirements are extensive but well established and any proposed alternative, for whatever reason, to recommended methods requires extensive discussion and international agreement. Thus the toxicologist has limited degrees of freedom in method selection in the regulatory environment.

After, and maybe during, the regulatory phase, however, relevant mechanistic studies may be commissioned. For example, these studies may address observed species differences in toxicity. In this way toxic hazard and risk to man may be more precisely determined. Of necessity, these studies will be tailor-made to the chemical, the effect and the species concerned. In vitro techniques will frequently be used in these investigations, especially when human tissue response needs to be determined (Volans et al, 1989). Whilst the majority of animal studies provide relevant, informative data, there are a number of examples in the literature of rodent specific toxicity which is unimportant in relation to human hazard and risk assessment (Short & Swenberg, 1988; Lock et al, 1989; Green, 1990).

Prior to the regulatory phase, elective toxicology screening can be incorporated into the development programme to provide information which can contribute to the optimal selection of a chemical for progression as a product to the market place. Clearly any methods which will help to predict at this stage the likely outcome of subsequent lengthy, costly, regulatory studies will be attractive. Because of the constraints on development at this point (principally time but also costs and compound supply) then only short term, toxicity tests are pragmatic.

Decisions based on test results will remain 'in-house' and therefore regulatory sanction or approval for a test method is unnecessary. Providing the results from any methods can be interpreted within an acceptable error rate, then the toxicologist has a free hand to utilise those methods he judges appropriate. In many cases the development and utilisation of short term screening assays has been pioneered in this "early screening" elective environment with initial validation against conventional animal methods restricted to well-defined chemical series or analogues. Subsequent publication and screening of chemicals of more varied structure may then lead to more widespread use of the method.

ADVANTAGES AND DISADVANTAGES OF IN VITRO SCREENING METHODS

The theme of 'reduction, refinement and replacement' of animals first introduced by Russell and Burch (1959) is applauded within toxicology and a significant advantage of in vitro methods is that they help to realise this theme. In addition, in vitro techniques are rapid and require relatively small amounts of chemical. These characteristics can be particularly important in the early development phase. Finally, in vitro techniques offer the opportunity for extending investigations into human tissue which may remove the element of species extrapolation and lead to a more accurate assessment of toxicity. Thus there are potential ethical, economic and specificity benefits, none of which is mutually exclusive, for developing and using in vitro techniques in toxicology.

However, there are many difficulties and disadvantages which have retarded the introduction and utilisation of these methods. Principally, and in brief, the major shortcoming of in vitro techniques is their inability to mimic complex biological tissues and processes. An holistic response in animals is a consequence of many inter-relating factors which can be conveniently characterised as either pharmacokinetic (the absorption, metabolism, distribution and excretion of a substance) and/or pharmacodynamic (the initial and sequential consequences of chemical interaction with specific tissue functions and structures) (Goldberg and Frazier, 1989). Knowledge of the mechanism of toxic action is usually the surest foundation for the success of an in vitro method and establishing structure activity relationships. However, the influence of pharmacokinetics and the multiplicity of mechanisms for most given end toxic effects may diminish the performance of an in vitro method. This is best illustrated by the history of short-term tests for mutagenicity, the evolving tiered strategies for assessing genotoxicity and the recognition of epigenetic cancer mechanisms (ECETOC, 1987; Tennant, et al, 1987; Purchase, 1990).

Finally, in vitro techniques may not readily allow comparison of different routes of exposure or indicate reversibility of effects which may be critical to qualifying the toxic hazard.

VALIDATION OF NEW METHODS

All new in-vitro methods must be validated to determine how accurately they can predict the result which would be obtained in vivo, usually in animal tests but occasionally, if the data base is sufficient, against the known incidence of human response (Scala, 1987). The accuracy of a model can be determined in terms of sensitivity (number of correct positives in the test) and specificity (the number of correct negatives in the test). The higher the sensitivity the lower the number of false negative results; the higher the specificity the lower the number of false positives (Cooper et al, 1979). Various factors will influence test accuracy, particularly the range and number of chemical structures examined. It is possible that an acceptable test performance, when screening a closely analogous chemical series, will fade as more diverse chemicals are examined.

The extent of any validation programme will depend on how the test is to be applied. In-house validation with a small number of closely related analogues may suffice for elective, early screening. Extensive validation

is usually required before an in vitro method can gain regulatory acceptance (Scala,1987). The consequences of making the wrong decision about toxic hazard are different in each case. In-house screens aid optimal selection of candidate chemicals or formulations; a false positive result may lead to unnecessary restriction or rejection of an otherwise promising chemical whereas a false negative result will be highlighted later in regulatory testing. Both of these outcomes are clearly unacceptable and costly in terms of resources. For regulatory approval, if the in vitro test is to be an equivalent substitute to accepted methods then sufficient scientific evidence must be produced by selective intra- and inter laboratory trials to verify that a hazard evaluation from either the in vitro or the in vivo method has equal merit. Because of this stringent requirement, in many cases in vitro tests may eventually occupy a middle ground in the public domain as adjuncts or prescreens but not replacements of animal tests. This situation is understandable. Whilst a false positive result may lead to unnecessary constraints on the use of a compound there is no back-stop after the regulatory phase; a false negative result may lead to unwarranted risk in an exposed population.

APPLICABILITY OF IN VITRO METHODS IN INDUSTRY

Some of the points and principles discussed above are illustrated below with reference to two in vitro models, one for assessing skin effects per se, the other for measuring rates of absorption through the skin.

(a) Skin Corrosivity Model

Occupationally, skin is a major target organ, the skin being the most common route of exposure to industrial chemicals, pesticides and their formulations. Invariably, new chemicals will be assessed for their direct effect on skin tissue in well established animal tests. The most severe effect is termed "corrosion" and is similar to a chemical burn, involving overt tissue destruction resulting in permanent damage or residual tissue scarring. On the assumption that corrosive chemicals would "dissolve" the skin's natural outer protective barrier, the stratum corneum, we developed an in vitro method based on skin slices taken from humanely killed animals (Oliver et al, 1988). Corrosive action in vitro was measured by a fall in the inherent electrical resistance, below a determined threshold, across a skin slice due to the loss of normal stratum corneum integrity and function. Initial validation of the model resulted in high sensitivity (>90%) and lower specificity (approx 75%) indicating a tendency for the model to provide some false positive results but few false negative results. This was subsequently realised using the technique as a preliminary to animal studies. Of 101 chemicals, 78 were negative in vitro and non-corrosive; 23 were positive in vitro and 11 of these corrosive in vivo (Oliver, 1990). Overall the value of this model is as a prescreen (Oliver et al, 1989) which can identify those chemicals which are more likely to be corrosive and a decision can be made on whether to proceed to a full in vivo evaluation or modify subsequent animal testing (eg. use one animal, reduce contact periods, increase clinical observation periods). Steps are in progress to decrease the false positive incidence, but in its current form, the model allows rapid screening, with refinement and reduction in the use of animals.

However, an added benefit of this approach is that human skin can be examined in vitro in a similar way. Comparative testing revealed that human cadaver skin was less sensitive to some apparent animal corrosive agents (Oliver and Pemberton, 1986). To verify this in vitro finding, the in vivo hazard of "corrosive" chemicals which did not affect electrical resistance of human stratum corneum in vitro was studied in human volunteers (Table 1). Three chemicals with specific in vitro/in vivo

TABLE 1 Putative corrosive chemicals selected for in vivo human studies

Chemical	Corrosive Category (in-Vivo Rabbit)	Physico-chemical Effect on Skin In-Vitro**		Result of Human Volunteer Study
		Rat	Human	
Aromatic Solvent	III*	+	-	Slight irritation
Proprietory washing-up liquid	III	+	-	Slight irritation
Fungicide Formulation	III	+	-	Slight irritation

* III - Corrosive In-Vivo after 4 hrs contact

** Reduces skin electrical resistance below normal threshold after 24 hrs contact In-Vitro

profiles were assessed under exposure conditions similar to those used in the in vivo animal test. No clinical signs consistent with severe inflammation or corrosion were seen in any volunteer (Oliver el 1989). In this case, the utilisation of an in-vitro technique enables the direct evaluation of human tissue and results in a more accurate description of the real hazard to man.

(b) In Vitro Skin Permeability Technique

The second example of an in vitro technique which has proved effective in product, and particularly formulation selection is the skin permeability technique (Dugard and Scott, 1984). This technique allows the quantification of hazard by assessing the absorption of a chemical through the skin following exposure and, therefore enabling an estimation of systemic dose.

Regulatory agencies recommend in vivo rat experiments to assess absorption as a percentage of dose over a 24 hour exposure period (Farber and Zendzian, 1990). The in vitro technique measures absorption over time through skin epidermal sheets prepared from humanely killed rats. Fewer (by 80%) animals are needed to supply tissue for experiments when compared with the in vivo study. Rate of absorption, total dose absorbed and the

absorption profile with time can all be measured in the same tissue sample at multiple, selected timepoints.

Since a toxic effect is not being assessed, the in vitro model cannot be characterised simply in terms of sensitivity and specificity. However, direct comparison of chemicals under similar exposure conditions by the in vivo and in vitro methods indicates similar levels of absorption (Table 2).

TABLE 2 Comparison of in vivo and in vitro skin absorption through animal skin.

Chemical	% Absorption in vivo	% Absorption in vitro	Exposure Period Hours	Reference
Cypermethrin[1]	19	20	20	Scott, 1989
Carbaryl[1]	20	20	10	"
Benz(α)pyrene	36	38	96	Yang et al, 1989
DDT[2]	25	28	48	Grissom et al, 1987
Fenvalerate[2]	19	19	48	"
Permethrin[2]	27	17	48	"
Vamidothion[2]	21	24	48	"
2,4-D[2]	41	28	48	"
2,4-Dinitro-chlorobenzene[3]	52	48	24	Bronaugh and Maibach, 1985
Nitrobenzene[3]	4	6	24	"

1 - rat skin; 2 - mouse skin; 3 - monkey skin.

It is well-documented from in vivo experiments that the rate of skin absorption is different in man when compared with a number of other animal species (Moody et al, 1990). This difference is obviously relevant to any quantitative hazard assessment in man based on eventual systemic dose. Clearly, the routine measurement of skin absorption in man in vivo in controlled volunteer studies is as impractical as it is desirable. However, human cadaver epidermal sheets can be relatively easily prepared and in-vitro skin absorption measured. The available data indicates reasonable agreement between in vitro and in vivo measurements of absorption in man (Table 3) and as noted above for other animal species.

TABLE 3 Comparison of *in vivo* and *in vitro* skin absorption through human skin

Chemical	% Absorption *in vivo*	% Absorption *in vitro*	Exposure Period Hours	Reference
Carbaryl	6.2	4.5	48	Scott, 1989
Fluazifop-butyl	3.1	3.0	8	Ramsey et al, 1990
2,4-Dinitro-chlorobenzene	53	33	24	Bronaugh and Maibach, 1985
Nitrobenzene[3]	1.5	7.8	24	"
Benzoic acid	46.5	60.6	24	Bronaugh and Franz, 1986
Caffeine	40.6	40.6	24	"
Testosterone	39.4	49.5	24	"

The absorption of chemicals *in vitro* can be easily compared between species; the consequence of the results to a quantitative hazard assessment is readily exemplified in Table 4. The relative absorption rate of paraquat in various species compared to man differs by a factor of 40 to 1400 (Scott et al, 1986).

TABLE 4 Comparison of the permeability of human and animal skin to paraquat dichloride

Species	Permeability rate (cm/hr x 10^5)	Permeability ratio
Man	0.732	1
Rat	26.7	40
Mouse	97.2	135
" (hairless)	1066	1460
Guinea Pig	195.6	270
Rabbit	79.9	110

Thus this *in vitro* technique reduces considerably the numbers of animals used, and allows rapid, extensive and comprehensive measurements to be made which describe the absorption process. Absorption rates of active ingredients from different vehicle environments can be easily compared and ranked in animal species and man. This allows the preferential selection of formulations from which absorption, and therefore systemic dose is mimimal. Specifically, the use of human tissue allows a more accurate assessment of absorption in man following inadvertant exposure and therefore improves quantitative hazard and risk calculations (Chester, 1988).

CONCLUSION

In vitro techniques hold many potential attractions in industrial toxicology which will stimulate their continued development. In reality progress will be slow and utilisation will proceed with caution. The reasons for this conclusion stem from the fact that (i) many complex biological processes will be difficult to emulate by simplified *in vitro* methods (ii) significant decisions may ride on the results of toxicity testing (iii) *ergo*, confidence through appropriate validation will be needed to define the utility of new techniques.

Nevertheless, whereas few, if any, regulatory test methods have been replaced by *in vitro* short-term methods there are many occasions where these approaches are applied effectively within industry for in-house prescreens to animal tests and for product selection as well as for the more accurate quantitation of human hazard.

Robust and predictive *in vitro* techniques will increase their contribution to hazard evaluation in the future; industrial toxicology laboratories will provide a breeding and nurturing environment for these new methods; optimal development and utilisation will, as always, be realised by collaboration and cooperation between industrial and academic toxicologists, regulators and those interested in animal welfare.

REFERENCES

Balls, M.; Rydell, R.J.; Warden, A.N. (1983). *Animals and alternatives to toxicity testing*. Academic Press, London.

Bronaugh, R.L.; Maibach, H.I. (1985). Percutaneous absorption of nitroaromatic compounds: *in vivo* and *in vitro* studies in the human and the monkey. *Journal of Investigative Dermatology*. 84, 180-183.

Bronaugh, R.L.; Franz, T.J. (1986). Vehicle effects on percutaneous absorption: *in vivo* and *in vitro* comparisons with human skin. *British Journal of Dermatology*. 115, 1-11.

Chester, G. (1988). Pesticide Applicator Exposure - Towards a predictive model for the assessment of hazard. *Aspects of Applied Biology*. 18, 331-343.

Cooper, J.A.; Saraachi, R.; Cole, P. (1979). Describing the validity of carcinogen screening tests. *British Journal of Cancer*. 39, 87-89.

Dugard, P.H.; Scott, R.C. (1984). *Absorption through Skin. In Chemotherapy of Psoriasis*. (Ed), Baden, HP pp 125-144. Pergammon Press, Oxford 1984.

ECETOC (1987). Monograph No.9. *Assessment of mutagenicity of industrial and plant protection chemicals*. Brussels.

EEC (1984). Commission Directive adopting for the 6th time Council. Directive 67/548/EEC. Part B. *Official Journal European Community*. 1, (L251) 271.

EPA (1984). Data Requirements for Pesticide Registration. *Federal Register*. 49 No.207, 42857-42891.

Farber, T.; Zendzian, R. (1990). Pesticide registration in the United States. *In Prediction of Percutaneous Penetration*. pp 279-285. (Eds), Scott R C, Guy R H and Hadgraft J, IBC Technical Services, London.

Goldberg, A.M.; Frazier, J.M. (1989). Alternatives to Animals in Toxicity Testing. *Scientific American*. 261, 16-22.

Green, T. (1990). Chloroethylenes: A mechanistic approach to human risk evaluation. Annual Review of Pharmacology and Toxicology. 30, 73-89.

Grissom, R.E.; Brownie, C.; Guthrie, F.E. (1987). In vivo and in vitro dermal penetration of lipophilic and hydrophilic pesticides in mice. Bulletin of Environmental Contamination and Toxicology. 38, 917-924.

Lock, E.A.; Mitchell, A.M.; Elcombe, C.R. (1989). Biochemical mechanisms of induction of hepatic peroxisome proliferation. Annual Review of Pharmacology and Toxicology. 29, 145-163.

Moody, R.P.; Franklin, C.A.; Ritter L (1990). Dermal absorption of the phenoxy herbicides 2,4-D, 2,4-D amine, 2,4-D iso-octyl and 2,4,5-T in rabbits, rats, rhesus monkeys and humans: a cross species comparison. Journal of Toxicology and Environmental Health. 29, 237-245.

OECD (1981). Guidelines for testing of chemicals. Paris.

Oliver G J A, Pemberton M A and Rhodes C R (1988). An in vitro model for identifying skin corrosive chemicals. Initial validation. Toxicology In vitro 2. 7-17.

Oliver, G.J.A.; Pemberton, M.A. (1986). The identification of corrosive agents to human skin in vitro. Food and Chemical Toxicology. 24, 513-514.

Oliver, G.J.A.; Pemberton, M.A.; Leeser, J.E. (1989). In vivo validation of an in vitro skin corrosivity test in human volunteers. Toxicologist. 9, (1), 1041.

Oliver, G.J.A. (1990). The evaluation of cutaneous toxicity, past and future. In Skin Pharmacology and Toxicology, Recent Advances. (Ed), by Galli C L, Hensby C N, and Marinovitch M pp147-163. Plenum Press, NY and London.

Paton, W. (1984). Man and Mouse: Animals in Medical Research. Cambridge. Oxford University Press.

Purchase, I.F.H. (1990). Risk Assessment of Chemical Carcinogens. Polish Journal Toxicology. In press.

Ramsey, J.D.; Woollen, B.H.; Auton,T.R.; Batten, P.L.; Leeser, J.E. (1990). Percutaneous absorption of the lipophilic herbicide fluazifop-butyl in man. In Prediction of Percutaneous Penetration. pp 49-57 (Eds), Scott R C, Guy R H and Hadgraft J. IBC Technical Services, London.

Reinhardt, Ch.A.; Bosshard, E.; Schlatter, Ch. (1985). Irritation Testing of Skin and mucous membranes. Food and Chemical Toxicol. 23 (2).

Russell, W.M.S.; Burch, R.L. (1959). Principles of humane experimental technique. Methuen.

Scala, R.A. (1987). Theoretical Approaches to Validation. In Alternative Methods in Toxicology. No.5 (Ed), Goldberg A.M. pp 1-9, Mary Ann Liebert Inc, N.Y.

Scott, R.C.; Walker, M.; Dugard, P. H. (1986). A comparison of the in vitro permeability properties of human and some laboratory animals. International Journal of Cosmetic Science. 8, 189-194.

Scott, R.C. (1989). Percutaneous Absorption: In vitro technique as an alternative to in vivo assessments. In Biological Monitoring for Pesticide Exposure. pp 158-169. (Eds),Wang, R.G.M.; Franklin, C.A.; Honeycutt, R.C.; Reinert, J.C. American Chemical Society, Washington.

Short, B.E.; Swenberg, J.A. (1988). Pathologic investigations of the mechanism of unleaded gasoline-induced renal tumours in rats. CIIT Activities Bulletin. 8, (7) 1-6.

Tennant, R.W.; Margolin, B.H.; Shelby, M.D.; Zeiger, E.; Haseman, J.K.; Spalding, J.; Caspary, W.; Resnick, M.; Stasiewicz, S.; Anderson, B. (1987). Prediction of Chemical Carcinogenicity in Rodents from in vitro genetic toxicity assays. Science. 236, 933-941.

Volans, G.N.; Sims, J.; Sullivan, F.M.; Turner, P. (1989). V International Congress of Toxicology. Basic Science in Toxicology. Taylor and Francis Ltd. London.

Yang, J.J.; Roy, T.A.; Krueger, A.J.; Neil, W.; Mackerer, C.R. (1989). In vitro and in vivo percutaneous absorption of benzopyrene from petroleum crude-fortified soil in the rat. Bulletin of Environmental Contamination Toxicology. 43, 207-214.

PESTICIDE EPIDEMIOLOGY

D. COGGON

MRC Environmental Epidemiology Unit, Southampton General Hospital, Southampton. S09 4XY

ABSTRACT

Assessment of the health risks from pesticides depends largely on laboratory investigation in vitro and in animals, but epidemiology has an important complementary role. It provides a necessary check on the validity of extrapolation from experimental models. This paper reviews the main epidemiological methods used to study pesticides, and examines their applicability to the various contexts in which human exposure to pesticides occurs.

INTRODUCTION

The regulation of pesticides requires that their economic advantages and any benefits which they bring to the public health be balanced against their toxicity and the risks which they pose to the environment. Information about the toxicity of pesticides comes largely from laboratory investigation, both in vitro and particularly in experimental animals. Laboratory studies have the advantage that they can be conducted relatively quickly and give warning of potential hazards before any substantial human exposure has occurred. However, they cannot be guaranteed to predict all adverse effects in man. For example, arsenic is an established cause of lung cancer in sheep dip manufacturers, but its carcinogenicity has proved difficult to demonstrate experimentally (IARC, 1980). Because of the uncertainties inherent in the extrapolation from laboratory studies, epidemiology provides an important complement to routine toxicity testing. It offers a safeguard against missed hazards, and in the event of such a hazard coming to light, may indicate ways in which toxicological screening could be improved.

TYPES OF EPIDEMIOLOGICAL STUDY

Three types of epidemiological study are commonly used in the investigation of pesticides.

Cohort studies

Cohort studies are analogous to laboratory experiments in that subjects exposed to a known or suspected hazard are followed over time, and their mortality or disease incidence compared with that of unexposed controls. Importantly, however, the investigator does not originate the exposure. He observes subjects as he finds them, and they may differ not only in relation to the hazard of interest but also in other ways which independently influence their risk of disease. This complicates interpretation. For example, exposed subjects might smoke more than their controls with a resultant spurious increase in their risk of lung cancer. Such 'confounding' effects are a concern in all observational studies, although they can often be taken into account through appropriate design and analysis.

A problem with the cohort method, particularly when applied to diseases of low incidence such as cancers, is that large numbers of subjects must be followed for a long time in order to achieve statistically viable results. This difficulty may sometimes be overcome by identifying exposed and unexposed populations retrospectively, for example from old company records, and comparing their disease incidence up to the time of the study. Another way of making investigations more efficient is to use disease rates in the general population for control purposes rather than following up a specially selected control group. This approach is legitimate where exposure in the population at large is negligible in comparison with that in the study group.

Case-control studies

Case-control studies examine the link between exposure and disease in the reverse direction to cohort studies. The starting point is a group of patients who have developed the disease of interest. Their past exposure to hazards which are suspected of causing the disease is ascertained and compared with that of controls who do not have the disease. The method provides information more quickly and cheaply than the cohort technique, but is often limited by difficulties

in obtaining reliable information about subjects' past exposure. Most case-control studies rely on people's memories, and while recall of attributes such as occupation and smoking habits may be reasonably accurate, there is wider scope for error in the retrospective measurement of variables such as diet and exposure to specific chemicals.

Cross-sectional studies

Cross-sectional studies measure the distribution of disease and/or its determinants in a population at one point in time. For example, levels of cholinesterase might be ascertained in a sample of operators applying an organo-phosphorus insecticide, and related to the spraying techniques which they were using when the survey was carried out. Because cross-sectional investigations give only a 'snapshot' picture, there are limits to their value in studying the relation between exposure and disease. When an association is found it is not always clear what is cause and what is effect. If subjects with dyspeptic symptoms are shown to consume a food more frequently than others who are symptom-free, is that because the food causes gastric dysfunction, or has the illness led people to modify their diet? Furthermore, where an exposure leads to disability, its effects may be underestimated in a cross-sectional investigation because sufferers have been selected out of the study population. For example, the liability of a product to cause asthma might not be apparent in a cross-sectional survey of spray operators if sensitive individuals tended to leave employment when their symptoms developed.

Despite these limitations, however, cross-sectional studies do have an important role in the monitoring of pesticides. They are particularly useful in the assessment of more common, shorter term and less severe effects on health.

EPIDEMIOLOGY IN THE STUDY OF PESTICIDES

The study methods that have been described can be used to look for associations between suspected hazards and disease, and to estimate risk in relation to patterns of exposure. Human exposure to pesticides occurs in several contexts. The applicability of epidemiology to each of these is now discussed.

Exposure in manufacture

Studies of operatives exposed to products in their manufacture and formulation have been the main source of epidemiological data on pesticides to date. In particular, cohort studies have been used to explore the mortality and cancer incidence of pesticide producers in many countries. Manufacturing populations are well suited to this type of investigation in that companies often hold historical records of employment which enable large groups of exposed workers to be identified retrospectively. Interpretation of positive findings requires care if subjects have been exposed to a range of chemicals (as is usually the case), but clues may come from laboratory studies and from epidemiological investigations in other populations. For example, the International Agency for Research on Cancer is currently coordinating an international collaborative survey of workers exposed to phenoxy herbicides and chlorophenols (Johnson ES et al., in press). A total of 15 manufacturing populations is being studied, and while subjects in each individual population have worked with a variety of chemicals, these potentially confounding exposures are different in different factories. By analysing for each factory separately it should be possible to discriminate confounding effects. Multiple exposure is not a problem when no excess of disease is demonstrated. Any reassurance from the negative findings applies to all chemicals insofar as they have been encountered.

Cross sectional surveys of pesticide manufacturers have been carried out to look at less serious morbidity, but there is scope to use this approach more than at present. Examples of health effects which could be examined in this way include anaemia, skin irritation and disturbance of thyroid function. Studies may be particularly worthwhile when animal testing has suggested the possibility of a hazard, but estimates of risk are uncertain.

Occupational exposure during application

Exposure to pesticides in their use is often higher than during manufacture because chemical contact in the factory is more easily controlled by engineering and work practices. However, pesticide spraying tends to be carried out by small contractors and by self-employed farmers, and this reduces the opportunity for epidemiological studies, especially those which rely on

historical records of exposure. Such records are usually either non-existent, or held in such small numbers that to assemble a retrospective cohort of adequate size would be impractical. Where cohort studies of pesticide sprayers have been carried out, subjects have worked for the occasional larger employer. Again, the interpretation of findings is frequently complicated by the problem of multiple exposure, but this does not present an insuperable obstacle.

Because of the difficulty in finding suitable cohorts, the health effects of pesticide application have been examined more often by the case-control approach. Exposure to pesticides has usually been ascertained from memory, and this gives scope for error. In particular, spurious associations may arise if cases are highly motivated to find out why they became ill, and therefore recall their past exposure more completely than controls. To reduce the likelihood of such bias, controls may be selected from patients with other diseases who also have reason to explore their past carefully. The possibility of error is also lessened if the analysis is based on broad classes of compounds (eg insecticides) rather than specific products which are harder to remember. In one study carried out in a farming community in the mid-west of the United States reported exposures were verified for a sample of subjects by reference to their suppliers' records (Hoar et al., 1986), but usually such objective corroboration is not feasible.

The constraints imposed by the decentralisation of spraying operations are less in relation to cross-sectional studies of more minor, short-term morbidity. These do not usually require such large numbers of subjects, and as in the field of pesticide manufacture, are currently an under-used resource.

Non-occupational exposure during application

Complaints from members of the public that they have become ill through contact with recently sprayed crops or spray drift are not uncommon. However, the epidemiological investigation of such complaints is fraught with difficulty. Often the alleged health effects cannot be measured objectively (eg headache, nausea), and the possibility of biased reporting cannot be adequately assessed. Moreover, direct pharmacological effects cannot be distinguished from psychogenic responses. If objectively measurable

disease does occur, it should normally be more readily apparent in spray operators because their exposure is generally higher. However, the possibility of unusual sensitivity in special groups such as children and pregnant mothers may need to be considered.

Domestic use of pesticides has been examined in some case-control studies, and again perhaps the greatest interest is in the risk to children and other potentially vulnerable groups who are not included in occupational investigations.

Dietary exposure

The other major source of human exposure to pesticides is contaminated food. Pesticide residues in food are tightly controlled, and individual exposure by this route is therefore much lower than during manufacture and application. However, many more people are affected. Unfortunately, the opportunity for epidemiological investigation of dietary pesticides is minimal because it is virtually impossible to measure long-term exposure. Some assessment might be possible if use of a product were restricted to a few unusual foods that could be used as markers of intake, or if a bioassay were available for the pesticide or one of its metabolites. In most cases, however, decisions about the safety of food residues must rest on extrapolation from laboratory data and from the effects of human exposure in other circumstances.

FUTURE DEVELOPMENT

Epidemiology should not be viewed as an alternative to toxicity testing in the laboratory which will remain the mainstay of risk assessment in pesticide regulation. Rather, it augments the experimental data base and provides an important check on the validity of extrapolations from animals to man. Opportunities for pesticide epidemiology will increase in the future, particularly in relation to occupational exposure. Greater emphasis on industrial hygiene monitoring and better record keeping will make it easier to identify and characterise populations for study of both short and long-term health outcomes. In Britain, the recently enacted Control of Substances Hazardous to Health (COSHH) regulations require employers to retain records of workers exposed to more hazardous substances for a minimum of 30 years.

It is important that these opportunities be exploited. A public increasingly concerned about risks to health from environmental pollution and contamination of food is unlikely to be reassured by laboratory findings alone. Regulatory authorities should look more often for epidemiological data to support toxicity testing, particularly in the review of older products.

REFERENCES

Hoar, S.K.; Blair, A.; Holmes, F.F.; Boysen, C.D.; Robel, R.J.; Hoover, R.; Fraumeni, J.F. (1986) Agricultural herbicide use and risk of lymphoma and soft tissue sarcoma. *Journal of the American Medical Association*, **256**, 1141-1147.

International Agency for Research on Cancer (1980) Arsenic and arsenic compounds. In: *Monographs on the evaluation of the carcinogenic risk of chemicals to humans. Volume 23. Some metals and metallic compounds*, Lyon : IARC, pp 39-141.

Johnson, E.S.; et al. (in press)Phenoxy acid herbicides and contaminants : Description of the IARC international register of workers. *American Journal of Industrial Medicine*.