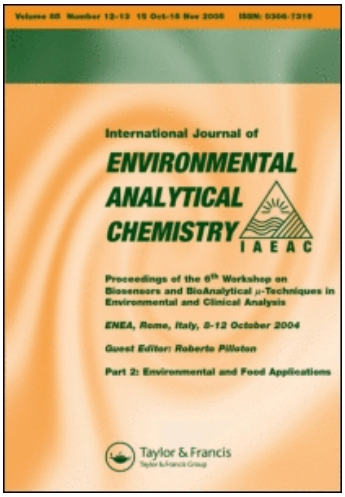

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Metabolism and the Pesticide Residue Analyst

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Metabolism and the Pesticide Residue Analyst†

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KEY WORDS: Significance of pesticide metabolites, pesticide metabolism, pesticide residue analysis, microsampling for infrared analysis.

Pesticide metabolism studies and residue analytical studies have some common objectives and some basically different ones. The metabolism chemist determines what metabolites are present in a biological environment after exposure to a pesticide but is not able to determine their concentrations under actual realistic field conditions. The residue analyst, after being informed what significant metabolites he must determine, then measures them after field exposure. This paper explores how a metabolism chemist achieves his identification and the significance of the metabolites he identifies.

It might be appropriate to look back briefly at the history of interest in residues of pesticides on food crops and in the environment. The first concerted effort to study and regulate pesticide residues on food crops came in the 1930s with the concern over residues of arsenic and lead which were used for control of codling moths on apples. This interest was not only for residues on the fruit, but there also was apprehension about the build-up of arsenic in the soil resulting from spraying of the trees. There was a natural spillover

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of interest from lead arsenate residues to those of other inorganic pesticides and of organic pesticides of natural origin. However, with the advent of synthetic organic pesticides, beginning with DDT during World War II, there has been an exponential increase in interest in residues on food crops, and we are still observing this rate of increase of interest. About the early 1960s there began a revival of interest of pesticides in the environment and this interest is now increasing logarithmically.

During the pre-war days rather crude analytical procedures were used, with titrimetric and color-comparison techniques predominating. With the influx of the synthetic organic pesticides, there was development of sophisticated colorimetric procedures, which incorporated not only sensitivity but also considerable specificity. However, these procedures lacked some versatility for regulatory screening purposes and, therefore, gave way to paper, thin-layer and gas chromatography.

All of this historical recitation involved mostly studies of the residual behavior of the parent pesticide on or in the biological environment of interest. Academic and regulatory interest in residual metabolites of these pesticides lagged behind that for the pesticide itself, due mostly to lack of adequate data and the technical difficulty in gathering these data. In the early days of DDT, there was not a complete lack of information or interest about the chemical alteration of the pesticide when exposed to the physical, chemical and biological forces found in plants and soils. In 1948 Gunther *et al.* reported on a study of the fate of DDT on citrus foliage.[1] Mature navel orange trees were sprayed with DDT and allowed to weather until chemical and biological assays showed that only 4–5% of the DDT remained. Microscopic evaluation of the foliage clearly indicated a white, crystalline deposit. After surface extraction of one hundred thousand leaves and subjecting the extract to classical column chromatography and to diagnostic chemical methods, they identified the compounds as shown in Figure 1. Thus, in 1948, Gunther had clearly indicated DDE as being the key metabolite in the metabolic scheme of DDT on citrus foliage.

For all practical purposes, the real beginning of pesticide metabolism studies dates back to the early 1950s when radiolabeled versions of the organophosphorus pesticides became available. Such workers as Casida and coworkers at the University of Wisconsin, O'Brien and Spencer at London, Ontario, Canada, Hartley and Heath of Pest Control Ltd. Laboratories, Cambridge, England, and Metcalf and March at the Citrus Experiment Station at Riverside, California, did pioneering work elucidating the metabolism of schradan, parathion and demeton. Since these early days, when the academic workers preempted this field, metabolism research has slowly shifted to the industrial laboratories, which now are performing by far the major portion of the work. Although the work in the early days was of

excellent quality, the sophisticated techniques now employed have resulted in a greater definition of the metabolic pathways of pesticides and is accomplished in shorter time.

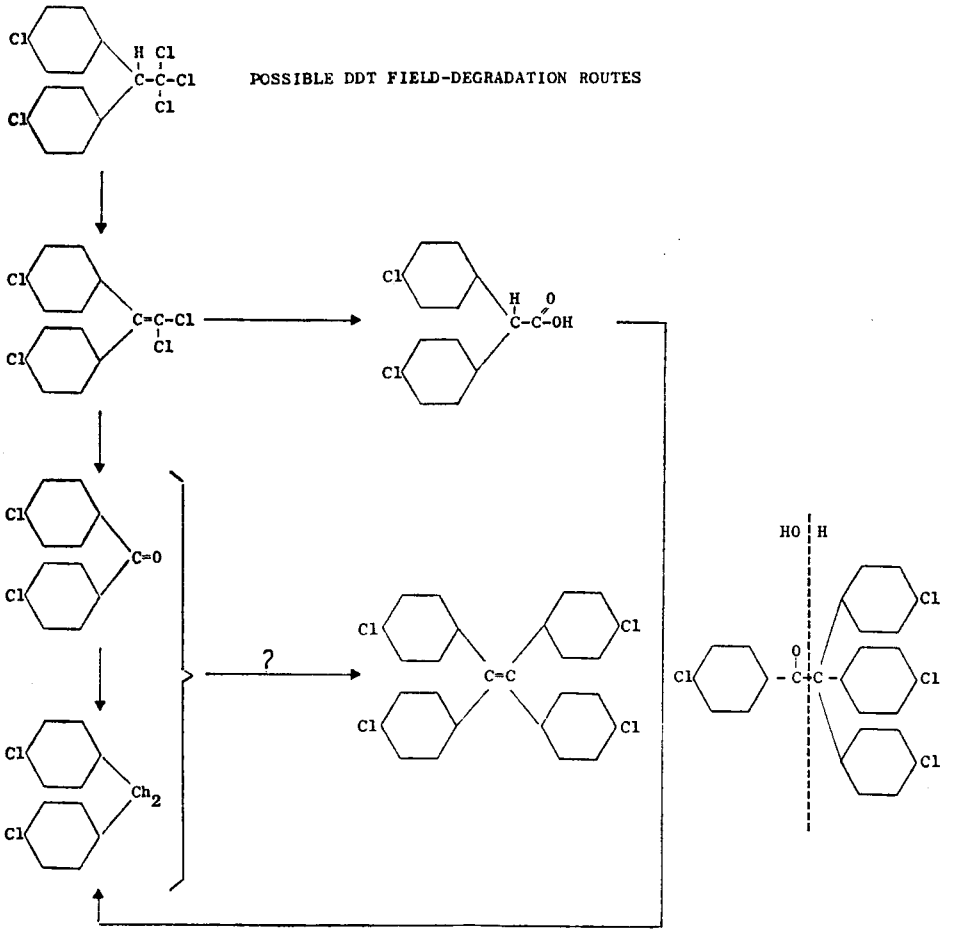

FIGURE 1 Suggested degradation routes for the major part of field decomposition of DDT on citrus foliage.[1]

METABOLISM STUDIES REQUIRED FOR REGULATORY PURPOSES

The early workers in the pesticide metabolism field concentrated on studying the fate of insecticides in plants, animals and insects. Their objective was often to define the mode of action or the site of action of the toxicant or its effective

metabolite. Their findings of metabolic products with toxicities equal to or greater than the parent insecticides, such as paraoxon, heptachlor epoxide and demeton oxygen analog sulfone, resulted in a natural concern of the regulatory officials for the possible hazards presented by these metabolites. This concern has grown at an exponential rate over the last few years. At present in the United States, the Environmental Protection Agency (EPA) requires data defining the metabolic fate of a pesticide in animals, plants, soil and the environment.

For animal metabolism studies, if the pesticide or its plant residues are not expected to be ingested by food animals, studies with the rat as the test animal are usually sufficient to aid the toxicologists in their evaluation of the data. If the pesticide is to be used directly with food animals, the metabolic fate of the pesticide in these animals is required.

Plant metabolism studies must be conducted on all crop groupings for which a registration is desired. Thus, the metabolism chemist may be required to determine a pesticide's metabolic fate on a leafy vegetable such as lettuce; a deciduous fruit, such as apples; a citrus fruit such as lemons; a root vegetable, such as carrots; and so forth. I personally believe that a simple exposure of the radiolabeled pesticide to the plants of interest and two-dimensional TLC of the extract will suffice for most crop groupings; of course after, in most cases, a thorough study on one crop has been completed.

Recently, the United States EPA has issued a set of requirements, referred to by the name "PR 70–15", for data on environmental impact which must be satisfied before a registration for use of a pesticide can be granted. These requirements include several which can be considered as metabolism studies. The metabolic fate of the pesticide must be elucidated in soil, and in soil microorganisms. In addition, the photolytic fate must be determined at the soil-atmosphere interface. The soil microorganism and the photolysis studies are, in my opinion, of justifiable academic interest; however, I personally do not believe that these studies add any additional relevant information to the soil metabolism study that will aid the regulatory officials in evaluating the safety of the use of the pesticide or how to minimize hazards, if any. After all, the soil metabolism study, if performed properly, will define the metabolites and/or degradation products, no matter what forces are responsible for their presence.

In addition, there has just recently been voiced a regulatory concern for the fate of pesticides in the aquatic environment, including the anaerobic soil metabolism of a pesticide, its fate in fish, and its aqueous photolysis.

Actually, all of these environmental studies now are more expensive and consume more of the scientist's time than the total for all of the more conventional animal and plant metabolism studies which, until recently, were all that were required.

RATIONALE FOR METABOLISM STUDY VERSUS ANALYTICAL RESIDUE STUDY

At this point, I think it is important to differentiate the rationale for doing a metabolism study from that of a residue study. There is a significant difference in the objectives, and in how each study is performed. A metabolism study is designed to elucidate the qualitative nature of the total residue resulting from the pesticide on and in the crop of interest, in appropriate animals, and in the environment. The metabolism study and the ensuing toxicological studies on the metabolites determine the compounds to be analyzed in the residue assays which follow. It is not designed to determine the quantitative relationships of these metabolites under field conditions. The fact that the use of radiotracers requires small and confined plots and extremely careful control of the radioisotope makes realistic field conditions virtually impossible. Therefore, any quantitative relevance placed on a metabolism study is fallacious. The residue study, however, is designed to determine the quantitative aspects of the residues of the parent and its *significant* metabolites under field conditions. The importance of the words "significant metabolites" as used here, will be explored in more depth later. Therefore, the precautions and techniques so essential for one study may well be of little importance for the other study.

METABOLISM STUDY

In order to achieve most easily a total accounting for a compound placed in a metabolizing environment as well as the metabolite complex resulting from the metabolic forces, a radioisotopic version of the compound should be used. Without achieving such a reasonably complete accounting, the possibility exists that an important portion of the metabolite complex is missing, which can affect any evaluation of the study. Traditionally, carbon^{14}, tritium, sulfur^{35}, phosphorus^{32} or chlorine^{36} have been used for pesticide studies; whichever radioisotope is employed it is important to use as high a specific activity of the radiolabeled compound as is feasible. High specific activity enables chromatographic evaluation of urine, bile, blood or an extract with very little or no clean-up. It is important to note that administration of *large* amounts of a low-specific activity compound can create artifacts.

Clean-up procedures can, on occasion, result in alterations of sensitive metabolites. If this artifactual alteration is not discovered, there can be a serious mis-evaluation of the metabolism picture.

In order to evaluate initially a metabolite complex in a tissue or excretory product, the various radiolabeled metabolites must be separated from each

other and detected. In our experience, two-dimensional TLC, followed by autoradiography has proven the most universally useful technique for this purpose. We usually use the commercially available silica-gel abrasion-resistant 8 × 8-in. thin-layer plates; autoradiography in our laboratory consists of exposure of Eastman Kodak Royal Pan photographic film to the thin-layer plate, followed by development in Baumann's diafine two-bath

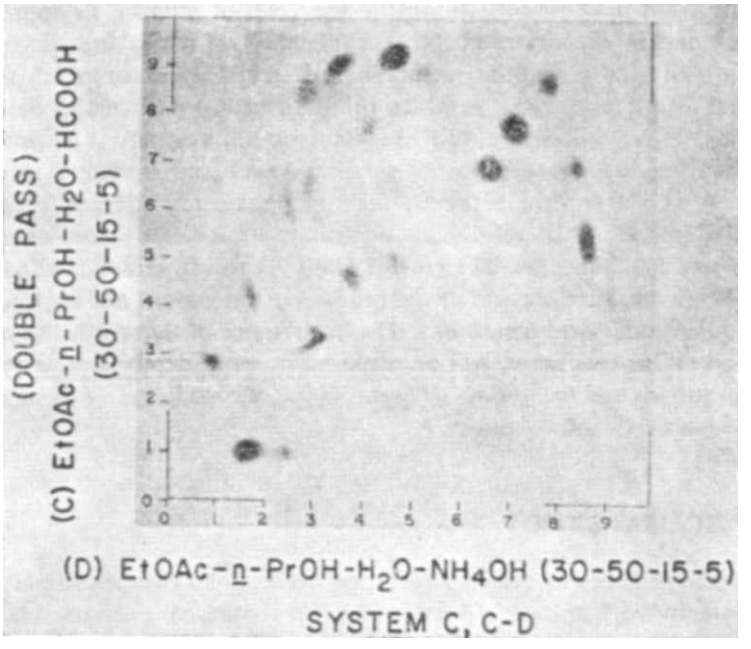

FIGURE 2 High-resolution two-dimensional thin-layer chromatogram of carbon-14 labeled urinary metabolites from rat treated with radiolabeled TRAMISOL® levamisole.[3]

developer.[2] Figure 2 shows an illustration of the definition of a metabolite complex using these described techniques. These are rat urinary metabolites of the anthelmintic agent, TRAMISOL® levamisole.[3] Of course, such a complicated picture is not usually encountered for pesticides, but when it does occur, the techniques described can best define the complexity of the problem.

APPROACHES FOR METABOLISM STUDY

When one is confronted with the prospect of conducting a metabolism study, with the attendant requirement of identifying the various metabolites, there

are two principal approaches. One approach consists of consulting the excellent review on pesticide metabolism compiled by Menzie,[4] the monumental book on "Detoxication Mechanisms" by Williams,[5] or other literature sources on the metabolism of foreign compounds. If this search of the literature reveals studies of compounds possessing structures similar to the compound under study, one can then postulate structures of probable metabolites. After these candidate materials have been synthesized, they can be compared with the actual radiometabolites by co-chromatography and isotope dilution to confirm or deny their presence. This approach certainly promises to achieve results quickly, particularly if the time required for synthesis is ignored. That is true if one has postulated correctly and if the postulated compound proves easy to synthesize. If, however, the metabolizing organism has "thrown you a curve" and very few, if any, of the postulated metabolites are actually present, one must either make further postulations or try the second or "direct" approach.

The second approach involves the isolation and purification of the principal radiometabolites from the biological source for chemical and instrumental characterization, whether this source be animal excreta or tissues, plant parts, or soil or other environmental media. Such characterization allows a more pertinent postulation of structure for ultimate synthesis and comparison. This latter approach is much more difficult and certainly more time-consuming, but it is the only alternative to the first approach if one has either guessed wrong or found no reasonable basis for postulation. After all, it has been my experience that the metabolizing organism can be "unreasonable". In many studies, both approaches are needed: the postulation approach to account for a portion of the radiometabolites and the isolation-and-characterization approach for the remainder. I will concern myself solely with the second approach, the isolation and characterization of the radiometabolite.

ISOLATION

Since the achievement of adequate isolation is the key to success in the second approach, it is appropriate to discuss a way to accomplish it. In our laboratory, the metabolite complex is first partitioned between a non-polar solvent and water; the non-polar solvent probably being chloroform or ethyl acetate. If possible, it is preferable then to convert as many of the water-soluble metabolites as possible to less-polar derivatives by chemical or enzymatic hydrolysis, treatment with diazomethane or acetic anhydride, or other appropriate reaction. They can then be partitioned into the non-polar solvent. The remaining polar, water-soluble metabolites can, unfortunately, then only be isolated by the use of various combinations of columnar gel filtration, ion exchange, adsorption and partition chromatography, by thin-layer and paper

chromatography, or by high-voltage electrophoresis. Frankly, there is no easy way of isolating polar metabolites, but the isolation of the chloroform or ethyl acetate-soluble metabolites is usually not so difficult. This is so because only small amounts are usually required for their adequate characterization, usually 50–100 mcg, and because requisite small amounts can be isolated by preparative TLC. For most isolations four or five sequential thin-layer separations are needed, using at least three different solvent systems so that co-chromatographing natural extractives with the derived metabolite in one system can be separated from the radioactive zone in another. Of course, occasionally radiometabolites, such as some phenols, may be unstable to TLC conditions; in these instances, column chromatography often offers a successful isolation procedure.

Once a metabolite has been adequately isolated, it must be characterized. I will discuss several of these techniques, but as an example will describe micro-infrared techniques in some detail.

INFRARED SPECTROPHOTOMETRY

One important function of infrared spectrophotometry is to monitor the purity of the isolated radiometabolite. After separation from all other radiometabolites is achieved, non-radioactive contamination originating in the sample, adsorbents, solvents, reagents, atmosphere, and the like must be removed before a meaningful instrumental characterization is possible. The infrared spectrum is a very good indicator of purity. When, after sequential isolations, there is no significant change in the infrared spectrum, the isolated radiometabolite can be assumed to be clean enough for further instrumental characterization.

The other function of infrared spectrophotometry in metabolism research is, of course, to characterize the isolated radiometabolite. The information obtained from an infrared spectrum can be sufficient in some cases to allow the structure of the radiometabolite to be logically deduced, or even identified. By use of comparative infrared spectroscopy of the isolated radiometabolite with a synthetic compound, confirmation of structure can sometimes be assured. Thus, infrared spectrophotometry can be a valuable aid to the metabolism chemist in assessing the purity of his isolated metabolite and in characterizing and identifying it.

Micro-sampling for infrared spectrometry

At this point, it is important to restate that mass isolation of radiometabolites in multi-milligram amounts is not usually necessary, especially if these metabolites are relatively non-polar. If amounts in the 50–100 mcg range can be

isolated, this is usually sufficient to achieve an adequate spectrum for structure evaluation, and it is in this range that microsampling techniques for infrared spectrophotometry are most valuable. The two microsampling techniques that have proven to be most useful are the potassium bromide micro-pellet technique and multiple internal reflectance spectroscopy. Both provide adequate sensitivity and are reliable for achieving a successful spectrum with minimum training.

Potassium bromide micro-pellet techniques

Of all of the micro-sampling techniques described for infrared spectrometry, the use of potassium bromide pellets of 1–2 mm in diameter seems to offer the best opportunity for placing the maximum number of molecules of the unknown in the usable energy beam of the spectrophotometer. Harrick[6] disputes this premise, claiming that "multiple internal reflectance is basically more sensitive than transmission for detection of minute samples". "The reasons for this," he states, "are that the coupling of the electromagnetic wave to a thin-film absorber is stronger for internal reflection, more efficient use of the sample can be made, and the signal can be enhanced by employing multiple reflection and multiple sampling". However, he admits that with present-day technology the micro-pellet technique offers the greatest sensitivity until further advances are made on multiple internal reflectance accessories. The key to sensitivity with the micro-pellet technique is the ability to transfer the maximum amount of compound to the minimum amount of potassium bromide to be pressed into the micro-pellet. Conventional mixing procedures using a mortar and pestle are subject to very large losses of compound to the surface area of the mixing vessel when only 10 mg or less of potassium bromide is used. One of my first attempts to resolve this problem was the procedure illustrated in Figure 3.

With this technique, powdered potassium bromide was packed tightly in the 1.5-mm orifice of the stainless steel disk. The sample in solution of a volatile solvent was added dropwise to the potassium bromide, allowing the solvent to evaporate between additions. After the additions are completed, the pellet was pressed in the conventional manner for infrared evaluation. This procedure theoretically offers complete transfer of the sample to the energy beam of the spectrophotometer. Practically, this usually does not happen since a portion of the solution is preferentially drawn from the potassium bromide due to the greater surface tension of the metal disk Often the losses are very great, but with due care and steady nerves, excellent sensitivity can be realized with this technique.

Another technique,[7] shown in Figure 4, consists of "dispensing" about 0.5 mcl of a chloroform solution of the sample to the tip of the syringe needle,

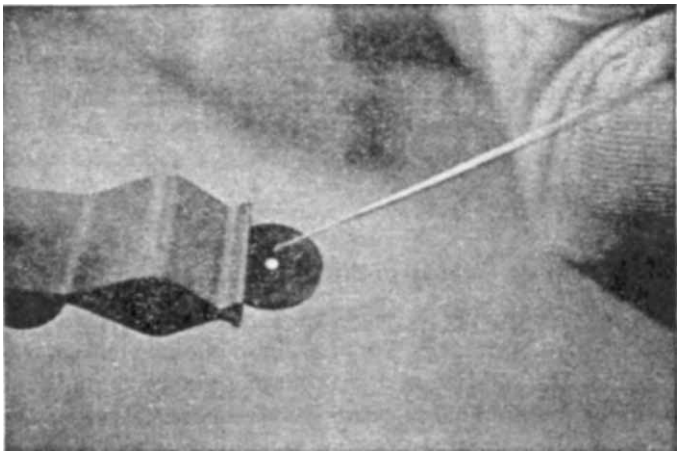

FIGURE 3 Application of solution of pesticide metabolite directly to potassium bromide in 1.5-mm orifice prior to pressing into disk.

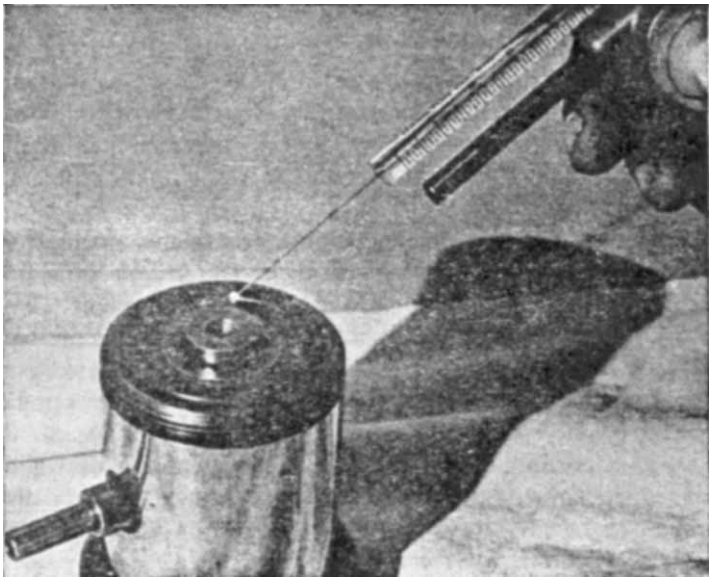

FIGURE 4 Application of solution of pesticide metabolite directly to powdered potassium bromide adhered to syringe needle.[7]

"picking-up" powdered potassium bromide by adhering action on the needle tip, dispensing another aliquot of the solution to the powder, and evaporating the chloroform. The "dispensing" of the solution is continued until the entire sample is transferred to the powder, which is then pressed into a micro-pellet. This procedure is theoretically sound but would seem to be nerve-wracking in operation. Actually, the greatest difficulty is in achieving the initial adhesion of the powdered potassium bromide to the syringe needle. Once adhered, the powder usually will stick to the needle tip until completely dry of solvent.

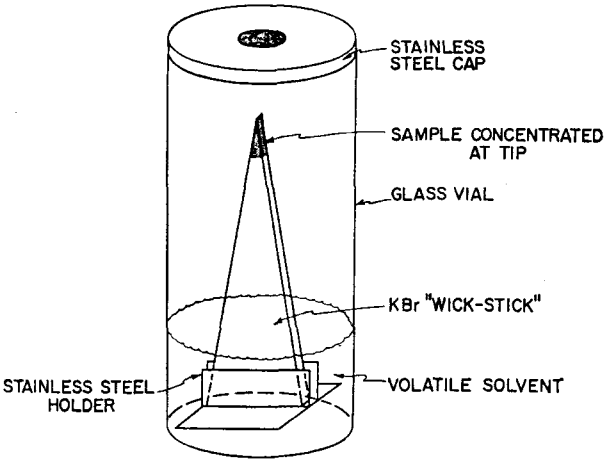

FIGURE 5 "Wick Stick" method for concentrating pesticide metabolite in tip of potassium bromide wedge.

What I believe is a better technique, shown in Figure 5, involves using the commercially available "Wick Stick". The sample is applied to the wedge of potassium bromide, which is then dipped at its base into a volatile solvent. The solution migrates up the wedge to the tip, where it evaporates, and the compound is concentrated at the tip. Just this tip is then cut or broken from the wedge and pressed into a micro-pellet. With proper care in using only the very tip of the wedge containing the sample, and in preparing the micro-pellet, I can recommend the use of "Wick Sticks" as a reliable and "fool-proof" manner in preparing micro potassium bromide pellets.

Several workers have proposed that the absolute lower limit of sensitivity is 10 ng of a compound with moderately strong absorptivity values. However, due to the difficulty in placing all, or most, of the sample in the usable energy beam of the spectrophotometer, an actual lower limit of sensitivity is about 0.1–0.5 mcg, i.e., about 100–500 ng. For most workers the lower limit will be 5–10 mcg. This last point must be emphasized—that one does not have to be a

professional infrared spectroscopist to attain success in the microgram range. Students who have never previously used an infrared spectrometer have achieved excellent spectra with 10 mcg or less of a pesticide after a few hours of instruction and practice. Thus, micro-techniques in the infrared *are usable* by the metabolism chemist.

The disadvantages of the micro-pellet technique are several. Of great importance to the metabolism worker is that the radiometabolite incorporated into the potassium bromide pellet can not feasibly be used for other instrumental evaluation. The pellet may be dissolved in water and the radiometabolite extracted into a solvent, but mechanical losses, introduction of interfering substances, and exposure to possible chemical alteration are probable. Another disadvantage is the difficulty in eliminating or minimizing contamination during preparation of the micro-pellet. Water in the potassium bromide is extremely difficult to eliminate and handling interferences do inevitably occur when working in the microgram range. Finally, losses of compound to the surface of any vessel used for grinding the potassium bromide sample mixture are inevitable and usually quite significant. Therefore, the micro-pellet technique is best used when the amount of sample is very limited and extreme sensitivity is required.

Multiple internal reflectance spectrometry

Figure 6 shows the principle by which infrared spectra are obtained by multiple internal reflectance. The infrared energy is directed into the entrance face of the infrared-transparent crystalline plate. This energy is reflected repeatedly from the inner faces of the top and bottom surfaces as it transverses the plate and then emerges through the exit face and is directed into the spectrophotometer. The sample is plated as a film on the top and bottom surfaces of the plate.

One of the most useful applications of multiple internal reflection spectrometry (variously referred to as ATR and FMIR) is in micro-sampling. It is a convenient and sensitive method for examining micro-samples that can be spread over a considerable area, such as the residue from evaporation of a solution or condensed vapor phase chromatographic fractions.

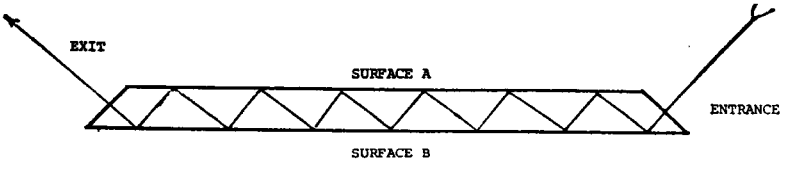

FIGURE 6 Principle by which infrared spectra are obtained by multiple internal reflectance A. Entrance face. B and C. Totally reflecting interfaces. D. Exit face.

Multiple internal reflectance spectroscopy depends for its existence on the very small penetration of a light wave beyond a totally internal reflecting interface. If a sample is placed in contact with this surface, the reflected beam is attenuated at the characteristic absorption frequencies of the sample. The actual depth of this penetration is difficult to specify since the intensity of the penetrated wave falls off exponentially with distance from the interface.

In Figure 7 we can picture the effective depth of penetration as being reflected at an imaginary surface, but parallel with the interface. This penetration is dependent upon the wavelength of the incident energy. For example,

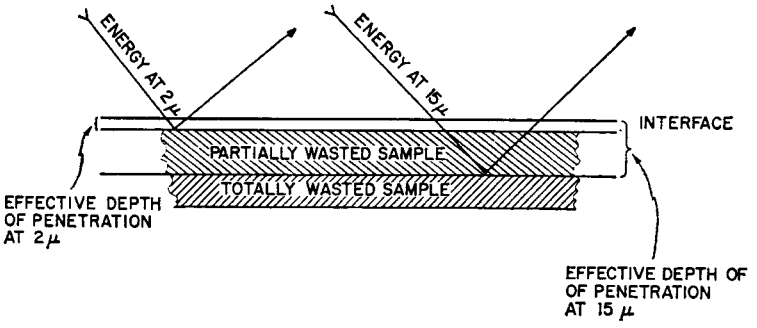

FIGURE 7 Effective penetration depth varies with wavelength.[8]

using an internal reflectance plate made from KRS–5, a thallous bromide-iodide mixture, with a 45° angle of incidence, the effective depth of penetration ranges from less than one micron at short wavelengths to several microns at longer wavelengths. If the sample is as thick as the penetration at the longer wavelengths, the internal reflectance spectrum will show enhancement of the low-frequency bands as compared to the transmittance spectrum.

What, then, are the ways to achieve as much sensitivity as possible in multiple internal reflectance spectrometry? First, the sample must be spread as a film on a reflectance plate so that it is at least as thin as the effective penetration depth at the shortest wavelength, thereby eliminating totally wasted sample. Second, the reflectance plate should be as thin as feasible and the angle of incidence as small as possible so as to maximize the number of reflections. Finally, the sample should be spread over only the effective sample area,[8] as shown in Figure 8.

When using a 50 × 20 × 1-mm plate, sensitivities of about 15–20 mcg can be routinely achieved with stronger absorbing compounds. Using a micro-version of this technique, with a 12.7 × 5 × 1-mm plate, sensitivities of about 3–5 mcg are achievable. The great advantage of multiple internal reflectance

is the ease of applying the sample to the surface of the reflectance plate. It is just streaked or dotted as a solution on to both surfaces and the solvent is allowed to evaporate. After the spectrum is recorded, the sample can be washed from the surfaces for any further evaluation desired. The only real difficulty with sample preparation is with those crystalline compounds where intimate contact with the surface is difficult to achieve and with those materials which react with the thallous bromide or iodide. Therefore, because of all of its advantages, I strongly recommend that the metabolism chemist use multiple internal reflectance spectroscopy for most of his infrared needs.

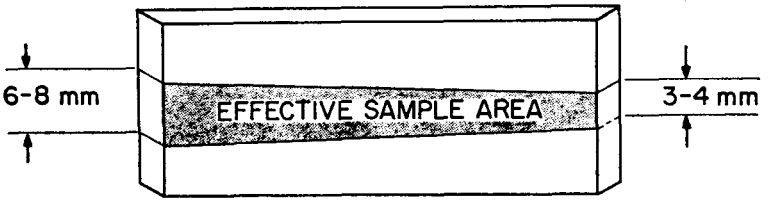

FIGURE 8 The light which reaches the spectrophotometer detector passes down the central part only of the multiple reflection plate.[8]

I want to emphasize that four factors are very important when using micro-techniques in infrared spectrophotometry. First, in order to gain sensitivity, an efficient transfer of the sample to the usable energy beam of the spectrophotometer must be achieved. Second, micro-techniques strictly limit the amount of allowable contamination from such sources as the sample, solvents, sorbents, reagents, atmosphere, handling and the like. Third, start the isolation procedure with sufficient sample so that there will be enough of the finally isolated metabolite to allow a spectrum. Remember that each handling step in the isolation procedures and the micro-infrared techniques results in some loss of the sought metabolite. Fourth and last, infrared spectrophotometry is only one tool used by the metabolism chemist and he cannot be expected to become as skilled as the professional spectroscopist, but he can achieve successful results in the 5–10 mcg range if he uses the simplest techniques commensurate with the objectives of the problem.

MASS SPECTROMETRY

It has been said that mass spectrometry can yield more information per microgram of compound than can be gained by any other instrument. Certainly this statement is very difficult to refute in the light of present-day

instrumentation. If determining the molecular ion were the only information that could be gained by the mass spectrometric examination of an unknown, this knowledge by itself could be immensely helpful in structure determination. The low-resolution instrument, such as is commonly used with the gas chromatograph-mass spectrometer combination, can give this molecular weight information as well as the fragmentation pattern. However, one of the more valuable uses of the low-resolution instrument is for the confirmation of identity of the isolated radiometabolite by comparison with a synthetic compound postulated to be the metabolite. Another valuable use is to monitor the isolated radiometabolite after each clean-up step, so as to assess purity and suitability for scrutiny by the high-resolution instrument. These two uses by themselves can fully justify the purchase of the low-resolution instrument.

The quadrupole mass spectrometer is a low-resolution instrument that has the added benefit of nanogram-range or lower sensitivity. Although I do not recommend conducting a metabolism study in the nanogram range, the sensitivity of the quadrupole instrument can adequately characterize a very small amount of an isolated metabolite. This could be extremely important when a larger supply of the isolate could be gained only through the excessive expenditure of valuable time and effort. It is sometimes possible to identify a metabolite from material scraped from one spot on a high-resolution, two-dimensional, thin-layer chromatogram. This use, too, can fully justify the purchase of a low-resolution quadrupole mass spectrometer.

High-resolution mass spectrometry offers a great deal more information which is very useful in the characterization of an isolated unknown radiometabolite. If high-resolution mass spectral photoplate data with computerized reduction of the data are available, the exact elemental composition of the parent ion and its fragments can often be determined and this information may well be the key to the unknown's identity. With ideal conditions of sample vapor pressure, stability, and purity, only micrograms or less of the sample are required for high-resolution mass spectrometry. With unknown compounds isolated from biological material, these ideal conditions cannot always be assured and the entire sample can be lost from further scrutiny without achieving any positive clues. It is for this reason that monitoring of the radiometabolite with a low-resolution instrument is so valuable. Also, it is important that data should be accumulated by all non-destructive means prior to mass spectrometry. Not only does this assure that complete loss of the metabolite in the mass spectrometer does not result in the loss of all possible clues as to identity, but it will also aid the mass spectrometrist in setting instrumental conditions and in interpreting his mass spectral data. Although clean-up conditions for mass spectrometry are quite rigid, use of deuterium isotope dilution can be useful in order to locate the mass peaks of interest among the multitude of extraneous ones.

Because of the excessively high cost of adequate instrumentation and the need for highly qualified personnel to interpret data, high-resolution mass spectrometry is not yet a practical tool for most working metabolism chemists. However, the coupling of low-resolution mass spectrometers with gas chromatography will help overcome many of these difficulties. In addition, heavily computerized MS–GC units can sometimes handle *mixtures* of metabolites.

NUCLEAR MAGNETIC RESONANCE SPECTROMETRY

Nuclear magnetic resonance spectrometry (n.m.r.) can offer much valuable information about the structure of an unknown compound. In fact, the chemists having the responsibility of the synthesis of organic compounds find n.m.r. invaluable in confirming the structures of their products. This same type of information is also extremely helpful to the metabolism chemist in solving his problems. Unfortunately, n.m.r. requires relatively larger amounts of sample when viewed in the terms of a metabolism study. Also, the sample must be reasonably pure so that the resulting spectrum will be meaningful. With present instrumentation without accessories, sample sizes of 100 mg or so are desirable. With microcells and suitable techniques, one can obtain reasonably good spectra on about 200 mcg. In order to get into the low-microgram range, use of computer data smoothing is required. The relatively new Fourier transform instruments may prove to be a further improvement. Because of these severe sample size requirements, n.m.r. is only occasionally an integral part of the metabolism chemist's arsenal of identification weapons.

DIAGNOSTIC TESTS

One means of characterizing an isolated radiometabolite which is available to metabolism chemists, no matter how tight his budget and how modestly equipped is his laboratory, is, of course, the use of diagnostic chemical reactions. Derivatization techniques have been developed which are particularly useful for gas chromatography. These techniques are valuable in characterizing an unknown metabolite found as a peak on a gas chromatogram. When dealing with a radiometabolite, the effects of these same tests can be very easily observed and evaluated using two-dimensional TLC followed by autoradiography. In addition, many other diagnostic chemical reactions, such as acid, base, and enzymatic hydrolysis, oxidation, reduction, esterification, and halogen addition can be conducted in the submicrogram range. The information gained can be used in conjunction with instrumental data in aiding the metabolism chemist in arriving at an identification. These diagnostic tests

alone do not usually result in an identification, but the information gained with little expenditure of cost or effort is quite valuable.

RECYCLING EXPERIMENTS

Similar in scope and objective to the diagnostic tests are recycling experiments, i.e., the subjecting of a radiometabolite to the same metabolizing forces that were used with the parent radiolabeled compound. Recycling requires a greater amount of isolated radiometabolite than is needed for diagnostic tests and the information gained is not as comprehensive. However, recycling can be useful in elucidating the routes of metabolism. In addition, there can sometimes be an increased concentration of a secondary metabolite, resulting from one of the primary metabolites, and this can ease the job of its isolation.

ISOTOPE DILUTION CONFIRMATION OF STRUCTURE

After utilizing some or all of the characterization procedures described, hopefully a structure of the radiometabolite can be postulated with considerable confidence. When this compound is synthesized by an unequivocal route, it can be compared to the isolated radiometabolite by any of the instrumental or diagnostic tests, and a tentative identification can be realized. In order to remove doubt as to the validity of this identification, confirmation by isotope dilution is recommended. This is accomplished by adding the synthetic material to either the isolated radiometabolite, the original biological sample, or the extract of the biological sample. This mixture is then subjected to a series of isolation procedures until a constant specific radioactivity is achieved. If the synthetic material is crystalline, then simple recrystallizations are usually all that is required as the isolation procedure. Or, if desirable, the compound can be chemically derivatized into a solid compound for recrystallization. Whichever segregation method is used, achieving a valid constant specific radioactivity is very good evidence that your identification is correct.

TOXICITY EVALUATION OF METABOLITES

Once positive identification of a pesticide metabolite has been achieved, an evaluation of the toxicological properties of the synthesized compound must be made. With residues on food crops, this usually takes the form of acute

oral dosing of a small mammal, such as a rat or mouse, in order to determine an LD_{50} value. Those metabolites which possess toxic properties, especially in comparison to the parent pesticide, and which occurred in significant concentration in the metabolism study, are then singled out for more extensive surveillance by the residue analytical chemist.

As stated earlier, there is a basic difference in the objectives, the approach, and the results between a pesticide metabolism study and a pesticide residue analytical study. Because of the atypical nature of a metabolism study, which is necessitated by the restrictions imposed by the use of radioisotopes, the quantitative aspects of this study should be considered with reserve. It is the purpose of the metabolism study to determine *what* metabolites are present in the metabolite complex, not the concentration of the metabolites. The metabolism chemist's only interest in metabolite concentration in his study is to aid evaluation of his results and to speculate which metabolites are formed in significant amounts and are likely to be sufficiently persistent in the field to make more careful evaluation by residue analysis desirable. The pesticide residue analyst, however, has the responsibility of determining the persistence and residue levels of the pesticide and its significant metabolites as they exist under actual realistic field conditions, and he must rely on the metabolism chemist to tell him which of the metabolites are significant.

SIGNIFICANCE OF METABOLITES

There has been a dramatic change from the previous tendency to minimize or ignore the presence of pesticide metabolite residues to the present overemphasis of their contribution to possible hazards to the environment in which they exist. The fact is that we really do not know how to interpret adequately a pesticide metabolism study in terms of its effect on many mammals, birds, fish, soil micro-organisms, and other segments of the environment. This uncertainty has manifested itself as an ever-increasing demand for more data, the assumption apparently being that the problem can be solved by sheer bulk of data. These studies require more and more time to be accomplished. Since time is money to a pesticide manufacturer, the cost of finding, developing, and marketing a pesticide is increasing at a prohibitive rate. With these increasingly higher costs the pesticide manufacturer is becoming less willing to invest in developing the new biodegradable and pest-specific pesticides that are so desirable. When do we stop these increasing demands for data that are often neither interpretable or relevant to the protection of the environment resulting from the application of useful pesticides?

Now what are the criteria that would, in my opinion, constitute significance of a metabolite in the environment? By a metabolite I mean, of course, a

fragment, a derivative, or an alteration of the parent pesticide that is not a normal chemical constituent of the environment. One criterion would be the persistence of a metabolite or the accumulation as a pool of the metabolite. I believe that each of the compounds, parent pesticide or metabolites, resulting from the field use of the pesticide must be considered sufficiently persistent to warrant development of procedures for analytical surveillance if the radiotracer study indicates it constitutes more than about 10% of the applied dose to the environment at the end of the growing season. If a metabolite constitutes about 10% or less of the applied dose at these times and is decreasing at a reasonable rate, it should *not* be considered persistent and worthy of surveillance.

Since the purpose of surveillance is to warn against significant accumulation of the pesticide or metabolite, the analytical methods need not necessarily be of sufficient sensitivity to measure the quantities indicated by the criteria just discussed. However, the required sensitivity must be decided upon by the application of sound scientific judgment to the total body of information available. Where possible, it is advantageous for the surveillance method to determine the parent pesticide and all of its persistent metabolites to a sensitivity of about 0.1 ppm.

Another criterion which might obviously seem to be of equal importance is the toxic properties of the metabolite. Of course, the question might validly be asked—toxic to what organism under what conditions? The usual and practical answer for food crop residues has been the acute toxicity to a small mammal, such as the mouse or rat. Of course, if a metabolite is present in significant quantity and possesses high mammalian toxicity, it should be considered suspect and surveyed for its residues. If, however, this toxic metabolite is proven by surveillance to be present in inconsequential concentrations as a residue, it should no longer be considered suspect.

Therefore, of the two criteria for significance of a residue of a pesticide metabolite, its persistence and accumulation is of much greater consequence than mere mammalian toxicity. This point is emphasized by the present furor over residues of DDE in the environment. As mentioned earlier, Gunther *et al.* had shown unequivocally the presence and persistence of DDE residues in citrus foliage in 1948.[1] Other workers had also known of this persistence on other crops during this early period. However, because the mammalian toxicity of DDE is quite low, lower than for DDT, this was considered a detoxication of the DDT residue. Therefore, its unusually persistent nature, although of some concern, was really considered by most authorities to be of little importance. With the refinement of analytical techniques and the concomitant finding of its residues virtually everywhere, and with the thin egg shell controversy, the current concern over its residual persistence has magnified immensely. Yet even today it would be almost

impossible to predict such an obscure effect of a metabolite on such a little-studied phenomenon as the thickness of bird egg shells.

So, what is the answer? Should a pesticide which has proven useful be banned because of the persistence of a metabolite whose biological impact on its environment is unknown? Certainly if a pesticide or its metabolites have been demonstrated to have a detrimental effect upon the local environment, the potential hazards should be weighed very carefully against the potential benefits before it is either denied or allowed registration for widespread use. However, I firmly believe that if a pesticide or its persistent metabolites have been shown by reasonable tests to appear innocuous, its use should be allowed under careful surveillance. Only such widescale use can allow a valid evaluation of its impact on the environment at large. If after a period of usage an adverse effect is discovered, the hazards of use versus the benefits most certainly should be re-evaluated. In this way, the maximum benefit can be realized by use of the pesticide with a minimum of insult to the environment.

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