THE RELEVANCE OF PESTICIDE METABOLISM

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Summary

Pesticides can degrade in plants and soils by metabolic (enzyme-mediated) or chemical (spontaneous, non-enzyme catalysed) reactions. Such reactions lead to the formation of new molecules with different chemical, physical and biological properties.

Studies of pesticide metabolism provide a basis for the residue analyst to estimate the residues likely to arise in practice. This estimate is in turn needed by the toxicologist to plan his investigations. Accordingly the metabolic chemist must maintain close contact with the residue analyst and the toxicologist to ensure that the best decisions are made on:

a) How far to pursue metabolite identification in laboratory studies

b) How do design follow-up field studies

c) How to deal with firmly bound, unextractable residues

d) Which metabolites require additional toxicological study.

Some criteria for these are discussed.

When a pesticide is applied to a plant or to a soil it can disappear by a variety of processes including evaporation, leaching, chemical decomposition and metabolism. The physical processes, evaporation and leaching, can be important but are outside the scope of the present discussion. The chemical and metabolic processes have a common feature in that they both result in the loss of the pesticide by its transformation into other compounds which have different chemical, physical and biological properties from those of the compound originally applied.

Metabolic reactions can be defined as reactions that are mediated by living organisms and catalysed by enzymes. Chemical reactions, on the other hand, occur spontaneously or involve catalysts that are not enzymes. Metabolic processes usually predominate over chemical processes in transforming a pesticide in a plant or soil. However, in many studies of pesticide degradation it has not been proved satisfactorily whether the reaction is metabolic or chemical. Furthermore the definitions that are given above are debatable and alternatives are possible which make the distinction between chemical and metabolic processes less obvious. In spite of the title the present discussion will thus include both metabolic and chemical transformations of a pesticide that can occur in plants or soils. Particular and general aspects of these transformations will be considered and some attention will be paid to the relevance of these transformations to the residue analyst and to the toxicologist.

Nowadays metabolic studies are almost invariably pursued using radioisotope techniques. The parent pesticide, usually labelled with carbon 14, is applied to the crop or soil in the laboratory or glasshouse or sometimes under outdoor

conditions which attempt, usually unsuccessfully, to simulate normal agricultural conditions. The soils and plants are sampled at intervals of time. The radioactive components are extracted with suitable solvents and these components in the extracts are separated by a range of chromatographic techniques.

Typical preliminary results of such a study are shown in Figure 1 and this example will serve to illustrate many of the basic principles involved. Radiochemical techniques can be particularly sensitive and with greater diligence the list of unknown compounds could be extended considerably, down to Unidentified Z if one wishes to pursue components present in even smaller amounts than any of those listed.

The identification of these components involves a range of physical and chemical techniques. Such techniques showed (Figure 2) that in this case compound A was TALCORD itself. Compound B was the oxime formed by hydrolysis of TALCORD. The process could have been either chemical or biochemical. Compound C was the isomer of TALCORD. Its formation could have been photochemically induced but this was not proven. Compound D was the sulphoxide of TALCORD formed by oxidation and the process was probably biochemical. Compound E was also formed by oxidation, again probably biochemical, which occurred at the N-methyl group.

The fraction F was a complex mixture of polar products which were almost certainly sugar conjugates of the oxime B and the hydroxy methyl compound E. Some components remained unidentified but some were almost certainly naturally-occurring compounds, probably sugars, which contained one of the radioactive carbon atoms derived from the TALCORD. The identification of such components can be difficult and their formation is best avoided by labelling the carbon atoms in parts of the molecule that are not readily degraded to small fragments which can be readily assimilated by the plant. This is not always feasible, however, and indeed was not so with TALCORD.

In every study such as this, one is faced with the problem of how far one pursues the identification of minor components. The toxicologist cannot always decide this since he will prefer the component to be identified, synthesised and examined toxicologically before he can be certain of its relevance. There are as yet no widely accepted rules for deciding how far to pursue an investigation. Indeed at this state of knowledge there is no basis for formulating rules to cover every case. One recommendation is to pursue the identification of all metabolites that exceed 10% of the total residue. However, one would not go even this far when the total residue is below 0.1 ppm. On the other hand one might even pursue components that are present in amounts less than 10% level if the chemistry involved indicates the possibility of the formation of traces of compounds which are known to be toxic or which are related chemically to known toxicants.

Whilst the metabolism of TALCORD illustrates many of the basic reactions it is not feasible to illustrate all of the possible reactions using just one compound. Before we get any further involved with the data in Figure 2 we should divert our attention for a moment from this particular case and review briefly the full range of biochemical and chemical conversions that have been established for pesticides. The list is not an unduly long one (Figure 3). The reactions encountered most frequently are oxidation and hydrolysis.

Oxidation reactions (Figure 4) can occur at phosphorus, sulphur, carbon or nitrogen atoms. These reactions are usually enzyme-mediated and occur readily in plants. The reactions can occur in soils although some of them are slower, particularly the oxidation of carbon.

The conversion of P-S into P-O derivatives is an important reaction for many of the well known thiophosphate pesticides (parathion, malathion etc). The P-O derivative is usually the active insecticide and is a more potent inhibitor of cholinesterase. The P-O compound is usually of a higher order of acute toxicity than is the P-S derivative. However, the P-O compound is frequently more readily degraded than the P-S compound, indeed that is why the P-S compound is usually used in the first place. Furthermore, the long term effects of the ingestion of small amounts of a chemical, and not the acute effects are of more significance in assessing the hazard from a residue. At intake levels arising from crop residues the mammalian toxicity of the P(O) compounds is often similar to or less than that of the P(S) analogues.

Oxidation at a sulphur atom is well exemplified by TEMIK (Figure 5). The sulphoxides and sulphones that are formed have insecticidal properties, as has the parent compound, and they are inhibitors of cholinesterase.

Oxidation at a carbon atom (Figure 6) occurs in many forms and is the mechanism for the demethylation of N-methyl group as in BIDRIN and AZODRIN (Figure 7). It could also be an alternative mechanism to hydrolysis in some cases for the conversion of P-O-R into P-O-H (Figure 8).

Turning now to reduction reactions the microbiological conversion (Figure 9) of parathion into aminoparathion is the example most frequently quoted but it does not occur to any great extent in plants under field conditions. For a herbicide, trifluralin, the conversion of NO_2 into NH_2 has been shown to predominate in waterlogged soils when anaerobic conditions exist.

Hydrolysis reactions occur readily (Figure 10) and whilst in some cases they can be chemical and not biochemical reactions, they are usually catalysed by the esterases which can exist in plants and soils. Hydrolysis of CN to $CONH_2$ occurs readily in soils, is enzyme-mediated, but is a reaction that does not occur readily with relevant pesticides in plants.

In the time available isomerisation reactions will not be discussed further except to say that such reactions can be enzyme-mediated but on plant surfaces at least are most likely to be photochemically induced.

The oxidation of the pesticide to a hydroxylated derivative is frequently followed in plants by a conjugation reaction with a sugar and this is an enzyme-mediated reaction (Figure 11). This reaction can occur with derivatives other than hydroxy-compounds and with molecules other than sugars. In many metabolic studies the sugar involved has been identified as glucose but the evidence for this is usually inadequate. In reality, a wider range of sugars are almost certainly involved (Kuhr and Casida, 1967; Beynon and Wright, 1969).

In animals sugar conjugates are normally excreted. In plants the sugar conjugates may possibly be hydrolysed eventually but may also remain unchanged at harvest or can react with, and become incorporated into, plant polysaccharides and lignin etc. (Stewart, 1960).

After the rapid survey of the general, it would be useful to return to the particular example discussed previously (Figure 2) and to make a few additional points concerning the relevance of the metabolism study and the metabolic process.

The first additional point is that one must not rely too much on the quantitative aspects of the data generated by metabolism studies of the sort shown. The procedure gives a good indication of the metabolic pathway but the residues obtained are usually much higher than those resulting from normal agricultural practice. Indeed one cannot always guarantee that even the relative values obtained in

metabolism studies reflect field conditions. Each of the various factors causing depletion (evaporation, leaching, chemical and biochemical degradation) affect each metabolite in a different way and field conditions can only rarely be simulated in small scale radiochemical experiments. Valid quantitative data on the amounts of the pesticide and metabolites can only be obtained by analysing field samples treated in accordance with good agricultural practice and in accordance with the recommended conditions for the use of the pesticide.

However, do we need to analyse field samples for every metabolite that has been identified in the laboratory studies? The decision as to which metabolites to analyse for will be based on a combination of factors including:-

(i) The relative amount found in the laboratory studies - bearing in mind that this need not always reflect the field situation.

(ii) Known toxicological data and mammalian biochemistry of compounds of related structure.

It is evident that these are not clear-cut criteria and the combined judgement of the residue analyst, the toxicologist and the metabolic chemist is needed in reaching a decision.

Having analysed field crops for the identified metabolites, long-term toxicity studies (3 month rat feeding studies and possibly 2 year studies) will normally take place with any metabolite that is detected on field crops in significant amounts. However, this is not normally considered necessary if the metabolite found in plants is also a metabolite of the pesticide in animals. In those cases where the metabolite in plants and animals is the same, toxicity data on the parent compound are normally considered to be sufficient to cover the toxicity of the metabolite as well.

Finally I would like to say a few words about the relevance of those metabolites that cannot be extracted and are thus unidentified. As far as possible of course one keeps trying to extract, using more extreme conditions, and to identify, but this is not always possible and usually some small amount of radioactivity will always remain unextracted.

In plants some radioactivity can be bound firmly as a result of reactions of the pesticide or of its metabolites with macromolecules such as proteins and polysaccharides, including lignin. In soils the pesticide and its metabolites can react with humic acids and become firmly bound.

It is not realistic to attempt the complete identification of such bound materials. However, they cannot be ignored completely. The debate as to what to do continues but at the moment reasonable advice is to consider whether such materials are bio-available or not. One way of doing this with plants is to treat them with the ^{14}C-pesticide and to feed the unextracted residues to animals. Still and Mansager (1975) have done this with the unextracted residues from the treatment of the herbicide isopropyl carbanilate, and have found that the radioactivity in the plant material was efficiently eliminated by rats. It was thus not bio-available and was probably of no further concern.

For soils it is desirable to examine the bio-availability of the bound residues by demonstrating whether the residues can be taken up by plants subsequently grown in the soil. It is also desirable to evaluate the possibility of the eventual degradation of these bound residues in soil. It will usually be a slow degradation but accelerated tests, such as soil perfusion, can be used.

During this brief survey I have attempted to summarise the chemical and biochemical reactions that change a pesticide following its application to a plant or to a soil. Sometimes these reactions increase the insecticidal and mammalian toxicity temporarily but even such changes are followed by detoxification processes. I have attempted to discuss how far we should pursue the identification of metabolites and the problem of residues that become firmly bound in plants and soils. I hope that I have made it sufficiently evident that the metabolic chemist, the residue analyst and the toxicologist cannot work in isolation from each other and that their combined judgement is needed to produce data on pesticide metabolites that are relevant.

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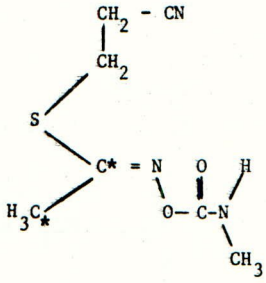
Figure 1 The metabolism of the insecticide TALCORD on apples and cotton (Beynon et al)



*Radiolabelled

Compound	ppm (as TALCORD equivalents)	
	Apples at 24 days from treatment indoors at ~ 40 ppm	Cotton leaves at 95 days from treatment indoors at ~ 50 ppm
Unidentified A	31	0.8
Unidentified B	0.07	-
Unidentified C	0.07	-
Unidentified D	0.1	-
Unidentified E	-	0.4
Unidentified F	-	11
Total of other unidentified components	0.34(a)	3.1(b)
Unextracted residue	0.40(c)	3.7(d)
Total	32	19

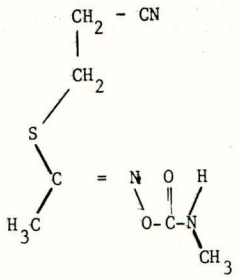
(a) This includes at least 5 compounds with the two majors present at 0.17 and 0.14 ppm

(b) This includes at least 3 compounds with the major present at 2.5 ppm

(c) After extraction with acetone followed by 50% v/v water in methanol

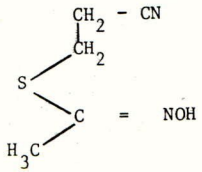
(d) After extraction with 20% v/v aqueous methanol.

Figure 2 The metabolism of the insecticide TALCORD on apples and cotton (Beynon et al)

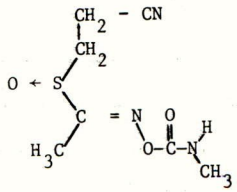


Compound	ppm (as TALCORD equivalents)	
	Apples at 24 days from treatment indoors at ~ 40 ppm	Cotton leaves at 95 days from treatment indoors at ~ 50 ppm
A TALCORD	31	0.8
B Oxime	0.07	-
C Anti-isomer	0.07	-
D Sulphoxide	0.1	-
E Methylol	-	0.4
F Sugar conjugates of A and E	-	11
Unidentified components	0.34	3.1
Unextracted residue	0.40	3.7

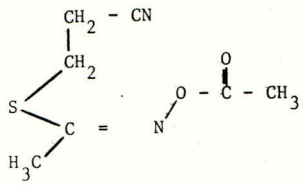
(B)



(D)



(C)



(E)

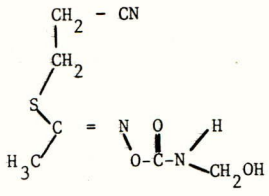


Figure 3 Chemical and biochemical conversions of pesticides in plants or soils

OXIDATION

REDUCTION

DEHYDROHALOGENATION

HYDROLYSIS

ISOMERISATION

CONJUGATION

Figure 4 Oxidation reactions of pesticides in plants and soils

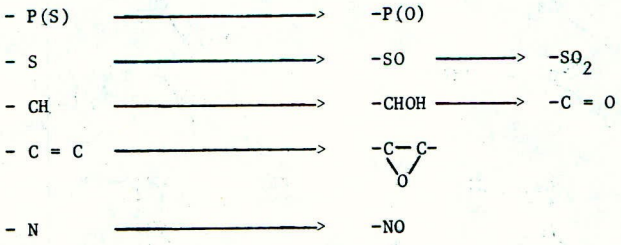


Figure 5 Degradation pathway of TEMIK in cotton

(Probst et al, 1967)

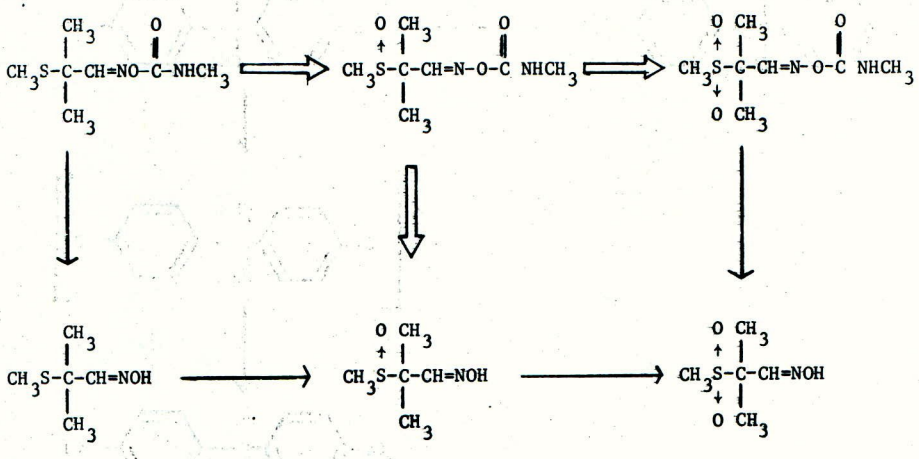
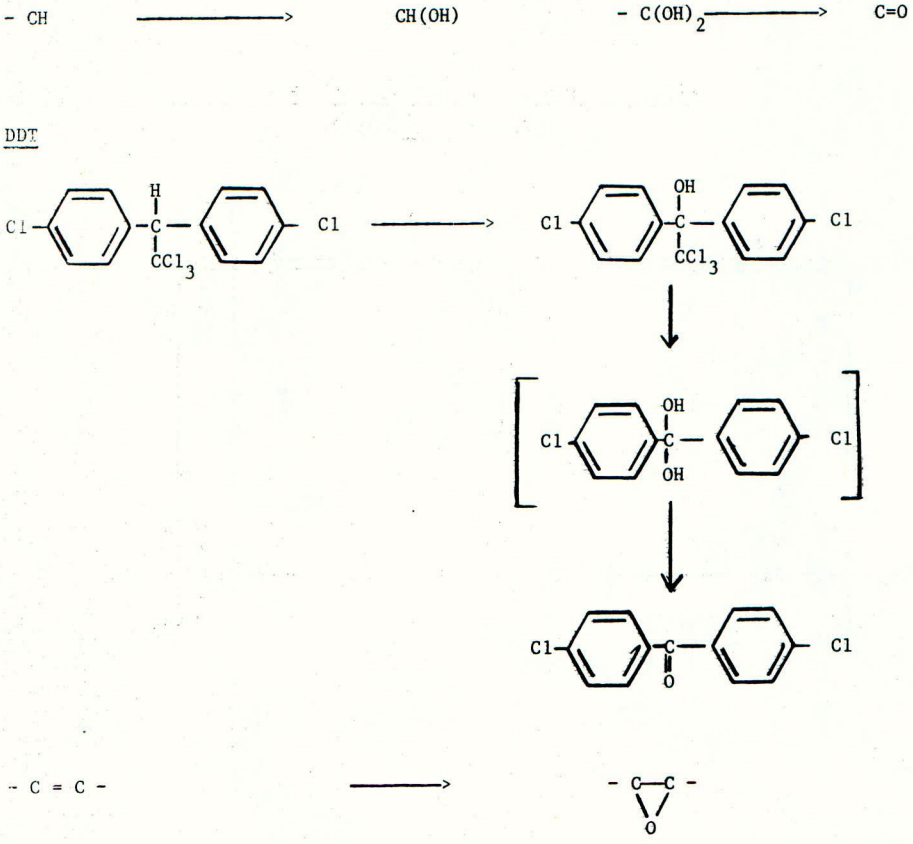


Figure 6 Metabolic oxidation at carbon atoms



Aldrin ——> dieldrin

Heptachlor ——> heptachlor epoxide

Figure 7 Oxidative dealkylation reactions of BIDRIN

(Bull and Lindquist 1964; Menzer and Casida 1965)

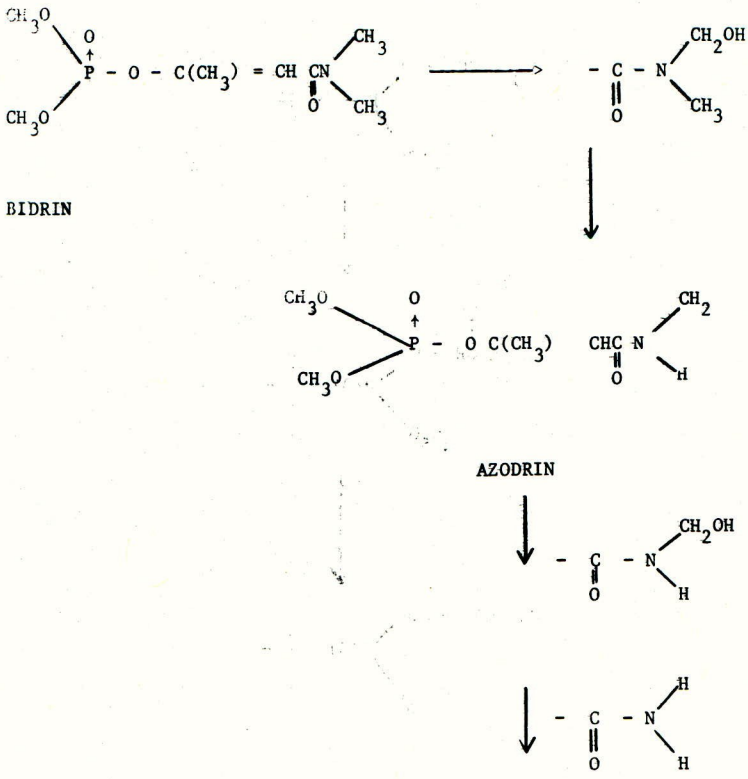


Figure 8 Oxidative de-esterification

(Donninger et al, 1972)

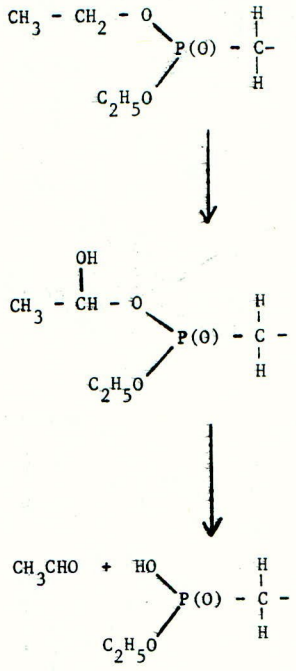


Figure 9 Reduction of parathion

(Miyamoto et al, 1966)

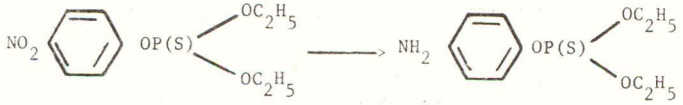
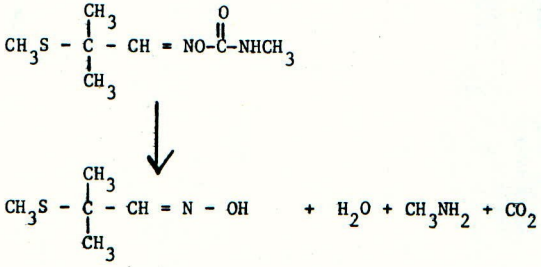
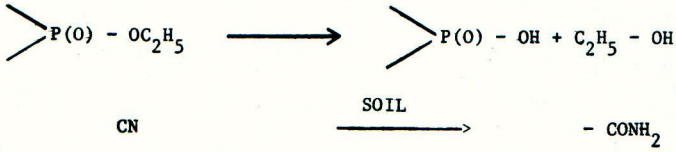


Figure 10 Hydrolytic reactions

TEMIK (Metcalf et al)





TALCORD (Beynon et al)

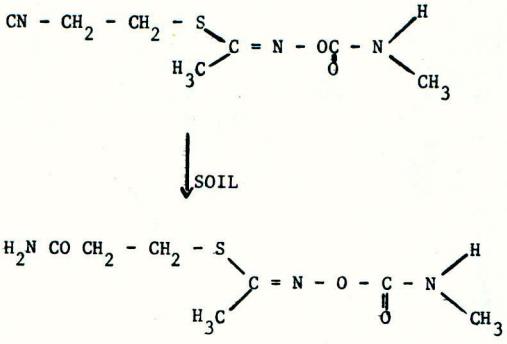
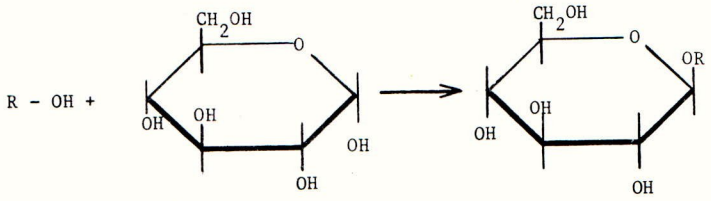


Figure 11 Conjugation reactions



R - N + Sugar ——> R - N - Sugar

R - Cl + Glutathione ——> R - glutathione

NOTES

Proceedings 8th British Insecticide and Fungicide Conference (1975)

RESIDUES - THE DETERMINATION OF NOTHING IN EVERYTHING

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Summary The determination of the behaviour and distribution of pesticide residues, including breakdown products, is dependent upon the ability of the residue analyst to detect and accurately determine minute amounts of these chemicals. The routine determination of pesticides at ng (10^{-9}g) and pg (10^{-12}g) levels in field samples is a result of the discovery and development of extremely sensitive detection systems. In particular, the Electron-Capture, Alkali Thermionic, Flame Photometric, and other selective detectors in combination with the efficient resolving power of gas-liquid chromatography (GLC), have been of prime importance.

Some recent developments in residue analysis are discussed including the newer techniques of Mass Fragmentography (GLC combined with Mass Spectrometry) and High Pressure Liquid Chromatography.

INTRODUCTION

For a pesticide to be acceptable it is as essential to prove that it is safe to use, as it is to show that control of the target pest is achieved. Regulatory authorities require proof that the pesticide usage will not leave harmful chemical residues in food for human and animal consumption; and that residues will not persist in and contaminate the environment or unduly affect non-target organisms.

Laboratory and small scale field studies, using compounds which contain a traceable radioactive atom, provide useful and essential information about the behaviour and the distribution of a pesticide and its breakdown products. Ultimately however, it is necessary to determine the levels of pesticide and its metabolites in field samples, which have been treated in accordance with the recommendations for use of the particular pesticide.

A prerequisite of such studies is the ability to detect and determine, with acceptable precision, the minute quantities of the pesticide and its breakdown products which may occur. Generally we wish to attain limits of detection of 0·1 - 0·01 ppm in most samples, and sometimes it is necessary to work at the parts per billion level as in milk and water analysis.

Fortunately, the residue analyst has been partner, during the past 20 years, to considerable advances in analytical instrumentation. This is particularly true in the combination of the technique of gas chromatography with selective detectors; thus the electron-capture and the phosphorus/nitrogen sensitive detectors are now routinely used for the detection of nanogram (10^{-9}g) and picogram (10^{-12}g) quantities of pesticide residues.

The main steps in residue analysis are: sampling (including storage), extraction, clean-up, and detection and determination. Each of these topics can be discussed at length but it is in the detection and determination step that probably the most

significant advances have occurred, and which is the main topic for consideration in this paper.

THE ELECTRON-CAPTURE DETECTOR

Lovelock and Lipsky (1960) described the extremely simple, electron-capture detector which is still the most widely used detector for the determination of chlorinated hydrocarbons. The ability to detect nanogram and picogram amounts of the chlorinated insecticides was revolutionary and allowed a more detailed understanding of their environmental behaviour. It also stimulated probably unjustifiable public concern over insignificant residues of these and other compounds.

Over the years, major developments have markedly improved several of the practical shortcomings of the electron-capture detector, particularly with respect to its limited linear range and operating temperature. The application of simple but effective electronics by Maggs et al (1971) has produced the so-called 'linear' or 'constant current' detector with a linear range of over four decades. This compares with a linear range of less than 100 in many conventional EC detectors. This facility offers several useful advantages to the analyst including:- reduction of errors which arise from co-extracted materials eluting with the pesticide and which move its response out of the linear calibration range; also the ability to analyse solutions of quite different concentrations without excessive re-calibrations. The increased linearity is particularly useful in automated analysis.

The most commonly used radioactive source in the ECD was the tritium foil, which imposed a temperature limitation because significant loss of tritium occurred above 200°C. Nowadays commercial detectors using Nickel-63 radioactive sources operate at temperatures of up to 350°C. This has extended the range of usable chromatographic conditions and can be useful in minimising detector contamination.

The extreme sensitivity of the electron-capture detector to substances with a strong affinity for electrons has been widely applied in the determination of residues of the chlorinated hydrocarbon insecticides. However the detector was of no value for the analysis of the new and important group of organophosphate and carbamate insecticides not containing halogen. Fortunately for the analyst there were corresponding developments in the selective detection of phosphorus and nitrogen.

ALKALI FLAME IONISATION DETECTORS

Karmen and Giuffrida (1964) showed that the introduction of a sodium salt into the flame of a flame ionisation detector produced a selective response to phosphorus compounds. These alkali flame (or thermionic) detectors, have found wide application for the detection of the organophosphate pesticides. The response of such detectors (particularly using Rubidium salts eg Rb SO4, Rb Br) to nitrogen-containing compounds is also well known, (Aue et al., 1967) together with the knowledge that nitrogen detection requires a much stricter control of various parameters such as detector temperature, the height of the alkali source above the flame, and particularly the rate of hydrogen flow to the flame. Undoubtedly the problems of limited stability and reliability which have been encountered by numerous workers have affected their acceptance for routine analysis of nitrogen compounds.

A most interesting recent advance in nitrogen detection has resulted from the use of an involatile alkali source. The latter, which is a rubidium silicate glass bead fused onto platinum wire, is mounted between the flame jet and the collector electrode of a normal flame-ionisation detector. The hydrogen flow to the detector is extremely low at 1 - 5 ml/minute. Under these conditions a normal flame cannot be produced and the silicate bead is electrically heated to red heat. A cool reducing flame plasma is produced, around the glowing bead, in which nitrogen-containing compounds are pyrolysed to stable intermediate cyanide radicals. The

detection mechanism proposed by Kolb and Bischoff (1974) suggests an interaction of the cyanide radicals with volatilised neutral rubidium atoms to form cyanide ions which migrate to the collector electrode. Compounds, which cannot form cyanide radicals, including ammonia, nitrogen oxide, amides and nitrate esters will not give a signal. The detector also responds to phosphorus compounds.

The major difficulties associated with nitrogen detection such as the need for critical control of hydrogen flow to the flame, mechanical adjustment of the alkali source etc. have been removed and the system is extremely stable and repeatable. It promises to be particularly useful for the routine analysis of nitrogen-containing pesticides.

Thus, some 10 years after the first introduction of the selective thermionic detector, the continuing evolution and development in detector design has produced a significant and needed advance in the field of nitrogen detection.

FLAME PHOTOMETRIC DETECTOR

Concurrent with the development of the thermionic detector was the introduction by Brody and Chaney (1966) of the outstandingly simple but extremely effective Flame Photometric Detector (FPD) for the detection of phosphorus and sulphur. In this device, the effluent from the gas chromatograph is mixed with air and oxygen and is burned in a hydrogen rich flame. Excited species which are formed in the flame (HPO species from phosphorus compounds and S_2 species from sulphur compounds) emit radiation which passes through selective filters (526 nm for phosphorus and 294 nm for sulphur) to a photomultiplier tube.

The detector is easy to use, stable, extremely selective and sensitive. Modifications include dual filter and photomultiplier assemblies for the simultaneous detection of phosphorus and sulphur (Bowman et al., 1968). Because the detector is so selective it has been found that many pesticides in many sample types can be analysed with little or no clean-up of the sample extracts - although clean-up is sometimes required merely to prevent deterioration in the performance of the GLC column.

From the above examples it can be seen that the residue analyst is equipped with quite effective devices for detecting volatile compounds containing halogens P, S and N. But these systems cannot deal with compounds which do not contain such atoms and although they can indicate the elemental composition of a molecule they cannot establish its structure.

MASS FRAGMENTOGRAPHY

The value of mass spectrometry for determining the structure of organic molecules is well recognised. In a conventional mass spectrometer the introduced sample is bombarded by a beam of high energy electrons (50 - 70 eV) and breaks up into a variety of fragements or ions. The pattern of fragmentation, which is recorded as the mass spectrum, is highly diagnostic and characteristic of the original molecular structure.

Gunther (1962) indicated the potential of mass spectrometry for evaluating metabolic pathways of pesticides. The combination of the efficient separatory powers of gas chromatography with the structural determinations of the spectrometer pointed to a very powerful analytical tool. This is utilised in the relatively new technique of mass fragmentography where the mass spectrometer is employed as a sensitive and highly specific detector. The effluent from the chromatograph can be monitored continuously for characteristic fragments - in current commercial instruments up to eight ions can be monitored simultaneously.

This ingenious combination of gas chromatography with mass spectrometry probably provides the residue chemist with the ultimate specific detector and considerably enhances his ability to detect and identify pesticide residues. However the technique is expensive and requires a fair degree of operational skill; hopefully its assured wider acceptance and application must promote positive developments in design to make it cheaper and easier to operate.

HIGH PRESSURE LIQUID CHROMATOGRAPHY

Let us now consider those chemicals which, because they are involatile or are thermally unstable, cannot be determined by gas-chromatography. This group contains many pesticide breakdown products and metabolites which are often extremely polar and water soluble chemicals; in fact it is an area which often presents severe problems to the analyst. Obviously there will continue to be determinations based upon the formation of suitable derivatives - which are themselves either volatile or whose colour or fluorescence can be measured instrumentally. However a much more useful approach to the analysis of such compounds is possible with the fairly recent development of the technique of high pressure liquid chromatography (HPLC).

The separation of compounds by chromatography, on columns or layers of solid adsorbents, dates back to 1905 with the separation of plant pigments by the Russian biologist Tswett. Analysts have found the technique indispensible in their work; in residue analysis it is of particular importance mainly as a means of separating co-extracted interfering components before the final determination of the pesticide. For many years it was known that the efficiency of separation of different columns improved as the particle size of the adsorbent diminished. But it was not until the late 1960's that a radical change in column chromatography occurred through the development and introduction of suitable small particles.

Modern High Pressure Liquid Chromatography uses small diameter columns (1 - 3 mm) filled with small particles of adsorbent (5 - 50 um diameter). The solvent is pumped through the columns at relatively high flow rates (1 - 5 ml/minute) at high pressure (up to 3000 atmos.) In such systems it is possible to achieve separation efficiencies and analysis times equivalent to those found in gas chromatography. The range of possible conditions for separation is much greater than for gas chromatography because of the positive effect of the solvent upon the chromatography. Also there is a much wider range of adsorbent and other particles (eg ion exchange, gel permeation etc) of different separation characteristics for the analyst to choose from.

The usefulness of HPLC to pesticide analysis, particularly for involatile or thermally unstable compounds was obvious. The first reported use was for the determination of Abate insecticide in water by Henry et al (1971) and since then numerous papers have been published (Moye, 1975). But the realisation of its full potential for residue analysis awaits the introduction of sensitive and selective detectors. Currently the most widely used detectors measure ultra violet absorption or refractive index, and although they are fairly sensitive they are not selective and do not cope adequately with the problems of background interference commonly encountered by the residue analyst. Here we see a very interesting example of a potentially powerful analytical technique waiting for the discovery of a suitable detection system - analogous with the situation in gas chromatography before the introduction of the selective electron-capture detector. Recent papers describing novel detectors indicate the awareness of this need - we look forward impatiently to our selective LC detectors.

Nowadays we accept casually the ability to detect and determine picograms (10^{-12}g) and even femtograms (10^{-15}g) of pesticides in a wide range of substrates. This routine determination of 'nothing in everything' has depended upon the discovery and application of ingenious detection systems; some of which I have described briefly in this paper.

The impressive advances in detection capability are unfortunately not matched in some other areas of residue analysis. The removal of interfering components from sample extracts at the 'clean-up' stage before determination is still based upon an essentially empirical approach. We need the discovery of more generally applicable clean-up systems, possibly combined with a greater knowledge of the identity of materials co-extracted from substrates by various extraction solvents. The increasing use of the combination of gas chromatography with mass spectrometry (GC-MS) indicates one way of acquiring structural information on compounds which interfere with residue determinations. More widely applicable automatic analysis systems, which would significantly increase the ability of the residue laboratory to efficiently cater for increasing demands, also depend markedly upon advances in the pre-detection analytical steps. To date, the development and introduction of novel pesticides has required the development and introduction of novel analysis and undoubtedly this will continue into the future.

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METABOLISM OF [6-^{14}C] CARBOXIN IN BEAN PLANTS

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Summary Steeping and root treating experiments were made with [6-^{14}C] Carboxin to study its uptake, translocation and decomposition in plant cultures Phaseolus vulgaris. The seeds were treated with the fungicide by wet and dry steeping, or via the roots in nutrient solutions of Knop. The experiments ran for various time periods. Analysis of the parts of the plants, i.e. leaves, stem and root showed the distribution and translocation of the decomposed systemic fungicide as a function of the cessation of treatment time/CTT/.

Résumé Pour étudier l'absorption, la translocation et le métabolisme de [6-^{14}C] Carboxin dans la plante Phaseolus vulgaris, nous avons effectué des essais par macération et avec une solution nutritive. On a soumis les grains à une macération humide, les autres fois nous avons étudié l'absorption grâce à un traitement de solution nutritive Knop. Au cours des expériences cinétiques nous avons examiné la distribution et la translocation - dans les différentes parties feuille, tige, racine de la plante - du fongicide et des métabolites en fonction du temps.

INTRODUCTION

Since Carboxin has been reported by Schmeling and Kulka (1966) various isomers of the oxathiin have recently been shown to have a broader spectrum of fungistatic properties. To fully understand the mode of action and specificity of a systemic biocide, it is necessary to know how fast such materials decompose and move in the plant organ and are built into the plant material. Snel and Edgington (1970) studied the metabolism of Carboxin labelled with ^{14}C in the anilino moiety. Wei-Tsung Chin et al. examined its degradation in water and soil (1970a) and its metabolism by Barley and Wheat plants (1970b) using Carboxin labelled with ^{14}C in both aniline and hetero moieties.

The aim of our experiment was to study the metabolism of Carboxin in shorter time periods and hopefully separate the formation and translocation of its metabolites; also to obtain information about the kinetics of its uptake and the translocation of metabolites in the bean plant.

METHOD AND MATERIALS

Carboxin (5,6-dihydro-2-methyl-1,4-oxathiin-3-carboxanilide) was labelled with ^{14}C in the hetero moiety, i.e. in the carbon position 6. The labelled compound was supplied by the Isotope Institute of Hungarian Academy of Sciences. Purity of the labelled chemicals was ascertained by means of thin layer (TLC) and radiochromatography. (Solvent: 1. Benzene-methanol, 9:1: 2. chloroform.) The

specific activity of ^{14}C-Carboxin was 11,8 uCi/mg. Plant culture used was Phaseolus vulgaris.

The expected metabolites were prepared for identification and used for TLC. The fungicide was formulated with talcum powder to 47% w/w. After shaking the seeds for 2 hours with the fungicide, 0•7 mg Carboxin was taken up by 1 g bean seed, which was the approximate weight of one seed

In the case of wet steeping, the bean seeds were steeped for 24 hours in distilled water saturated with Carboxin. The concentration of this solution was 17 mg Carboxin/100 ml, according to radio-activity measurements. The steeped seeds were incubated in the greenhouse and after the necessary time period they were cut into root, stem and leaves. These were analysed separately and their acetone extractions checked for radioactivity and used for TLC.

To separate the metabolites Merck Kieselgel F 254 TLC sheets were used and to develop the spots use was made of UV light and Dragendorff solution. For the detection of radioactive spots on TLC plates Berthold radio-chromatogram scanner was employed.

To check the radioactivity Packard 3380 and Nuclear Chicago Mark II. liquid scintillation spectrometers were used. Quench corrections were made employing the channels ratio method.

For the time course experiments the 2 week old untreated seedlings were transferred into hydroponics containing 150 ml half strength Knop nutrient solution and labelled Carboxin of 0•00234 mg Carboxin/ml.

RESULTS

Dry steeping Fig. 1 shows the autoradiographic pictures of 14 day old seedlings. The time of exposure was: 2 days, with ORWO X-Ray Film. The pictures show that the radioactive fungicide was transferred with the xylem transport and accumulated in the marginal sites of the leaves. In the case of better developed plants (Fig. 2), during the same germinating time, i.e. 14 days, the autoradiograph shows that the stem and the veins of the leaves do not contain radioactive materials due to the lack of supply of the radioactive fungicide. In the pictures they appear white, and the black spots refer to the radioactivity.

Wet steeping The autoradiograph in (Fig. 3) shows that the 3 week old plants contain the radioactive fungicide or its metabolites mostly in the marginal sites and in the root. The root, stem and leaf parts of the plant were separately extracted with acetone. The distribution of the radioactivity in these acetone fractions were: 17•7% in the root, 4•7% in the stem and 77•5% in the leaves. According to the absolute activity this amounts to 3•4 ug Carboxin/plant.

Fig. 1 - 3. Autoradiographs of Phaseolus vulgaris seedlings after dry steeping (Fig. 1 - 2), and wet steeping (Fig. 3).

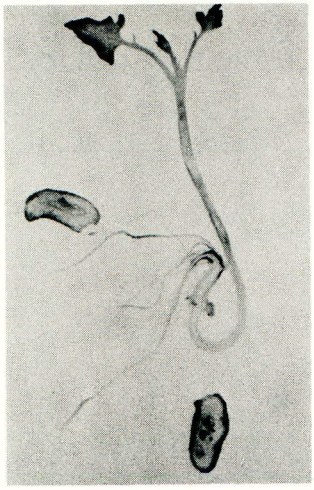


Fig. 1.

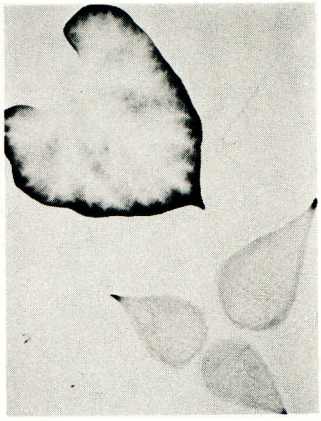


Fig. 2.

Fig. 3.

Time course experiments Table 1 presents the distribution of the radioactivity in the bean plants as a function of treatment time. The results are also presented graphically in Figure 4.

Table 1

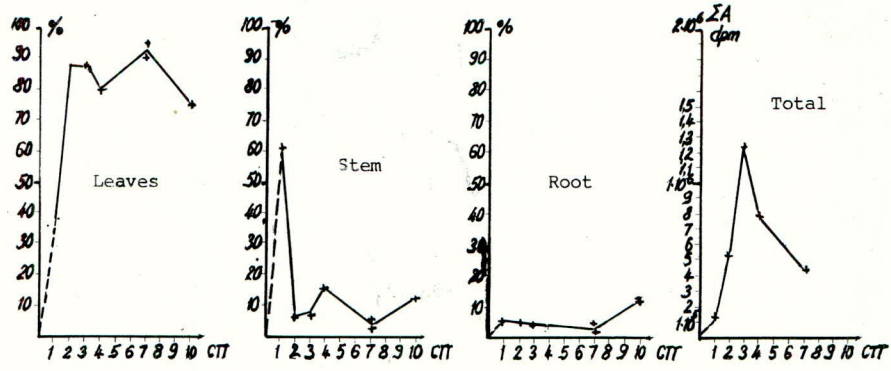
Distribution of [6-^{14}C]Carboxin and its metabolites in Phaseolus vulgaris as a function of root treatment time in nutrient solution of Knop

Treatment time Days	Relative activity %		
	Leaves	Stem	Root
1	35	60	5
2	88	7	5
3	88	8	4
4	80	15•5	4•5
5,5	75	13	12
7	90	5•5	4•5
7	90	3	2

Nutrient solution: Knop of half strength. Concentration of Carboxin: $2•34X10^{-3}$ mg/ml. Volume of nutrient soln: V = 150 ml.
Data of radioactivity: Specific activity: $2•78X10^{-2}$ uCi/ml.
Sum of activity: 4•169 uCi/150 ml.

Fig. 4

The relative distribution of the radioactivity in bean plants, in the acetone soluble fractions as a function of the root treatment, (cessation of treatment time = CTT, in days)

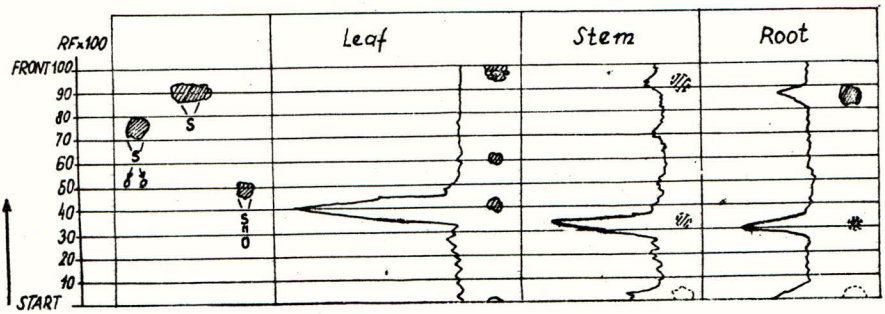


As it can be seen in Fig. 4 we have calculated the absolute activity of the acetone extractable parts of the plant, as ΣA, in dpm. It shows a decrease in function of the treatment time and from the third day it has a deficit in the activity. It may be caused by CO_2 loss by transpiration and/or radioactivity becoming bound in the plant in a form which cannot be extracted by acetone.

Metabolism To study the metabolism, root treatment experiments were carried out. Ten seedlings (2 week old) were treated hydroponically for 5•5 days in 150 ml half strength Knop nutrient solution. The roots, stems and leaves were ground separately with acetone and these solutions were used for TLC. The TLC spots were developed by UV light and also registered with a Berthold scanner. Dragendorff-reagent was also used. Fig. 5 presents the results. The radiochromatogram shows that there is a small amount of Carboxin in the root together with a large amount of the sulphoxide. In the stem there is no Carboxin with most of the radioactivity found as the sulphoxide. In the leaf fraction there is only one peak which belongs to the sulphoxide form. Comparing these results with the time course experiments, it can be explained why the leaf so quickly takes up the radioactivity. The root behaves as a pool for Carboxin which is able to supply the leaves with the fungicide through the stem by the apoplastic stream, but according to its "capacity" to take up the fungicide from the nutrient solution. Even though 10 plants were used to obtain a reasonable quantity of metabolites for the TLC, the sulphone could not be identified, although in the TLC of the stem fraction a small peak at the place of the sulphone can be seen.

Fig. 5

Results by TLC and radiochromatography scanning of acetone extracts of 2 weeks old seedlings.



CTT = 5•5 day, 10 plants processed
Carboxin applied:- ~ 23•16 uCi
TLC developing solvent:- Benzene/methanol (9:1 v/v)
Symbols:  Carboxin  Sulphoxide  Sulphone

During this series of experiments, following the acetone extraction, hydrochloric acid extracts were also obtained and tested. The yield of acetone extractable radioactivity was: Leaves: 97•7%; Stem: 95•5%; Root: 89•6%.

The radioactivity yield from IM HCl extraction following the acetone extraction was: Leaves: 0•4%; Stem: 8•0%; Root: 28•3%

DISCUSSION

The dry and wet steeping experiments show very quick movement of the fungicide with the apoplastic stream resulting in the marginal accumulation of activity as the sulphoxide metabolite of Carboxin. The root treatment time course experiments show that by the second day of the treatment in nutrient solution the leaves contain nearly at a constants level the ratio of the radioactive fungicide. The only form of Carboxin in the leaves is the sulphoxide. The root contains nearly at a constant level the radioactivity during the first 7 days. The form of the Carboxin in the root is sulphoxide of a higher ratio, but according to the TLC analysis, the form of Carboxin can be established, too, nearly in a ratio of 3:1. The TLC of the acetone soluble fraction of the stem shows that the predominant form is sulphoxide, a small peak may be taken as sulphone and the other one as Carboxin. In Fig. 4 it cannot be ignored that by the 10th day of the treatment of the root the radioactivity increases, which may be due to the lignin complex forming in accordance with the results of Wei (1973c).

The sum of the absolute activity of the whole plant decreases from the third day probably by the respiration of the plant leading to the activity loss in the form of CO_2.

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