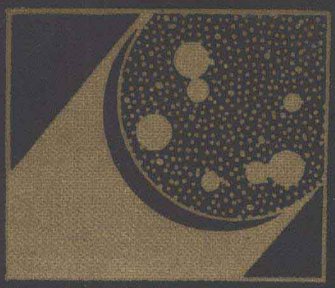
Xenobiotic Metabolism: In Vitro Methods



Xenobiotic Metabolism: In Vitro Methods

Gaylord D. Paulson,
D. Stuart Frear, and
Edwin P. Marks, EDITORS
U. S. Department of Agriculture

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ACS Symposium Series

Robert F. Gould, *Editor*

FOREWORD

The ACS SYMPOSIUM SERIES was founded in 1974 to provide a medium for publishing symposia quickly in book form. The format of the Series parallels that of the continuing ADVANCES IN CHEMISTRY SERIES except that in order to save time the papers are not typeset but are reproduced as they are submitted by the authors in camera-ready form. Papers are reviewed under the supervision of the Editors with the assistance of the Series Advisory Board and are selected to maintain the integrity of the symposia; however, verbatim reproductions of previously published papers are not accepted. Both reviews and reports of research are acceptable since symposia may embrace both types of presentation.

PREFACE

The beneficial effects of a wide variety of pesticides and other xenobiotics in eliminating or controlling certain insects, plants, and disease processes have been demonstrated conclusively. The standard of living that we now enjoy is attributable, at least in part, to an increased use of xenobiotics. However, there is also a growing awareness and concern that some xenobiotics may have adverse effects on both man and his environment. This concern has resulted in more extensive testing and evaluation of xenobiotics to determine whether they can be used safely. One type of information that is important in making this evaluation is an understanding of the metabolic fate of the xenobiotic in both target and nontarget organisms.

In the past, most xenobiotic metabolism studies were conducted with the intact plant or animal. Such in vivo studies have generated a wealth of useful information and will continue to be the method of choice for many investigations. However, there is a growing realization that in vitro studies may be superior for generating certain types of information. For example, in vitro techniques are often the methods of choice when there is a need to isolate and identify intermediate products of a multistep metabolic sequence. Cofactor requirements and other factors (inhibitors, activators, etc.) affecting the enzymes involved in xenobiotic biotransformations usually are determined by in vitro studies. Comparative studies to determine the effect of factors such as species, sex, age, tissue, subcellular fraction, nutritional factors, disease states, etc. on xenobiotic metabolism often are conducted most quickly and easily in vitro. Usually the mode of action and selectivity of xenobiotics are investigated most effectively by in vitro studies.

Although in vitro techniques are extremely useful and have broad application in studying the metabolic fate of xenobiotics, they also have definite limitations. For example, many in vitro techniques are not applicable to long-term studies because of the buildup of end products, microbial contamination, and other problems. Some investigators have used in vitro conditions (temperature, substrate concentrations, etc.) that had little or no relationship to conditions in the intact organism; therefore, the results obtained were of limited value. Endogenous inhibitors that are retained in subcellular compartments (and therefore have no effect on the metabolism of a xenobiotic in vivo) may be released

during the preparation of an in vitro system and result in misleading information. These and other potential problems cited in the proceedings of this symposium make it clear that in vitro methods must be used with caution. However, when properly used within the recognized limitations, in vitro techniques are extremely useful in studying the metabolism of xenobiotics.

This symposium was organized because of the growing interest in the use of in vitro techniques for xenobiotic metabolism studies. The primary objectives were to critically review, evaluate, and summarize: (1) how in vitro techniques are used in the laboratory with special emphasis on their application to xenobiotic metabolism studies; (2) advantages, disadvantages, and limitations of these techniques; and (3) examples of how in vitro techniques may be useful in future studies. It is our hope that the proceedings of this symposium will provide a point of departure for a more effective and efficient use of in vitro techniques in future research on the metabolism and fate of xenobiotics in the environment.

Metabolism and Radiation Research Laboratory
Agriculture Research,
Science and Education Administration
U.S. Department of Agriculture
Fargo, ND 58102

GAYLORD D. PAULSON
D. STUART FREAR
EDWIN P. MARKS

December 20, 1978

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Plants

Xenobiotic Metabolism in Plants: In Vitro Tissue, Organ, and Isolated Cell Techniques[1]

R. H. SHIMABUKURO and W. C. WALSH

Metabolism and Radiation Research Laboratory, Agricultural Research, Science and Education Administration, U. S. Department of Agriculture, Fargo, ND 58105

Interest in xenobiotic metabolism in plants has centered primarily on the fate of pesticides in plants. Although herbicides have been of predominant interest in plant metabolism studies, the methods and techniques discussed in this report are equally applicable to other classes of pesticides including insecticides and fungicides. In this report, the term "xenobiotics" refers to synthetic pesticides and not to other unnatural compounds. However, the discussion on in vitro techniques for xenobiotic metabolism in plants is based primarily on research with herbicides.

The metabolism of pesticides in plants is discussed extensively in several publications (1, 2, 3). Much is known on the metabolism of organic pesticide chemicals in plants, but the fate of most pesticides in plants is still unknown. It is important to know the identity of transitory intermediate products and the ultimate fate of these chemicals in plants since the intermediate products may be toxic. Knowledge of chemical identity and quantity of intermediate products of a herbicide in plants at different times after treatment is essential for the elucidation of the mode of action and basis for selectivity.

Whole plants treated with pesticides through their roots or foliage have been used extensively for metabolism and "terminal" residue studies. Useful quantities of metabolites may be generated from large-scale treatments of whole plants; such metabolites can then be purified for chemical characterization. The development of various chromatographic techniques and improved UV, IR, NMR and mass spectroscopy instrumentation

[1]Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the U. S. Department of Agriculture and does not imply its approval to the exclusion of other products that may also be suitable.

has made it possible to characterize chemically small amounts of metabolites. Excised plant tissues and organs and isolated cells may be used for pesticide metabolism studies for short periods where limited quantities of metabolites are generated. Results from selected reports are presented to illustrate the techniques and methods that may be used.

Metabolism of Xenobiotics

The successful isolation and chemical characterization of any xenobiotic biotransformation product require the generation of sufficient quantities of the metabolite. The degradation mechanisms in plants may be slower than those in animals (1). Plants also lack an excretory system comparable to the renal excretion system in mammals. Therefore, intermediate degradation products of pesticides cannot be concentrated from normal excretion products as with mammals. Plants metabolize significant amounts of pesticides ultimately to insoluble residues (3). The chemical nature and quantities of the metabolites in a plant are influenced by the site of absorption of the pesticide, translocation, and the residence time in the plant. The use of excised plant tissues, organs, and isolated cells for studies of xenobiotic metabolism is an attempt to modify the influence of the above physiological functions in order to optimize the conditions for maximum metabolite generation. Fundamental functions of the whole plant, including absorption, translocation, cell functions and senescence should be considered when *in vitro* techniques with isolated plant parts are used. The physiological significance of metabolism in isolated plant parts must be evaluated ultimately in terms of results in intact plants.

Absorption. Regardless of how a pesticide is applied to the plant, the chemical must penetrate the plant and be absorbed specifically into the cells where biotransformation reactions occur. The leaf surfaces and root tips are the primary sites of penetration into the plant (4, 5). The cuticle, a thin, lipoidal membrane that covers the entire surface area of the above ground parts of a plant, is the primary barrier to penetration by organic pesticides.

The penetration of nonpolar organic pesticides into leaves and roots is believed to be a two-stage process (4, 6). The first stage in leaf absorption involves passive penetration or partitioning of the nonpolar compound into the cuticle and desorption into the cell walls (apoplast) of the underlying cells. In roots, the first stage involves the inactive diffusion of the compound into the root "free space" (apoplast). The second stage in leaves is the active transport of the pesticides across the plasmalemma (cell membrane) into leaf cells (symplast), and in some cases into the phloem for

symplastic transport. Symplastic transport is an active, energy-requiring process occurring in the living cytoplasmic continuum of the plant. In roots, the second stage involves an active transport of the pesticides across the endodermis and into the stele where the differentiated vascular structure is located. Therefore, symplastic intercellular transport of pesticides occurs at one stage during the transport of pesticides from the external root solution into the xylem vessels in the stele for apoplastic transport to the shoot. Apoplastic transport from the root to shoot is an inactive, physical process occurring in the non-living extra-protoplasmic component of the plant under the influence of the transpiration stream. The symplast-apoplast concept is applied as defined by Crafts and Crisp (7). Unfortunately, little is known about the mechanisms of absorption and transport of organic pesticide molecules in roots. The above hypothesis is based mainly on information regarding the absorption of inorganic ions by roots.

Translocation. Once penetration into leaf cells or root tips is accomplished, the pesticide must be translocated symplastically from leaves and apoplastically from roots to different plant organs and distributed to specific tissues and cells where the target sites for biological activity may be located. The target sites of the pesticide may or may not be located in the same tissues or cells as the biotransformation sites. Detailed discussions on structure of the vascular system and mechanisms of transport are discussed in several publications (5, 7, 8).

It is generally recognized that phloem or symplastic transport occurs from "sources to sinks" or in broad terms from green leaves to active centers of growth and storage. This results in differential or selective translocation and accumulation of photosynthates in young leaves, buds, and meristematic regions of the plant. Most herbicides, and probably other pesticides, do not appear to translocate very readily in the symplast (2) although exceptions are known (5). Nonspecific translocation and uniform distribution to all parts of plants occur by apoplastic transport. This is generally observed when herbicides are translocated in the xylem from roots to shoots under the influence of the transpiration stream (9). The rates of pesticides translocated in the xylem appear to depend on the amount of material released by parenchyma cells to the xylem (9). Factors affecting transpiration also influence apoplastic transport of pesticides from roots to shoots.

The absorption and translocation functions in a whole plant reflect the functions of specific organs, tissues, and cells organized and integrated in their activities to meet the requirements of growth and maintenance. The implications of separating the organs, tissues, and cells for use in in vitro

xenobiotic metabolism studies are clearly evident.

Senescence. Senescence of plant tissues is a major factor that must be considered in xenobiotic metabolism studies with isolated plant organs and cells. In contrast to mammals, senescence in plants is not due to irreversible changes in the genome (DNA breakdown), but due to internal plant factors that inhibit cell metabolism or alter its direction toward autolytic pathways (10). Plant senescence is a hormonally controlled phenomenon (10, 11) that may be induced, retarded or reversed under different circumstances.

The process of leaf senescence begins as soon as leaves are excised or detached from the whole plant. Protein synthesis and chlorophyll content decline, and protease activity, respiration, and RNase levels increase in detached leaves (12, 13, 14, 15). In wheat leaves the levels of lipase, esterase, and acid phosphatase declined after detachment. However, the decline in the enzyme levels was retarded by treatment of leaves with kinetin (15). Light retards senescence in excised leaves (12). Light-induced retardation of senescence was not linked to phytochrome action, but was related directly to photosynthesis (12). However, evidence indicates that light retardation of senescence is not linked to CO_2 fixation or photochemical activity of PS II. Diuron [3-(3,4-dichlorophenyl)-1,1-dimethylurea] did not eliminate or reduce the effectiveness of light in retarding chlorophyll loss (16). Addition of sucrose also had no effect on chlorophyll loss when CO_2 fixation was inhibited by diuron (16). Glucose inhibited significantly the loss of chlorophyll in the dark (14). However, the effectiveness of glucose at the optimum concentration of 100 μM was still only half the effectiveness of kinetin at 10 μM (14). If a high-energy intermediate is required to delay senescence, the results indicate that light may be acting through its action on cyclic photophosphorylation, a system that is not inhibited by diuron.

Total protein synthesis declines in senescence but a specific proteinase with L-serine in its active center increases in activity as senescence progresses (17). Most of the total soluble protein lost in early senescence was accounted for by a decrease in ribulose-1,5-biphosphate carboxylase (13). Therefore, the chloroplast appears to be the organelle in which the initial senescence sequence begins.

Excised leaves or leaf discs have been utilized extensively to study senescence in plants. These systems have two advantages: 1) detached tissues senesce at a faster rate than when they are attached to the plant, and 2) regulatory compounds can be fed conveniently to the tissues through the cut surfaces. However, it is uncertain whether the biochemical and physiological changes in an excised leaf resemble those in attached leaves. These considerations in the study of senescence are

relevant also to studies on metabolism of xenobiotics in isolated plant tissues.

Isolated Plant Part and Cell Methods

Whole plants with pesticides applied through their roots or leaves or injected into stems and fruits are the most commonly used experimental material for xenobiotic metabolism studies. However, studies with intact plants are complicated by variables related to root and leaf absorption, translocation and transpiration. To overcome some of these variables, researchers have used excised plant parts and enzymatically separated mesophyll cells. Isolated plant protoplasts also may be useful for xenobiotic metabolism studies.

Results of xenobiotic metabolism studies and observed biochemical and physiological responses in separated plant parts or cells are usually extrapolated to reflect reactions occurring in complex whole plants. This may or may not be appropriate and caution must be exercised in evaluating results from separated systems. The different methods used for xenobiotic metabolism studies with plant parts and isolated cells together with results from selected reports are presented here.

Excised Leaves and Roots. Whole organs separated from the intact plant are used in this method. The leaves of both di- and monocotyledonous plants may be used.

Method. Dicotyledonous plants such as cotton (*Gossypium hirsutum* L.) (18, 19, 20), peanut (*Arachis hypogaea* L.) (21, 22), carrot (*Daucus carota* L.) (20) and soybean [*Glycine max* (L.) Merr.] are grown until their first true leaves are fully expanded. The petioles of the true leaves are excised under water to prevent disruption of the water column in the xylem by the introduction of air. This precautionary step minimizes permanent wilting of leaves due to the interruption of the transpiration stream.

Leaves of monocotyledonous plants lack petioles. Therefore, selected leaves may be excised near the base of the lamina or blade as described for the petioles of dicots. Leaves from corn (*Zea mays* L.), sorghum (*Sorghum vulgare* Pers.), and sugarcane (*Saccharum officianarum* L.) have been excised and used successfully (23, 24). Excised leaf blades of barley (*Hordeum vulgare* L.) and rice (*Oryza sativa* L.) have been used to study the metabolism of detergents (25). The entire shoot of young (2- to 3-leaf stage) barley, wheat (*Triticum aestivum* L.) and wild oat (*Avena fatua* L.) seedlings may be excised at the soil level by the same method and used for metabolism studies. Morphologically, the shoots of young cereals and other grasses consist of overlapping leaf sheaths of emerged and younger leaves. The vascular anatomy of the leaf sheath

would be comparable to that of the petiole. Excising the shoot of young cereal plants is nearly equivalent to excising several leaf blades.

The cut edges of either petioles or leaf blades are immersed in a solution of the xenobiotic compound (18, 25). Excised leaves are normally treated in a controlled-environment chamber with a definite photoperiod. Light not only retards senescence but it stimulates transpiration and increases the uptake of treatment solution.

Excised whole roots, separated from the shoots, have not been used extensively for metabolism studies. The root, a heterotrophic plant organ, is more conducive for use in tissue culture where a carbon source may be provided. Corn roots supplied with glucose in aseptic culture metabolized atrazine (2-chloro-4-ethylamino-6-isopropylamino-s-triazine) over a 72-hr period (26). Excised roots of several species, including corn, wheat, soybean, oats (Avena sativa L.) and barley were incubated with simazine [2-chloro-4,6-bis(ethylamino)-s-triazine] in Hoagland's nutrient solution for 6 hours (27). Excised roots and hypocotyls of soybean were incubated for 24 hours in the dark in a 0.1% Na_2CO_3 or distilled water solution of amiben (3-amino-2,5-dichlorobenzoic acid). The amiben metabolite, N-glucosyl amiben [N-(3-carboxy-2,5-dichlorophenyl)-glucosylamine], was isolated and characterized from these tissues (28).

Roots of dicotyledonous seedlings with large endogenous sources of carbon in their cotyledons may be cultured successfully after removal of their epicotyls (immature shoots). The growth of roots from pea (Pisum sativum L.) seedlings with their epicotyls removed was similar to growth of roots from intact seedlings for 11 days in nutrient solution (29). These roots were treated with atrazine for 9 days. The endogenous reserve in the cotyledons was the only carbon source for the roots.

Discussion. The use of excised leaves for xenobiotic metabolism has several advantages: 1) ease of treating plant material with the xenobiotic chemical, 2) rapid uptake of the chemical into plant tissues, and 3) elimination of root and leaf surface absorption as barriers. Some limitations of this technique include: 1) the treatment period for metabolism must be short, 2) senescence of the plant organ begins upon excision from the intact plant, and 3) reactions in excised plant organ may not be the same as those occurring in intact plants.

A relatively large number of excised leaves can be treated with a minimum volume of treatment solution to obtain maximum uptake and distribution of a xenobiotic in leaf tissues. Metabolites of fluorodifen (2,4'-dinitro-4-trifluoromethyl diphenylether) (22) and perfluidone [1,1,1-trifluoro-N-{2-methyl-4-(phenysulfonyl)phenyl}methanesulfonamide] (21) were

isolated and characterized from 300 and 3000 excised peanut leaves, respectively. Treatment solutions were prepared at physiological concentrations of 1 to 100 μM and sparingly soluble compounds may be prepared as aqueous solutions containing up to 1% acetone (22). Acetone at 1% did not cause severe injury to excised leaves. The use of surfactants and emulsifiers common to leaf surface applications is not necessary.

Absorption and transport of xenobiotics to cellular biotransformation sites are fairly rapid in excised leaves. Absorption by dicot leaves is rapid, usually within the first 3 to 5 hours of treatment. Peanut leaves absorbed approximately 3 to 4 ml of fluorodifen solution per leaf within 5 hours (22), and cotton leaves absorbed up to 5 ml of cisanilide (cis-2,5-dimethyl-1-pyrrolidinecarboxanilide) solution per leaf within 3 hours (20). Additional distilled water is required to maintain excised leaves for treatment periods exceeding 5 to 6 hours. The distilled water may be added as a pulse-chase and to replace treatment solution lost through transpiration by excised leaves. Generally, the rate of xenobiotic absorption declines significantly after the initial 3 to 5 hours.

Absorption of treatment solution by excised leaf blades of monocots is not as rapid as with excised leaves of dicots. The rate of treatment solution lost per unit leaf area was not determined, but excised leaf blades generally required little additional distilled water. Sufficient quantities of *s*-triazines (23), propachlor (2-chloro-*N*-isopropylacetamide) (24), and diclofop-methyl [methyl-2-{4-(2',4'-dichlorophenoxy) phenoxy}propanoate] (30) were absorbed within 24- to 48-hour periods by excised corn and sorghum leaves and wheat shoots, respectively, for metabolite isolation and characterization.

Excised leaves may be useful for investigating pesticide interactions. Rapid pulse-chase treatment of excised leaves is possible with a xenobiotic preceded or followed by a second compound. The pulse-chase technique with excised leaves also is useful in studies on product-precursor relationships. Isolated metabolites may be used in treatments. Simultaneous treatment with xenobiotics may be appropriate if the compounds are compatible as a mixture. However, before any treatments with two or more compounds are made, the absorption and translocation characteristics of each compound in excised leaves should be determined to insure proper evaluation of results.

Absorption and translocation in excised leaves. Absorption of a xenobiotic in solution occurs predominantly through the cut edges of petioles or leaf blades or sheaths. Therefore, any influence exerted by roots on the absorption and translocation of a xenobiotic is circumvented by the excised leaf method. This method allows direct uptake of the xenobiotic into the xylem (apoplast) and transport throughout the leaf under the influence of the transpiration stream. Maximum