Xenobiotic Metabolism: In Vitro Methods

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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

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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.

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

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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

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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

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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*

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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

number of degradation sites in the mesophyll cells of leaves should be exposed to the xenobiotic. This is often a distinct advantage of the excised leaf method. However, the apoplastic transport of xenobiotics, even when introduced directly into the xylem, may not be solely a function of transpiration. Several herbicides were selected (Figure 1) to illustrate translocation differences in excised leaves.

Figures 2 to 5 show the translocation of [^{14}C-ring] amitrole (3-amino-1,2,4-triazole) (A), [^{14}C-ring]atrazine (B), [^{14}C-phenyl]diclofop-methyl (C), and [$^{14}CF_3$]fluorodifen (D) in root-treated intact plants and excised leaves of soybean and oat. All herbicides were applied at 10 µM (spec. act. 0.54 mCi/mmol) concentration. Two excised soybean leaves and oat shoots were treated with 10 ml and 5 ml, respectively, of each herbicide for 24 hours (Figures 2 and 4). The roots of a soybean and oat plant were treated with 50 ml and 30 ml, respectively, of each herbicide for 48 hours (Figures 3 and 5). All plant materials were exposed to a 14-10-hour light-dark cycle at 13 klux light intensity, 26 C day and 20 C night temperatures. The relative absorption date of soybean and oat plants (Tables I and II) cannot be compared directly since the surface areas and the ^{14}C applied differed between the two species. However, apparent differences between a dicot (soybean) and monocot (oat), as discussed previously, are evident.

Table I. Absorption and translocation of [^{14}C]herbicides by excised soybean leaves and excised shoots of oat.[a]

	% of applied ^{14}C absorbed[b]		Distribution of absorbed ^{14}C(%)			
			soybean		oat	
Herbicide	soybean	oat	immersed[c]	leaf	immersed[c]	shoot
Amitrole(A)	99	32	6	94	53	47
Atrazine(B)	99	28	1	99	22	78
Diclofop-methyl(C)	92	77	36	64	75	25
Fluorodifen(D)	94	56	49	51	81	19

[a] Quantitative data for plants in Figures 2 and 4.

[b] ^{14}C remaining in post-treatment solution: A, B, D - 100% parent herbicide; C - soybean - 100% parent herbicide; oat - 30% parent herbicide, 70% acid metabolite (diclofop).

[c] Lower 3 cm of soybean petiole and excised oat shoot immersed in [^{14}C]herbicide solution. Length of soybean petioles was 8 cm.

CHEMICALS USED FOR TRANSLOCATION

AMITROLE

DICLOFOP—METHYL (HOE 23408)

ATRAZINE

FLUORODIFEN

Figure 1. C-14 herbicides applied to excised soybean leaves, excised oat shoots, and roots of both species for absorption and translocation

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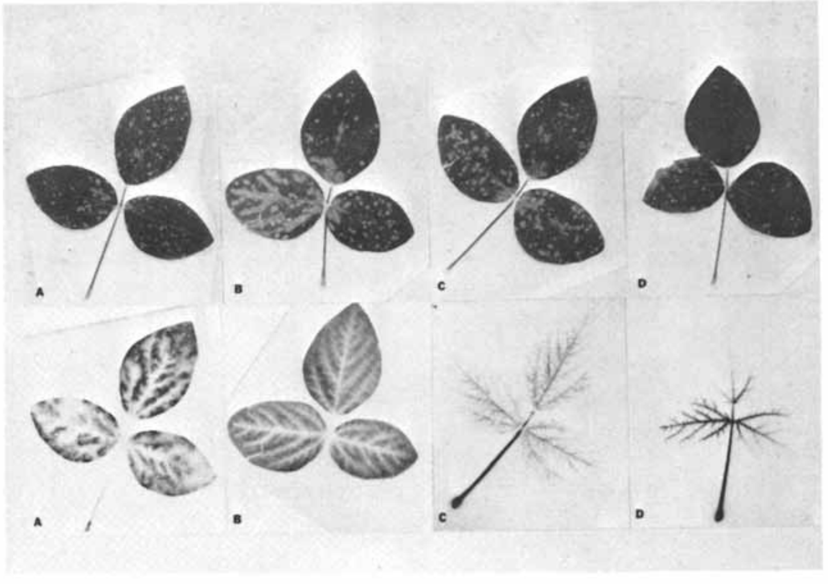

Figure 2. Radioautographs of excised soybean leaves treated with (A), ^{14}C-labeled amitrole; (B), atrazine; (C), diclofop-methyl; and (D), fluorodifen. The bottom are radioautographs of the leaves above. The lower 3-cm section of each petiole was immersed in ^{14}C herbicide solution for 24 hr.

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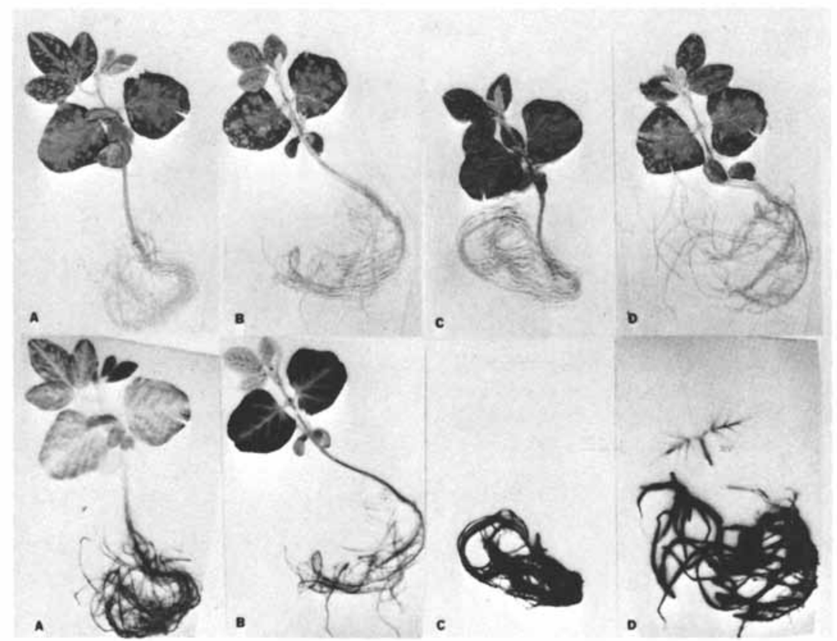

Figure 3. Radioautographs of soybean plants root-treated with (A), ^{14}C-labeled amitrole; (B), atrazine; (C), diclofop-methyl; and (D), fluorodifen. The bottom are radioautographs of the plants above. Plants were root-treated for 48 hr.

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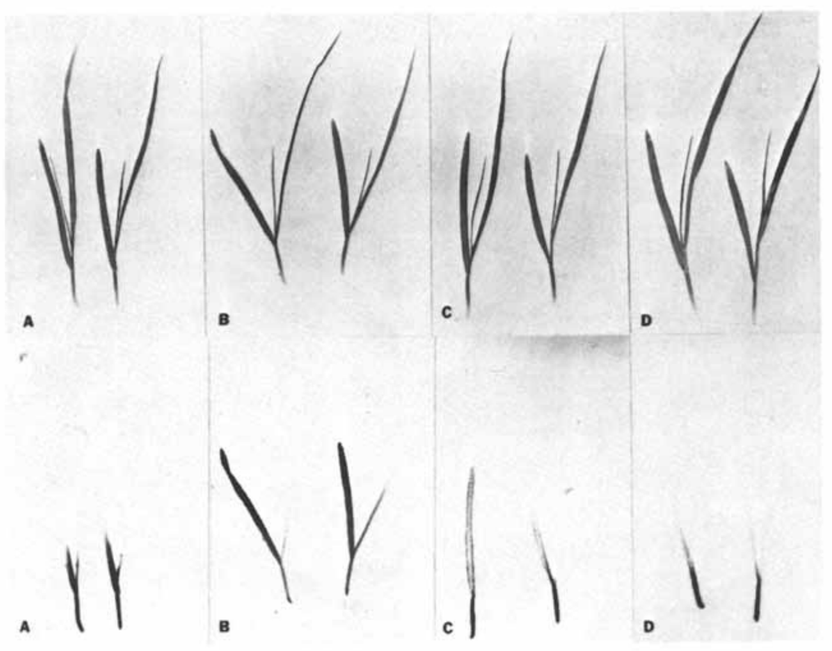

Figure 4. Radioautographs of excised oat shoots treated with (A), ^{14}C-labeled amitrole; (B), atrazine; (C), diclofop-methyl; and (D), fluorodifen. The bottom are radioautographs of the excised shoots above. The lower 3-cm section of each shoot was immersed in ^{14}C herbicide solution for 24 hr.

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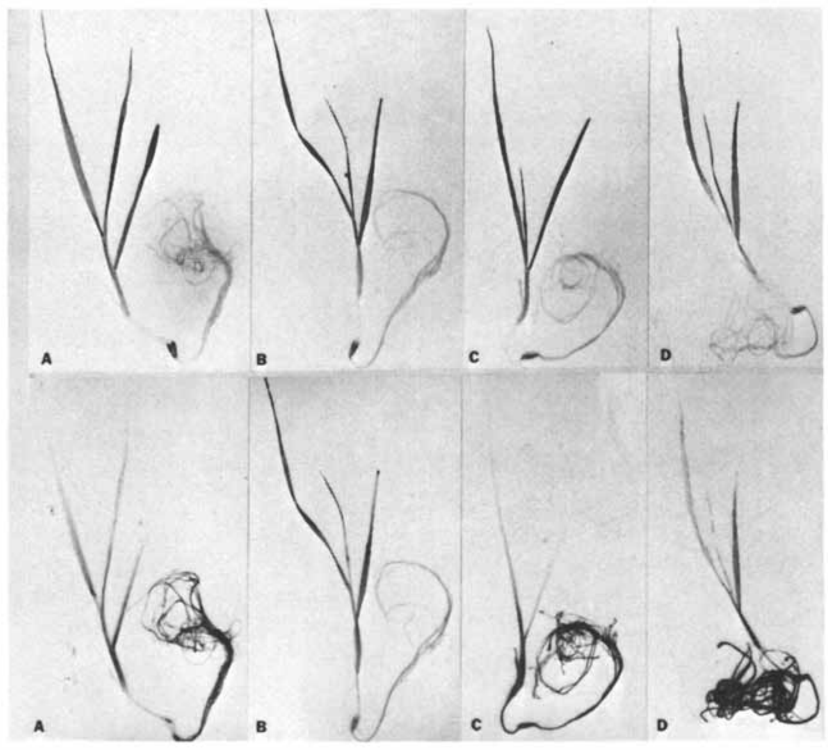

Figure 5. Radioautographs of oat plants root-treated with (A), ^{14}C-labeled amitrole; (B), atrazine; (C), diclofop-methyl; and (D), fluorodifen. The bottom are radioautographs of the plants above. Plants were root-treated for 48 hr.

Table II. Absorption and translocation of [^{14}C]herbicides by root-treated intact soybean and oat plants.[a]

Herbicide	% of applied ^{14}C absorbed[b] soybean	% of applied ^{14}C absorbed[b] oat	Distribution of absorbed ^{14}C(%) soybean root	soybean shoot	oat root	oat shoot
Amitrole(A)	27	14	52	48	60	40
Atrazine(B)	29	8	20	80	31	69
Diclofop-methyl(C)	34	16	98	2	63	37
Fluorodifen(D)	77	48	83	17	78	22

a/ Quantitative data for plants in Figures 3 and 5.

b/ ^{14}C remaining in post-treatment solution: A, B, D - 100% parent herbicide; C - 100% acid metabolite (diclofop).

Rapid transpiration rates increased the uptake of [^{14}C] herbicides in excised leaves, especially in soybean (Table I). Distilled water was added to excised soybean leaves after 5 hours. Excised oat shoots required no additional water over the 24-hour period. Root uptake varied according to the herbicide (Table II). No additional distilled water was added to root-treated plants over the 48-hour period.

The ^{14}C from atrazine was uniformly distributed in excised leaves and root-treated plants. This is typical of apoplastic transport under the influence of the transpiration stream. The distribution of ^{14}C from amitrole was less uniform than that from atrazine in both species. The differential localization of ^{14}C from amitrole in younger leaves and apex of soybean plants (Figure 3) indicates retranslocation or symplastic transport of ^{14}C out of older leaves. This contrasts to ^{14}C from atrazine which localized in the two unifoliate primary leaves with little ^{14}C in the younger developing leaves. Symplastic transport of [^{14}C]atrazine and/or its metabolites from older leaves does not occur as with amitrole (Figure 3). Retranslocation from leaves following initial apoplastic transport from roots is not as evident in a monocot (Figure 5) as in a dicot (Figure 3). The vascular transport of material into and out of leaves, a normal physiological process, is altered when leaves are excised (Figures 2 and 4). Therefore, the significance of results from excised leaves must be evaluated in terms of what may be occurring in an intact plant.

Diclofop-methyl and fluorodifen were readily absorbed by excised leaves and root-treated plants (Tables I and II).

Uptake was enhanced by transpiration, especially in excised leaves of soybean (Table I). However, transpiration appeared to have very little influence on transport. Unlike amitrole and atrazine, much of the ^{14}C from diclofop-methyl and fluorodifen was immobilized in the petiole of soybean leaf and the immersed section of excised oat shoot and the roots of both species (Tables I and II, Figures 2 to 5). Atrazine is not metabolized in significant amounts in soybean (26, 31) and probably in oat, an atrazine-susceptible species. Amitrole metabolism in soybean may be as rapid as in bean (*Phaseolus vulgaris* L.) (32) and oat (33). Fluorodifen metabolism is rapid in soybean (20) whereas diclofop-methyl metabolism is rapid in both oat and soybean (30).

Immobilization of xenobiotics in the xylem may be due to adsorption by xylem vessels or uptake and retention by parenchyma cells. The extent of immobilization may be a function of the physical and chemical properties of the molecular entities being translocated with only limited influence by the transpiration stream. Therefore, metabolism of a xenobiotic and the formation of products with differing physical and chemical properties may be a greater factor influencing translocation than species differences in the translocation mechanisms *per se*. The greater translocation of ^{14}C from [^{14}C]dipropetryn [2-ethylthio-4,6-bis(isopropylamino)-*s*-triazine] to the leaf tips in oat than in corn excised leaves was reported to be a factor in the higher susceptibility of oat to dipropetryn (34). This difference in translocation between excised leaves of oat and corn was attributed to an inherent mechanism regulating translocation of dipropetryne within the leaves of these species (34). However, such a conclusion is questionable since the molecular forms of the ^{14}C being translocated were not determined. The translocation differences between a resistant and susceptible species may simply reflect differences in metabolism of the xenobiotic and immobilization of its metabolites as a function of their physical and chemical properties.

Translocation of ^{14}C-labeled xenobiotics in excised leaves must be determined and not assumed to be typical of apoplastic transport. If immobilization occurs in specific parts of excised leaves, extraction of metabolites from these parts will maximize the concentration of metabolites and minimize the impurities and natural products that must be removed in the purification procedure.

Xenobiotic metabolism in excised leaves. The metabolism of xenobiotics in excised leaves appears to be similar qualitatively to metabolism in shoots of intact plants, but may differ quantitatively. The same water-soluble metabolites of perfluidone were detected in excised peanut leaves and leaves or shoots of intact peanut plants treated through their roots (21). However, excised leaves tolerated treatment with twice the highest

concentration used for root-treatment with 62% uptake of applied [^{14}C]perfluidone versus 25% uptake by roots. Only 31% of the root uptake was translocated to the shoots. Two water-soluble conjugates of perfluidone accounted for 31% of the ^{14}C in excised peanut leaves after 48 hours but the same metabolites accounted for only 8% of the ^{14}C in the shoots of root-treated intact plants after 8 days (21). Ethylenethiourea (ETU), a decomposition product of ethylenebis(dithiocarbamate) fungicides, was metabolized similarly by excised leaves and shoots of root-treated tomato (*Lycopersicon esculentum* Mill.) (35). However, metabolism in tomato shoots was slower than in excised leaves when ETU was stem-injected into intact plants. The metabolism of diclofop-methyl, a post-emergence herbicide, in wheat and wild oat was qualitatively similar in excised shoots and root-treated intact plants (29). Excised shoots of wheat and wild oat absorbed 81% and 87% of the applied [^{14}C]diclofop-methyl, respectively, and metabolized 77% and 72% of the absorbed ^{14}C to water-soluble conjugates within 24 hours. Intact root-treated wheat and wild oat absorbed 68% and 37% of applied [^{14}C]diclofop-methyl, respectively, but only 8% and 11% of the root uptake was translocated to the shoots. Therefore, the use of excised leaves (dicots) or shoots (monocots) to study metabolism of foliarly-applied xenobiotics such as diclofop-methyl may have distinct advantages over root application or stem-injection.

Metabolites of monuron [3-(4-chlorophenyl)-1,1-dimethylurea] were isolated and characterized from excised cotton leaves (18, 19). Within 24 hours, two β-*D*-glucosides constituted 20 to 25% of the methanol-soluble metabolites (18). These were identified as conjugates of the hydroxymethyl intermediates. Longer treatments of up to 4 days indicated that the rapid oxidative *N*-demethylation of monuron, occurring within the first 24 hours, was followed by slow oxidative aryl hydroxylation and subsequent conjugation (19). Both rapid and slow biotransformation reactions of monuron were elucidated by the use of excised leaves.

Qualitative differences in the metabolism of a xenobiotic between roots and shoots are not common. However, such differences have been observed for some herbicides (36, 37). Little or no glutathione conjugates of atrazine (GS-atrazine) and its derivatives were detected in the roots of intact root-treated corn plants (36). The major metabolite in corn roots was hydroxyatrazine (2-hydroxy-4-ethylamino-6-isopropylamino-*s*-triazine). Significant concentrations (10 to 20% of ^{14}C in intact plant) of GS-atrazine were found only in the shoots. GS-atrazine and its derivatives accounted for 60 to 70% of the atrazine absorbed directly into excised corn leaf blades within 24 hours. Very little hydroxyatrazine was detected (36). The qualitative differences in atrazine metabolism between roots and shoots of corn were due to the localization of glutathione S-

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transferase in the shoots and not roots of corn (37). The metabolites of chlorpropham (isopropyl-m-chlorocarbanilate) differed between roots and shoots in root-treated intact soybean plants (38). Conjugates of 2-hydroxy-chlorpropham and 4-hydroxy-chlorpropham were present in the shoots, but only the conjugate of 2-hydroxy-chlorpropham was found in root tissue (38). This suggests that leaf tissue probably formed both hydroxylated derivatives, but root tissues formed only 2-hydroxy-chlorpropham. Studies on chlorpropham metabolism in excised soybean leaves should confirm the above conclusion.

Leaf Discs and Leaf Sections. Leaf discs of uniform size, leaf sections of lamina (leaf blade) from grasses or thin leaf strips may be used for xenobiotic metabolism studies. The structural and metabolic integrity of the leaf tissues is maintained, but the normal vascular transport of materials to and from the leaf tissues is eliminated. Penetration of xenobiotics into the mesophyll and palisade cells is predominantly through the cells along the cut edges of leaf discs or sections (39). The cuticle, a major barrier to leaf penetration by surface-applied chemicals, is circumvented by the use of leaf discs or sections as in the use of excised leaves or shoots described previously. The uptake and efflux of organic compounds in plant cells are not well understood (40). The mechanism of uptake involving physico-chemical interactions at the plasmalemma and tonoplast (vacuole membrane) (41) is applicable to leaf discs and leaf sections.

Method. The size of leaf discs and leaf sections used, the incubation medium for the leaf tissue, and the method of applying the test chemicals vary according to the objectives of the specific experiments. Leaf discs are usually cut with a cork borer (8 to 15 mm diameter) from interveinal areas of the leaf blade (41, 42, 43, 44). Leaf sections or slices from monocots varied from barley leaves cut transversely at 0.75 mm wide strips (45) to sorghum lamina cut into 1 cm x 1 cm sections (46). Dicotyledonous cotton leaves were cut into 0.4 mm wide strips (47).

The incubation medium for leaf discs or sections may or may not contain an osmoticum. Sucrose (0.25 M, pH 6.2) (41) and mannitol at 0.26 M (pH 4 to 6) (45) and 0.35 M (42, 48) are commonly used osmoticums. Leaf tissues were also incubated in water (43, 44, 46, 49, 50). Other than an osmoticum and buffer, inorganic salts are not commonly added to the incubation medium. The simplicity of the incubation medium for leaf discs or sections is in marked contrast to the medium required for separated cells or aseptic tissue culture.

The leaf discs or sections are treated with xenobiotics or other test compounds by vacuum infiltration (41, 42, 43, 48) or by either floatation or submersion of the leaves in solutions

of the compounds (44, 45, 46, 50). Infiltration of the test chemical into leaf tissue may be accomplished by evacuating the leaf tissue in a solution of the compound to a given pressure (20 cm Hg) (49) before releasing the vacuum or maintaining a given pressure (40 cm Hg) for a specific period (15 min) (41). The most common practice is to evacuate to the maximum pressure with a water aspirator (42) and either maintain the vacuum or release it immediately. Successful infiltration of the solution into the intercellular air spaces of the mesophyll cells can be checked visually by the darker, water-soaked appearance of the leaf tissue.

Discussion. Leaf discs or sections have been used extensively for basic research on the uptake of inorganic salts and organic compounds by plant cells, and on photosynthesis and photorespiration. Use of this method to study metabolism of xenobiotics has been limited. Little is known about the penetration of plant cell membranes by xenobiotics (51). Unlike inorganic ions, amino acids, and sugars that appear to have specific membrane transport systems, the predominantly lipophilic and neutral xenobiotics, such as herbicides, probably cross cell membranes by diffusion (51). Therefore, vacuum infiltration of the xenobiotic should enhance penetration of the compound into mesophyll cells. The xenobiotic will penetrate more cells than without vacuum infiltration, thereby increasing the concentration of metabolites formed in leaf discs.

Vacuum infiltration of leaf discs has some detrimental effects. Although respiration in wheat, barley, and bean leaf discs was unaffected by vacuum infiltration of water, CO_2 fixation and O_2 evolution were totally inhibited (49). Carrier-mediated uptake of amino acids into leaf sections was dependent on respiration and photosynthesis for ATP as its energy source (45, 52). Amino acid uptake was inhibited by 2,4-dinitrophenol or anaerobic conditions in the dark. Light stimulated amino acid uptake under anaerobic conditions, but this stimulation was totally inhibited by 10 μM diuron (45). If uptake of compounds such as 2,4-D (2,4-dichlorophenoxyacetic acid) is an active process in leaf discs as it appears to be in root tissue (53), then vacuum infiltration of leaf discs may not be advisable for studies with such compounds. However, for compounds such as monuron which is taken up passively by diffusion in roots (53), vacuum infiltration for uptake by leaf discs may be advantageous. This may be true for metabolism studies but not for experiments on inhibition of photosynthesis by monuron. Studies comparable to those on amino acid uptake by plant cells have not been reported for herbicides and other pesticides. Therefore, the effect of vacuum infiltration on the uptake of specific herbicides in leaf discs is only speculative.

Little is known of the effects of senescence on uptake and

metabolism of xenobiotics by leaf discs or sections. The senescence processes described earlier may have significant effects on the processes described above.

Metabolism in leaf discs or sections. Selected examples are presented to illustrate the versatility of the leaf disc or section technique. The use of this method permits: 1) rapid assessment of pesticide degradation in plant tissues, 2) determination of physiological factors that influence metabolism of pesticides, and 3) evaluation of interaction effects between pesticides. A thorough study on the metabolism of phenylurea herbicides in leaf discs of resistant and susceptible plants (54, 55) demonstrates the above points. A time-course study on the metabolism of monuron showed rapid *N*-demethylation and metabolism to water-soluble conjugates within 3 hours after preincubation of resistant cotton and plantain (*Plantago major* L.) leaf discs in a buffered solution of monuron (54). Leaf discs of monuron-sensitive soybean and corn metabolized monuron to a much lesser extent over the same period than cotton or plantain. Active photosynthesis was not required for monuron metabolism since the rate of degradation was similar in the dark and light under CO_2-free conditions.

A flux of a chemical into and out of leaf discs may be expected (41, 51). The kinetics of uptake and efflux of nonelectrolytes have been described (41). However, in most of these studies it was assumed that the chemical was not altered between its uptake and efflux by leaf discs. Some evidence indicates that this may not be necessarily true. The efflux from monuron-resistant plantain leaf discs was nearly one-half of the initially absorbed herbicide within 2 to 3 hours (54). Subsequent reabsorption and metabolism occurred but 37% of the total ^{14}C was present in the efflux after 7.6 hours. More than 50% of the ^{14}C in the efflux was three metabolites with parent monuron accounting for the remainder (54). However, polar water-soluble conjugates, which constitute the major metabolites of monuron in leaf discs, were not detected in the efflux. Similarly, major water-soluble conjugates of atrazine (56) in corn leaf discs and diclofop-methyl (30) in wheat leaf sections were not detected in the efflux from these tissues. Therefore, a selective efflux of non-polar parent compounds or their metabolites appears to occur in leaf discs or sections with some xenobiotics. Any relationship between the efflux characteristics of leaf discs and release of material from parenchyma cells into the vascular system of intact plants is still obscure. However, efflux characteristics of specific compounds from leaf discs may be related to their immobilization or translocation in intact plants.

The evaluation of xenobiotic metabolism as a factor in selectivity, antagonism, or synergism is possible because of the rapid determination of metabolism in leaf discs. Metabolism and

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detoxication of herbicides in plants is probably the most important single factor in herbicide selectivity. Physiological responses coupled to time-course metabolism may be readily demonstrated in leaf discs for photosynthetic inhibitors such as monuron (55) and atrazine (44, 48, 56). Inhibition of photosynthesis and recovery within 8 hours was correlated with glutathione conjugation of atrazine in sorghum (44), corn (56), and several species of the subfamilies, Festucoideae and Panicoideae (48). Recovery of photosynthesis to nearly the control level occurred in monuron-treated cotton leaf discs within 4 to 5 hours with rapid N-demethylation of monuron (55).

The nature of interaction between xenobiotics at the molecular level may be demonstrated readily in leaf discs. The application of a mixture of the herbicide, propanil (3,4-dichloropropionanilide), with a carbamate or phosphate insecticide caused injury to resistant rice (57). The injury was due to the inhibition of an aryl acylamidase in rice by carbamate insecticides that prevented detoxication of propanil in the resistant species (58, 59). In resistant cotton leaf discs, certain carbamate insecticides such as carbaryl (1-napthylmethylcarbamate) strongly inhibited the degradation of the photosynthetic inhibitor, monuron. The inhibition of photosynthesis was enhanced and normal recovery to control levels of photosynthetic activity was delayed (55).

The interactions between several organophosphate and carbamate insecticides and herbicides were tested in leaf discs of several species (60, 61). Organophosphate insecticides, dyfonate (O-ethyl-S-phenyl ethylphosphonodithioate) and malathion [O,O-dimethyl-S-(1,2-bis-carbethoxy)-ethyl phosphorodithioate], strongly inhibited the metabolism of substituted phenylurea herbicides. Several carbamates [carbaryl, carbofuran (2,3-dihydro-2,2-dimethyl-7-benzofuranyl methylcarbamate), PCMC (p-chlorophenyl-N-methylcarbamate)] strongly inhibited propanil metabolism (60). The effects of several herbicides on the degradation of carbaryl, dyfonate, and malathion were not as severe as the effects of the insecticides on herbicide metabolism (61). Carbaryl metabolism was stimulated by chlorpropham while metabolism of dyfonate and malathion was inhibited by propanil (61). The nature of the interaction between carbofuran and the herbicides, alachor (2-chloro-2',6'-diethyl-N-methoxymethyl acetanilide) (62), chlorbromuron [3-(4-bromo-3-chlorophenyl)-1-methoxy-1-methylurea] (63), and butylate (S-ethyldiisobutylthiocarbamate) (64) was explained on the basis of whole-plant experiments. Carbofuran increased the absorption and inhibited slightly the metabolism of the herbicides. If the inhibition of herbicide metabolism by carbofuran is significant, it should be readily demonstrated in leaf discs over much shorter periods than is possible with whole plants.

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Isolation and characterization of metabolites. The identification of metabolites extracted from leaf discs is generally based on thin-layer cochromatography with known standards. Limited numbers of leaf discs or sections are treated with radiolabeled xenobiotics, extracted, and the radioactive metabolites are separated and quantitated. Leaf discs or sections also may be used to generate sufficient quantities of metabolites for purification and chemical characterization. However, use of leaf discs or sections for such a purpose has been limited.

The identification of glutathione conjugation as one of the major pathways for herbicide detoxication in plants was made through the use of sorghum leaf discs and sections. A water-soluble metabolite of atrazine was detected as a major metabolite in sorghum leaf discs during recovery of atrazine-inhibited photosynthesis (44). This metabolite was generated from a large-scale treatment of sorghum leaf sections bathed in a solution of atrazine for 20 hours (46). The metabolites, *S*-(4-ethylamino-6-isopropylamino-*s*-triazinyl-2)glutathione (III) and γ-glutamyl-*S*-(4-ethylamino-6-isopropylamino-*s*-triazinyl-2)cysteine (IV), were successfully isolated and characterized from sorghum leaf sections (46). Subsequent studies with whole plants elucidated the mercapturic acid-like pathway for atrazine metabolism in plants (65).

The limited periods that leaf discs or sections may be allowed to metabolize xenobiotics could be a disadvantage. Metabolites that are formed over longer periods cannot be generated in leaf discs or sections. The metabolite of atrazine, *N*-(4-ethylamino-6-isopropylamino-*s*-triazinyl-2)lanthionine (VII), was present in significant quantities in leaves of root-treated sorghum plants only after 5 days (65). Metabolites III and IV were major metabolites in sorghum shoots of whole plants after 14.4 hours of treatment but declined thereafter (65). Therefore, due to temporal requirements it is doubtful if metabolites such as VII can be successfully generated and isolated from leaf discs or sections.

Separated Leaf Cells. In this method the mesophyll and palisade cells from leaves are separated to give a homogenous liquid suspension of cells that can be manipulated much like unicellular algae. The separated cell system is useful for studying biochemical, physiological and cytochemical processes in plants. Use of individual cells in suspension permits equal exposure of all cells to test chemicals. This is unlike the techniques involving leaf discs or sections in which cells along the cut edges are exposed preferentially to the test chemicals. The presence of rigid cell walls permits repeated washing and centrifugation of cells with minimal damage. The metabolic function of the leaf tissue is maintained in the cell suspension but the structural integrity of the leaf and the

normal function of vascular transport are no longer retained.

Method. Morphologically intact mesophyll cells may be separated by: 1) gently grinding leaf tissue and separating individual cells from leaf debris by selective filtration and centrifugation (66, 67); and 2) digestion of leaf tissue with enzyme preparations containing polygalacturonase and cellulase (68, 69, 70, 71, 72). The enzyme separation methods presently used are basically modifications of those developed by Takebe et al. (68) and Jensen et al. (69).

Separated leaf cells are prepared by cutting leaves into strips (69) or squares (72) and vacuum infiltrating these tissues with a maceration medium containing the enzyme (0.5 to 3.0%), inorganic salts, buffer (pH 5.8), potassium dextran sulfate, a hypertonic solution of sorbitol (0.7 to 0.8 M), anti-senescent agents (2,4-D, benzyladenine), and antibiotics. The infiltrated tissue is digested with additional maceration medium for 10 to 30 min. The cells separated during this first and a second similar maceration periods are discarded. The cells obtained during the third maceration period (30 to 45 min) are most active metabolically and are used for experimentation. The wash and assay media contain similar salts as the maceration medium, 0.6 to 0.7 M sorbitol and are buffered at a higher pH (6.7 to 7.2).

Discussion. The discussion is limited to enzymatically separated cells since this is the system that has been used to study the effects of xenobiotics (herbicides) on separated plant cells.

Maceration of leaf tissue in a hypertonic environment that results in cell plasmolysis was necessary to yield photosynthetically active cells (69). Plasmolyzed cells with intact plasmalemmas were detected by phase contrast microscopy (69). Separated cotton mesophyll cells fixed CO_2 at linear rates of 50 to 100 μmoles/mg chlorophyll /hour for 4 to 8 hours (71). Uptake of ^{14}C-leucine and ^{14}C-uracil and incorporation into protein and RNA, respectively, occurred in separated tobacco (*Nicotiana tabacum* L.) cells (73). Uptake and incorporation of the percursors into macromolecules required light and active photosynthesis. Addition of ATP only partially substituted for the light requirement. A smaller percentage of the absorbed percursors was incorporated into macromolecules when cells were incubated in the dark than in light (73). Efflux of photosynthetic products amounted to 1 to 2% of total carbon fixed per hour (71). Efflux was inhibited by the addition of Ca^{2+} in the incubation medium.

The metabolic activity is similar between separated cells and cells of intact leaves and leaf discs described earlier. The separated cells were metabolically active for 20 to 30 hours (69, 73). However, critical experimental periods should

not exceed 5 to 12 hours since the metabolic activity in the cells may not be sustained beyond this period.

Metabolism in separated cells. Separated cells have been used to study primarily the effects of herbicides and surfactants on plant cell membranes (74, 75, 76) and selected metabolic reactions (70, 72, 77, 78, 79). The biochemical and physiological changes observed in the above studies were not correlated with metabolism of the xenobiotics in separated cells. Therefore, the studies are not complete. However, the potential usefulness of this technique has been demonstrated in the limited number of reports.

Cationic surfactants increased significantly the efflux of $^{14}CO_2$ fixation products in separated soybean, wild onion (*Allium canadense* L.) and cotton mesophyll cells (74, 75). In wild onion, CO_2 fixation was also inhibited by the same surfactants. Nonionic surfactants had relatively less effect on membrane permeability and photosynthesis than the cationic surfactants (74, 75). Wild onion cells were less sensitive to the action of a cationic surfactant than were soybean cells (75). Mixtures of oryzalin (3,5-dinitro-*N*^{4},*N*^{4}-dipropylsulfanilamide) with selected surfactants enhanced efflux in soybean cells above that of cells treated with oryzalin or surfactant alone (74). Oryzalin and surfactants caused greater efflux of intracellular material from cotton cells than from soybean cells (74). The synergistic effects between oryzalin and surfactants were not observed in cotton as with soybean cells. The differences observed between cells from different species in response to treatment with a chemical may not be due to inherent differences in their membranes. Such differences may be only a reflection of the differences in metabolism and detoxication of the biologically active compound between cells from different species. Nonionic surfactants like Triton X-100 are rapidly metabolized in plants when taken up by excised leaves or absorbed from leaf surfaces (25, 80).

The concept that herbicides have multiple sites of action rather than a single site has been supported by results from separated cells (72, 77, 78). A time-course effect of 13 major herbicides on photosynthesis, respiration, protein synthesis, RNA synthesis, and lipid synthesis was measured in separated kidney bean cells at various concentrations (72). Photosynthetic inhibitors such as atrazine, monuron, bromacil (5-bromo-3-*sec*-butyl-6-methyluracil), and dinoseb (2-*sec*-butyl-4,6-dinitrophenol) inhibited photosynthesis significantly but stimulated lipid synthesis at physiological concentrations of 0.1 to 1.0 μM. RNA synthesis also was inhibited at the same concentrations. At 100 μM, the same herbicides strongly inhibited lipid synthesis. The inhibition of macromolecule synthesis by photosynthetic inhibitors is probably a secondary effect since the uptake and incorporation of percursors require

active photosynthesis (73). The physiological significance of the reported effects on metabolic processes may be evaluated better if information on the metabolism and intracellular localization of the herbicides were known.

The metabolism of differentially labeled [^{14}C]fluorodifen in separated mesophyll cells from resistant peanut is shown in Table III. The cells were prepared using the method of Jensen *et al*. (69) except that Pectinol 41-P (crude pectinase and cellulase) was used in place of macerozyme, no 2,4-D was added to any media, K_2SO_4 was substituted for potassium dextran sulfate and the assay medium contained 0.6 M sorbitol buffered with 0.05 M HEPES (pH 7.0). Assays were conducted in 2 ml photosynthetic medium (69) that contained 10 μM [^{14}C]fluorodifen, 1% acetone, 7.5 mM $NaHCO_3$, and separated cells (238 μg chlorophyll per reaction flask). Reaction flasks were incubated in a differential respirometer at 25 C and illuminated from below with incandescent lamps (5000 lux). The cells were separated from the assay medium and extracted with 80% methanol. The cell extract and the assay medium were analyzed for fluorodifen and its metabolites as reported previously (22, 81, 82).

The uptake of fluorodifen by peanut mesophyll cells occurred readily in light or dark (Table III). The ratio of ^{14}C in the cell pellet to that in assay medium decreased with decreasing cell concentrations in repeated experiments. Maximum uptake and metabolism of fluorodifen occurred within 2 hours. Light appeared to have no influence on the uptake of fluorodifen. However, fluorodifen metabolism was enhanced by light in the 2-hour treatment period. Efflux of water-soluble metabolites (glucosides and peptide conjugates) occurred from separated cells. However, efflux of fluorodifen could not be determined. The role of light in the enhanced metabolism of fluorodifen is not clear. Oxygen evolution was not inhibited by fluorodifen and ATP was not a required co-factor for glutathione S-transferase, the enzyme that catalyzes the ether cleavage of fluorodifen (83).

Separated zinia (*Zinia elegans* Jacq.) cells absorbed [^{14}C]fluorodifen and [^{14}C]trifluralin (α,α,α-trifluoro-2,6-dinitro-*N*,*N*-dipropyl-*p*-toluidine) within 2 hours (70). After washing cells with solutions of the unlabeled herbicides, [^{14}C]trifluralin appeared to be bound more strongly than [^{14}C]fluorodifen. However, the significance of the results is not clear since metabolism of the two compounds was not determined.

The single example of xenobiotic metabolism in a separated cell system illustrates the importance of metabolism when studying the mechanism of action and selectivity of a compound. Separated cells can be used to measure rapid biochemical and physiological changes in response to treatment with a xenobiotic. However, the dynamic changes in uptake, metabolism,

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Table III. Metabolism of [$^{14}CF_3$]- and [^{14}C-p-NO_2-phenyl]-labeled fluorodifen in separated peanut mesophyll cells.

	Distribution of total ^{14}C (%)					
	2-hour treatment				4-hour treatment (light)	
	[$^{14}CF_3$][a]		[^{14}C-p-NO_2-phenyl][a]			
	light	dark	light	dark	[$^{14}CF_3$]	[^{14}C-p-NO_2-phenyl]
Assay medium:						
fluorodifen	18	31	15	29	16	13
conjugates[b]	9	1	11	5	10	16
Cell Pellet:						
fluorodifen	29	58	30	58	27	26
conjugates[b]	40	9	39	7	40	40
MeOH-insoluble residue:	4	1	5	1	7	5
Total	100	100	100	100	100	100

a/ Specific activities: [$^{14}CF_3$ fluorodifen] - 2.95 mCi/mmol; [^{14}C-p-NO_2-phenyl] fluorodifen - 2.7 mCi/mmol.

b/ [$^{14}CF_3$]fluorodifen: predominantly S-(2-nitro-4-trifluoromethylphenyl)-gluthathione with small amounts of S-(2-nitro-4-trifluoromethylphenyl)-N-malonylcysteine (82); [^{14}C-p-NO_2-phenyl]fluorodifen: mixture of p-nitrophenyl-β-D-glucoside and p-nitrophenyl-6-O-malonyl-β-D-glucoside (81).

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and efflux of the xenobiotic and its metabolites in plant cells also should be known before conclusions on mechanisms of action and selectivity are made.

Plant Protoplasts. Protoplasts are naked cells obtained by removal of their cell walls. This may be accomplished by: 1) microdissection, or 2) enzymatic digestion. The latter is the more effective and commonly used procedure.

The exposed plasmalemma differentiates protoplasts from isolated mesophyll cells whose rigid primary cell walls are still intact. The same advantages described for isolated mesophyll cells apply to protoplasts. Because of the exposed plasmalemma, it is possible to study membrane absorption and permeability effects without interference by the cell wall (84).

Protoplasts have been useful for studies on cell ultrastructure and functions. They have not been used for studies on metabolism of xenobiotics or their mechanisms of action. This may be due to the difficulties of preparing and maintaining protoplasts; they are extremely fragile and must be stabilized by osmotic stabilizers (sorbitol or mannitol) and Ca^{2+} salts (85, 86).

Method. Numerous methods for the isolation of plant protoplasts have been published, but no standard method exists since each species, type of tissue, cell-strain, environmental growth conditions, etc., require new adjustments in the isolation procedures (85, 86). Generally, the methods are modifications of that originally published by Cocking (87). Each special problem appears to require empirical adjustments to a general scheme outlined as follows (85): Leaves are selected from plants exposed to specific growth regimes and surface sterilized. All operations are performed aseptically. The lower epidermis of leaves is removed to expose mesophyll cells; the leaves are cut into sections and transferred to an enzyme solution. The enzyme solution for cell wall digestion may contain pectinase and/or cellulase and/or hemicellulase with an osmotic stabilizer and Ca^{2+}. Protoplasts, usually released within 6 to 24 hours, are washed with a solution of the osmoticum and used for experimentation or cultured under aseptic conditions for subsequent studies. Modifications to this basic procedure are illustrated in recent publications (88, 89, 90).

Discussion. Isolated plant protoplasts are generally spherical in appearance with cellular components arranged along the cell periphery (91). Protoplasts may undergo wall rejuvenation, cell division, and differentiation to produce new plants under suitable conditions (86, 92). One of the greatest potentials of protoplasts is the production of intra- and inter-generic hybrids through cell fusion. Fusion of naked protoplasts from two tobacco species has been reported (93).

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Protoplasts may not be suitable for large scale generation, purification, and characterization of metabolites from xenobiotics at present. However, if the techniques for preparation of protoplasts are further improved and standardized, protoplasts may become another important tool for the pesticide chemist.

Protoplasts may be useful for studying physiological and biochemical effects of xenobiotics in plant cells. Oat mesophyll protoplasts incorporated uridine and leucine up to 6 hours and thymidine up to 21 hours. Kinetin inhibited leucine incorporation with increasing concentration of the growth hormone, but 2,4-D, abscisic acid and gibberellic acid had no effect on the same process (94). Uridine incorporation was inhibited by 2,4-D at concentrations above 0.1 μM. None of the growth hormones had any effect on thymidine incorporation (94). The differential susceptibility of resistant and susceptible oat varieties to the phytotoxin, victorin, was demonstrated with isolated protoplasts (95). The cytokinin-like action of methyl-2-benzimidazole carbamate (MBZ), the fungitoxic metabolite of benomyl [methyl-1-(butylcarbamoyl)-2-benzimidazole carbamate], was confirmed in oat protoplasts (96). The decrease in nuclease activity, increase in leucine incorporation and decrease in uridine and thymidine incorporation were similar in protoplasts treated with kinetin or MBZ (96). The effect of MBZ on the plasmalemma of protoplasts was not resolved.

Tomato fruit protoplasts absorbed and retained significant amounts of fluorodifen and trifluralin (70). The short-term structural integrity of the plasmalemma from tomato protoplasts was unaffected by fluorodifen, trifluralin, fluometuron [1,1-dimethyl-3-(α,α,α-trifluoro-*m*-tolyl)urea], and chlorbromuron. However, complete membrane breakage and collapse of the protoplasts occurred after 30 minutes of treatment with paraquat (1,1'-dimethyl-4,4'-bypyridinium dichloride) (97).

The protoplasts have not been used to investigate metabolism of xenobiotics. However, it appears to be a convenient system to determine the metabolism of xenobiotics concurrently with a study on the phytotoxic and selective action of these compounds in plant cells.

Conclusion.

The survey of the literature on *in vitro* organ, tissue, and isolated cell techniques for xenobiotic metabolism in plants is not complete. Selected reports indicate that isolated plant systems may be used successfully by pesticide chemists to elucidate degradation pathways and interactions of xenobiotic compounds in plants.

Isolated plant systems are most useful for short-term investigations on metabolism, mechanism of action and selectivity.

One of the major advantages of the techniques discussed in this report is the ability to measure rapid biochemical and physiological changes in response to a chemical coupled to its metabolism under carefully controlled conditions. This may not be possible in whole-plant experiments. Reduced growth and other visible manifestations of injury in whole plants are usually much delayed secondary responses to a chemical. However, results from isolated plant systems must be carefully evaluated before they are extrapolated to intact whole plants. Also, conclusions based only on whole-plant experiments should be confirmed by in vitro techniques.

Abstract.

Whole plants have been used for xenobiotic metabolism and "terminal" residue studies. However, in vitro techniques using isolated leaves or roots, leaf discs or sections, and separated mesophyll cells have proven to be more useful for short-term investigations on xenobiotic metabolism, mechanism of action and selectivity. The in vitro techniques allow the treatment of large amounts of plant material with a minimum of chemical and facilitate rapid uptake of the chemical into plant tissues. The influence of root and leaf surface absorption is also circumvented by the use of in vitro techniques. Quantitative differences in xenobiotic metabolism between whole plants and isolated plant systems have been observed, but qualitative differences are not common. Results from isolated plant systems must be carefully evaluated before extrapolation to intact plants because of the unknown influence of senescence, absorption, and translocation on in vitro systems.

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2

Xenobiotic Metabolism in Higher Plants: In Vitro Tissue and Cell Culture Techniques

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Millions of pounds of xenobiotics have been applied to plants in our environment for the control of pests and plant growth. Some of these chemicals have recognized and well characterized biological effects on animals and plants, while other xenobiotics, such as oils, adjuvants, emulsifiers and inert materials, are applied in even greater quantity, but have been assumed to cause little ecological effect. It is important to understand what happens to all chemicals applied to our environment and to properly interpret the ecological significance of these chemicals and of their degradation products. If we cannot know this in detail, then a general knowledge of persistance and metabolic products is of importance. Since the target organism of these xenobiotics is often plants, it is of the utmost importance to understand the fate of these chemicals in plants. Ultimately, animals and even humans are exposed to these chemicals and/or their subsequent metabolites and degradation products. Most investigations of xenobiotic metabolism in plants have focused on biologically active pest control chemicals. Thus, this review will also focus primarily on plant metabolism of pesticides.

There are many ways to study the metabolism of xenobiotics by plants, but whatever technique is employed, it should predict what would actually happen under field conditions. Metabolism studies have involved whole plants, excised plant parts (meristems, shoots, stems, leaves, roots, leaf disks), plant cell cultures, subcellular particles, and isolated enzymes. Any metabolism study conducted in the laboratory is less than ideal because it is difficult to duplicate many factors that affect the degradation of xenobiotics under field conditions such as weather, light, microsymbionts, soil or method of application. Metabolism of xenobiotics by plant tissue culture offers the obvious advantages of sterility, space, economical use of labeled chemicals, less pigments, etc. These advantages will be discussed in detail later.

The metabolism of xenobiotics by plant tissue culture (1) and the metabolism of endogenous and exogenous chemicals (2) have been reviewed recently. Within the last few years, many new

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investigations of xenobiotic metabolism by plant tissue cultures have been reported. This review will focus on these more recent papers with an emphasis on pesticide metabolism. We will attempt to evaluate and compare the results obtained with tissue culture techniques versus those obtained with whole plants and to explore the many factors (limitations) that affect metabolism studies with plant tissue culture.

History and Principles of Plant Tissue Culture

Plant tissue culture refers to the growth of relatively undifferentiated plant cells or differentiated organs on solid or liquid nutrient medium. The undifferentiated plant tissue growing on solidified medium is usually referred to as a callus culture since they are frequently obtained from cut or wounded surfaces and maintain the appearance of wound callus tissue. When such tissues are placed in liquid medium with shaking, many small aggregates of cells and even some single cells may be obtained. Subcultures of small aggregates or cell clumps in liquid culture are usually designated as suspension cultures. The culture of excised differentiated organs is, of course, organ culture. Frequently, callus cultures will differentiate with the formation of xylem elements and sometimes buds and/or roots. In many callus cultures with this potential usually a high kinetin/auxin concentration ratio in the medium favors bud formation while the reverse favors root formation. In a few cases, by proper manipulation of the medium, thousands of pseudoembryos can be induced and grown into normal plants of the same genotype.

Although the culture of plant tissues was attempted in 1902 (3), success was not achieved until White cultured excised tomato roots in 1934 and tobacco callus in 1939 (4). Gautheret and Nobécourt also described culture of plant callus tissue at about the same time (5, 6). White and Gautheret both developed nutrient media that are used widely today (5, 7). In addition to essential salts and sucrose, White added thiamine, nicotinic acid, pyridoxine and glycine while Gautheret added thiamine, pantothenic acid, biotin, inositol and cysteine. It appears that only thiamine is essential; but, in some cases, better growth may be obtained by the addition of other vitamins. White did not add an auxin for culture of excised root organ cultures or tobacco tumor tissue, but Gautheret added naphthaleneacetic acid (NAA). Van Overbeek et al. (8) introduced the use of coconut milk and much later Miller et al. (9) found that kinetin was necessary to culture tobacco stem pith. In general, excised root organ cultures, tumor and some callus cultures (so called habituated) do not require either auxin or kinetin. Some tissues do not require kinetin especially if 2,4-dichlorophenoxyacetic acid (2,4-D) is used as the auxin (10). The addition of coconut milk is usually not essential, and a requirement for gibberellic acid has been reported rarely. It is probable that adaptation to the medium

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and the selection of tissue that grows well on a particular medium accounts for the wide variation in nutrient requirements for a tissue such as tobacco callus. Other media used widely for plant tissue culture include those of Murashige and Skoog (11), Nitsch and Nitsch (12) and Gamborg (13). In addition, some media formulations are now available from a commercial source (Flow Laboratories, P. O. Box 2226, 1710 Chapman Ave., Rockville, MD 20852).

In theory callus may be derived from any plant tissue containing parenchyma cells. Some species form callus readily and others do not. Tissue sterilization may be accomplished with 70% alcohol (1-2 min dip) and/or a similar treatment with 1 to 5 or 1 to 10 diluted commercial bleach (0.5-1% sodium hypochlorite) containing 0.1% Tween 20. In either case, the tissue is rinsed 2-3 times in sterile water. Seeds are germinated in sterile petri dishes and bits of root or stem tissue are transferred to solidified agar medium containing relatively high auxin levels (0.5-1.0 mg/l NAA or 2,4-D). Sterilized tissue from bud, leaves or stems may also be used. Formation of enough callus to subculture may vary from 2 weeks to 2 months. Considerable variation in appearance and texture between callus pieces from the same source may be observed. Three to 4 bits of callus (5-7 mm in dia.) are transferred to solidified agar medium (50 ml in a 125 ml Erlenmeyer flask). Difficult tissues may require the addition of 50 ml of autoclaved-filtered coconut liquid and/or 2 g of casein hydrolysate per liter. Growth rates vary, but it is convenient to subculture bits of callus onto fresh medium every 4-6 weeks. Temperature and light requirements may not be critical for most tissues. High temperatures (30°C or more) have caused the loss of kinetin dependence of tobacco callus (14) and inhibited growth. We have maintained cultures under continuous low level fluorescent light at 27°C.

Simple equipment is needed for plant tissue cultures; a temperature controlled incubator or culture room, an autoclave, and a shaker, if suspension cultures are grown. It is also desirable to have a sterile transfer hood.

The genetic and physiological stability of some plant tissue cultures is of concern. Tissue from the same original culture may change in appearance and growth rate over a period of time. It is commonly observed that the ability to regenerate normal plants by the formation of buds and roots is lost with time. It may even be necessary to reisolate callus from the same source, if the callus tissue appears atypical or slow growing. A low growth rate is observed frequently in August or September. Variations in growth rate and tissue appearance may be important factors in metabolism studies and should be examined carefully. A mitotic index at two weeks after transfer may give a good indication of growth rate (15) or fresh weights at the end of 4 or 5 weeks may be used. The source of callus tissue (root, stem, leaf, cotyledons, etc.) also may influence metabolism. The appearance of soybean cotyledon, leaf and root callus was similar and limited

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work on 2,4-D metabolism indicated no major qualitative differences (16). Of much more importance was the age or stage of growth of the cultures (15). Most callus cultures exhibit a lag in growth for 4-7 days after transfer, a log growth phase followed by a slower growth phase after 4-5 weeks. Cultures 5-9 weeks old are more active in metabolism of 2,4-D than 3 week old cultures (15). It should be noted that prolonged pesticide treatment of older callus in fresh medium may put the tissue into log phase growth again with probable changes in metabolism.

Metabolism of Xenobiotics by Plant Tissue Culture

Most investigations with plant tissue cultures and xenobiotics have been concerned with pesticide metabolism. Tables I and II illustrate the variety of herbicides, insecticides, and plant tissue cultures that have been used in metabolism studies. The metabolism of lindane probably represents one of the more extreme cases where fourteen different plant tissues were used. As is evident, the source of the plant tissue culture, the type of culture (suspension or nonsuspension) and the media used also varied. This variation in plant cultures and techniques makes it extremely difficult to critically compare metabolism studies. As will be pointed out later, there are also many other factors that affect metabolism studies with plant tissue cultures.

HERBICIDES

It is apparent that herbicides exert toxic or physiological effects on sensitive plant species and that some physiological effects can be expected on tissue cultures of these species. An exception may be photosynthetic inhibitors. Due to the sugar in the plant tissue culture medium, photosynthetic inhibitors should not be strongly inhibitory unless they have secondary sites of action. If tolerance is due to metabolism, this might be expected to lead to qualitative and quantitative differences in metabolism by plant tissue cultures from susceptible and resistant varieties or species. Of course, such tissue cultures should also show differences in actual tolerance.

The metabolism of the herbicide fluorodifen (p-nitrophenyl α,α,α-trifluoro-2-nitro-p-tolyl ether) has been investigated (21) with tobacco cells in suspension culture (42). The cells were incubated for 15 days with 1-2 ppm of either $^{14}C_1$- or $^{14}CF_3$-labeled fluorodifen. All of the applied fluorodifen was metabolized. Recovery of added radioactivity varied between 52 and 76%. The cells contained 60 to 80% of the recovered radioactivity and the remainder was found in the medium and cell wash. With $^{14}C_1$-labeled fluorodifen, 4-nitrophenol (7%) was isolated only from the medium. Aqueous-soluble conjugated forms of 4-nitrophenol (93%), primarily the β-D-glucoside and other acidic conjugates, were present in both the cells and the medium as summarized in Figure 1.

Table I. Metabolism of Herbicides by Plant Tissue Cultures.

Herbicide	Plant Tissue Culture: Species	Source	Medium[a] and Culture Type[b]	Reference
Cisanilide	Carrot (*Daucus carota* L.) Cotton (*Gossypium hirsutum* L.)	Leaves	B5, S	17, 18
Diphenimid	Soybean (*Glycine max* [L.] Merr. 'Wilkin')	Root tips	B5, S	19
Propanil	Rice (*Oryza sativa* L. var. Starbonnet)	Root	H, S	20

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Table I - page 2

Compound	Plant	Tissue	Culture	Reference
Fluorodifen	Tobacco (Nicotiana tobacum L. var Xanthi)		M, S	21
Metribuzin	Soybean (Cultivar Bragg)	Cotyledon	X, S	22
	Soybean (Cultivar Coker 102)	Cotyledon	X, S	
2,4-D	Soybean (*Glycine* max L. var Mandarin)	Root	B5	23
	Soybean (*Glycine* max L. var Acme)	Cotyledon	M, C	24, 25, 26, 27
	Jackbean (*Canavalia ensiformis*)	Pod	M, C	26, 27
	Sweet corn (*Zea mays*)	Endosperm	M, C	27
	Tobacco (*Nicotiana tobacum*)	Pith	M, C	27
	Carrot (*Daucus carota* var Sativa)	Pith	M, C	27
	Sunflower (*Helianthus annus*)	Pith	M, C	27
	Rice (*Oryza sativa* var Starbonnet)	Root	M, C	28
	Wheat (*Triticum monococcum* L.)		B5, S	29
	Field bindweed (*Convolvulus arvenis* L.)	Stem	X, C	30

Table I - page 3

Compound	Plant	Tissue	Media, Culture type	Reference
2,4,5-T	Soybean (*Glycine* max L. var Acme)	Cotyledon	M, S	31
IAA	Geranium (*Pelagonium hortorum* var Nittany Red)	Stem	W, C	32
	Boston ivy (*Parthenocissus tricuspidata*)	Crown-gall	W, C	33
	Apple (East Malling rootstock 3430)	Bark	W, C	34, 35
	Shamouti orange (*Citrus sinensis* Osb.	Ovular	MS, C	36

[a]Basic media: B5 - Gamborg's; H - Heller's; M - Miller's; MS - Murashige and Skoog's; W - White's; X - Others.

[b]Culture type: S - Suspension; C - Callus.

Table II. Metabolism of Insecticides by Plant Tissue Cultures.

Insecticide	Plant Tissue Culture			
	Species	Source	Medium[a] and Culture Type[b]	Reference
Carbaryl	Tobacco (*Nicotiana tobacum* L. var Xanthi)		M, S	37, 38
Lindane	Purple cockle (*Agrostemma githago* L.)		?, S	39
	Soybean (*Glycine max*)			
	Bedstraw (*Galium verum*)			
	Carrot (*Daucus carota*)			
	Clover (*Meliotus alba*)			
	Tobacco (*Nicotiana tobacum*)			
	Tobacco (*Nicotiana glutinosa*)			
	Tobacco (*Nicotiana sylvestris*)			
	Tobacco (*Nicotiana glauca*)			
	Lettuce (*Lactuca sativa*)			
	(*Beta vulgaris*)			
	Parsley (*Pstroselinum hortense*)			
	Potato (*Solanum tuberosum*)			

Table II - page 2

Aldrin	Bean (*Phaseolus vulgaris* var *Canadian Wonder*)	Roots & Shoots	MS, S	40
	Potato (*Solanum tuberosum* var Majestic)	Tuber	W, S	40
p,p'-DDT	Parsley (*Petroselinum hortense*, Hoffm.)		B5, S	41
	Soybean (*Glycine* max L.)			
Kelthane (Ascaroside)	Parsley (*Petroselinum hortense*, Hoffm.)		B5, S	41
	Soybean (*Glycine* max L.)		B5, S	41

[a]Basic media: M - Miller's; W - White's; MS - Murashige and Skoog's; B5 - Gamborg's; X - Other.

[b]Culture type: S - Suspension; C - Callus.

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Figure 1. Metabolism of fluorodifen by tobacco cell in suspension culture

These data from plant tissue culture studies are consistant with results obtained with whole plants where the glucoside of 4-nitrophenol has been reported as the major product of soybean and maize seedlings (43, 44). Whole plant metabolism studies with fluorodifen (43, 44) suggested that a small percentage of the applied pesticide may be reduced to 4-aminophenyl 2-amino-4-(trifluoromethyl) phenyl ether, 4-nitrophenyl 2-amino-4-(trifluoromethyl) phenyl ether or 4-aminophenyl 2-nitro-4-(trifluoromethyl) phenyl ether, but none of these compounds, including 4-aminophenyl, was detected in the tobacco tissue culture studies.

Propanil (3,4-dichloropropionanilide) is an herbicide used to control weeds in rice. The resistance of rice to propanil is attributed to the high levels of an arylacyl amidase (propanil amidase) that hydrolyzes propanil to 3,4-dichloroaniline and propionic acid. The enzymatic level of propanil amidase in rice plants and in rice root suspension cultures has been investigated (20, 45). The activity of the enzyme was found to be two to four times greater in older plants (four leaves) than in younger plants (less than four leaves). Propanil amidase was also demonstrated in the rice suspension culture, but interestingly, the enzymatic activity could only be demonstrated after the tissue culture had developed to stationary phase (5.5 days). This investigation is important because it documents a change in the biosynthetic capacity of plant tissue cultures with the age of the culture. The dependency of several enzymes, including phenylalanine ammonia lyase, upon illumination of parsley cell cultures also has been shown (46).

The metabolism of diphenamid (*N*-*N*-dimethyl-2,2-diphenylacetamide) by soybean root tip cell suspension cultures has been

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investigated (19) at different stages of growth and compared with whole plants. The metabolites found in both plants and suspension cultures were N-hydroxymethyl-N-methyl-2,2-diphenylacetamide (MODA), N-methyl-2,2-diphenylacetamide (MMDA), 2,2-diphenylacetamide (DA) and two polar glycosides. One of the glucosides was acidic and was identified as an ester of malonic acid (Figure 2).

Figure 2. Metabolism of diphenamid by soybean root cells in suspension culture

About 9-22% of the diphenamid was metabolized by the cell cultures at early log (3-7 days) and stationary phases (14-18 days), respectively. However, diphenamid metabolism per gram was about 2 times more rapid by early log phase cells than by stationary cells. Cultures of all ages formed the same metabolites with MODA and the dealkylated products predominating. The relative composition of the metabolites is presented in Tables III and IV. The hydroxylated metabolite, MODA, was found almost exclusively in the medium 92-99%, and the dealkylated products were found predominantly in the medium (68-94%).

Log phase and stationary phase cells produced larger amounts of the dealkylated metabolites per mg dry weight per day than did early log phase cells. Glycosides consisted of only 6-7% of the total metabolites in cell cultures but were the major metabolites (46-48%) of tomato, pepper and soybean plants.

These cell suspension cultures demonstrate clearly the same metabolic degradation pathways as intact plants, but significant quantitative differences do occur especially in the small amount of glycoside formation by the soybean root suspension cells. No qualitative changes occurred in the metabolism of diphenamid with age of the culture.

The metabolism of cisanilide (cis-2,5-dimethyl-1-pyrrolidinecarboxanilide), a selective preemergence herbicide, has been investigated in excised leaves and cell suspension cultures of

Table III. Diphenamid Metabolism by Growth Phase of Soybean Cell Suspension cultures (19)

Growth Phase	Diphenamid or Metabolite	nmol per Gram Cells	
		Cell Extract	Medium
Early Log (3-7 Days)	Diphenamid	12.8	444.8
	MODA	0.1	31.6
	Dealkylated	0.6	10.8
	Glucoside	0.6	1.8
Log (7-14 Days)	Diphenamid	41.9	99.4
	MODA	2.2	27.4
	Dealkylated	10.6	27.8
	Glucoside	1.9	1.1
Stationary (14-18 Days)	Diphenamid	42.5	144.3
	MODA	1.2	25.4
	Dealkylated	6.7	14.5
	Glucoside	0.9	1.9

Table IV. Relative Composition of Diphenamid or Metabolites in Cells and Medium in Early Log Phase (19)

Diphenamid or Metabolite	% Cell Extract	Medium
Diphenamid	2.8	97.2
MODA	0.3	99.7
Dealkylated	5.5	94.5
Glucoside	25.0	75.0
Acidic Glucoside	100.0	0.0

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carrot and cotton (17, 18). After 6 days, excised carrot leaves metabolized ca. 70% of the applied cisanilide, but carrot tissue cultures metabolized >95% of the applied herbicide in 3 days. Cotton leaves metabolized cisanilide to a greater extent than cotton suspension cultures. The metabolism of cisanilide by carrot and cotton plants is shown in Figure 3 and the relative composition of metabolites in plants and tissue culture is presented in Table V.

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Figure 3. Metabolism of cisanilide by carrot and cotton plants and cells in culture

In the excised plants, glycosides I and II were the major methanol soluble metabolites and only trace quantities of the aglycons were detected. In carrot and cotton cell suspension cultures, aglycon I or glucoside I was not detected and only trace amounts of glycoside II were detected. Aglycon II was a major metabolite in the medium of the cell suspension cultures. In the excised plants, less (20-25%) of the ^{14}C-label was found in the insoluble residue fraction compared to the cell suspension cultures (39-40%).

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Table V. Relative Composition of Cisanilide Metabolites (18).

Excised Carrot Leaves (6 Days)		Carrot Suspension Culture (3 Days)[a]	
Fraction	%	% Cells	% Medium
Total Metabolized	70	>95	
Methanol Soluble	50 (Glycoside I=II)	--	40 (Aglycon II)
Methanol Soluble (Unknown)	10	20	--
Insoluble Residue	20	40	

Excised Cotton Leaves (2 Days)		Cotton Suspension Cultures (7 Days)	
Fraction	%	% Cells	% Medium
Total Metabolized	>95	>76	
Methanol Soluble	55 (Glycosides I>II)	--	24 (Aglycon II)
Methanol Soluble (Unknown)	25	13	--
Insoluble Residue	18	39	

[a]Glycoside I was not detected and glycoside II was tentatively identified as a minor component.

At first approximation, it would be easy to conclude that the metabolic degradation pathways were different in the excised plants versus the cell suspensions since aglycon I and glucoside I were not detected in the tissue or the medium. This difference may indicate rapid incorporation of aglycon I into a methanol-insoluble fraction, because when aglycon I was administered to the cell suspension culture, it was rapidly converted to methanol insoluble products. Neither tissue is able to cleave appreciably the urea type structure of cisanilide. This is also the case for the metabolism of other urea herbicides by plants (43). Both tissues possess the ability to form the hydroxyl derivative, aglycon II, but the cell cultures evidently have a reduced capacity to form the glycoside conjugate and consequently aglycon II accumulates in the media. This hydroxylated metabolite is apparently not incorporated into the methanol insoluble product(s). Thus, the metabolic pathways may be somewhat similar except for loss of aglycons into the medium and the increased formation of an insoluble product.

The phytotoxicity and metabolism of the herbicide metribuzin (4-amino-6-*t*-butyl 3-[methylthio]-*as*-triazin-5-[4H]-one) has been investigated with dark-grown soybean cotyledon suspension cultures from susceptible ("Coker 102") and resistance ("Bragg") cultivars. Bioassays were based on population changes of viable cells during incubation with metribuzin. Viable cells were classified as cells with structural integrity and cytoplasmic streaming. Differential resistance to metribuzin was demonstrated by the cell suspensions from resistant and susceptible cultivars. Metribuzin had been reported to inhibit photosynthesis, but the demonstrated phytotoxicity toward both cultivars of dark-grown achlorophyllous suspension cultures indicated that phytotoxicity was not restricted to photosynthesis. Detoxification of metribuzin by soybeans has been attributed to formation of an N-glucoside. Enzymatic detoxification of metribuzin did not occur in the susceptible cultivar due to the accumulation of a substance which inhibited the enzyme. The resistant cultivar metabolized the inhibitor to a noninhibitory form. Therefore, metribuzin resistance by the Bragg cultivar was attributed to the ability of this cultivar to metabolize a common enzymatic inhibitor.

Plant tissue culture techniques have been used by numerous investigators (23 - 29) for 2,4-D metabolism studies. In 1968, the metabolism of 2,4-D-2-^{14}C by suspension cultures of soybean root grown under continuous light (2000 lux) was examined (23). Most of the ^{14}C-label in the tissue appeared as two spots on paper chromatography. The faster moving compound has the same Rf as 2,4-D and was assumed to be free 2,4-D. The slower moving spot was a glycoside that yielded glucose and free 2,4-D when treated with emulsin. These investigators assumed that 2,4-D was metabolized only to the β-D glucose ester of 2,4-D. Presumably, the amino acid conjugates, which have been subsequently identified as metabolites of 2,4-D, did not separate from 2,4-D in the

chromatographic solvents used. The addition of glutamine to the media increased the uptake of 2,4-D, but did not change the apparent metabolic products when analyzed by their chromatographic solvent systems. Some 2,4-D was also associated with protein.

Two clones of field bindweed (Convolvulus arrensis L.) that differed in their susceptibility to 2,4-D under field and greenhouse conditions also exhibited similar differences when stem cells were cultured in liquid and agar media (30). When amino acids were added to the culture media, the response to 2,4-D was altered. The absorption of 2,4-D was increased with glutamine and decreased with glutamic acid. Glutamic acid increased the tolerance of the susceptible clone, but reduced the tolerance of the resistant clone. Glutamine increased the susceptibility of the susceptible clone to a much greater degree than it did the resistant clone. There was a correlation between 2,4-D susceptibility and nitrate reductase activity.

When soybean (Glycine max L.) cotyledon callus was incubated for 32 days with 2,4-D-1-^{14}C, metabolites changed qualitatively and quantatively with time (24). The water soluble fraction from the tissue increased in radioactivity. When it was treated with β-glucosidase, at least eight aglycons that changed with time were released (Fig. 4). 4-Hydroxy-2,5-dichlorophenoxyacetic acid (4-OH-2,5-D) was the most abundant aglycon and 4-hydroxy-2,3-dichlorophenoxyacetic acid (4-OH-2,3-D) was identified as a minor component. Free 2,4-D also was liberated following enzymatic treatment. The presumed presence of 2,4-dichlorophenoxyacetyl-β-O-D glucose, a metabolite reported previously (23), was suggested.

The ether soluble fraction reached a maximum after 2 days and consisted of seven different regions of components on paper chromatography (Et_1-Et_7) that varied with time (Fig. 5). The major component (Et_4) was identified as the glutamic acid conjugate of 2,4-D and its relative composition was maximal after one day. The aspartic acid conjugate of 2,4-D (Et_2) increased gradually in relative composition. Surprisingly, free 2,4-D (Et_7) did not reach its maximum until eight days. These data imply that contrary to some previous studies, the metabolism of 2,4-D by plant tissue is quite complex. Subsequently, five additional amino acid conjugates of 2,4-D have been identified from soybean (27). These include the alanine, valine, leucine, phenylalanine and tryptophan conjugates. A typical distribution of 2,4-D metabolites isolated from 4-week-old callus tissue is presented in Table VI.

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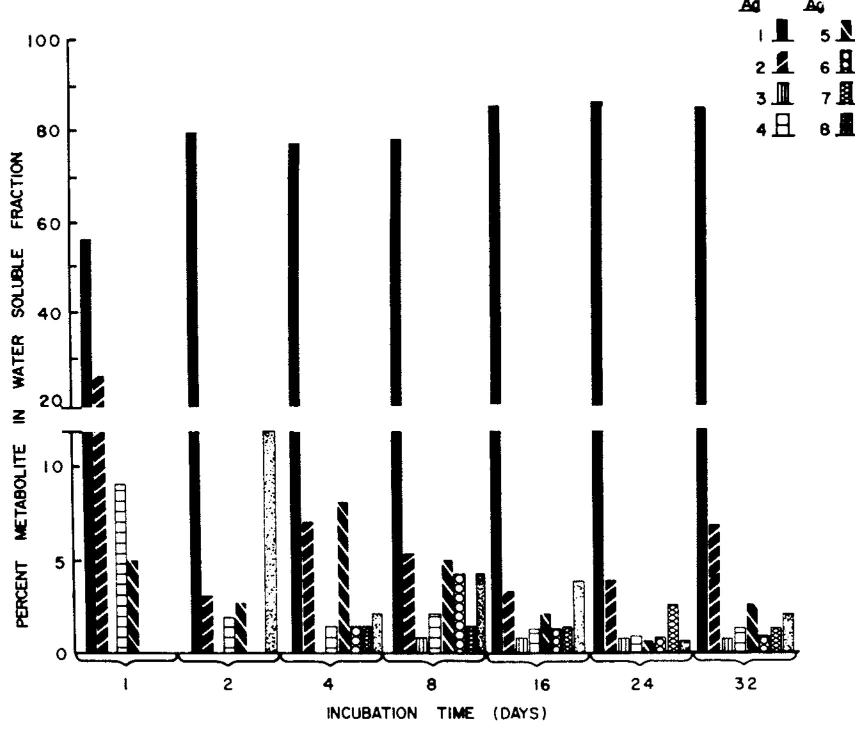

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Figure 4. The relative amounts of the aglycons obtained from the water-soluble fractions of soybean callus tissue incubated with 2,4-D-1-^{14}C: (Ag_1), primarily 4-OH-2,5-D and 4-OH-2,3-D; (Ag_7), 2,4-D.

Table VI. Relative Percentage of 2,4-D Metabolites in Soybean Callus Tissue[a] (27).

Ether-Soluble Metabolites		Aglycons (emulsin)	
Metabolite	% In Tissue	Metabolite	% In Tissue
Unk	1.2	(4-OH-2,5-D, 4-OH-2,3-D)	26.3
2,4-D-Asp	3.7	Unk	2.5
2,4-D-Gly	12.9	Unk	1.1
Unk	1.4	Unk	1.0
2,4-D-Ala,-Val)	5.3	Unk	0.8
2,4-D	33.7	Unk	0.9
2,4-D-Leu,-Phe -Try)	4.0	2,4-D	0.8
		Unk	0.4
TOTAL	62.2%		33.8%

[a]Four-week-old callus tissue (10g) incubated for 8 days with 2,4-D.

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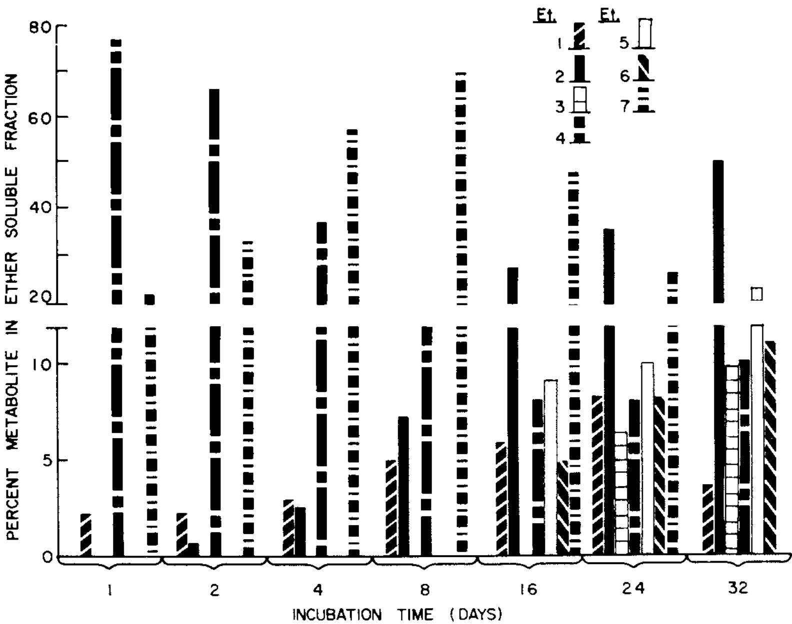

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Figure 5. Relative amounts of ether solubles isolated from soybean callus tissues incubated with 2,4-D-1-^{14}C: (Et_2), aspartic acid conjugate; (Et_4), glutamic acid conjugate; and (Et_7), free 2,4-D.

Additional minor aglycons also have been identified tentatively from corn endosperm callus as 3-hydroxy-2,4-dichlorophenoxyacetic acid (3-OH-2,4-D), 4-hydroxy-2-chlorophenoxyacetic acid (4-OH-2-Cl) (27), and from wheat suspension cultures as 6-hydroxy-2,4-dichlorophenoxyacetic acid (6-OH-2,4-D) and 2-hydroxy-4-chlorophenoxyacetic acid (2-OH-4-Cl) (29). The ethyl ester of 2,4-D has been isolated from the glycoside fraction of rice callus tissue culture following β-glucosidase treatment (28). The ethyl ester was presumed to be an artifact of the isolation procedure probably being derived from the glucose ester that exists in high concentration in this tissue.

The glutamic acid conjugate of 2,4-D is not an end product of 2,4-D metabolism (25). When ^{14}C-2,4-D-glutamic acid was incubated with soybean callus tissue, free 2,4-D, the aspartic acid conjugate, and other products were found. These data suggest that amino acid conjugates may represent a reservoir of bound 2,4-D

that may be involved in the regulation of 2,4-D levels in the tissue.

Comparative metabolism studies of 2,4-D in carrot, jackbean, sunflower, tobacco, corn and rice callus tissue cultures (27, 28) and a wheat suspension culture (29) are shown in Tables VII and VIII. All tissues examined, except rice, formed amino acid conjugates and all formed glycosides (phenolic glycosides as well as glucose esters). The dicots formed a higher relative percentage of amino acid conjugates while the monocots produced a higher percentage of glycosides.

The metabolism of 2,4-D in soybean and corn plants has been compared with 2,4-D metabolism in soybean callus tissue (26). These data are presented in Tables IX and X. In this experiment, the 2,4-D-1-^{14}C was directly injected into the callus tissue growing on agar rather than into fresh liquid medium with suspended callus. The callus, therefore did not revert into log phase growth and its nutrient status was not changed. The metabolites found in the plants are also present in the callus tissue, but differ quantitatively. Of particular note is the fact that free hydroxylated 2,4-D exists in both callus and in plants in contrast to earlier tissue culture experiments. Free hydroxylated 2,4-D also has been reported in bean plants (48). These experiments also suggest that the unknown compounds, Unk_1 and Unk_2, are amino acid conjugates of hydroxylated 2,4-D metabolites (primarily the glutamic acid conjugate of 4-OH-2,5-D) and are common to all tissue examined. A summary of the metabolism of 2,4-D in plants is presented in Figure 6.

Recently, the metabolism of 2,4-D has been reported in six intact plants: wheat, timothy, green bean, soybean, sunflower and strawberry (47). The relative percentage of amino acid conjugates is low compared to the previously cited work with tissue culture experiments (Table XI). Of particular notice is the unusually high percentage of the dichlorophenol glycoside in strawberry.

In contrast to use of true suspension cultures by most other laboratories, Feung et al. (24-28) usually incubated a fairly large amount (~10 gms) of small pieces of 4-5 week old callus taken from solid medium in 25-40 ml liquid medium with shaking during treatment with 2,4-D-1-^{14}C. This technique results in nearly total uptake of the applied 2,4-D within 48 hours, the accumulation of significant amounts of glycoside metabolites in the tissue (phenolic glycosides and glucose ester), insignificant amounts of primary hydroxylated products and no accumulation of products in the medium. In cell suspension cultures, absorption also is rapid but metabolites often accumulate in the medium. In the study (26) where callus tissue was injected directly with 2,4-D-1-^{14}C, additional metabolites were found. Thus, the method of pesticide administration may be important.

Data with intact plants (Tables IX, X and XI) are consistant with metabolism data obtained with plant tissue cultures, but significant quantitative differences do occur. The monocots

Table VII. Relative Percentage of Water-Soluble 2,4-D-1-^{14}C Metabolites Isolated from Seven Species of Plant Tissue Cultures as the Aglycons.

	% Total in Tissue[a]						
Metabolites	Carrot	Jackbean	Sunflower	Tobacco	Corn	Rice	Wheat
(4-OH-2,3-D, 4-OH-2,5-D)	6.9	6.9	8.3	17.4	31.7	0.4	↑
Unk	3.2	2.6	4.1	8.3	19.4		
Unk	0.7	0.4	0.8	2.0	0.5		
Unk	0.4	0.3	0.5	1.9	1.2		10.2
Unk	0.1	0.3	0.8	2.3			
Unk					1.6		↓
Unk	0.9	1.1	0.6	2.6			
2,4-D	1.0	1.2	3.2	7.7	10.2	14.9	23.9
Ethyl-2,4-D						12.9	
Others						1.5	
TOTAL	13.2	12.8	18.3	42.2	64.6	29.7	

[a] All were callus tissue cultures (27, 28) except wheat (29).

Table VIII. Relative Percentage of Ether-Soluble 2,4-D-1-^{14} Metabolites Isolated from Seven Species of Plant Tissue Cultures as the Aglycons.

Metabolites	% Total in Tissue[a]						
	Carrot	Jackbean	Sunflower	Tobacco	Corn	Rice	Wheat
Unk					1.9		↑
Unk					1.7		
2,4-D-Asp		1.2		0.8	1.6		
2,4-D-Glu	23.8	32.5	5.0	6.7	1.3		21.0
Unk	0.8	1.1	0.7	1.8	3.2		
Unk	5.8	5.2	5.7	5.5	0.8		↓
2,4-D	51.7	45.4	51.7	34.3	12.5	14.7	15.7
Unk	1.7		15.3	4.5	2.6		
Others						1.2	
TOTAL	83.8	85.4	78.4	53.6	25.6	15.9	36.7

[a]All were callus tissue cultures (27, 28) except wheat (29).

Table IX. Relative Percentage of Water-Soluble 2,4-D-1-^{14}C Metabolites Isolated from Soybean Callus, Soybean Plant, and Corn Plant as the Aglycons (26).

	% In Tissue		
Metabolites	Soybean Callus	Soybean Plant	Corn Plant
Unk	5.18	0.39	0.94
Unk	1.67		
Unk	1.79	0.15	0.73
(4-OH-2,3-D* 5-OH-2,4-D)	8.27	1.47	2.45
4-OH-2,5-D	16.28	3.20	1.67
2,4-D	4.02	9.05	24.15
Unk	0.94	2.46	1.64
Ethyl-2,4-D		1.22	7.70
TOTAL	38.15	17.94	39.28

*Major metabolite.

Table X. Relative Percentage of the Ether-Soluble (at pH 2) 2,4-D-1-^{14}C Metabolites Isolated from Soybean Callus, Soybean Plant, and Corn Plant (26).

	% In Tissue		
Metabolites	Soybean Callus	Soybean Plant	Corn Plant
Unk	0.50	3.14	Trace
(5-OH-2,4-D 4-OH-2,3-D* 4-OH-2,5-D*)	1.48	3.50	7.08
Unk_1	9.59	8.87	0.69
Unk_2	1.49	1.94	0.25
Asp-2,4-D	0.62	Trace	0.16
(Asp-2,4-D Glu-2,4-D*)	11.15	4.02	0.28
Unk	2.67	2.22	0.50
Unk	2.00	1.59	0.62
Unk	1.73	1.48	0.36
(Ala-2,4-D Val-2,4-D*)	4.07	1.37	0.86
(Val-2,4-D 2,4-D*)	11.91	43.55	29.60
(Phe-2,4-D) Trp-2,4-D Leu-2,4-D*)	0.51	1.63	5.60
TOTAL	47.72	73.31	46.00

*Major metabolite

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Figure 6. Summary of 2,4-D metabolism by plants (26)

Table XI. Radioactivity of 2,4-D and its Metabolites, Represented as Percentage of Total ^{14}C Absorbed, in Plants (47).

Fraction or Substance	Wheat	Timothy	Bean	Soy-bean	Sun-flower	Straw-berry
2,4-D	29.9	30.9	4.0	10.5	85.0	7.0
2,4-D-Asp and 2,4-D-Glu	0	0	3.2	5.6	1.5	0
4-OH-2,5-D and 4-OH-2,3-D	0.8	0	3.5	3.0	0	0
2,4-Dichlorophenoxy-acetyl/β-D-glucose	23.9	21.7	24.8	5.7	3.7	2.0
Glucosides of 4-OH-2,5-D and 4-OH-2,3-D	10.2	3.3	50.1	53.0	0	12.0
Glycoside of 2,4-Dichlorophenol	2.2	2.4	—	—	—	65.0
Unknown 2,4-D Metabolite	15.0	12.6	2.5	8.0	0	0

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consistantly produced large quantities of the glucose ester. Although the amino acid conjugates were present in the dicots, their concentration in intact plants was not as high as in plant tissue cultures.

The lack of substantial amounts of amino acid conjugates in the plants may be explained partially by recent investigations (15). In these studies, it was shown that the age of the callus tissue and the concentration of the applied 2,4-D greatly affected the metabolism of 2,4-D. In young soybean root callus tissue (3 weeks), 2,4-D was metabolized (2 days) to ether soluble metabolites (amino acid conjugates) and to aqueous soluble (ether insoluble) metabolites (glycosides). As the concentration of herbicide increased uptake into the ethanol fraction increased and the amount of free 2,4-D increases proportionately while only a slight increase in metabolites is observed (Fig. 7). In older soybean callus tissue (5-9 weeks), a major biochemical change occurs such that the level of free 2,4-D in the tissue is regulated at ca. 4 nm per gram fresh weight. As the concentration of 2,4-D administered was increased, a greater amount of 2,4-D was converted to amino acid conjugates so the level of free 2,4-D in the tissue was regulated (Fig. 8).

This same phenomena, the regulation of free 2,4-D levels through the formation of amino acid conjugates, was also demonstrated in soybean leaf callus tissue cultures. Although the regulation of the 2,4-D concentration within the tissue is controlled by the formation of the ether soluble metabolites, the aqueous soluble fraction is twice as big in leaf callus as it is in root callus tissue. Differentiated soybean root culture behaves biochemically like old callus tissue. The 2,4-D levels are regulated at ca. 2 nm per gram fresh weight (Fig. 9).

These experiments with 2,4-D have shown the importance of the types of tissue, the age of the tissue, the concentration of the pesticide, and the method of incubation used in plant tissue culture studies. Since each laboratory seems to adopt its own procedures, which may affect results, it is time to standardize some of the procedures used in tissue culture studies. Although the incubation of the pesticide with callus tissue cultures has been used only by a few laboratories (24-28, 31-36, 57), this technique should be examined in more detail with other pesticides to determine whether it may be more representative of whole plant studies.

The metabolism of 2,4,5-T by soybean root callus tissues has been examined recently (31). Studies showed that soybean callus tissue cultures also convert 2,4,5-T into amino acid conjugates (glutamic acid [90%] and aspartic acid [10%]). No glucose ester formation was demonstrated.

Although the metabolism of indoleacetic acid (IAA) by plant tissue cultures has not been studied extensively, the metabolic pathways appear similar to those of intact plants, or excised plant parts. One pathway is the peroxidase-IAA oxidase

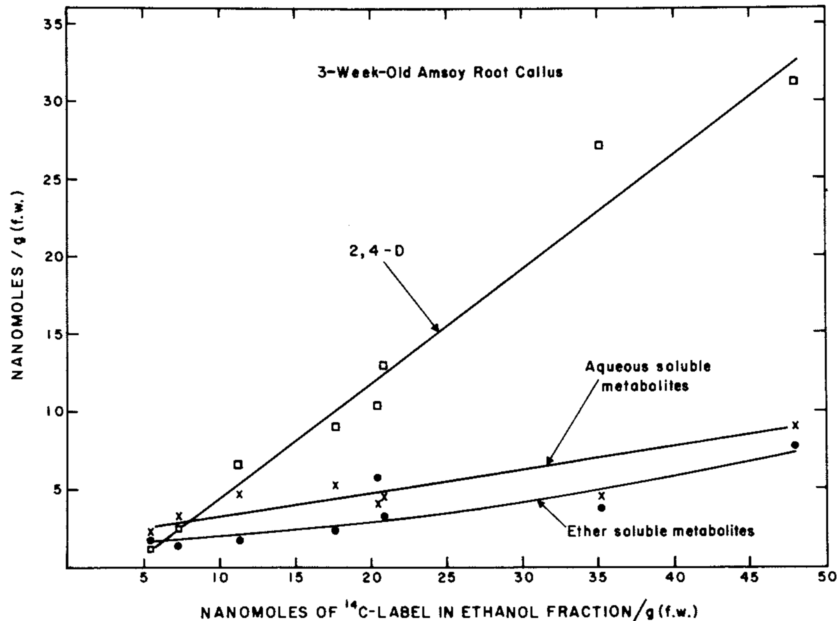

Figure 7. Concentration of 2,4-D or 2,4-D metabolites found in various subfractions vs. total concentration in the ethanol extract of 3-week-old soybean root callus tissue following incubation for 48 hr with various levels of 2,4-D-1-^{14}C (1.8 $\times$ 10^{-6} to 1.1 $\times$ 10^{-5}M). The free 2,4-D content was subtracted from the total ether-soluble metabolites (mostly amino acid conjugates).

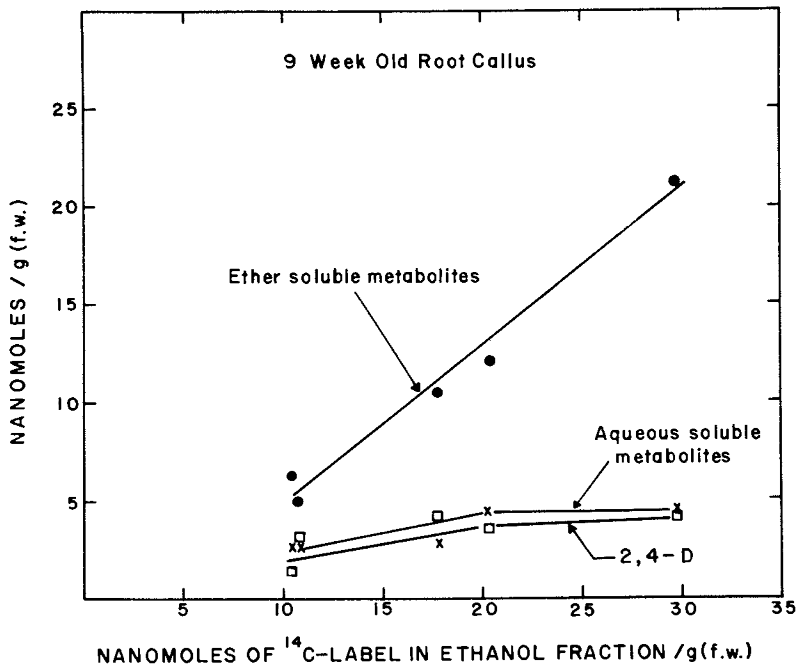

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Figure 8. Concentration of 2,4-D or 2,4-D metabolites found in various subfractions vs. total concentration in the ethanol extract of 9-week-old soybean root callus tissue following incubation for 48 hr with various levels of 2,4-D-1-^{14}C (1.8 $\times$ 10^{-6} to 1.1 $\times$ 10^{-5}M). The free 2,4-D content was subtracted from the total ether-soluble metabolites (mostly amino acid conjugates).

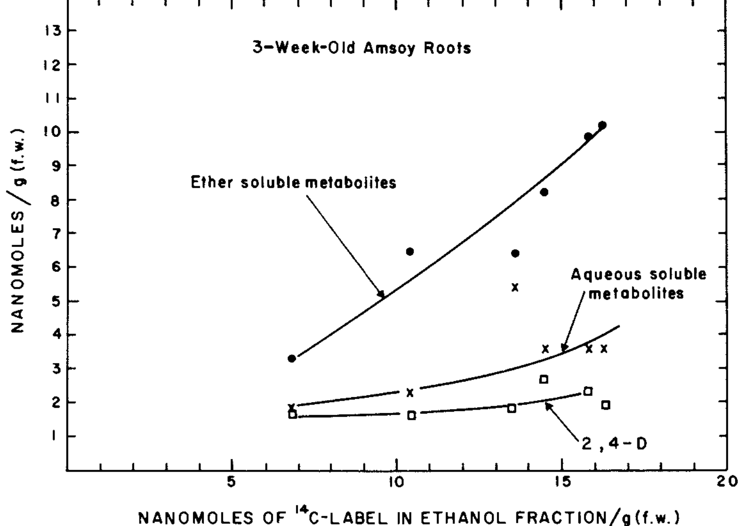

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Figure 9. Concentration of 2,4-D or 2,4-D metabolites found in various subfractions vs. total concentration in the ethanol extract of 3-week-old differentiated soybean roots following incubation for 48 hr with various levels of 2,4-D-1-^{14}C (9×10^{-7} to 2.3×10^{-6}M). The free 2,4-D content was subtracted from the total ether-soluble metabolites (mostly amino acid conjugates).

oxidation of IAA in the presence of Mn^{+2} and phenolic cofactors. The second pathway (in common with 2,4-D) is the conjugation of IAA with amino acids or sugars.

It now appears that peroxidases are as widely distributed in plant tissue cultures as they are in intact plants (49). These so-called isozymes that are observed on gel electrophoresis often change with the stage of development or hormonal treatment (49, 50, 51) of plants. Since genetic information is not available (52) and peroxidase contains carbohydrate (53), the term isozyme is probably a misnomer. These "isozymes" have varying activities on peroxidase substances or act as an IAA oxidase.

An IAA oxidase has been found in *Picea glauca* callus cultures (54) and the first extra-cellular IAA oxidase was detected in *Parthenocissus tricuspidata* crown gall cultures (55). An extra-cellular IAA oxidase and the products of an intracellular IAA oxidase from this tissue have also been characterized (56, 57). Although the enzymatic products were 3-hydroxymethyloxindole and ultimately its dehydration product 3-methyleneoxindole, neither metabolite was found in the tissue (57). However, small amounts of 3-methyloxindole were found in the medium in confirmation of its reported occurrence in pea seedlings (58). Extensive decarboxylation of IAA-1-^{14}C supplied to this tissue (50% in 24 hours) suggests the degradation of IAA by IAA oxidase and conversion of the oxindole to unidentified products. In 12 week old apple callus, 90% decarboxylation of IAA-1-^{14}C was found in 4 hours compared to 20% decarboxylation in 4 hours by 6 week old apple callus (35). Six week old apple callus accumulated elevated IAA levels in the tissue, and two metabolites, thought to be oxindoles, were detected in 12 week and 6 week old apple callus (34).

The conjugation of IAA to aspartic acid has been reported in geranium stem callus (32) and in embryogenic habituated ovular callus of orange (36) where it is suggested to maintain levels favorable to embryogenesis. The major conjugates found in *Parthenocissus tricuspidata* crown gall callus were the glycine, alanine, and valine conjugates (33). Minor amounts of the aspartic and glutamic acid conjugates also were found. Chromatographic separation of the glycine conjugate from IAA as well as the aspartic conjugate from the glutamic conjugate is difficult (59). Thus, the glutamic and glycine conjugates may have remained undetected in intact plants or excised tissue.

The glucose ester of IAA has been detected in many plant tissues (60, 61, 62, 63, 64), but does not appear to have been identified in plant tissue cultures.

INSECTICIDES

The metabolism of carbaryl (1-naphthyl methylcarbamate) by tobacco cells in suspension culture has been reported (37, 38). The first report (37) indicated that up to 16% of the total metabolites of carbaryl might be conjugates of N-hydroxycarbaryl

(1-naphthyl N-hydroxy-N-methyl carbamate) a potential mutagen. In a more thorough investigation (38) no N-hydroxy carbaryl or derivatives could be detected. In these later studies, ^{14}C-labeled C_1-naphthyl, carbonyl and N-methyl carbaryl (9 ppm) were incubated in the dark with tobacco cell suspensions for 14 days. The cells metabolized 36% of the carbaryl of which 7.9% represented nine metabolites. The only metabolite found in the medium was N-CH_2OH-carbaryl. It represented 18.38% of the metabolites. Free N-CH_2OH-carbaryl and its glycoside were found in the cells, but in very low concentrations. The major tissue metabolite was the β-D-glucoside of α-naphthol. This metabolite was found present only in trace amounts in intact bean plants (65). Small amounts of conjugated 4-OH-(0.3%), 5-OH-(0.3%) and 7-OH-(0.82%) carbaryl were also identified in the suspension cultures. These conjugates were the major metabolites in intact bean plants (65). Of considerable interest was the tentative identification of two new metabolites of carbaryl, 1,4-dihydro-1,4-epiperoxynaphthalene (0.39%) and O-1-naphthylcholesterol (3.0%), the latter metabolite being a unique ether. A summary of the metabolism of carbaryl by tobacco suspension culture is presented in Figure 10. The relative composition of metabolites in tobacco cell in suspension culture (38) and intact bean plants (65) are compared in Table XII.

Metabolites that have been identified in whole plants are also found in the tobacco tissue culture, but there is a large quantitative difference between bean plants and tobacco suspension cultures. A more detailed study of the metabolites found in intact plants of the same species should be made to see if any of the newly discovered metabolites found in tobacco cell suspension cultures also exist in intact tobacco plants. In bean plants, the glycosides of 4-OH, 5-OH and N-CH_2OH-carbaryl predominate. The primary hydroxylated product N-CH_2OH-carbaryl is perhaps excreted into the suspension culture media before the glycoside is formed. Strangely, the α-naphthol was found only as the glycoside in tobacco suspension culture cells. In contrast, the glycoside of N-CH_2OH-carbaryl was present in the cells in only minor amounts.

The metabolism of ^{35}Cl-labeled lindane (α-hexachlorocyclohexane) by various plant cell cultures has been compared to its metabolism in intact plants (39). With intact plants (28 day incubation), very little ^{35}Cl was liberated, but plant suspension cultures (12-28 days incubation) metabolized from 0% (soybean) to 6.8% (wild carrot) of the applied lindane. Trace quantities of 1,2,4-trichlorobenzene (1%) were identified in tobacco cultures, but were not detected in the other cultures. The main metabolite of carrot suspension cultures had not been reported previously in plants and was tentatively identified as a glucoside of trichlorophenol.

Tissue cultures showed a higher uptake and metabolic rate for lindane than intact plants. No degradation to CO_2 was detected, and lettuce plants were not able to metabolize lindane to pentachlorophenol as has been reported (66). These studies also showed

Table XII. Percentage of ^{14}C-Carbaryl Metabolites Isolated from Bean Plants and Tobacco Suspension Cultures.

Carbaryl or Metabolite	Percentage of ^{14}C-Labeled Compound			
	Bean Plants[a]		Tobacco Suspension Culture[b]	
	Ether Soluble	Conjugated (Aglycons)	Ether Soluble (Medium)	Conjugated (Aglycons) (Cell)
Carbaryl	+	1.0	64.72	0.
1-Naphthol	0	Trace	0	5.72
4-Hydroxy Carbaryl	0	33.0	0	0.02
5-Hydroxy Carbaryl	0	25.0	0	0.02
7-Hydroxy Carbaryl	0	0	0	0.06
N-Hydroxymethyl Carbaryl	0	18.1	1.47	0.20
Dihydrodihydroxy Carbaryl	0	15.6		

[a] Data taken from (65)
[b] Data taken from (38)

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Figure 10. The metabolism of carbaryl by tobacco cell suspension cultures

that intact plants were contaminated with epiphytic microorganisms that could metabolize lindane.

The metabolism of aldrin has been investigated in suspension cultures from bean roots, bean shoots and potato tubers (40). The effect of media variations on metabolism also were studied. In intact plants, aldrin was converted to dieldrin, photodieldrin, dihydrochlorodene dicarboxylic acid and aldrin trans-diol (Fig. 11). Aldrin (40%) was metabolized mainly to dieldrin by the plant suspension cultures (28 days). The dieldrin was located primarily in the cells. Aldrin trans-diol was also isolated in small amounts, but only in the bean shoot and potato tuber cultures.

Small quantities of photodieldrin were detected when larger quantities of aldrin (10 mg) were added to bean root cultures. Conversion of aldrin to photodieldrin was only found in mixed bean root and shoot cultures although it was formed from dieldrin in all cultures. These data support the direct formation of photodieldrin by plants in the dark rather than by a light induced photochemical reaction or microorganisms.

The metabolism of aldrin by plant tissue cultures is in good qualitative agreement with similar intact plant experiments, except that the dicarboxylic acid isolated in low levels from intact plants grown under field conditions was not detected in plant suspension cultures. Variation in the culture media affected the quantitative distribution of metabolites. Differences also existed between bean root and shoot cultures and between potato tuber cultures. Aldrin trans-diol was only found in cultures incubated with aldrin, never with dieldrin, and suggest that aldrin trans-diol is directly formed from aldrin.

The metabolism of DDT and Kelthane has been examined in cell suspension cultures of parsley and soybean (41). After 44-48 hr incubation, only 0.6 to 2.2% of the applied DDT was metabolized. DDE (1,1-dichloro-2,2-bis-[4-chlorophenyl]-ethylene) was identified as the major metabolite of DDT. Other metabolites were detected but were not identified. Parsley suspension cultures converted Kelthane to a nonpolar (0.1%) and a polar (0.3%) metabolite.

Phytotoxicity and Bioassays

A number of investigators (22, 67-74) have used plant tissue cultures as a means of studying phytotoxicity. These investigations usually have not focused on the metabolism of the xenobiotic directly but have concentrated on the physiological affect of the xenobiotic on the plant tissue. Indirectly, these investigators may be measuring the ability of the tissue culture to metabolize the xenobiotic to nontoxic substances. An important example is the prior mentioned investigation (22) of metribuzin phytotoxicity in soybean suspension cultures where the principle of resistance was the ability to metabolize an inhibitor of an enzyme that metabolized metribuzin to a nontoxic form.

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Figure 11. Metabolism of aldrin and dieldrin. The dicarboxylic acid metabolite could not be detected in plant cell suspension cultures.

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The effects of organic solvents on growth and ultrastructure of plant cell suspensions have been studied recently (75). These studies have bearing on most metabolism studies. The phytotoxic effects of solvents increased in the following order: chloroform, dimethyl sulfoxide, methanol, acetone, isopropanol, and ethanol. At concentrations of 0.25%, chloroform had no apparent affect on cell growth, however, ethanol inhibited growth at concentrations as low as 0.05%. These data suggest that small amounts of organic solvents, often used to introduce the xenobiotic to the plant tissue culture, must be carefully considered.

Plant tissue culture techniques have been used to evaluate the biological activity of growth stimulating herbicides and their metatolites or potential metabolites. Amino acid conjugates of 2,4-D (76), 2,4,5-T (77), and IAA (78) were added to culture media (10^{-4}-10^{-8} M) of soybean callus tissue and evaluated for their ability to stimulate cell division and growth as measured by increase in weight. Amino acid conjugates of 2,4-D and IAA stimulated the growth of callus tissue and some conjugates of 2,4-D stimulated growth even better than 2,4-D itself. Surprisingly, the amino acid conjugates of 2,4,5-T did not stimulate growth of soybean root callus.

Production of Secondary Substances

Plant tissue cultures have been evaluated as a potential means of production of plant products sometimes referred to as secondary products. Plant products of potential commercial use such as steroids, alkaloids, glycosides, vitamins and others have received some attention of investigators, especially those of medicinal importance (79-83). The metabolism of three alkaloids, vindoline, catharanthine HCl, and vincaleukoblastine sulfate have been examined with suspension cultures of *Catharanthus roseus* (L.) (84). Vindoline formed two metabolites, desacetylvindoline and dihydrovindoline. No metabolites of catharanthine HCl were detected. Vincaleukoblastine sulfate formed three metabolites.

The biosynthesis of alkaloids by suspension cultures may be enhanced greatly by incubation of the plant tissue with selected intermediates (85). In this way, the cell media would be supplemented with key intermediates that would be metabolized further to the desired alkaloid.

The biosynthesis of edulinine and furoquinoline alkaloids from selective quinoline derivatives by *Ruta graveolens* cell suspension cultures has been studied (86). The biogenetic synthesis of the alkaloids in the tissue culture experiments were consistant with pathways discovered in rutaceous plants. The metabolic intermediates differed quantitatively from intact plants and varied with the age of the culture and with different culture isolates.

Berberine and plamatine, two medicinally important alkaloids, are produced by plant tissue cultures of *Coptis japonica* (79). The production of ginseng glycosides has been studied in plant

tissue cultures of *Panax ginseng* (79). Nitroquanidine and X-ray treatments were used to obtain mutants that had higher titers of saponins. These genetic mutation techniques may also be useful in the selection of resistant and nonresistant cultivars by altering their metabolism of xenobiotics. So far, large scale commercial drug production using plant tissue cultures has not been achieved.

It has been suggested (87) that through proper selection of plant species, tissue culture techniques may be useful for the preparation of metabolites of xenobiotics in quantities needed for standards and for structural characterization. For example, the glucose ester of 2,4-D can be mass produced with rice callus tissue (28) and amino acid conjugates of 2,4-D can be mass produced from soybean callus tissue when culture conditions are selected for optimization of amino acid conjugates (15).

Advantages and Disadvantages

In general, all studies indicate that the same metabolic pathways of degradation appear to be operating in intact plants and plant tissue cultures. Thus, xenobiotic metabolism by plant tissue cultures is a desirable model for estimating metabolic pathways in the intact plant. However, at the present time, work with intact plants (or parts) is also necessary to confirm and evaluate the quantitative aspects of xenobiotic metabolism. Plant tissue cultures are sterile, homogenous, rapidly growing and do not require extensive facilities. Because of ease of standardization of conditions, reproducibility should be achieved more readily, data are amenable to statistical analysis and results could be compared directly in different laboratories. Use of tissue cultures obviates the difficulties of poor penetration, translocation, and perhaps there is less compartmentation of metabolic pools than encountered with intact plants. Plant tissue cultures permit the use of lower levels of radiolabeled material and the isolation of metabolites that are free of many interfering substances so troublesome with intact plants. Metabolism by different plant species or by different tissues of the same plant can be compared readily. However, it is not at all clear that callus started from different tissues will show major differences in metabolism. However, one would expect organ culture to exhibit clear differences in the metabolism of xenobiotics. Plant tissue cultures are also useful for studying the mode of action of phyto-active xenobiotics because of ease of controlling the various parameters of growth. Cultures have been used as a technique for the bioassay of chemicals that cause physiological changes such as growth stimulation, cell division and phytotoxicity. In addition, plant tissue culture techniques offer great potential in the mass production of metabolites as well as medicinally important chemicals.

Obvious disadvantages do exist in the use of plant tissue cultures. The technique does not show the importance of cuticular

penetration, of root absorption, of microorganisms associated with the intact plant, or of vascular transport. Tissue cultures would not indicate the importance of environmental factors such as wind, nutrition, disease, water stress, temperature or sunlight on metabolic products and pool sizes. In tissue cultures, the photosynthetic apparatus is often absent or only marginal. If photosynthesis is important for the metabolism of the xenobiotic, then tissue culture technqiues might be undesirable. Experiments with tissue cultures have shown the importance of culture age and the method of xenobiotic administration in determining metabolite levels. These conditions would be different with intact plants.

Conclusions

Xenobiotic metabolites isolated from plant tissue cultures are qualitatively the same as metabolites found in whole plants, but with quantitative differences. Therefore, metabolic pathways operating in whole plants also seem to be operating plant tissue cultures. With some metabolites, such as glycosides and aglycons, there may be a large quantitative difference between the two techniques. This is especially true with plant suspension culture technqiues which often produce quantitatively lower amounts of glycosides and higher amounts of aglycons than studies with intact plants. Apparently the aglycons can be excreted from the cells before the glycosides are formed. Perhaps the level of carbohydrates in the media affects glycoside synthesis.

Common reactions found in plant tissue cultures are oxidations at both aliphatic and aromatic carbons, dealkylations, hydrolytic enzymes such as amidases, and esterases, dehydrochlorinations, dechlorinations, hydrogenations and conjugations with sugars, amino acids and acids. Plant tissue cultures can reflect the tolerance of the parent plant, e.g., cultivars showing resistance to phytotoxic xenobiotics also exhibit resistance in tissue culture.

Many factors affect xenobiotic metabolism by plant tissue cultures. The source of the tissue is important. Different tissues from the same plant show minor variations, and tissue from different species (or even varieties) of plants show greater variations (e.g., monocot and dicot plant tissue cultures may exhibit major differences in metabolism). The tissue culture method is also very important. Suspension cultures may give rise to signigicant amounts of metabolites in the medium and low relative proportions of glycosides. In the few cases reported, incubations with callus tissue results in rapid absorption of the xenobiotic, no significant metabolites in the medium and accumulation of glygoside conjugates similar to that found in intact plants. The method of administrating the xenobiotic to the culture may also be important. Direct injection of 2,4-D into the callus tissue seemingly gave different results from callus tissue incubated in solution with 2,4-D (26). Another factor, of some importance, is the composition of the medium (40). In intact plants, illumination

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has been shown to stimulate the formation of the glucose ester (60) and enzymes such as phenylalanine ammonia lyase (46). This parameter has not been examined thoroughly in plant tissue cultures and needs further study. The physiological stage or age of the tissue culture has been demonstrated to show a significant effect on metabolism (15). In some cases only older tissues demonstrated enzymatic activity (20) and controlled metabolism (15). In spite of these limitations, investigations of the metabolism of xenobiotics by plant tissue cultures provides a reasonable approximation of the metabolism expected with intact plants.

With so many factors affecting metabolism and each research laboratory using different tissues and culture conditions, there is a need for the standardization of some tissue culture parameters. Perhaps certain well studied plant tissues should be used by all laboratories as a base of comparison. Selected plant tissues should include some important dicot and monocot plants. Tobacco tissue has been suggested as at least one possibility for routine use (38). Perhaps a plant tissue bank, similar to those established for microorganisms, could be established for laboratories working with plant tissue cultures.

Additional research is needed to determine which of the many factors affecting metabolism in plant tissue cultures are most important before we can suggest standardization of these factors. Really, only a few xenobiotics and pesticides have been examined, e.g., few compounds from the important organophosphate or triazine pesticides have been examined. Fungicides appear to have been neglected. Therefore, additional investigations with other pesticides and xenobiotics are desirable before we can properly evaluate the relative merits of studying metabolism with plant tissue cultures and the extrapolation of these results to intact plants.

ABSTRACT

Xenobiotic metabolism by plant tissue cultures are reviewed with an emphasis on pesticide metabolism. The results obtained with tissue culture techniques are evaluated and compared with results using whole plants. Some factors affecting metabolism studies with plant tissue culture techniques are also considered.

Some advantages of the plant tissue culture techniques include: sterility, a rapidly growing homogenous tissue with low pigment content, moderate cost and space requirements, ease of duplication of treatment conditions, rapid uptake and metabolism of xenobiotics, and ease of isolation of metabolites. It offers the opportunity to mass produce desired metabolites as well as evaluate phytotoxicity or physiological changes of the plant tissue. Obvious disadvantages of this technique are its failure to evaluate such factors as: penetration, absorption, microorganisms, vascular transport and environmental influences. Other factors affecting xenobiotic metabolism by plant tissue cultures are: culture method, source of the tissue, method of treatment and concentration of xenobiotic, composition of medium and physiological age of tissue.

Although quantitative and perhaps qualitative differences may be found, it is concluded that xenobiotic metabolism by plant tissue culture provides a useful approximation of metabolic pathways in intact plants.

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Pesticide Metabolism in Higher Plants: In Vitro Enzyme Studies[1]

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The study of xenobiotic metabolism in plants has developed primarily as a result of the use of pesticides to protect plant crops from damage by weeds, insects and other pests. Great stimulus for research in this field was experienced in the early 1960's when increased concern was raised over the possible hazards of pesticide residues to man and his environment.

The complexity of xenobiotic metabolism studies is a continuum with *in vivo* studies conducted in the natural environment at one end, and the use of isolated enzymes to study molecular reactions at the other. Each technique in this continuum has its own particular limitations, but can be used to great advantage under the proper conditions. In the previous two reports, the use of cell cultures, tissue cultures, isolated cells and isolated plant organs were considered.

This report will examine the use of isolated plant enzyme systems in xenobiotic metabolism studies. The primary topic will be a discussion of the enzymes involved in the four basic metabolic reactions of xenobiotics in plants: oxidation, reduction, hydrolysis and conjugation. The literature regarding key enzymes within these classes will be reviewed. Specific examples of *in vitro* plant enzyme systems used to study xenobiotic metabolism will be presented. A detailed discussion of various techniques will not be attempted.

OXIDATION REACTIONS:

Oxidations are among the most important reactions in the metabolism of pesticides because they are frequently the primary

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reaction that results in detoxication or activation of a pesticide. In mammals and insects, oxidation reactions have been the subject of many in vitro studies. It is now evident that mixed function oxidases are responsible for many of the oxidation reactions in these organisms. Although plants have mixed function oxidase systems, their role in pesticide metabolism has not been widely demonstrated. In addition to mixed function oxidases, peroxidases, lacasses and polyphenol oxidases are found universally in the plant kingdom and some of these enzymes may also play a role in oxidative pesticide metabolism. At the present, our knowledge of the enzyme systems involved in many of the pesticide oxidation reactions that occur in plants is limited and there is great need for more research.

Peroxidases:

The plant peroxidases catalyze two general types of oxidation reactions, the classical peroxidative reaction that requires hydrogen peroxide and the oxidative reaction that utilizes molecular oxygen. The horseradish peroxidase (HRP) peroxidative reaction normally proceeds by the following mechanism (1, 2):

$$HRP + H_2O_2 \rightarrow HRP\text{-}I$$
$$HRP\text{-}I + AH_2 \rightarrow HRP\text{-}II + AH\cdot$$
$$HRP\text{-}II + AH_2 \rightarrow HRP + AH\cdot$$
$$2\ AH\cdot \rightarrow Products$$

The oxidative reactions are not as well understood as the peroxidative reactions. In Mn^{++} inhibited reactions, the oxidative hydroxylation of several substrates can be catalyzed by peroxidase in the presence of dihydroxyfumaric acid (3). Other oxidative reactions require an aromatic co-factor such as 2,4-dichlorophenol and Mn^{++} (4). In some oxidative reactions, a catalytic amount of hydrogen peroxide is needed as an initiator, but oxygen is used in stoichiometric amounts (5).

Plant peroxidases catalyze the oxidation of a large and diverse class of endogenous and exogenous substrates such as phenols, aromatic amines, enediols, ascorbate, ferrocyanide, cytochrome C, indole-3-acetic acid, and the leuco form of many dyes. Phenols and aromatic amines are among the most commonly used substrates and the reaction catalyzed is generally an oxidative condensation of the substrate (6). In addition to oxidative condensations, decarboxylations, sulfur oxidations, *N*-demethylations, ring hydroxylations, carbon-halogen bond clevages, and oxidation of aromatic methyl groups have all been attributed to peroxidases (3) (Figure 1). Peroxidases are ubiquitous in the plant kingdom (3). They occur throughout the plant cell and have been found in the cytoplasm, cell wall, membranes, nuclei, mitochondria and ribosomes (7). Peroxidase isozymes have been demonstrated in horseradish (4, 8-11) and several other species (4, 8-13). Striking differences have been

1) OXIDATIVE CONDENSATIONS OF PHENOLS AND AROMATIC AMINES

2) DECARBOXYLATION

3) SULFUR OXIDATIONS

4) N-DEMETHYLATIONS

5) RING HYDROXYLATIONS

6) OXIDATIONS OF AROMATIC METHYL GROUPS

7) CARBON—HALOGEN BOND CLEAVAGES

F^- + COMPLEX CONDENSATION PRODUCTS

8) IODIDE OXIDATION

$I^- \rightarrow I_2$

Figure 1. Types of reactions attributed to plant peroxidases (3, 22, 188, 189, 190)

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noted in the substrate specificity of several HRP isozymes (14).

In spite of their widespread occurrence, the physiological role of peroxidases is uncertain. Their ability to utilize indole-3-acetic acid (15-18) and flavonoids (19) as substrates suggests that peroxidases may be important in the metabolic regulation of these endogenous substrates. Peroxidases are also thought to be involved in the lignification process (20). The high concentration of peroxidase activity associated with the cell wall (12) is consistent with this theory.

Several reports have associated peroxidase activity with pesticide metabolism. The oxidation of parathion to paraoxon and the hydrolysis of both parathion and paraoxon can be catalyzed by HRP (21) (Figure 2). The reaction occurs under both oxidative and peroxidative conditions. A peroxidase was also isolated from bean hypocotyl that was approximately 50% as active as HRP in the oxidation and hydrolysis of parathion (21). This enzyme was equal to HRP in the oxidation of guicacol. The *in vitro* reaction of ten [^{14}C-carbonyl]carbamate insecticides with hydrogen peroxide in the presence of HRP has been examined (22). Conversion to ether-soluble products that could be differentiated from the reactants by TLC or conversion to water-soluble products were used as the criteria for a reaction. Of the ten substrates tested (banol, baygon, carbaryl, HRS-1422, isolan, mesurol, UC-10854, matacil and zectran), only matacil and zectran reacted. The highest percentage of radioactivity was in the form of unidentified water-soluble products; however, several ether-soluble products were identified (Figure 3). The ether-soluble products represented various stages of N-dealkylation. This may suggest a minor role for peroxidases in *N*-dealkylation. Mesurol, a carbamate insecticide that contains an arylmethylsulfide, and cysteine, thioglycolic acid, mercaptoethanol, thiourea and thiouracil were not substrates for HRP (22, 23); however, chloropromazine, a heterocyclic sulfide, was a substrate (3). The precise role of peroxidases in sulfur oxidation is thus uncertain.

Anilines are known degradation products of phenylcarbamates, phenylureas, and acyl anilide herbicides; therefore, the fate of anilines in the environment is an important consideration. A number of workers have shown that various anilines are converted to azobenzenes and other products by the action of HRP and hydrogen peroxide (3). However, it is of particular note that the chloroanilines that formed azobenzenes *in vitro* as the result of HRP and hydrogen peroxide were the same as those that formed azobenzenes in the soil as a result of microbial action (24) (Figure 4). The HRP system served as a model to predict the likelihood of azobenzene formation in the soil. In these studies, no effort was made to identify products other than the azobenzene analogs or to quantitate the reaction; therefore, the full scope of products produced and the yields are not known.

BEAN HYPOCOTYL PEROXIDASE (10 ug) pH 4.7

DIHYDROXY MALEIC ACID

+ HYDROLYSIS PRODUCTS

4% 14%

36% HYDROLYSIS AND 10% OXIDATION OBTAINED WITH 50μg HRP

Figure 2. Oxidation and hydrolysis of parathion by plant peroxidases (21)

ZECTRAN

H_2O_2/HRP 1 hr

MATACIL

H_2O_2/HRP 1 hr

Figure 3. N-*Demethylation of substituted phenylcarbamate insecticides by horseradish peroxidase (22)*

Figure 4. HRP catalyzed formation of azobenzenes from various chloroanilines (3, 24)

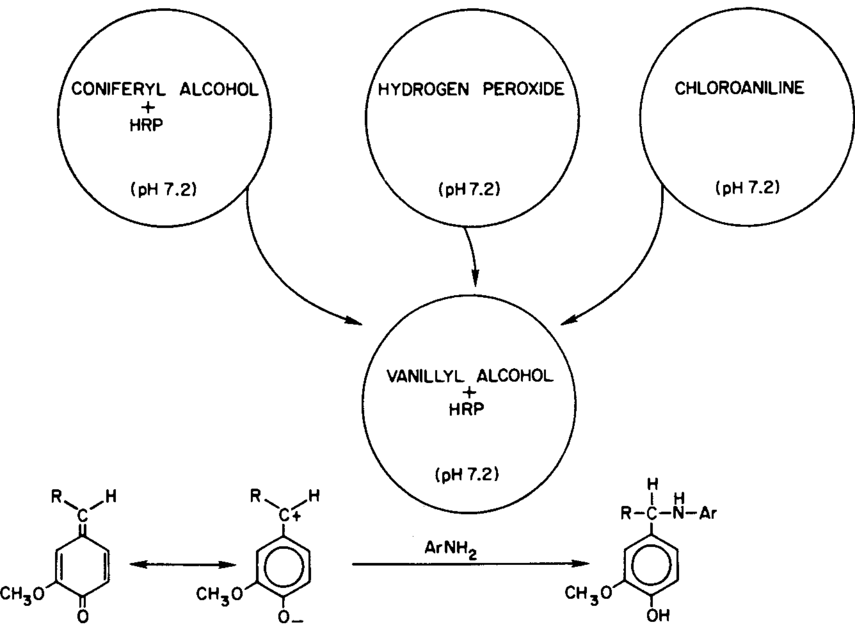

Figure 5. Model system for incorporation of chloroanilines into synthetic lignin (26)

Although azobenzenes do not appear to be metabolites of chloroanilines in higher plants, chloroanilines may be incorporated into lignin or converted to lignin-like products (25). In an effort to determine the chemical nature of the chloroaniline residue in the lignin fraction, a model system was developed that uses HRP to catalyze the free radical formation of a synthetic lignin-like material (26). In this system, the ^{14}C-labeled pesticide metabolite, 3-chloroaniline or 3,4-dichloroaniline, was co-polymerized with coniferyl alcohol, a building block of natural lignin (Figure 5). The polymerization was accomplished by simultaneously pumping solutions of (a) coniferyl alcohol and HRP in pH 7.2 buffer, (b) hydrogen peroxide in pH 7.2 buffer, and (c) the ^{14}C-labeled chloroaniline in pH 7.2 buffer into a buffered solution of vanillyl alcohol and HRP. After 10 hr in the dark, the insoluble ^{14}C-labeled polymeric product was removed by centrifugation, washed and subjected to gel permeation chromatography. Based upon an average molecular weight of 1,000, 1.19 residues of 3-chloroaniline or 1.68 residues of 3,4-dichloroaniline were incorporated per molecule of polymer formed. *In vivo* incorporation into rice lignin was also greater for 3,4-dichloroaniline than for 3-chloroaniline. When acetylated anilines were used in the model HRP system, the incorporation rate was reduced by over 50%. This may indicate the involvement of the aniline nitrogen in the co-polymerization reaction.

A number of techniques were used for the analysis of the synthetic lignin. The most successful method was pyrolytic GC/MS. Pyrolysis released over 80% of the radioactivity in a volatile form. Over 60% of the released ^{14}C was 3-chloroaniline or 3,4-dichloroaniline. Similar results were obtained with natural lignin isolated from chloroaniline-treated rice, but with much lower yields. It was concluded that a high percentage of the chloroaniline in the lignin-like material was covalently bonded between the aniline nitrogen and the α-carbon of the polymer (Figure 5).

HRP was also used to study the metabolism of botran (27). Botran was not a direct substrate for HRP, but its reduction product (2,6-dichlorophenylenediamine) was readily converted to at least 8 products upon treatment with HRP (Figure 6). One of the most abundant products was identical to a soil metabolite of botran. High resolution mass spectrometry of the soil metabolite indicated a molecular formula of $C_{12}H_6N_4Cl_6$. A botran metabolite, *N*-(4-amino-3,5-dichlorophenyl)malonamic acid, was found in soybean plants and in soybean callus cultures (28). The presumed intermediate of this metabolite (2,6-dichlorophenylenediamine) was not detected; however, plants contain a system that is capable of reducing arylnitro groups (29). The diamine of botran could thus be formed in the plant and serve as a substrate for a malonyl transfer reaction, a peroxidase-catalyzed oxidation or lignin incorporation.

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Figure 6. A possible role for peroxidases in botran metabolism (27, 28, 29)

Table I. Relationship between resistance to iodide and absence of peroxidase capable of oxidizing iodide.*

Species	Peroxidase Activity: Guaiacol donor (units/mg protein)	Peroxidase Activity: Iodide donor (units/mg protein)	Iodide accumulation (mg/g dry weight)	Resistance
tomato	2.6	0	70	short term
buttercup	2.0	9	13	resistant
cabbage	6.7	26	4	resistant
bean	12.7	28	15	susceptible
mettle	15.3	75	5	susceptible
pea	82	123	8	susceptible

*Adapted from (32).

The finding that ioxynil liberated iodide when exposed to ultraviolet light or when subjected to plant or animal metabolism resulted in an examination of the herbicidal properties of iodide. Subsequently, iodide was shown to possess some selectivity as a herbicide (30). The ability of plant peroxidases to oxidize iodide to iodine (31) varied greatly with the plant species (32) and this ability was related to resistance to iodide toxicity (Table I). Iodine was shown to be 10^5 times more powerful than iodide as an inhibitor of the Hill reaction (33). This suggested that iodide toxicity was due, in part, to intracellular oxidation of iodide to iodine. When 30 iodo-benzoic acids were assayed for herbicidal activity, only those that liberated iodide *in vivo* were toxic to *Phaseolus vulgaris* seedlings. It was suggested that liberation of iodide might be one factor in the herbicidal activity of these compounds (30).

In addition to *in vitro* studies that suggested an active role for peroxidases in pesticide metabolism, several studies have indicated that certain pesticides may increase the level of peroxidases in plants. EPTC was reported to increase peroxidase activity and lignification in corn seedlings; these increases could be alleviated by treatment with a protectant, N,N-diallyl-2,2-dichloroacetamide (9). Stimulation of peroxidase activity was also observed when *Phaseolus radiatus* seedlings were treated with sodium diethyldithiocarbamate (34).

A microsomal hydroperoxide-dependent oxidizing system (hydroperoxidase) was recently isolated from pea seeds (35, 36) (Figure 7). This enzyme hydroxylated indole, phenol, α-naphthol, and aniline to indoxyl, hydroquinone, α-napthylhydroquinone, and *N*-hydroxyaniline, respectively. Hydrogen peroxide, *tert*-butyl hydroperoxide, cumene hydroperoxide, or linoleic acid hydroperoxide served as sources of oxidizing power. A well-defined pH optimum at pH 7.2 was observed with linoleic acid hydroperoxide, but a broad pH optimum around 8.7 was observed with the other hydroperoxides. An allosteric effect was noted with linoleic acid hydroperoxide, but not with the other hydroperoxides. It was speculated that the natural hydroperoxides for this system were produced by the action of lipoxygenases on substrates such as linoleic acid. Studies with O^{18}-labeled linoleic acid hydroperoxide indicated a direct oxygen transfer from the hydroperoxide to the hydroxylated substrate. No participation of molecular oxygen was observed. Pre-treatment with *p*-chloromercuriobenzoate resulted in a slight promotion of the hydroperoxidase reaction, indicating no involvement of P450. Other properties of this system also indicated that activity was distinct from that of P450.

Although none of the *in vitro* peroxidase studies discussed proves that these enzymes play an important role in pesticide metabolism in plants, it is clear that the spectrum of substrates utilized by these enzymes does encompass many pesticides or pesticide metabolites. The apparent universal occurrence of

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Figure 7. Hydroperoxide-dependent enzyme system from pea (35, 36)

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peroxidases in the plant kingdom and throughout the cell contributes greatly to the possible involvement of these enzymes in pesticide metabolism. A number of possible roles for peroxidases in the metabolism of specific pesticides or classes of pesticides have been pointed out. The utility of HRP in model systems to produce metabolites found in the soil and to aid in the study of the pesticide residues found in the plant lignin fraction have been clearly demonstrated.

Mixed Function Oxidases:

Extensive research over the last 28 years has shown that mixed function oxidases are of great importance in xenobiotic metabolism in insects and animals (37). The presence of mixed function oxidases in higher plants has been established in the last 10 years by experiments conducted with both endogenous substrates (38-42) and xenobiotics (43-48). Based upon light reversible CO inhibition (39, 44) and spectral properties (49), several mixed function oxidase (mfo) systems in plants appear to resemble the cytochrome P450 mfo systems found in animals and insects. Other plant mfo systems, however, appear to be quite different (38, 40).

Based upon our knowledge of insect and mammalian mfo systems and our knowledge of metabolites produced in plants, a number of reactions in plants should be considered as possible mixed function oxidase-catalyzed reactions. Some of these are N-dealkylation, O-dealkylation, aromatic hydroxylation, alkyl oxidation, epoxidation, desulfuration, sulfur oxidation, ester hydrolysis, and nitrogen oxidation (Figure 8). The demonstrated mfo reactions in plants are more limited.

There are several examples of mfo-catalyzed N-dealkylations in plants. A mfo system from cotton that catalyzes the N-demethylation of phenylurea herbicides (47, 48, 50) and mfo systems from castor beans (51) and avocado pear (46) that catalyze the N-demethylation of p-chloro-N-methylaniline have been reported. The O-demethylation of p-nitroanisole has been detected with an in vitro system from avocado pear (46). A similar mfo-catalyzed dealkylation might explain the presence of the phenol of 2,4-D that has been reported as a metabolite in certain plant systems. The formation of the phenol of 2,4-D does not seem to involve an initial decarboxylation (52). The reported in vivo alkyl oxidation of the plant growth regulator flurenol butyl ester might also be explained by a mfo (53). Several mixed function oxidase systems that catalyze aromatic ring hydroxylations have been studied in vitro with both xenobiotic (44-46) and endogenous substrates (38-41, 45, 54-57). Because of the large number of potential substrates, this reaction is of particular interest. The epoxidation aldrin has been demonstrated by in vitro systems isolated from pea and bean (43, 58-62). The aldrin epoxidase systems have not been clearly

1) N-DEALKYLATION (MONURON)

2) O-DEALKYLATION (P-NITROANISOLE)

3) ALKYL HYDROXYLATION (FLURENOL BUTYL ESTER)

4) AROMATIC HYDROXYLATION (2,4-D) (NIH SHIFT)

5) EPOXIDATION (ALDRIN)

6) DESULFURATION (PARATHION)

7) SULFUR OXIDATION (PHORATE)

8) HYDROLYSIS (PARATHION)

9) NITROGEN OXIDATION (MORPHINE)

Figure 8. Reactions for which a mfo mechanism is known or might be expected

demonstrated to be mfo systems. The conversion of organophosphorothioates to phosphates in plants has been well established. This conversion involves mixed function oxidases in animals and insects (63). Mixed function oxidases may also catalyze this reaction in plants, but the possibility that other enzyme systems may be involved should be considered (21). The oxidation of a number of thio-ethers to sulfoxides and sulfones has been reported in plant metabolism studies; again, the enzymes responsible for these reactions have not been well characterized. An enzyme that catalyzes the sulfoxidation of phorate has been isolated from soybean root (64). Additional studies are needed to characterize this system. In animals, organophosphate ester hydrolysis can be catalyzed by both mixed function oxidases and glutathione S-transferases (63). In plants, this reaction may be catalyzed by esterases or peroxidases (21, 65, 66), but the reaction mechanism needs further study at the *in vitro* level. In plants, *N*-oxide formation does not appear to be a common reaction in pesticide metabolism. However, an enzyme that catalyzes the formation of the *N*-oxide of morphine has been detected in *Papaver somniferum* latex (67). Since peroxidases and polyphenol oxidases are found in high concentrations in plant latex, the possibility that this reaction may be catalyzed by one of these enzymes should also be considered.

The *N*-demethylase from cotton (47) and the cinnamic acid hydroxylase from cucumber (44) are two mfo systems that have been shown to utilize pesticides as substrates. The *N*-demethylase system appears to have limited substrate specificity, only *N*-methyl substituted phenylureas were found to be active substrates. The *N*-demethylation reaction was strongly and competitively inhibited by carbamate insecticides that had high electron density at the position ortho to the carbamate group (50, 68). Inhibition by substituted phenylureas was also demonstrated. This inhibition was dependent upon the presence of a proton on the aniline nitrogen atom (50). Field observations (69, 70) and an *in vitro* study (71) indicated an antagonistic interaction of carbamates with dimethylphenylurea herbicides. A positive correlation between resistance to phenylurea herbicides and the presence of an active *N*-demethylase system was supported by *in vitro* determinations of *N*-demethylase activity in 12 plant species. In cotton, the specific activity of the mfo system varied over a 10-fold range, depending upon the age of the plant and the specific tissue.

A recent study has shown that 2,4-D is hydroxylated by an *in vitro* mfo system from cucumber leaves (44). This appears to be the first reported ring-hydroxylation of a pesticide catalyzed by an isolated plant mfo system. Cinnamic acid was also hydroxylated by this system. Hydroxylase activity was increased 2- to 3-fold by spraying the cucumber leaves with 2,4-D before enzyme isolation. Other studies have shown that certain plant mfo systems were induced or stimulated by light

(39, 72). The NIH shift was observed in the hydroxylation of 2,4-D. The primary product was 4-hydroxy-2,5-dichlorophenoxyacetic acid. The NIH shift was also demonstrated in the 4-hydroxylation of cinnamic acid by a mfo system from pea (73). These studies suggest that additional investigations on the induction of plant mfo systems may be warranted. They also suggest the need to conduct kinetic studies on the cinnamic acid 4-hydroxylase reaction with 2,4-D and related pesticides as possible inhibitors. Since microsomal mixed function oxidases play an important role in the metabolic pathway of some endogenous substrates (39, 41), the induction or inhibition of these reactions by pesticides could have important implications.

In animals, the microsomal fraction has typically displayed a broad specificity range (37) that can be explained by the presence of a series of P450 cytochromes (74). In contrast, mixed function oxidases from plants appear to have a fairly narrow substrate specificity range with some tissue specificity. The mfo system from castor bean (51) displayed 4-hydroxylase activity for cinnamic acid and *N*-demethylase activity for *p*-chloro-*N*-methylaniline, *N*-methylaniline, and *N*,*N*-dimethylaniline, but displayed no activity for 15 other compounds that contained *N*-, *O*-, or *S*-methyl groups. The system from avocado pear may have one of the broadest specificity ranges of the isolated plant systems. It apparently has hydroxylase activity for biphenyl and aniline, *O*-demethylase activity for *p*-nitroanisole, and *N*-demethylase activity for *p*-chloro-*N*-methylaniline (46).

Mixed function oxidase systems isolated from plants typically display very low levels of activity. Specific activities are frequently in the range of 1- to 10-nmol product/mg protein/hr. This low level of activity can be explained by the report that P450 concentrations in plants range from 0.007 to 0.02 nmol P450/mg microsomal protein as compared to 0.9 to 1.2 nmol P450/mg microsomal protein in rat liver (49). Because of the low levels of mfo activity in plant tissues, the use of radioactive substrates in assays is often necessary.

The properties of isolated plant mfo systems are highly variable (Table II). Some mfo systems are microsomal, undergo light reversible CO-inhibition, require NADPH as a co-factor and behave much like mammalian P450 mfo systems (39). Others require illuminated chloroplasts as a co-factor and may be soluble (38). Some systems require only one co-factor (47), but other systems require several (40). Ascorbic acid, NADH, NADPH, dithionite, illuminated chloroplasts, and an unidentified natural product (probably a pteridine) have all been utilized in various *in vitro* plant mfo studies (38, 39, 40, 47). The response of these systems to various inhibitors is also quite variable. Mercaptoethanol was an inhibitor of phenylalanine hydroxylase (40) and gibberellin A_1 hydroxylase (41), but it was necessary to demonstrate 4-hydroxylase activity with cinnamic acid (39). Chelating agents inhibited an *N*-demethylase (47) and a

Table II. Properties of plant enzymes with mfo type activity.

Enzyme	Co-factors	Activators or factors used to demonstrate activity	Inhibitors	Reference
N-demethylase	NADH or NADPH	NaCN, isoascorbate, polyclar AT	CO, ionic detergents sulfhydryl reagents, chelating agents and electron acceptors.	47
aldrin epoxidase	NADPH (activity present at reduced level without)	chelating agents and detergents	electron acceptors, CN, phenols, aniline.	43
2,4-D hydroxylase and cinnamic acid hydroxylase	NADPH	mercaptoethanol	CO-light reversible.	44
biphenyl hydroxylase (microsomal)	NADPH	safrole and 3,4-benzopyrene are stimulatory to 2-hydroxylation.	weak with CO, SKF 525A, Cu^{++}, NADH.	45,46
pyroloxygenase	illuminated chloroplasts, or dithionite		chelating agents, dithiothreitol and mercaptoethanol.	38
phenylalanine hydroxylase	NADPH or NADH, and an unidentified product or THFA	ascorbate	aminopteridine, sulfhydryl-containing compounds; i.e., mercaptoethanol.	40
gibberellin A_1 hydroxylase	NADPH, ascorbate Fe^{++}		mercaptoethanol, EDTA	41
cinnamic acid hydroxylase	NADPH	mercaptoethanol	CO-light reversible, azide.	39
cinnamic acid, hydroxylase and N-demethylase	NADPH (or NADH with 2nd substrate)	EDTA	CO lipases, menadione, 1,4-napthoquinone, riboflavin.	51

pyroloxygenase (38), but slightly stimulated an aldrin epoxidase (43). Safrole and 3,4-benzopyrene stimulated a biphenyl 2-hydroxylase from avocado, but other mfo activities were unaffected (46). Castor bean N-demethylase was strongly inhibited by Kp_i buffer and was assayed with tricine buffer (51). In sharp contrast, N-demethylase from cotton was inhibited by tricine, but not by Kp_i (47). Based on differences in co-factor requirements, responses to different inhibitors and responses to different buffers, it would appear that a variety of different plant mfo systems exist.

Although most mfo systems in plants are associated with particulate (microsomal) fractions, a gibberellin A_1 hydroxylase (41) and a biphenyl hydroxylase (45, 46) are soluble systems. The nature of several other mfo systems (38, 40) is uncertain. The solubilization of Swede root cinnamic acid 4-hydroxylase with Triton X-100 has been reported (75) and both soluble and particulate aldrin epoxidase (60, 61) and biphenyl hydroxylase (45, 46) have been isolated from the same tissues. It appears that isolated active fractions may not always be a true indication of the nature and location of the enzyme in the native state, but may also be a function of the combination of techniques and tissue used.

A number of different procedures and special precautions were used to isolate mfo activity from plant sources (Table III). In the isolation of N-demethylase activity from cotton, the tissue was ground under liquid nitrogen (47, 48) and in the isolation of aldrin epoxidase from pea (43), totally anaerobic conditions were used. Most extraction and isolation procedures utilized pH 7.5 buffers that contained either polyvinyl pyrrolidine or polyclar AT. The buffer used to extract N-demethylase activity from cotton contained isoascorbate, polyclar AT and sodium cyanide. Mercaptoethanol, chelating agents, sucrose, and bovine serum albumin have also been used with varying frequency to help protect mfo systems during isolation. Differential centrifugation has been the most commonly used fractionation method, but DEAE chromatography, sephadex chromatography, ammonium sulfate fractionation and density gradient centrifugation have also been employed.

Based on this brief survey, it is apparent that the number of xenobiotics shown to be metabolized by *in vitro* plant mixed function oxidases is very limited. Some of these oxidative systems have not been well defined. Considerable effort is needed to isolate and characterize these systems. The methods used for enzyme isolation have been highly variable. Because of the extremely low levels of enzyme activity associated with many of the mfo systems, they are frequently difficult to study and special methods are often needed to measure reaction rates. This is further complicated by the presence of endogenous inhibitors and the instability of many of these systems.

Table III. Methods used to isolate mixed function oxidase-like activity from higher plants.

Enzyme	Tissue Source	General Isolation Procedure	Special Precautions	Reference
N-demethylase (microsomal)	*cotton leaves	pulverized in liquid N_2, extract (pH 7.5), filter, differential and density gradient centrifug.	NaCN, polyclar AT, isoascorbate	47
aldrin epoxidase (microsomal)	pea root	extract (pH 7.5), filter, Sephadex G-100 and differential centrifug.	anaerobic isolation, polyclar AT	43
2,4-D and cinnamic A. hydroxylase (microsomal)	*cucumber leaves	extract (pH 7.5), filter, differential centrifug.	PVP	44
biphenyl hydroxylase (microsomal and soluble)	avocado pear	extract (pH 7.4), filter, differential centrifug.	none apparent	45,46
pyroloxygenase	*wheat germ	water-extract, $(NH_4)_2SO_4$ fractionation, DEAE and Sephadex G-100	remove high m.w. inhibitor	38
phenylalanine hydroxylase	spinach leaves	pulverize frozen tissue, water-extract, filter, acetone fractionation, DEAE-cellulose and calcium phosphate gel adsorption.	none apparent	40
gibberellin A_1 hydroxylase	snap bean	homogenize decoated imbibed seeds (pH 6.5), differential centrifug.	sucrose, PVP	41
cinnamic acid hydroxylase (microsomal)	castor bean	pulverize in pH 7.5 buffer, filter, differential centrifug. or density gradient centrifug.	EDTA, sucrose	51
cinnamic acid hydroxylase (microsomal)	aged Swede root	homogenize in pH 7.5 buffer, filter, differential centrifug.	EDTA, sucrose, mercaptoethanol	75
cinnamic acid hydroxylase (microsomal)	wounded Jerusalem artichoke tuber	homogenize in pH 7.5 buffer, filter, differential centrifug. and density gradient centrifug.	mercaptoethanol, EDTA, serum albumin, polyclar AT, mannitol.	76

*Also isolated from other sources.

REDUCTION REACTIONS:

Although reductive reactions have not been demonstrated to play a major role in xenobiotic metabolism in higher plants, several investigators have reported the reduction of aromatic nitro groups in plants (77-82) and callus culture (82). A reductive dehalogenation (83) and the reduction of a sulfoxide to a sulfide (84) have also been reported. The reported arylnitro reductions of pentachloronitrobenzene (PCNB) (77) and fluorodifen (p-nitrophenyl-α,α,α-trifluoro-2-nitrophenyl-p-tolyl ether) (79, 80, 85, 86) were in competition with glutathione conjugation. In studies with fluorodifen, arylnitro reduction was a minor metabolic pathway. However, in studies with pentachloronitrobenzene, about 28% of the pesticide was converted to pentachloroaniline.

The only reductive reactions involving pesticides that appear to have been studied in detail in plants are those catalyzed by aryl nitroreductases. Aryl nitroreductase activity has been isolated from peanut (77) and pea (78). The soluble arylnitroreductase system from peanut was isolated in conjunction with studies on GSH S-transferase activity. Magnesium chloride was used in the isolation procedure to precipitate the microsomal fraction (87) and to avoid high speed centrifugation. With PCNB as the substrate, reductase activity was detected with enzyme preparations from roots and hypocotyls of 7-day-old etiolated peanut seedings. Aryl nitroreductase activity was detected only when the reaction was run under a nitrogen atmosphere in the presence of both FAD and NADPH. The aryl nitroreductase isolated from soybean seedling roots catalyzed the reduction of dinoben (2,5-dichloro-3-nitrobenzoic acid) when the system was incubated under nitrogen at pH 8.2 with either NADPH or NADH (78). When this enzyme system was purified further, FAD or FMN was required in addition to NADH or NADPH. The fact that *in vitro* reduction could be demonstrated only under a nitrogen atmosphere suggests that these reactions may become more important *in vivo* under conditions of low oxygen tension.

In many cases in which reduction reactions have been reported, plants have been grown and treated in such a manner that microbial action could have accounted for the reaction. This possibility should be considered in any study in which reductive reactions are reported in xenobiotic metabolism in plants. The reduction of the aryl nitro groups of PCNB and dinoben with *in vitro* enzyme systems isolated from plants and the apparent reduction of the aryl nitro group of 2,6-dichloro-4-nitroaniline in soybean callus culture suggests, however, that aryl nitro reductions may result from plant metabolism.

HYDROLYTIC REACTIONS:

Aryl Acylamidases:

In Xenobiotic Metabolism: In Vitro Methods; Paulson, G., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 1979.

Aryl acylamidases that hydrolyze the herbicide propanil (3',4'-dichloropropionanilide) have been isolated from bacteria (88), fungi (89), birds (90), mammals (91, 92) and plants (93-110) (Figure 9). Of the hydrolytic enzymes that play important roles in the metabolism of pesticides in plants, the aryl acylamidases appear to be the most thoroughly studied. Studies with homogenates of rice plants (101, 105, 106) established the enzymatic nature of propanil hydrolysis. Hydrolytic activity was associated with a particulate fraction (93, 94). The enzyme was stable, required no co-factors, and had a pH optimum of 7.5-7.9. A comparison of the enzyme activity in rice and barnyard grass showed a 60-fold higher level of enzyme activity in rice (94). This suggested that propanil resistance was based on the presence of the aryl acylamidase in sufficient concentration to detoxify the herbicide before sensitive sites could be attacked. Enzyme activity in rice seedlings was a function of the developmental stage of the plant and maximum activity was reached at the fourth leaf stage. In field studies, however, seedlings in the third and fourth leaf stages were more sensitive to propanil than were younger seedlings (98). Aryl acylamidase activity was also reported in rice root callus suspension cultures (98), but *in vitro* activity was demonstrated only in cultures that were at least 120 hr old. This did not correspond with the much earlier appearance of 3,4-dichloroaniline in propanil-treated cultures.

Inhibitor studies showed that carbamate insecticides were powerful competitive inhibitors of the aryl acylamidase from rice (94). The phosphorothioate insecticides, parathion and sumithion were much weaker inhibitors of the enzyme, but their oxidized analogs, paraoxon and sumioxon, were respectively 100X and 200X more effective as inhibitors (95). Phosphorothioate insecticides are partially converted to their oxygen analogs in plants (103). The strength of paraoxon as an inhibitor of the hydrolysis reaction *in vitro* was correlated with its synergistic effect in reducing the fresh weight of propanil-treated rice seedlings (95). The synergistic effect between propanil and the organophosphorthioate and carbamate insecticides (101, 107) can thus be explained on the basis of competitive inhibition of the aryl acylamidase enzyme necessary for the detoxication of propanil.

A particulate aryl acylamidase enzyme has also been isolated from red rice (108) and soluble aryl acylamidases have been isolated from tulip (99) and dandelion (109). Substrate specificities of these enzymes are shown in Tables IV and V. Significant and potentially useful differences exist in the substrate preferences of these enzymes for different halogenated propionanilides. 2',3'-Dichloropropionanilide was the preferred substrate for the amidase from cultivated rice, but it was the poorest substrate for red rice. In contrast, propanil was the best substrate for red rice, but had a relative rate of metabolism of only 42% in cultivated rice. Substrate preferences for

Figure 9. Hydrolysis of 3',4'-dichloropropionanilide (propanil) by an aryl acylamidase

different alkyl side chains were similar for these enzymes, each had a marked preference for the propionic side chain. These aryl acylamidases were not capable of hydrolyzing carbamate or urea herbicides. Some differences were found in the response of these enzymes to inhibitors (Table VI). These differences between the aryl acylamidases suggest that substrate specificity tests might be used to develop more selective compounds or compounds with more desirable biological stability. Differences in response to sulfhydryl inhibitors suggests that inhibitor studies with isolated enzyme systems could provide an effective means for the development of selective pesticide synergists.

Table IV. Substrate specificity of aryl acylamidase from red rice (108), rice (94), tulip (99) and dandelion (109): the effect of chlorine ring substitution.

	Relative Activity (%)			
Substrate	Red Rice	Rice	Tulip	Dandelion
2',3'-dichloropropionanilide	29	100	41	14
2',4'-dichloropropionanilide	47	84	100	49
2'-chloropropionanilide	29	60	66	37
3'-chloropropionanilide	73	42	27	51
3',4'-dichloropropionanilide	100	42	100	100
3',5'-dichloropropionanilide	33	30	--	--
2',5'-dichloropropionanilide	27	27	--	--
4'-chloropropionanilide	38	21	99	54
2',6'-dichloropropionanilide	--	1	2	0
propionanilide	58	--	--	--

Table V. Substrate specificity of aryl acylamidases from red rice (108), rice (94), tulip (99) and dandelion (109): the effect of various 3',4'-dichloroanilide alkyl analogs.

Substrate	Relative Activity (%)			
	Red Rice	Rice	Tulip	Dandelion
3',4'-dichloroacetanilide	82	59	49	49
3',4'-dichloropropionanilide	100	100	100	100
3',4'-dichlorobutyranilide	18	32	18	37
3',4'-dichlorovaleranilide	40	39	3	12
3',4'-dichloro-2-methyl propionanilide	--	2	--	--
3',4'-dichloro-2-methyl acrylanilide	0	0	2	8
3',4'-dichloro-3-methyl butyranilide	--	0	--	--

Thirty-eight different horticultural and agronomic crop species representing 10 different plant families were assayed for aryl acylamidase activity with propanil as the substrate (96). Enzyme activity was reported in over half of the species. Only one family, leguminosae, was devoid of activity. A similar study was conducted with 19 genera of weeds. Enzyme activity was assayed with propanil, 1,1-dimethyl-3-phenylurea (fenuron), and isopropylcarbanilate (propham) (97). Propanil was hydrolyzed at widely varying rates by approximately 70% of the species. Fenuron and propham, however, were hydrolyzed by the enzyme preparation from only one species, wild cucumber. The distribution of aryl acylamidases in other members of the plant kingdom has also been reported (100, 110).

Extensive studies with aryl acylamidases have shown that these enzymes are widely distributed in the plant kingdom. Activity varies widely within different plant tissues and among different plant species. Resistance to propanil is dependent upon the presence of these enzymes. Detailed substrate specificity studies with four aryl acylamidases revealed subtle differences in substrate specificity and response to inhibitors. Inhibitor studies with carbamate and organophosphate insecticides have clearly shown that these compounds are strong competitive inhibitors of the aryl acylamidases from rice. Interactions observed in the field between propanil and these insecticides can be attributed to the inhibition of aryl acylamidases.

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ACS Symposium Series; American Chemical Society: Washington, DC, 1979.

Table VI. Inhibition of aryl acylamidases from red rice (108), rice (94), tulip (99) and dandelion (109).

Inhibitor	Source of Enzyme							
	Red Rice		Rice		Tulip		Dandelion	
	Conc.	% Inhib.	Conc.	% Inhib.	Conc.	% Inhib.	Conc.	% Inhib
o-iodosobenzoate	--	--	0.5mM	74	0.25mM	5	0.25mM	27
Na_2AsO_2	--	--	1.0mM	74	--	--	--	--
$HgCl_2$	--	--	0.1mM	74	0.25mM	100	0.5mM	89
$CuSO_4$	0.2mM	60	0.2mM	59	0.25mM	16	0.5mM*	86
p-chloromercuribenzoate	0.2mM	4	0.5mM	50	--	--	0.25mM	61
p-benzoquinone	0.2mM	35	0.5mM	47	--	--	0.50mM	50
N-ethylmaleimide	0.2mM	6	0.5mM	44	0.50mM	9	1.0mM	23
iodoacetate	0.2mM	6	0.5mM	18	0.50mM	2	1.0mM	2
2,4-dichlorophenoxyacetic	0.2mM	2	--	--	--	--	--	--
pyrocatecol	0.2mM	29	--	--	--	--	0.5mM**	73
$CoCl_2$	--	--	--	--	0.50mM	1	1.0mM	16
$FeCl_3$	--	--	--	--	0.50mM	0	1.0mM	6

*$CuCl_2$ used instead of $CuSO_4$

**Catechol used instead of pyrocatechol.

Esterases:

Many carboxylic acid ester pesticides are readily hydrolyzed to free acids in vitro. In most cases, the free acid form of the pesticide is presumed or known to be the active agent. Esterases that hydrolyze carboxylic acid ester pesticides are important, not only because they play a role in the degradation of the pesticide, but also because they may be involved in activation, selectivity or detoxication. Some esterases may be inducible (111). Common inhibitors of these enzymes include a number of the organophosphate and carbamate insecticides (112-114). The possibility of pesticide interaction between carboxylic acid ester herbicides and certain insecticides exists. Research on the induction, activation, inhibition, and substrate specificity of esterases should have important and direct application to the improvement and better use of pesticides.

Esterases are probably ubiquitous and have been isolated from many plant species. The stability of plant esterase preparations varies with the source and may be related to the presence of phenol oxidases and polyphenols. Gel electrophoresis has been a valuable tool in studying plant esterases and has shown that these enzymes are a complex family of isozymes with differences in substrate preference and susceptibility to inhibitors (115-118). Polyacrylamide-gel electrophoresis separated 7 esterases from pea and 14 esterases from green bean (114). Separated isozymes responded differently to the assay substrates, α-naphtylacetate, α-naphthylbutyrate, and α-naphthylpropionate. Differential response to inhibitors such as parathion, paraoxon and diisopropylphosphorofluoridate was also observed. Gel electrophoresis of enzyme preparations from cabbage indicated the presence of 6 esterases (113). With 2-naphthylacetate as the substrate, inhibition was demonstrated with carbofuran, eserine and several other compounds. The partially purified esterase from cabbage had a pH optimum of approximately 7, and an estimated molecular weight of 69,000. This enzyme lost 50% of the original activity when it was stored for 22 days at -22°.

A few studies have reported the hydrolysis of pesticides by in vitro esterase systems (Figure 10). Recent studies showed that apple and cucumber leaves contain esterases that hydrolyze fungicidal nitrophenyl esters (115). Esterase activity was detected with 18 different substrates. Hydrolysis rates varied 100-fold, depending upon the substrate. Selective inhibition with paraoxon and several other compounds was also demonstrated. Eserine and EDTA were not inhibitory. Differences in isozyme patterns were observed between the two species.

The carboxylic acid ester herbicide chlorfenprop-methyl (methyl 2-chloro-3-(4-chlorophenyl)propionate) is hydrolyzed in vivo and in vitro to an active herbicide (119). Active esterases were isolated from 2 cultivars of oat, wild oat, wheat and beet

Figure 10. Hydrolysis of fungicides and herbicides by plant esterases (115, 119)

Figure 11. Hydrolysis of organophosphorus insecticides by plant esterases (127, 128)

(119). Estimates of in vivo rates of hydrolysis based upon in vitro data indicated that herbicide selectivity was not related to esterase activity. Two related herbicides, flamprop-isopropyl [(±)-N-benzoyl-N-(3-chloro-4-fluorophenyl)alanine isopropyl ester] and benzoylprop-ethyl [(±)-N-benzoyl-N-(3,4-dichlorophenyl) alanine ethyl ester] are also hydrolyzed to active herbicides, presumably by plant esterases (120, 121). Very low levels of activity have been reported for the in vitro hydrolysis of bifenox [methyl 5-(2,4-dichlorophenoxy)-2-nitrobenzoate] in homogenates of velvetgrass (122).

These in vitro and in vivo studies have shown that plant esterases can catalyze the hydrolysis of a number of pesticides. These studies do not, however, answer important questions regarding the localization of the enzymes within the cell or the distribution of the enzymes in various tissues of the plant. In the case of herbicides, these factors may play an important role in selectivity.

A number of organophosphorous compounds are metabolized in higher plants to products that are consistent with the involvement of hydrolytic enzymes (123-129). In vivo and in vitro studies showed that wheat and sorghum grains rapidly degrade dimethoate [O,O-dimethyl-S-(N-methylcarbamoylmethyl)phosphorothiolothionate] to a number of products including O,O-dimethylphosphorothionate, mono-O-methyl S-N-methyl-carbamoylmethylphosphorothiolothionate and mono-O-methyl-S-carboxymethyl-phosphorothiolothionate (127) (Figure 11). The apparent hydrolysis of malathion (O,O-dimethyl-S-bis(carboethoxy)ethyl phosphorodithioate) to dimethylphosphorothionate and dimethylphosphorothiolthionate with crude extracts from wheat germ was also demonstrated (128). Unfortunately, these studies do not establish the significance of esterases or phosphohydrolyases in the hydrolysis of organophosphorous pesticides. In animals and insects, glutathione S-transferases and mixed function oxidases play important roles in the metabolism of organophosphorus compounds (126). These enzymes may form some of the same products produced by esterases. Unless experiments are conducted properly, it may not be possible to discern whether GSH S-transferase, esterase or mixed function oxidase-related reactions are responsible for ester hydrolysis. In addition, plant peroxidases may also catalyze the cleavage of organophosphorous compounds (21).

In vivo and in vitro studies have shown that chloramben (3-amino-2-5-dichlorobenzoate) is rapidly metabolized to a stable N-glucoside in resistant plant species (130). In addition to the N-glucoside, susceptible species also form the chloramben glucose ester (130, 131). The glucose ester was not stable in vivo and appeared to act as a chloramben reservoir (Figure 12). Crude homogenates from cucumber and barley tissues hydrolyzed the chloramben glucose ester quite rapidly. Since other benzoic acid or phenoxy herbicides may form glucose ester conjugates, these hydrolytic enzymes could play an important role in regulating

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Figure 12. Chloramben conjugation and hydrolysis (130, 131)

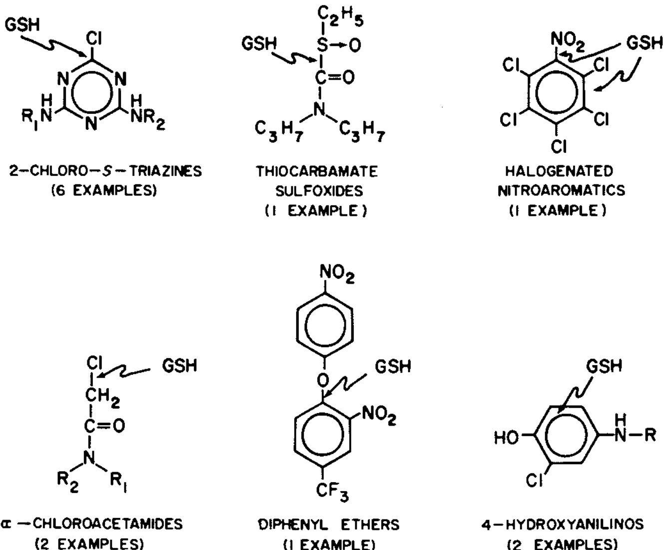

Figure 13. Agricultural chemicals known to be enzymatically conjugated to GSH in plants

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the levels of these herbicides *in vivo*.

CONJUGATION:

Glutathione S-Transferase:

In animals, glutathione (GSH) conjugation is an important reaction in the metabolism of a wide range of xenobiotics and has been the subject of a number of reviews (132-136).

```
                 HN-CH2-COOH                                HN-CH2-COOH
                 |                                          |
                 C=O                                        C=O
                 |           GSH S-transferase              |
R-X    +    CH2-CH       ------------------------->    CH2-CH    +   HX
            |    |                                      |    |
            SH  HN-C=O        COOH                      S   HN-C=O        COOH
                    |           |                       |       |           |
Substrate          CH2-CH2-CH                           R      CH2-CH2-CH
                                |                                           |
                               NH2                                         NH2

                   GSH                                       GSH Conjugate
```

Recent findings (137-146) have established that GSH conjugation is also important in plants. Although the range of xenobiotic compounds that have been demonstrated to undergo GSH conjugation in plants is limited (Figure 13), it can be assumed that GSH conjugation of additional classes of compounds will be shown as more studies are conducted. Glutathione conjugation should be considered as a possible metabolic reaction with any xenobiotic that possesses an electrophilic center and an appropriate leaving group, or with xenobiotics that can be activated by oxidation or by some other means to afford an active substrate. Glutathione conjugation is of particular importance in plants (a) because of the wide range of potential substrates, (b) because it may determine the nature of the terminal residues in the plant, and (c) because it may be a major factor in pesticide detoxication or herbicide selectivity.

Glutathione *S*-transferases have been found in 21 plant species (Table VII) and appear to be widespread in the plant kingdom. Glutathione *S*-transferases from animals (133) and plants (140, 142, 143, 145, 146) appear to be soluble enzymes. Glutathione *S*-transferase activity may not be detected in crude enzyme preparations until after gel chromatography (140) or may have limited stability when partially purified (142).

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Table VII. Species which have been examined *in vitro* for GSH *S*-transferase activity and cysteine *S*-transferase activity.

	Substrate Used to Assay for Activity				
Plant	Atrazine (143)	Fluorodifen (142)	PCNB (146)	EPTC (144)	4-hydroxy chlorpropham (191)
barley	N. D.				
corn	H	H	H	H	L
cotton		H	H		
crabgrass			H		
foxtail			H		
lambsquarter			H		
johnsongrass	H				
oat	N. D.			L	H
okra		H			
pea	N. D.	H	H		
peanut		H	H		
pigweed	N. D.		H		
soybean		H	H		L
sorghum	H				
sudangrass	H				
sugarcane	H				
squash		L			
tomato		L			
wheat	N. D.		H		
cucumber		L			L
rice					L

The first demonstrated GSH conjugation of a pesticide in plants was reported in sorghum (137). Atrazine (2-chloro-4-ethylamino-6-isopropylamino-s-triazine) was isolated as a GSH conjugate. The conjugation reaction was enzymatic (143) and active enzyme systems were subsequently isolated from atrazine-resistant corn, sorghum, johnsongrass, sudangrass and sugarcane. Atrazine-susceptible species (pea, oats, wheat, barley and pigweed) contained no detectable enzyme activity. The finding that GSH S-transferase activity was easily isolated from resistant species, but not from susceptible species, indicated that resistance to atrazine was based on the presence of this enzyme. Subsequent studies with an inbred corn line showed that GSH S-transferase activity was present in high concentrations in the resistant line, but not in the susceptible line. This was correlated to the production of the atrazine-GSH conjugate and lack of photosynthetic inhibition in the resistant line (147) (Table VIII). It was further shown that a number of resistant corn lines contained high concentrations of GSH S-transferase activity. These studies confirmed the importance of GSH S-transferase activity in the selectivity of atrazine.

In the tolerant species, corn and sorghum, GSH S-transferase activity was concentrated in the foliar tissue. Partially purified enzyme preparations from corn leaves were stable and could be stored with little loss in activity. Unfortunately, attempts to further purify the enzyme were not successful (148). Substrate specificity studies with several 2-chloro-s-triazine, 2-methoxy-s-triazine, and 2-methylmercapto-s-triazine herbicides showed that the 2-chloro-s-triazines were the only effective substrates. A later study showed that the sulfoxide of a 2-methylmercapto-s-triazine was a good substrate for GSH conjugation (149). The isolated corn enzyme was specific for GSH. Dithiothreitol, mercaptoethanol, 2,3-dimercaptopropanol or L-cysteine did not function as sulfhydryl substrates. Results from substrate specificity studies with the corn enzyme were comparable to those obtained with excised sorghum leaves (Table IX). Although *in vitro* studies indicated that 2-chloro-4-amino-6-isopropyl-amino-s-triazine was a poor substrate for GSH S-transferase in corn, *in vivo* studies with sorghum (150) showed that this substrate was conjugated with GSH. This difference may be attributed to differences in the substrate specificity of the GSH S-transferases from sorghum and corn.

The GSH S-transferase mediated cleavage of fluorodifen (p-nitrophenyl α,α,α-trifluoro-2-nitro-p-tolyl ether) to S-(4-tri-fluoro-2-nitrophenyl)glutathione and 4-nitrophenol appears to be the first demonstrated cleavage of a diphenylether by a GSH transferase system (142). When S-(4-trifluoromethyl-2-nitro-phenyl)glutathione was first detected *in vivo*, the nature of this metabolite was not understood. A crude enzyme that produced the same product in the presence of GSH was isolated from pea epicotyl tissue. Subsequent large-scale enzyme incubations

Table VIII. Relationship between GSH-transferase activity, formation of GSH conjugates, and inhibition of photosynthesis in atrazine resistant and susceptible corn.**

Corn line	Response to atrazine	GSH transferase* activity *in vitro*	% GSH conjugate in leaf disc after 5 hours	% Inhibition of photosynthesis in leaf discs after 2 hours
GT112RfRf	resistant	1.63	43.1	8.6
GT112	susceptible	0.03	0.4	64.9
6 other resistant lines	resistant	2.62 ± 1.0	---	---

*nmoles/mg protein/hour.
**adapted from (147).

produced S-(4-trifluoromethyl-2-nitrophenyl)glutathione in good yield and greatly facilitated the development of analytical techniques that were used in the isolation and identification of the in vivo metabolite (141, 142).

Table IX. Comparison of the relative substrate specificity of excised sorghum leaves (138, 150) with the substrate specificity of the glutathione S-transferase from corn (143).

Substrate	Relative activity in vitro (corn)	Relative activity in vivo (sorghum)
atrazine	0.68	1.00
GS-13529	1.00	0.93
cyprazine	0.52	0.92
propazine	0.42	0.90
simazine	0.05	0.62
2-chloro-4-amino-6-isopropylamino-s-triazine	0.01	active*
2-hydroxy-4-ethylamino 6-isopropylamino-s-triazine	0.01	--

*Glutathione-related conjugates were the primary products produced, but experimental conditions were different from those used with the other S-triazines.

The isolated GSH S-transferase from pea was stable, but crude enzyme preparations from corn, peanut and cotton underwent irreversible inhibition when stored for several hours at 4°. Enzyme activity was detected in higher concentrations in resistant species (cotton, corn, peanut, pea, soybean and okra) than in susceptible species (cucumber, tomato and squash). Fluorodifen selectivity appeared to be based on enzyme distribution and concentration. A broad substrate specificity study was not conducted with this enzyme, but two other diphenylether herbicides were tested and found to be inactive. It was hypothesized that only diphenylethers that were highly activated at the C-1 position would act as substrates. Mercaptoethanol, 2,3-dimercaptopropanol, dithiothreitol and cysteine would not function as the sulfhdryl substrate with the enzyme from pea.

Recent studies have shown that the fungicide PCNB (pentachloronitrobenzene) is also converted to GSH conjugates with an enzyme system isolated from pea (145). Additional studies will be needed to determine if the same enzyme is involved in the metabolism of both PCNB and fluorodifen. A unique feature of the

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PCNB GSH *S*-transferase assay system was the inclusion of *tert*-butanol to increase PCNB solubility and enzymatic activity (Figure 14). The use of *tert*-butanol in enzyme systems has been previously reported (151). This system has not been completely studied with respect to the PCNB-GSH conjugation reaction.

Detailed inhibitor studies were conducted with the GSH *S*-transferases isolated from both corn and pea (Table X). Both enzymes gave similar responses to sulfhydryl compounds and to the classical inhibitors of mammalian GSH *S*-transferase activity, sulfobromophthalein and 1,2-dichloronitrobenzene. Inhibition by sulfobromophthalein was competitive in mammalian systems and also appeared to be competitive in both plant systems. Propachlor (2-chloro-*N*-isopropylacetanilide) and barban (4-chloro-2-butynyl-*m*-chlorocarbanilate) were inhibitors of the enzymatic reactions with atrazine and fluorodifen. Inhibition by alkylating agents was also observed. The fact that sulfobromophthalein was a competitive inhibitor of both enzymes suggested some commonality of the active site(s). On the other hand, the fact that atrazine was neither a substrate nor an inhibitor of the pea enzyme suggested important differences. Inhibition of the pea enzyme by other diphenylether compounds, phenylureas and acetamide herbicides suggested the possibility of pesticide interactions with these compounds. A number of *s*-triazines were tested as inhibitors of the corn enzyme. These studies suggested that the *bis*(alkylamino)-methoxy-*s*-triazines and the methylmercapto analogs were probably competitive inhibitors capable of binding at the active site, but incapable of undergoing reaction. Hydroxytriazines and the dealkylated triazines were not effective inhibitors. It is of particular interest to note that a known synergist of atrazine, 2,3,6-trichlorophenylacetic acid, was an inhibitor of the GSH S-transferase from corn.

EPTC (*S*-ethyl dipropylthiocarbamate) does not appear to be a GSH *S*-transferase substrate. However, after oxidation to the sulfoxide, it readily undergoes conjugation in the presence of GSH *S*-transferases isolated from rat liver (152) or corn root (144).

$$\underset{\text{EPTC}}{C_2H_5\text{-}S\text{-}\overset{O}{\overset{\|}{C}}\text{-}N(C_3H_7)_2} \longrightarrow \underset{\text{EPTC Sulfoxide}}{C_2H_5\text{-}\overset{O}{\overset{\uparrow}{S}}\text{-}\overset{O}{\overset{\|}{C}}\text{-}N(C_3H_7)_2}$$

$$\underset{\text{EPTC Sulfoxide}}{C_2H_5\text{-}\overset{O}{\overset{\uparrow}{S}}\text{-}\overset{O}{\overset{\|}{C}}\text{-}N(C_3H_7)_2} \xrightarrow[\text{GSH-transferase}]{\text{GSH}} \underset{\text{S-Carbamyl-GSH}}{GS\text{-}\overset{O}{\overset{\|}{C}}\text{-}N(C_3H_7)_2}$$

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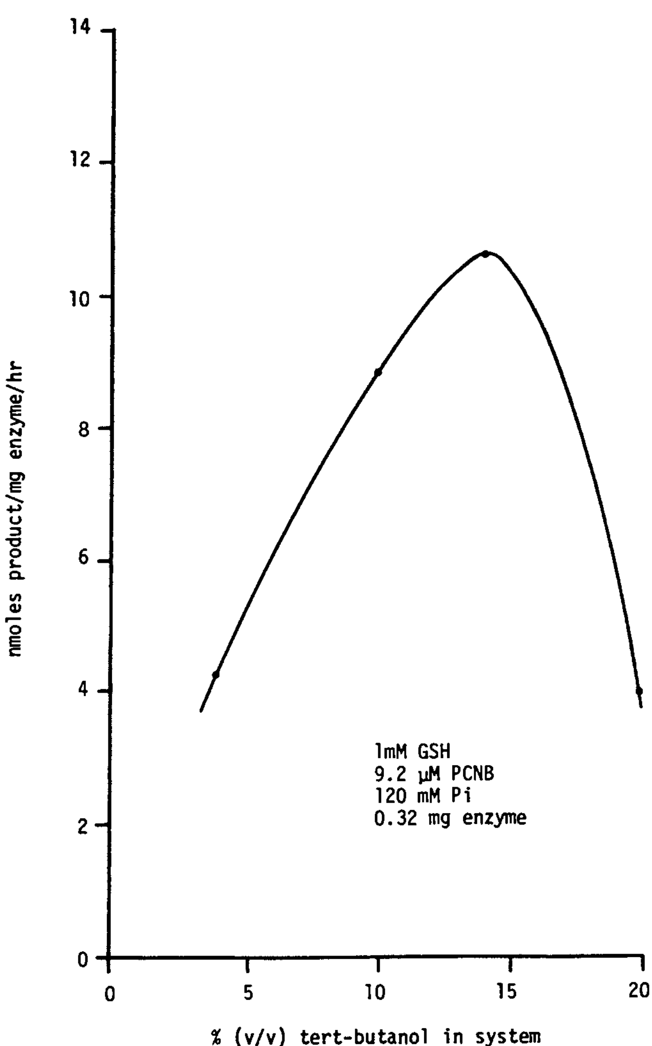

Figure 14. Effect of tert-*butanol on GSH conjugation of PCNB catalyzed by an enzyme system isolated from pea* (192)

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Table X. Inhibitor studies with the glutathione S-transferases isolated from corn (143) and pea (142).

Inhibitor	Inhibitor concentration (mM)	% Inhibition of GSH-transferase (pea)	% Inhibition of GSH-transferase (corn)
dithiothreitol	1	14	40
S-methyl glutathione	1	6	11
2,3-dimercaptopropanol	1	86	72
cysteine	1	0	0
atrazine	0.1	3	N.A.
2,4-bis(isopropylamino-6-methyl-mercapto-s-triazine*	0.06	--	61
2,3,6-trichlorophenyl acetic acid	0.1	--	29
propachlor	0.1	28	29
barban	0.1	60	34
sulfobromophthalein	0.1	88	41
1,2-dichloro-4-nitrobenzene	1	79	54
nitrofen*	0.03	55	--
diuron*	0.1	57	--
propanil	0.1	68	--
1-chloro-3-tosylamido-7-amino-L-2 2-heptane·HCl* (alkylating agents)	1.0	58	--

*Related compounds were tested with similar results.

This is comparable to a previous observation that a methylmercapto-s-triazine would not undergo conjugation until after oxidation to the sulfoxide (149). Recent studies have also shown that chlorpropham (isopropyl m-chlorocarbanilate) is not a substrate for GSH S-transferase activity in oat, but its oxidation product, 4-hydroxychlorpropham, readily undergoes an enzymatic reaction with cysteine or GSH (139, 140). These observations illustrate the need to consider the possible role of activating reactions in GSH conjugation.

When corn is treated with N,N-diallyl-2,2-dichloroacetamide (R-25788), herbicidal injury due to EPTC is greatly reduced (153). Glutathione S-transferase activity and the concentration of GSH were increased 2- to 3-fold by treatment with 0.3 to 30 ppm of R-25788. It was concluded that decreased herbicidal injury was due to an increased rate of GSH conjugation brought about by the elevated levels of GSH and GSH S-transferase activity (152). In EPTC-susceptible oat seedlings, the levels of GSH and GSH S-transferase did not increase in response to R-25788. The action of R-25788 appears to be selective. No increase in activity was noted when the isolated corn system was treated with R-25788; therefore, R-25788 does not appear to be a simple activator. Increased levels of GSH S-transferase activity were observed in both crude and partially purified enzyme preparations after treatment with R-25788. Although it was not proven, the results suggest that enzyme induction or possible removal of endogenous inhibitors may be responsible for the observed increases in enzyme activity. Twenty-eight compounds were compared to R-25788 for their effectiveness in increasing GSH S-transferase activity and GSH content in corn seedling roots. Although significant exceptions were noted, the effectiveness of these compounds as antidotes generally correlated with increased GSH and GSH S-transferase levels.

Chlorpropham (isopropyl m-chlorocarbanilate) and cisanilide (cis-2,N-phenyl-1-pyrrolidinecarboxanilide) are metabolized to hydroxylated derivatives in certain plant species (139, 154). Recent evidence indicates that the 4-hydroxylated derivatives of chlorpropham and cisanilide are converted to GSH and cysteine conjugates in oat shoot sections (139, 140) (Figure 15). The soluble enzyme complex that catalyzes conjugate formation was isolated from oat. When cysteine and 4-hydroxychlorpropham were incubated with the enzyme, a polar metabolite was formed. When GSH was substituted for cysteine, a more polar product was formed. The in vitro enzyme system was used to produce sufficient metabolite from the reaction with cysteine and 4-hydroxychlorpropham to allow isolation and partial characterization of the product (139). Because of the low yield of this product in the in vivo system and difficulties encountered in its isolation, the use of an in vitro system for product formation greatly facilitated the characterization of this product. In the characterization of the cysteine conjugate, cysteine C-S lyase

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CYSTEINE

HO, Cl, H, O, N–C–OCH(CH_3)$_2$

S–CYSTEINE

HO, Cl, H, O, N–C–OCH(CH_3)$_2$

4–HYDROXYCHLORPROPHAM

S – TRANSFERASES
(OAT)

GSH

HO, Cl, H, O, N–C–O–CH(CH_3)$_2$

SG

Figure 15. Reactions thought to be catalyzed by a GSH/cysteine S*-transferase from oat* (139, 140)

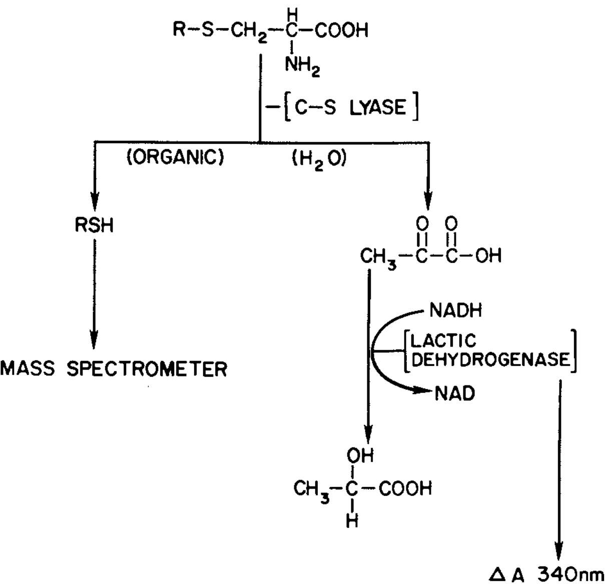

Figure 16. Cysteine C–S lyase cleavage of cysteine conjugates (139, 150)

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was used to cleave the cysteine moiety from the aryl group to yield pyruvic acid and a thiophenol (Figure 16). The reaction was quantitative and the liberated pyruvic acid was measured by coupling the cysteine C-S lyase reaction to a lactic acid dehydrogenase reaction. These coupled reactions were previously used in the characterization of a GSH-related conjugate of atrazine (150).

Glutathione S-transferase activity with GSH and 4-hydroxychlorpropham was not demonstrated until the crude enzyme was partially purified by Sephadex gel chromatography. This behavior suggested the presence of endogenous inhibitors. Detailed inhibitor studies showed that several naturally occurring aromatic compounds and 3-chloro-4-hydroxyaniline were powerful inhibitors of the cysteine S-transferase activity (155). It was suggested that these enzyme systems may use naturally occurring hydroxylated aromatic compounds or aryl hydroxylated xenobiotics as substrates. Two transferase enzyme systems were apparently present in oat shoots. One exhibited nearly comparable activity with either cysteine or GSH and the other displayed much greater activity with cysteine. The latter enzyme also functioned as a GSH S-transferase when the ethyl-ester of cysteine was added to the reaction mixture (140). The nature and the significance of this activation is not understood.

These are the first studies to suggest that cysteine may be utilized as a substrate much like GSH in a transferase reaction (139, 140, 155). This system should be studied in greater detail to better evaluate its significance to xenobiotic metabolism.

Glutathione S-transferase activity was recently isolated from 10 agriculturally important plant species and screened for activity with 8 different pesticide substrates (146). Of the substrates examined, GSH S-transferase activity was demonstrated in all species with PCNB, propachlor and CDAA (N,N-diallylchloroacetamide). The results suggested that certain types of GSH S-transferase activity may be widely distributed in higher plants.

These limited studies have clearly shown that GSH S-transferases play an important role in xenobiotic metabolism in plants. Some GSH S-transferases appear to be widely distributed in the plant kingdom, but others appear to be more limited in their distribution. Glutathione S-transferase enzymes play an important role in the selectivity of certain herbicides, such as the 2-chloro-s-triazines, fluorodifen and EPTC sulfoxide, but their role in the selectivity of herbicides such as the α-chloroacetamides is uncertain. The possibility that herbicidal selectivity may be increased by selectivity stimulating or inducing GSH S-transferase levels has been raised. Additional studies are needed to determine the distribution of GSH S-transferases in higher plants and to better determine the properties of the individual transferases.

Glucose Conjugation:

The reports that ethylenechlorohydrin was converted to a β-O-D-glucoside and a gentiobioside in wheat (156) and tomato (157) were among the first indications that plants had the ability to convert certain xenobiotics to glucosides. It was later shown that most higher plants (158, 159) had the ability to convert exogenous phenols to β-O-D-glucosides. This ability was apparently lacking in algae, fungi and certain aquatic plants (158). The formation of O-, N-, and S-glucosides, acylated glucosides, gentiobiosides, and glucose esters have all been demonstrated either *in vitro* or *in vivo* with xenobiotics or natural substrates (160). The formation of glucosides is extremely important in pesticide biochemistry for the following reasons: there is a wide range of potential substrates for conjugation, glucoside formation may affect the nature of terminal residue, and glucosylation may play a role in pesticide selectivity or detoxication. The most common types of glycoside reactions encountered in pesticide metabolism appear to involve an initial UDPG-dependent glucosyl transfer reaction (Figure 17).

The formation of simple O-glucosides from polyhydroxylated phenols was demonstrated with an *in vitro* system from wheat germ (161). Substrate specificity tests showed that the wheat germ glucosyltransferase could use a number of polyhydroxy phenols as substrates, but was not active with simple phenols. After purification of this enzyme, activity for certain substrates was lost; thus, the presence of more than one transferase was indicated. The *in vitro* synthesis of 14 phenolic glucosides by crude enzymes from wheat germ and bean was compared with the *in vivo* synthesis in bean (162). The only products detected *in vitro* were generally the primary products formed *in vivo*. The enzyme systems from wheat germ and bean could not utilize simple mono-hydroxylated phenols as substrates; it is, therefore, questionable whether these enzymes are involved in the formation of β-O-D-glucosides from pesticides or pesticide metabolites. A number of UDPG:sterol glucosyltransferases have been isolated from various plant sources (163-166). These enzymes are usually associated with the particulate fraction (164). For some phenolic xenobiotics, the possibility should be considered that UDPG:glucosyltransferase activity may be membrane bound.

A UDPG-dependent enzyme that catalyzes the formation of β-O-D-glucosides with a variety of phenols, alkyl alcohols and other substrates has been isolated from germinating mung bean (167). Attempts to demonstrate the presence of this enzyme in seedlings were not successful. The ammonium sulfate fractionated enzyme from germinating mung beans could be stored in liquid nitrogen with little loss in activity, but the more highly purified enzyme lost all activity upon freezing. This enzyme utilized UDPG as the glucosyl donor, had an estimated M.W. of 62,000 and had a pH optimum of approximately 10. The pH optimum

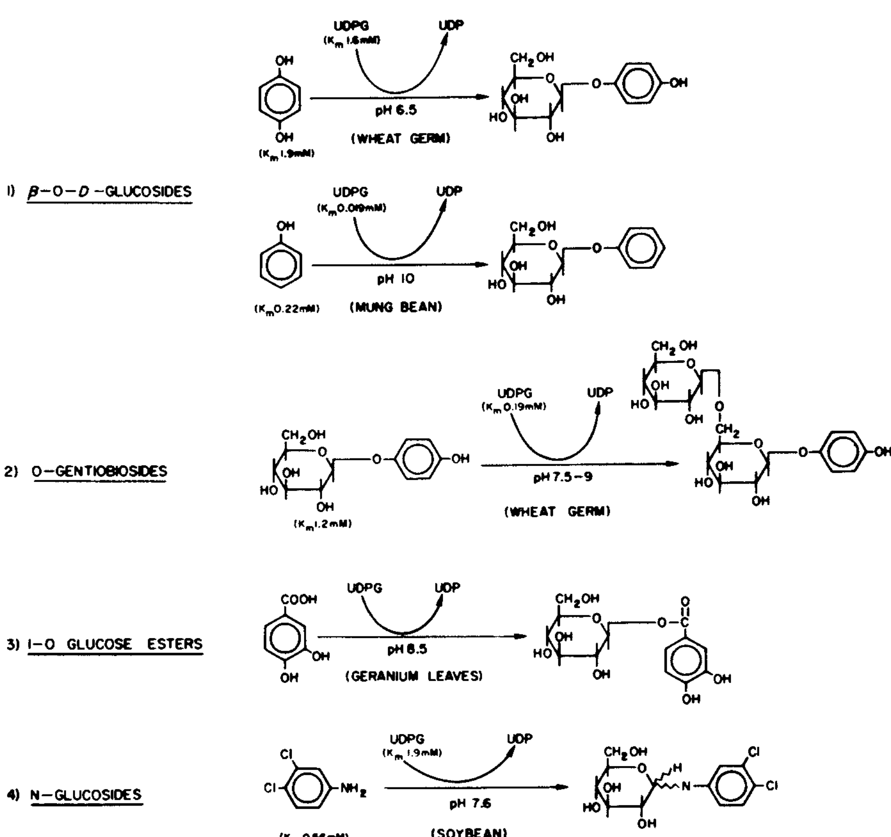

Figure 17. Glucosyl transferase systems that use UDPG as the glucosyl donor

for most other glucosyl transferases is between 6.5 and 9. Detailed substrate studies showed that reaction rates with the mung bean enzyme were primarily dependent upon the size of the acceptor substrate. n-Butanol was the most active alkyl acceptor and phenol was the most active aryl acceptor. The K_m values observed with a number of low molecular weight substrates suggested that this enzyme was non-specific and could play an important role in the metabolism of pesticides and other xenobiotics.

A crude enzyme system from wheat germ was shown to contain an enzyme that utilized arbutin as a substrate in the formation of the corresponding gentiobioside (168). This enzyme was separated from UDPG:polyhydroxyphenol glucosyl transferase activity and was partially purified. A broad range of phenolic β-glucosides were substrates for this enzyme, but it was not active with free phenols. This type of enzyme may be involved in the formation of pesticide gentiobioside conjugates such as diphenamid (169). A similar enzyme that catalyzes the formation of an O-α-L-rhamnosyl-(1-O)-β-D-glucosyl conjugate of quercetin has been isolated from mung beans (170). Pesticide conjugates of this type have not been reported.

The in vitro formation of the 1-O-glucose ester of anthranilic acid was demonstrated with a UDPG-dependent transferase from lentils (171, 172). The formation of 1-O-glucose esters of several 4-hydroxy cinnamic acid derivatives (p-coumaric, caffeic, ferulic, and sinapic acids) and several hydroxybenzoic acids (vanillic, isovanillic and syringic acid) was demonstrated with an acetone powder from geranium leaves (173). The crude enzyme system from geranium leaves required UDPG for activity, had a pH optimum of approximately 8.5 for ester formation, and was also capable of forming β-O-glucosides with phenols at pH 7.4. The 1-O-glucose esters of both chloramben (131) and naphthelene-acetic acid (174) have been reported in plants. These products may also be formed by UDPG-dependent systems such as those from lentils or geranium leaves. The formation of 1-0-glucose ester conjugates of herbicides may not be a permanent detoxication mechanism since these products are readily hydrolyzed in vivo and in vitro as previously discussed.

The formation of 2-O, 4-O and 6-O glucose esters of carboxylic acid appears to involve a different mechanism (Figure 18). A soluble enzyme from sweet corn kernels was shown to catalyze the formation of 2-0, 4-0 and 6-0 glucose esters of indole-3-acetic acid in the presence of ATP, CoA, and Mg^{++} (175). Products such as these may also be important in pesticide metabolism in higher plants (160).

The in vitro formation of substituted aniline N-glucosides was demonstrated with enzyme systems from soybean (176, 177) and pea (178) (Figure 17). The UDPG:arylamine glucosyltransferase from soybean utilized UDPG or TDPG as the glucosyl donor for 15 different arylamine acceptors (176). During the isolation of this

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Mg^{++}, ATP, CoA

pH 7.6

(SWEET CORN KERNELS)

Figure 18. Formation of 2–0, 4–0, and 6–0 glucose ester conjugates (175)

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enzyme, activity was demonstrated with arylamines and hydroquinones; however, the ratio of these activities changed dramatically during purification. It appears that more than one transferase was originally present.

In vivo studies indicated a direct relationship between chloramben N-glucoside formation and resistance (179). Several arylamine herbicides are metabolized directly to N-glucosides in plants and a number of other herbicides have been reported to be metabolized to arylamines by plants (160). These metabolites might also be expected to form N-glucosides. Based upon our knowledge of the metabolism of herbicides to aniline derivatives and the broad specificity of the UDPG:arylamine glucosyltransferase from soybean, it would appear that this enzyme plays an important role in xenobiotic metabolism.

The formation of 6-O-malonyl-β-D-glucosides of endogenous substrates (180-182) as well as the pesticides fluorodifen (183) and flamprop (184) have been reported. The reaction, with flavone glycosides as substrates, has been studied in vitro with an enzyme isolated from illuminated cell suspension cultures of parsley (185). The enzyme is a malonyl coenzyme A:flavone glycoside malonyl-transferase. The enzyme did not appear to be specific for the few glycoside substrates tested and no cofactors other than malonyl coenzyme A were required.

In addition to the UDPG-dependent glucosyl transferase systems, an enzyme that utilizes an endogenous glucoside, isosuccinimide β-glucoside, as the glucosyl donor has been characterized from pea (186) (Figure 19). This soluble enzyme has a pH optimum of 5.5 and converts ethanol and isopropanol to their corresponding β-O-D-glucosides. Isotope studies showed that the reaction was essentially irreversible. Various phenolic glucosides also functioned as glucosyl donors, but at a reduced rate. UDPG, glucose-1-P and related compounds did not function in this capacity. A high K_m for ethanol, 0.5 M, suggested that ethanol and isopropanol were probably not the natural substrates for this enzyme.

A similar or identical system from pea was recently shown to utilize polyethoxylated alkylphenol detergents as substrates (187). An unidentified endogenous glucoside and several exogenous phenolic glucosides functioned as glucosyl donors. Additional studies on the substrate specificity, distribution, and kinetics of this enzyme are needed to properly evaluate its importance in the metabolism of detergents and other xenobiotics.

SUMMARY:

Based on the enzyme studies considered in this report, it can be concluded that in vitro enzyme techniques can be used to great advantage to study pesticide metabolism in plants. Important advantages that may be offered by these techniques are as follows:

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Figure 19. Non-UDPG-dependent glycosyl transferase system from pea (186, 187)

Advantages:

(1) Specific metabolic reactions can be isolated from competing reactions and studied in a simplified system. Details of the reaction mechanism can be examined. Transitory or unstable intermediates can be detected or isolated.

(2) Extensive inhibitor and activator studies can be efficiently conducted. Possible pesticide interactions may be identified and nonpesticidal agents can be screened for use in combinations with pesticides to change their selectivity or improve their efficacy.

(3) Broad structure-activity studies can be conducted easily to determine the structural requirements for a specific metabolic reaction.

(4) Screening studies can be conducted with enzymes isolated from many different plant species and varieties to obtain basic information on genetic differences in metabolism or the mechanism of herbicide resistance.

(5) Enzyme induction studies can help in the development of protectants and other useful chemicals that function by stimulating the formation of key enzymes involved in metabolism.

(6) Isolated enzymes may be used effectively for the biosynthesis of specific metabolites in quantities that simplify their isolation and identification.

(7) Specific enzymes can be used to characterize and degrade more complex metabolites.

(8) Model enzyme systems can be developed to help solve difficult problems such as those associated with the bound residues of pesticides.

(9) Certain metabolic reactions may be studied in vitro without the need for radioactive substrates.

(10) Enzyme concentrations as a function of age or tissue type can be studied.

(11) Enzyme kinetic studies can be used to determine reaction mechanisms and to evaluate the importance of competing metabolic reactions.

The use of isolated enzymes to study xenobiotic metabolism is not without certain disadvantages. In addition to specific problems associated with the isolation of enzymes from plants, such as low levels of enzyme activity within the plant and the presence of phenolics and their oxidative enzymes that can result in enzyme inactivation or degradation during extraction and isolation, there are a number of disadvantages or problems associated with in vitro enzyme techniques in general. Those most relevant to xenobiotic metabolism are as follows:

Disadvantages:

(1) Results of isolated enzyme studies must usually be verified by in vivo studies.

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(2) The enzyme may be extremely difficult to isolate in an active form or may be labile and require reisolation for each study.

(3) The nature of the enzyme system may be unknown and it may be difficult to determine the proper co-factors.

(4) If the desired enzyme activity is found in several fractions, the researcher must decide whether to study only one fraction, study each fraction separately, or study the crude system.

(5) Endogenous inhibitors may be present. If not properly handled, they can yield misleading results.

(6) The system may be susceptible to substrate or product inhibition or the kinetics may be dependent upon the substrate concentration.

(7) The solubility of the substrate may be too low to obtain reliable kinetic data and it may be necessary to use detergents or solvents to solubilize the substrate. These agents may alter the kinetics or inhibit the enzyme.

(8) The methods necessary to isolate a specific enzyme activity from different plant species may vary sufficiently to make it difficult to conduct broad screening studies.

(9) It may be difficult to obtain the enzyme in sufficiently pure form to conduct meaningful kinetic studies.

(10) Low enzymatic activity generally makes necessary the use of radioactive substrates or other more sensitive assay methods.

This brief survey has documented the role played by various isolated enzyme studies in answering important questions related to pesticide metabolism in plants. It has shown that when proper consideration is given, *in vitro* techniques can be used to great advantage. Currently, our ability to use isolated enzymes to study certain areas of pesticide metabolism is limited by our basic knowledge of the enzymes involved. As this knowledge becomes available, our ability to use *in vitro* enzyme techniques to study additional areas of pesticide metabolism will be increased.

Abstract

The four basic biochemical reactions commonly involved in pesticide metabolism in higher plants (oxidation, reduction, hydrolysis and conjugation) are discussed in relation to the enzyme systems capable of catalyzing these reactions. The literature regarding the use of enzymes from these classes to study pesticide metabolism is reviewed. The following enzymes are considered: peroxidases, mixed function oxidases, hydroperoxidases, aryl nitroreductases, aryl acylamidases, esterases, glutathione *S*-transferases, cysteine *S*-transferases and various glucose-conjugating enzyme systems. The advantages and disadvantages of *in vitro* enzyme techniques as they related to pesticide metabolism studies are also discussed.

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Techniques for Studying the Metabolism of Xenobiotics by Intact Animal Cells, Tissues, and Organs In Vitro

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Researchers studying the fate of xenobiotics in mammals have tended to concentrate their efforts either on the whole animal or on liver homogenate systems such as microsomes. Each, of course, has its advantages. The response of a whole animal most closely represents what is likely to occur upon exposure of man to a compound. Liver homogenates are useful in that the liver is the principal organ responsible for the degradation and elimination of xenobiotics from mammalian systems, and liver microsomes appear to be the principal site within the mammal where metabolic conversions take place. These systems allow a rapid, inexpensive evaluation of metabolic events which are likely to take place in the mammal for any xenobiotic. The combination of studies in whole animals and liver microsomal systems generally provides a good understanding of the fate of a xenobiotic in a mammalian system.

Occasionally, however, a compound will be investigated in which the events taking place in a liver microsomal system do not entirely mirror what occurs in the whole organism. Furthermore, the use of microsomal systems does not provide information on the pharmacodynamics involved in the absorption, distribution, and elimination of a xenobiotic from an organism. Other techniques are available which will provide additional information on the fate of a xenobiotic in a mammal that would be difficult to obtain either with liver microsomal systems or in whole organisms. In this paper we will examine the use of perfused organ systems, with particular emphasis on liver and lung perfusion; tissue slices, particularly from lung and liver; cell culture systems, both primary cell cultures and established cell lines; and finally, the promising new technique of isolated hepatocyte preparations. For each technique we will examine the methodology currently in use and evaluate the ease by which an investigator inexperienced in the use of the technique would be able to adopt it for studies on specific compounds. The application of each of these techniques to the study of xenobiotics, with particular emphasis on pesticides, will be illustrated.

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Organ Perfusion

As is the case with most *in vitro* mammalian preparations, organ perfusion was originally developed to study the physiology and biochemistry of the organ itself and the functional position of the organ in the metabolism of the normal animal. Organ perfusion studies are an intermediate step between studies in the whole animal and experiments with isolated subcellular preparations. By observing the response of a viable organ isolated from the system in which it resides, one is able to assess the part which the organ plays in the metabolism of any given system. It is frequently possible to observe specific steps in metabolic processes in an isolated organ when in the whole animal only substrate and final product are observable. It is in the ability of the researcher to isolate individual reactions that perfused organs have their principal utility. Another advantage of perfused organ preparations is that blood flow, gas exchange, and temperature are under direct control. This enables the researcher to deliberately manipulate these parameters to assess the effect of, for example, reduced blood flow, anoxia, hypothermia, changes in blood pH, or osmolarity on the metabolic process being studied. For background information and descriptions of the methodology available for organ perfusion one may refer to a number of books dealing with the subject. Two examples are Ritchie and Hardcastle (11) and Ross (12).

Perfusion may be defined as the passage of a fluid medium or blood through the vascular bed of an organ. It is easy to see how one can utilize a perfusing organ to study the metabolism of a xenobiotic by the simple introduction of the chemical into the perfusate and observation of the effect which the organ has on the chemical. The possibilities for continuous sampling of the perfusate, continuous addition or pulse addition of the chemical to the perfusate, and studies of the interactions of more than one chemical in organs are readily apparent.

For workers studying xenobiotics the two most important organ perfusion systems are those for the liver and the lungs. The liver is probably the most frequently perfused organ and is used for a wide variety of studies. The techniques for liver perfusion presently most frequently used are those developed by Miller *et al.* (7) as illustrated in Figure 1. The apparatus designed by Miller and co-workers is now commercially available.

Techniques for perfusion of the lung have been more recently developed, the system of Niemeier and Bingham (8) being most frequently referred to by workers in this area (9). Niemeier and Bingham developed a system for the perfusion of rabbit lungs which allows the use of undiluted autologous whole blood as the perfusate. Lung perfusion is more complicated than liver since one is able to provide both a circulating liquid perfusate as well as being able to ventilate the isolated lung with a gas system. In

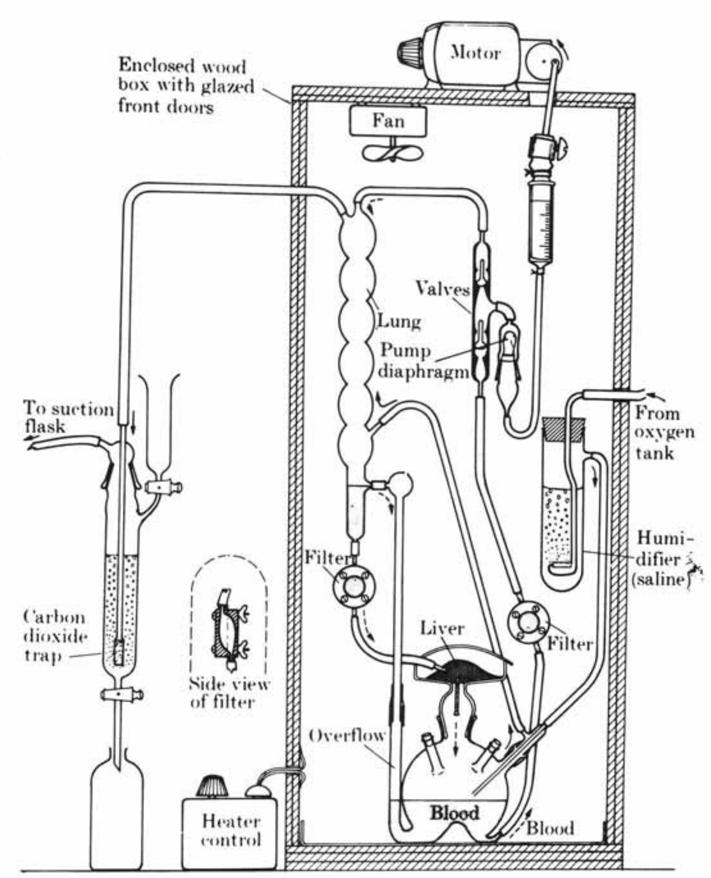

Figure 1. Liver perfusion apparatus. The apparatus is enclosed in a temperature-regulated cabinet and is composed of a system for pumping the perfusate (blood) at constant hydrostatic pressure and a system for oxygenating the blood (7, 12).

a lung, therefore, one can introduce a xenobiotic to the system either through the perfusion system itself or through the gas ventilating system, thus approximating the introduction of the xenobiotic either through the circulatory system or the respiratory system in the whole animal. The use of lung perfusion is particularly advantageous because the estimation of substrate utilization by the lung is very difficult *in vivo*. Lung tissue slices or other *in vitro* preparations do not seem to approximate the physiological state of the whole organ as well as comparable preparations from the liver; the need for oxygen and the problem of diffusion are particular problems in the lung. Isolated perfused lung systems overcome these problems and will be particularly useful in the future for the study of the metabolism by the lung of xenobiotics introduced by inhalation.

Xenobiotic metabolism in perfused liver may be illustrated with parathion. Both parathion and paraoxon were studied to assess the metabolic relationships of these compounds in the liver (3). It was shown that 68% of the administered parathion was metabolized to water soluble compounds. These water soluble compounds were found to be conjugates of *p*-nitrophenol, the bulk of which was in the circulating perfusate, not associated with the liver tissue or excreted via the bile. An additional 2.5% was paraoxon and there were traces of unconjugated *p*-nitrophenol. Administered paraoxon was degraded almost entirely (98.5%) to water soluble compounds, which again were conjugates of *p*-nitrophenol.

Another type of experiment possible using perfused organ systems is illustrated by a study of mirex-induced suppression of biliary excretion of polychlorinated biphenyls (5). In this study it was shown that 50 mg/kg/day of mirex-pretreatment of the rats whose livers were perfused suppressed the biliary excretion of 4-chlorobiphenyl and its metabolites by 92%. Furthermore, the rate of metabolism of 4-chlorobiphenyl was decreased slightly by mirex pretreatment. The reason for this phenomenon was theorized to be that transport of otherwise readily excretable metabolites from the hepatocytes into the bile canaliculi was affected by mirex. The fact that mirex causes changes in the ability of the liver to excrete xenobiotics has implications for the possible effect of this compound on the toxicology of other compounds.

A number of recent studies of xenobiotics in perfused lung systems have been reported: aldrin and dieldrin (6), parathion, methadone, imipramine, chlorcyclizine, and pentobarbital (4), trichloroethylene (2), and carbaryl (1). These studies illustrate well the potential for important results which can be obtained from organ perfusion studies.

Perfused rabbit lungs were used to study the metabolism and binding properties of aldrin and dieldrin. The compounds were added to the system via the perfusion medium and samples were withdrawn at several intervals. It was noted that aldrin was epoxidized to dieldrin, but dieldrin was not further metabolized

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in the system; no epoxide hydrase activity could be detected. The uptake of aldrin and dieldrin was by diffusion. The rate of uptake was biphasic, consisting of an initial rapid phase followed by a slower one, related to conversion of aldrin to dieldrin in the case of aldrin. These studies show that the lungs are not a significant storage site for either compound (6).

The metabolism of parathion, methadone, imipramine, chlorcyclizine, and pentobarbital was compared in a rabbit perfused lung preparation with rabbit lung and liver microsomal preparations. In the perfused lung parathion, methadone, and pentobarbital were oxidatively metabolized. Parathion was extensively metabolized to paraoxon and water soluble metabolites, which were not further identified. No accumulation of parathion was observed in the system. With increasing perfusion time there was a decrease in the appearance of parathion, methadone, and pentobarbital metabolites. This was more likely associated with decreasing substrate or depletion of cofactor than to denaturation or destruction of the lung system. No significant differences were observed between the drug metabolizing activities of the microsomes of lung or liver and the perfused lung system (4).

An interesting example of the use of the perfused lung system to study the metabolism of a xenobiotic by the inhalation route is given by Dalbey and Bingham (2). They studied the metabolism of trichloroethylene in a rabbit perfused lung system. Trichloroethylene was generated into the air supplied to the isolated perfused lungs, and the compound and its metabolites were measured periodically in the perfusate and in the lung tissue following a three-hour perfusion period. Trichloroethylene was extensively metabolized to trichloroethanol, trichloroethanol glucuronide, and trichloroacetic acid. It was postulated that chloral hydrate was an intermediate in the metabolism of trichloroethylene but it was not isolated in this system.

Carbaryl metabolism in the perfused rabbit lung was shown to be rapid. The pharmacokinetics of carbaryl uptake demonstrated simple diffusion. After 30 minutes of perfusion 1-naphthol was seen in the perfusate extracts. Since its concentration in the perfusate decreased during the course of the experiment, it was concluded that the 1-naphthol which was taken up by the lungs was formed by nonenzymatic hydrolysis of carbaryl in the perfusate. 4-Hydroxycarbaryl appeared in the perfusate at 30 minutes and increased in concentration until 60 minutes, after which it decreased. Other metabolites were isolated but no attempt was made to identify them (1).

Comparative studies (10) have shown that perfused organs, especially the liver, parallel changes which occur in the whole organism. Thus, the technique can be a useful bridge between other *in vitro* studies and *in vivo* studies.

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Tissue Slices

The techniques for tissue slice studies of metabolism were probably first introduced by Otto Warburg in 1923 (19). The method caught on rapidly and was used by many workers so extensively that Krebs and Henseleit, writing in 1932 (15), noted that between the two methods available for studying metabolism in animal tissues, they preferred tissue slices over perfusion. This judgment probably represented a reaction to the lack of reproducibility of perfusion techniques and the complicated systems then in use. By comparison, tissue slices coupled with manometry offered a simple, producible, flexible method for studying metabolism. While the judgment made by Krebs and Henseleit would probably not be valid today, the intervening years have seen a great deal of fine work using the isolated tissue slice technique.

Again, the liver is the principal organ which has been studied using tissue slices. However, many other organs have also been used in a variety of studies. Of particular note for xenobiotic metabolism studies in addition to the liver are kidneys, lungs, and intestines. The key to the preparation of viable tissue slices is to obtain reproducible, thin slices, generally less than 0.5 mm thick. This is most frequently done at present using a microtome or similar instrument. The viability of the tissue and standardization to determine reproducibility are frequently evaluated using Warburg respirometry (16).

Thin slice techniques have been used extensively for the study of pesticide metabolism, and examples of a variety of studies follow. One of the earliest establishments of the need for conversion of an organophosphorus insecticide to an active anticholinesterase metabolite used the liver slice technique in combination with Warburg respirometry (14). Liver slices were used to demonstrate the conversion of dimefox, a phosphoramidate, to an active inhibitor using rat brain cholinesterase as the substrate. Inhibition of cholinesterase was measured in the Warburg apparatus, and inhibition of cholinesterase was taken as indirect evidence for metabolism of dimefox by the liver slice. Liver slices 0.5 mm thick and 5 mm square, washed twice with 0.9% sodium chloride, were used in each Warburg flask. Substrate and enzyme sources were added from side arms, and cholinesterase inhibition was assayed by standard methodology.

The herbicides propham and chloropropham were studied in the rat *in vivo* and metabolism was compared in liver slices and kidney slices (13). These herbicides were metabolized *in vivo* to two major and three minor metabolites; both oxidative and hydrolytic mechanisms were evident. Liver and kidney slices, however, did not hydrolyze the chain moiety as observed *in vitro*. Only liver slices converted the herbicides to their oxidative metabolites.

Rat renal cortical slices and rat liver slices were used to assess the mechanism for excretion of dichlorodiphenylacetic acid (DDA) from animals (18). It was hypothesized that DDA is excreted

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via the organic acid system. It was shown that DDA was avidly accumulated by liver and by kidney slices. DDT or organic bases did not inhibit the accumulation of DDA, indicating that the organic acid excretion system is indeed responsible for the elimination of DDA from the mammalian system. This ultimately, of course, is the method for the elimination of DDT from the system since DDT is first converted to DDA before it is excreted.

An interesting study of carbaryl metabolism has been reported using an isolated intestinal tissue (17). For this study the small intestine between the bile duct and the cecum was removed from male rats, rinsed in isotonic saline, and divided into three approximately equal parts. The sections were everted and maintained over ice in fresh saline plus glucose solution until the serosal compartment was filled with serosal fluid. The filled sacs were then transferred to a flask containing mucosal fluid and incubated with agitation for one or two hours. Analysis of the products of carbaryl incubation indicated the production of 1-naphthol, again apparently by nonenzymatic mechanisms. At least seven metabolites were identified. The principal water soluble metabolite (60%) was 1-naphthol glucuronide.

Although much useful information about xenobiotic metabolism has been obtained with liver slice techniques, most workers today prefer to use other methods, probably because of difficulties of reproducibility using the technique.

Cell Culture

Mammalian cells in culture have been used for over one-half century to study various aspects of biology. Since Harrison first successfully propagated medullary tissue *in vitro* in 1907, cell cultures have been utilized in the study of radiobiology, cell division, and genetic cytology as well as other areas of cell biology. Mammalian cell cultures have also been used to study the correlation of cytotoxicity of drugs with other pharmacological attributes. The primary use of cell cultures has been to provide a method for investigating the direct action of drugs and other chemicals on cells in the absence of the complex interactions which apply in the whole animal. A high correlation, for example, has been found between *in vitro* cytotoxicity and *in vivo* antitumor activity of a number of chemotherapeutic agents screened for anticancer activity.

A wide variety of cell types have been cultured and maintained *in vitro*. Both normal cells from many different organs and tissues of many different animal species and abnormal cells isolated from tumors or other abnormal tissues have been utilized. Cells have been successfully explanted from animals in all stages of development, from the embryo to the adult.

One of the problems which must be recognized in working with cell cultures is the difference which is likely to exist between these cell strains and the tissue from which they originated.

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Cell strains or cell lines, so called, consist of cells which may have been growing *in vitro* for a considerable length of time and which have undergone many subcultures or dilutions of cell numbers. Generally they have a different pattern of metabolism, are capable of supporting the growth of a wide variety of viruses and microorganisms, and are frequently polyploid. Such divergences from normal tissue must be considered when evaluating experimental results.

Some of the difficulties in working with cell lines are eliminated by working in primary cell cultures. These are cells which have not undergone even a single passage or subculture since having been explanted from the donor animal. Such cultures have been shown to retain enzymatic activities similar to those of the *in vivo* donor tissue. The activity usually lasts through a few initial subcultures before receding to decreased levels.

Cells of both types, cell lines and primary cell cultures, are commercially available. The techniques for maintaining and using cells are simple, although rigorous sterile technique is absolutely necessary to avoid contamination of cell cultures by invading microorganisms of all types. The media used for cell cultures are ideal for the growth of microorganisms, which, if they contaminate the cultures, lead to artifacts in the experimental results. Rigorous attention to sterile technique is a prerequisite to accurate interpretation of the results of research using cell cultures.

Only a few papers appear in the literature reporting the results of studies of pesticide metabolism in cultured mammalian cells. The various studies have used human embryonic lung L-132 cells, HeLa S cells, mouse fibroblast L-929 cells, and mouse L-5178 lymphoma cells. All of these are established cell lines. In addition, studies have been conducted using primary human embryonic lung cells.

DDT metabolism has been studied in HeLa S cells (22), mouse L-5178 lymphoma cells (29), and primary human embryonic lung cells (28). The variation in the susceptibility of a compound to metabolism by various cell types is illustrated in these studies. Mouse lymphoma cells grown for 72 hours in the presence of DDT failed to metabolize the compound. At the other end of the spectrum, HeLa S cells metabolized DDT to DDD, DDE, DBM, and DBP. DDE was the metabolite present in the highest quantity after 24 hours of incubation and was postulated to be the terminal metabolite in this system. It is noted in the paper, however, that the conversion of DDT to DDE could have been enhanced by the iron porphyrin complexes in the medium. Primary human embryonic lung cells metabolized DDT only to DDD (38%) and DDA (4%). No other metabolites were found in this cell culture system.

Carbaryl has been studied in both a human embryonic lung cell line, the L-132 strain (20, 21) and in primary human embryonic lung cells (23). The HEL cell line was apparently active in conjugating carbaryl metabolites. The water soluble aglycones re-

sulting from beta-glucuronidase and aryl sulfatase treatment were 4-hydroxycarbaryl and 5,6-dihydro-5,6-dihydroxycarbaryl. In addition, hydroxylation at the C-4 position in conjunction with hydrolysis of the carbamate group resulted in the formation of naphthalene-1,4-diol. The interesting metabolite, 1-naphthyl methylcarbamate-*N*-glucuronide, was also reported in this study. The results obtained in primary HEL cells agreed very closely with those from the HEL cell line. In the primary cell cultures 1-naphthol was the principal metabolite isolated. Others included naphthalene-1,4-diol, naphthalene-1,5-diol, 4-hydroxycarbaryl, 5-hydroxycarbaryl, and 5,6-dihydro-5,6-dihydroxycarbaryl. In addition significant amounts of the administered carbaryl were present in extracts as conjugates. Acid hydrolysis freed 4-hydroxycarbaryl, naphthalene-1,4-diol, and 5,6-dihydro-5,6-dihydroxycarbaryl. On the other hand, beta-glucuronidase treatment of the aqueous material did not free aglycones. This result was in agreement with the earlier work in which Baron and Locke postulated the formation of an *N*-glucuronide in the cell culture system. The use of both primary and established HEL cells is an important link in understanding the metabolism of carbaryl in mammalian systems.

The utility and value of cell culture studies is illustrated well by a study of dimethoate metabolism in primary human embryonic lung cells (27). In this study the cells were shown to oxidize dimethoate with no interference from competing hydrolytic reactions. Thus, the progression of dimethoate metabolism from the phosphorodithioate to the phosphorothioate, concomitant with oxidative *N*-dimethylation of both the phosphorodithioate and phosphorothioate, could be demonstrated (Figure 2). The relationships existing between these three metabolites and their parent compound could not be established so well in a system in which hydrolytic reaction were competing with the oxidative ones.

Studies of the acaracide chlorphenamidine (24), and the related phenylurea herbicides, chlorotoluron, fluometuron, and metobromuron, (25) revealed striking differences in the susceptibility of these materials to metabolism by the cells. On the one hand, chlorphenamidine was very susceptible to oxidative metabolism in HEL cells with the formation of nearly 82% of the *N*-formyl metabolite and 2% of 4-chloro-*o*-toluidine as well as small quantities of other metabolites. While the herbicides were very resistant to metabolism (less than 2% of the applied compound was metabolized in 72 hours in each case), the small quantities of metabolites that were formed were the result of oxidative reactions in cells. It was in this cell culture system that the formation of *N*-formyl derivatives of chlorotoluron was first observed, a result later corroborated in liver microsomal preparations and in rats *in vivo* (26). The chromatographic behavior of the formyl derivatives made their detection difficult in systems which more actively metabolized the compound because of interfering materials on thin layer chromatographic plates.

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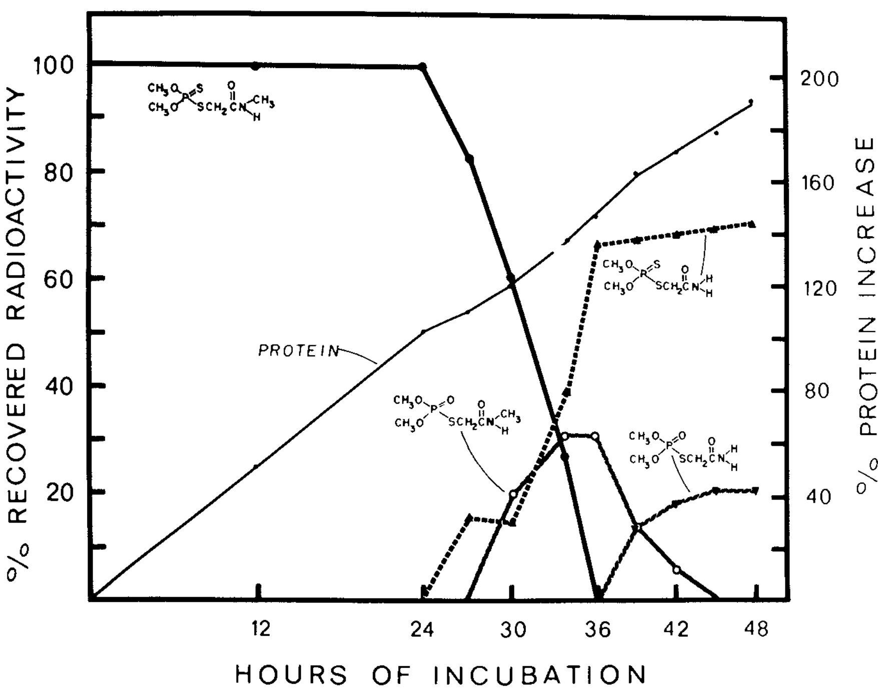

Pesticide Biochemistry and Physiology

Figure 2. Dimethoate disappearance, organoextractable metabolite formation, and percentage of protein increase in HEL cell cultures incubated with ^{14}C-dimethoate. Disappearance of dimethoate corresponds with the appearance of the des-N-methyl metabolite and the oxygen analog, which in turn disappears corresponding with the increase of its des-N-methyl derivative (27).

Cell culture techniques are useful in the study of xenobiotic metabolism. They cannot be used to establish quantitative relationships between xenobiotics and their metabolites, particularly when reference to the whole animal is desired, but they can be invaluable in studying the mechanisms of metabolism and in characterizing metabolites which may appear in only minor quantities in other mammalian systems.

Isolated Hepatocytes

The preparation and use of isolated hepatocytes is a recent innovation in the study of xenobiotic metabolism and should prove to be most useful. The technique is basically a simple one. Liver cells are dissociated from the protein matrix of the organ and are isolated in viable condition for use much as if they were isolated cells in culture as described earlier. A variety of methods have been developed for the isolation of hepatocytes, although the techniques have not yet reached the routine stage so that all researchers agree on the same basic methodology for their preparation. Earlier techniques involved either (1) the perfusion of liver with calcium chelators or alkaline hyperosmolar salt solutions, (2) the digestion of liver pieces in tetraphenylboron, a potassium chelator, (3) the use of enzymes as dissociating agents, notably trypsin for fetal or neonatal material, and (4) collagenase/hyaluronidase digestion. Various combinations of these techniques have also been tried (40). Currently there appear to be basically two methods for the preparation of hepatocytes in general use. One involves the perfusion of isolated liver with Hank's buffer for approximately 15 minutes followed by the addition of collagenase to the perfusate for an additional 10 to 15 minutes. This treatment collapses the liver, after which it is minced, centrifuged, washed, and resuspended in Hank's buffer for immediate use (45, 46, 47). The other technique eliminates the necessity for perfusion and uses enzymatic dissociation by treatment of liver with collagenase/hyaluronidase in Hank's balanced salt solution followed by centrifugation, washing, and resuspension (32, 40). The latter technique has the advantage of simplicity and low cost since no special perfusion apparatus is needed. Furthermore, it would be possible to prepare hepatocytes from pieces of fresh liver which might, for example, be available from biopsies without the need for the whole organ as required in the perfusion technique. Both techniques appear to give good yields of viable cells which can be used for metabolism studies. As for most *in vitro* methods, most of the early developmental work on isolation of viable hepatocytes has been done with rat livers. However, there is no reason why the techniques developed cannot be applied to the livers of other species as well, perhaps with very little modification. In fact chicken hepatocytes have been isolated using the rat liver techniques and were shown to carry out normal biochemical functions (37, 38).

The criteria of viability of isolated liver cells are a matter of considerable concern for metabolism studies since reproducibility demands a technique for standardization of preparations made at different times or by different methods. The most universally used technique to assess viability of isolated hepatocytes is the trypan blue exclusion test (31, 45). The test depends on the fact that the intact plasma membrane excludes dyes such as trypan blue, but damaged cells are stained, particularly intensely in the nucleus. Unfortunately, this actually measures structural integrity, not viability *per se*. Other tests have been used in addition, including electron microscopy, the content of adenine nucleotides, the activities of various enzymes, and the ability to synthesize various compounds. It seems to this reviewer that one must establish for his own type of experiments whether the cells are viable using criteria particularly applicable to the type of research being conducted. For example, in a metabolism study one might choose a particular substrate which could be used as a positive control in all preparations, whose metabolism could be conveniently measured and quantified for comparison between various preparations.

It is somewhat surprising to report that no studies have yet been published involving the metabolism of a pesticide in isolated hepatocytes. However, the technique has been widely applied in studies of drug metabolism and several reports on the metabolism of air pollutants and industrial chemicals have now appeared. Examples of drug metabolism studies are the reports of Billings *et al.* (33) on α-1-acetylmethadol, propoxyphene, butamoxane, ethinimate, 8-methoxybutamoxane, and *p*-nitrophenol; Erickson and Holtzman (39) on ethylmorphine; Hayes and Brendel (42) on quinine sulfate, dansylamide, and antipyrine; and Aarbakke *et al.* (30) on antipyrine. In general, the drugs studied were metabolized by the isolated hepatocytes by *N*- and *O*-demethylation, aromatic and aliphatic hydroxylation, and sulfate and glucuronic acid conjugation. These studies show that the metabolism of drugs in isolated hepatocytes correlates with *in vivo* drug metabolism better than does the liver homogenate 9000*g* supernatant or microsomal fraction. The results obtained from hepatocytes were more comparable to liver perfusions than to subcellular fractions in terms of the relative rates of individual reactions, which were sometimes faster and sometimes slower in hepatocytes than in microsomes.

Aromatic hydrocarbons are readily hydroxylated by isolated hepatocytes (34, 35, 43). Bock and co-workers showed that naphthalene was converted to 1-naphthol and to 1,2-dihydro-1,2-dihydroxynaphthalene and its sulfate and glucuronic acid conjugates (34). The isolated hepatocytes were more efficient in carrying out these conversions than microsomes, the reason being that the enzymes responsible, mixed function oxygenase, epoxide hydratase, and glucuronyl transferase, are all located in the same membranes. Benzo(a)pyrene was converted into arene oxides, phenols,

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quinones, and dihydrodiols as initial products and later sulfate, glucuronide, and glutathione conjugates were isolated (35). The monohydroxylated compounds and sulfate esters accumulated intracellularly, the 4,5- and 7,8-dihydrodiols were distributed evenly between the cells and the medium, and the 9,10-dihydrodiol accumulated in the medium. In these experiments significant amounts of radioactivity were bound irreversibly to cellular macromolecules (43).

The sequential formation of metabolites by isolated hepatocytes is illustrated by studies on the 4-hydroxylation of biphenyl and the subsequent conjugation of the metabolite (44). Figure 3 illustrates the fact that hydroxylation preceded glucuronide formation and that the removal of 4-hydroxybiphenyl by conjugation was necessary to stimulate a second phase of hydroxylation.

Fry *et al.* (41) have compared isolated cells from rat liver and kidney in terms of their ability to metabolize ethoxycoumarin, biphenyl, benzo(a)pyrene, 4-methylumbelliferone, and benzoic acid. The level of metabolism in the kidney cell suspension was extremely low compared with liver cells for initial oxidative reactions, but the pattern and extent of conjugations were very similar between the two types of cells. The difference in the ability of these cells to metabolize these compounds may relate to a different specificity of the renal cytochrome P-450, which is not adapted to xenobiotic metabolism.

The ability to retain active cytochrome P-450 in isolated hepatocytes is crucial to the use of this system to study xenobiotic metabolism. Cytochrome P-450 declines rapidly in isolated hepatocytes, but recently it was discovered that the addition of certain hormones to a primary hepatocyte culture retained the cytochrome P-450 activity at optimum levels for up to 24 hours (36). These workers used primary hepatocyte cultures derived from collagenase preparation with added hormones to study the metabolism of aflatoxin B_1.

Although isolated hepatocytes have not as yet been extensively used in metabolism studies, they clearly offer great promise in this area.

Conclusion

When one compares the various *in vitro* techniques used to study the fate of xenobiotics in mammals, one must be impressed by the fact that no one of the methods available is adequate to a complete understanding of the metabolism of a compound. However, when all methods are taken together, a rather complete picture can be assembled. Using carbaryl, one of the most extensively studied pesticides, as an example, one can see that the total *in vitro* results rather completely mirror *in vivo* metabolism. In fact, certain metabolites found *in vitro* have not been isolated following *in vivo* studies, the *N*-glucuronide noted from cell

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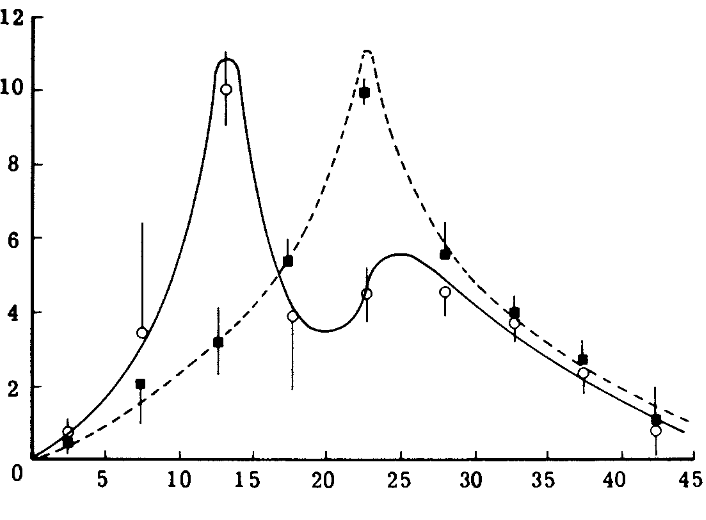

Figure 3. Rate of formation of 4-hydroxybiphenyl and its glucuronide conjugate in viable isolated hepatocytes. The rates of formation of (○), 4-hydroxybiphenyl and (■), its glucuronide conjugate are average rates for the respective 5-min intervals. Each point represents the mean of three values obtained from different experiments (± S.D.) (44).

cultures. The principal metabolite, 1-naphthol, is found in all systems studied. The 5,6-dihydro-5,6-diol, 5-hydroxy, 1,5-diol sequence is observed in cell cultures, while the 4-hydroxy metabolite is observed in perfused organs and cell cultures. The 1,4-diol was also isolated from cell cultures as were several glycoside conjugates.

One might compare the relative merits of the four techniques considered in this paper, adding microsomes to complete the picture, from four points of view: (1) ease of preparation, (2) flexibility of experimental use, (3) reproducibility of results, and (4) the extent to which the results obtained mirror *in vivo* results (Table I).

Table I. Relative merits of *in vitro* techniques for studying the metabolism of xenobiotics.

	Microsomes	Organ Perfusion	Tissue Slices	Cell Culture	Isolated Hepatocytes
Ease of Preparation					
Requirement for Special Equipment	-	-	o	+	o
Requires special training of personnel	+	-	+	o	-
Time for preparation	-	o	+	+	-
Expense	+	+	+	-	+
Flexability of Experimental Use	-	o	-	+	+
Reproducibility of Results	+	+	o	+	+
Mirrors *in vivo* results	-	-	o	-	+

\+ Favorable, positive aspect of this technique
o Neutral
\- Negative aspect of this technique

Although the assignment of values for each category is arbitrary and subjective, one must conclude from this exercise that each technique will be useful for some purposes in some researcher's hands. Ultimately our understanding of the fate of xenobiotics in mammals will be enhanced by accumulating data from all sources and applying each bit of data to obtain the complete picture.

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Abstract

Techniques considered include isolated whole living cells; cell, organ, and tissue culture; organ slices; and isolated perfused organs. Whole cell techniques serve as an important link between studies using purified enzymes and subcellular fractions and studies using whole organisms. The use of cells, tissues, and organs in culture is growing since they allow the researcher to explore the nature of metabolites and to research an understanding of the mechanisms of metabolism taking place within the cells or organs without the complicating regulatory influences of the whole organism. Both primary cells in culture and established cell lines have been used to study xenobiotic degradation. Conversely, the effects of xenobiotics on the cell can also be conveniently studied. The combination of the two types of studies allows one to ascertain whether the cell's metabolism of a xenobiotic is accomplished by a healthy cell or as the result of or in combination with some cellular defect. The use of whole organs, such as perfused liver, provides the opportunity to extend a metabolism experiment over a longer period of time than is possible with either subcellular fractions or isolated cells. Whole organs or organ slices allow the introduction of a higher degree of cellular organization and differentiation than the single cell, but without the complications of external regulation. The use of isolated hepatocytes is a recent innovation in the study of xenobiotic metabolism and should be most useful.

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The Use of Animal Subcellular Fractions to Study Type I Metabolism of Xenobiotics[1]

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The genius of man has resulted in the synthesis of a vast number of organic compounds during the last century. The quest to seek new drugs, synthetic polymers for industrial use, insecticides and pesticides -- to name but a few areas of chemical development -- has provided both beneficial as well as detrimental results which touch the lives of each of us. The last decade has seen a surge of activity directed toward the evaluation of a wide array of organic chemicals which have the potential of negatively modifying the homeostasis of cells and organs. Nearly every day the popular press identifies yet another agent which can be shown in experimental animals or bacterial test systems to cause mutagenesis and cellular dysfunction or neoplasia. It is now well established that many of these chemicals are merely precursors of metabolic products which are the true causative agents of cellular changes. Therefore considerable effort is now being expended to identify and characterize the requisite enzyme systems responsible for the metabolic transformation of such chemicals.

The purpose of this presentation is to review the methodology employed and the results obtained when studying one such enzyme system -- an enzyme complex which has a broad specificity for the oxidative alteration of a variety of both natural and synthetic organic chemicals. Central to this enzyme complex is a family of hemeproteins called cytochromes P-450. It is the properties and reactions of this type of hemeprotein and its associated electron transport carrier proteins which will serve as the primary emphasis of the present report.

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The Characterization of Cytochrome P-450

Cytochrome P-450 is widely distributed in nature spanning the phylogenetic tree from bacteria to the human organism. It has been observed in yeast, flowering plants, insects, reptiles, birds and animals (1,2). Indeed, it is interesting that the first report of cytochrome P-450 occurred as a consequence of studying the respiratory pigments of the mid-gut of the silkworm (3). The ease of detecting cytochrome P-450, using spectrophotometric methods to assess the characteristic absorbance band of the carbon monoxide adduct of the reduced hemeprotein, has greatly facilitated the quantitative evaluation of this cellular pigment (4,5). In organisms other than some specialized bacteria, cytochrome P-450 is a membrane bound protein -- a property which until recently has rendered it refractory to isolation and purification. Methodologies are now available, however, which permit the solubilization and stabilization of the hemeprotein permitting its characterization as a homogeneous protein species.

It is possible to generalize and identify two basic types of cytochrome P-450 depending on the associated electron transport carrier proteins responsible for transferring electrons (reducing equivalents) from reduced pyridine nucleotides to the hemeprotein. As shown in Figure 1, we have designated these as Class A or B. The Class A type of cytochrome P-450 is found associated with the mitochrondria of many cell types where it functions together with an iron-sulfur protein and a flavoprotein (6). The reactions of cholesterol metabolism in steroidogenic organs such as the adrenal, testis, and placenta are catalyzed by this class of cytochromes P-450.

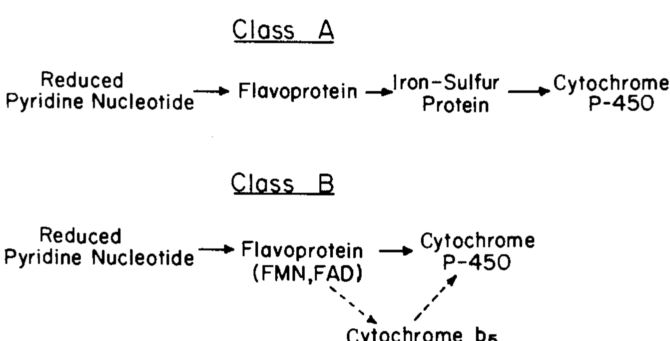

Figure 1. Two general classes of cytochrome P-450 based on the type of electron carriers functional in the transfer of reducing equivalents from reduced pyridine nucleotides

Best characterized (7,8) are the reactions for the oxidative degradation of camphor by the bacterium *Pseudomonas putida* where this class of cytochrome P-450 plays a key role in the initiation of the metabolism of this complex molecule.

The Class B type of cytochromes P-450 is generally associated with the endoplasmic reticulum (or its membrane equivalent) where it functions in concert with a flavoprotein which contains both FMN and FAD as prosthetic groups (9-11). This class of cytochrome P-450 is generally found associated with a second hemeprotein, cytochrome b_5. Although the role of cytochrome b_5 in the cyclic function of cytochrome P-450 (see below) is controversial, the parallelism of cellular association lends credence to its possible action as an electron transport carrier required for reduction of an intermediate formed during the course of cytochrome P-450 reactions.

The description provided in a later section of this presentation will emphasize those reactions of cytochrome P-450 which occur during its interaction with the substrate to be metabolized as well as molecular oxygen. A common pattern has emerged for the sequence of reaction steps involving cytochrome P-450 whether it is the pigment of either Class A or B. The principal difference resides in the manner of donation of electrons transported from reduced pyridine nucleotide rather than the reaction intermediates formed during oxygen activation and substrate transformation.

Methodologies of Study

An evaluation of the reactions occurring during the mixed function oxidation of many xenobiotics requires a suitable source of the enzyme system as well as appropriate instrumental apparatus. The most frequently employed enzyme system which has been studied in greatest detail is the one associated with the microsomal fraction of liver. This organ source is rich in the electron transport carriers which function in the activation of oxygen for xenobiotic metabolism, in part because of the role of the liver in serving as the primary recipient of chemicals absorbed from the intestinal tract. Further the requisite enzyme constituents, notably cytochrome P-450, are rapidly synthesized in liver as a result of exposure of animals to various inducing agents such as barbiturates or polycylic hydrocarbons. Thus, the content and composition of cytochromes and flavoproteins associated with liver microsomes can be readily altered to permit biologically differing conditions which assist in the delineation of the pattern of enzymatic reactions occurring. Studies with liver microsomes have served as the pattern for comparable studies of xenobiotic metabolism in other tissues such as the lung, kidney, intestine, etc.

The Preparation of Liver Microsomes. Rather standard techniques of differential centrifugation of liver homogenates have been developed for routine use in the preparation of microsomal fractions for study.

Most important is the need to recognize the multitude of factors which can influence the subsequent experimental results obtained when using this source of the enzyme system. The investigator should be cognizant of the variations introduced when using animals that are not controlled for alterations resulting from diet, inadvertent exposure to inducers (such as insecticides commonly used indiscriminately in animal quarters) and stress to name but a few. Further, the choice of age, sex, species, and genetic characteristics all introduce variables which lead to differences in the enzymatic characteristics measured.

In general animals are decapitated and the livers perfused _in situ_ with cold isotonic saline to remove as much blood as possible. After homogenization in the presence of a polyhydroxy compound, such as 0.25 M sucrose, the microsomal fraction is isolated by differential centrifugation after first removing unbroken cells, nuclei, and mitochondria. If precautions are taken, the enzyme activity of microsomes isolated in this way remains relatively stable for 48 to 72 hrs.

The Spectrophotometric Analysis of Microsomal Pigments. The microsomal fraction is composed of membrane vesicles originating from disruption of the endoplasmic reticulum. Therefore special instrumentation should be employed to accurately evaluate the spectrophotometric properties of the hemeproteins associated with these turbid membrane suspensions. A number of different types of commercial spectrophotometers are available for the measurement of difference spectra when using samples that have high light scattering characteristics. A schematic diagram for one such instrument is shown in Figure 2. Basically, the principal involved requires light from a tungsten filament lamp to be appropriately attenuated and dispersed by a split grating to generate two beams of monochromatic light which can impinge on a pair of cuvettes containing the membrane fragments to be analyzed. These cuvettes are positioned near the surface of the light detector to include the greatest solid angle of scattered transmitted light. Suitable electronic demodulation permits the accurate evaluation of differences in light absorption between the pigments in a sample cuvette minus those in the reference cuvette. Coupled together with wavelength scanning capabilities, the difference absorbance spectrum can be readily obtained. In addition to the capability of recording difference spectra, using the "split-beam" mode, such an instrument can be modified to measure the kinetics of absorbance changes at a predetermined wavelength, relative to a

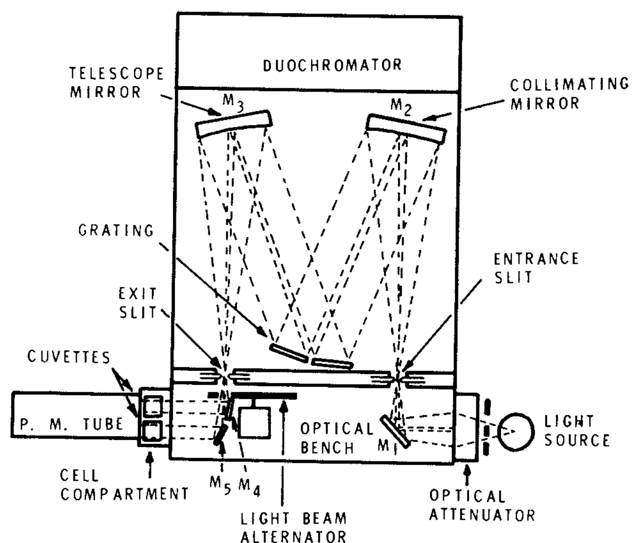

Figure 2. Representation of the duochromator optical configuration of the Aminco-DW2a spectrophotometer in the split-beam mode (12)

reference wavelength (usually an isosbestic point), as a function of time. A number of articles have been written describing the application of this methodology and many of the pitfalls and limits which may influence the interpretation of such spectrophotometric techniques (13,14).

Determination of Reaction Products. Measurements of the overall rates of xenobiotic metabolic transformations have been limited in the past to the colorimetric evaluation of products that can be readily derivatized or made reactive in coupled enzymatic oxidation-reduction reactions. For example, formaldehyde is easily measured colorimetrically during the N- or O-demethylation of many drug substrates (15). Other compounds, such as polycyclic hydrocarbons, form fluorescent products during metabolism and these can be readily quantitatively measured (16,17,18). In other cases radioactive compounds can be employed and use of differential solubility, as a consequence of the more hydrophilic characteristics of the metabolic products, provides an additional means of evaluating reaction rates (19).

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In recent years the introduction of high pressure liquid chromatography has opened a new and powerful method for product identification. This method is rapid, sensitive, and, with proper precautions, quantitative. One of the major new contributions to our knowledge of xenobiotic metabolism, which has resulted from the application of HPLC methods, is the recognition that multiple products are formed and that initial products formed may be rapidly further metabolized to secondary or tertiary products (20,21,22). The more general application of this methodology will undoubtedly have a profound influence on the current rather simplistic viewpoint which prevails regarding the oxidative transformation of many organic compounds.

The Cyclic Function of Cytochrome P-450

The primary function of cytochrome P-450 is to interact with molecular oxygen in a manner that permits the cleavage of the oxygen molecule to form an atom of oxygen with electrophilic characteristics. This "activated oxygen" is then presumed to react with a molecule of the organic substrate, which is at or near the site of oxygen bound to the hemeprotein, so that the substrate molecule is transformed to a higher oxidation state. This may be evaluated, for example, by the formation of an epoxide with aromatic compounds, by the N- or O-dealkylation of secondary or tertiary amines as well as ethers, or by the incorporation of oxygen into alkanes. Many of the reaction steps for the mixed function oxidation of substrates have been delineated and the reactive intermediates characterized. The following description briefly summarizes our current state of knowledge of these reactions.

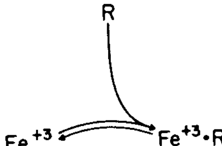
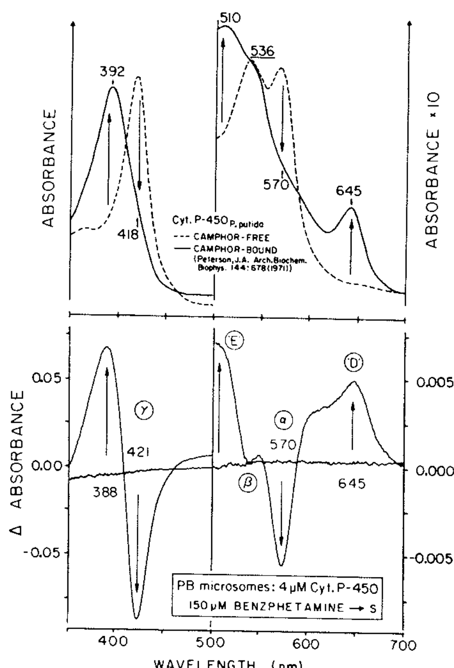


Figure 3

The Interaction of Substrate. The initial step in the function of cytochrome P-450 is the binding of the organic substrate to be metabolized to the oxidized form of the hemeprotein, as illustrated in Figure 3. Associated with this interaction is a change in the properties of the heme of cytochrome P-450. In the absence of a substrate molecule, the heme is in the low-spin, hexacoordinate form and it is characterized by an optical absorbance spectrum with a major absorbance band whose maximum is about 418 nm (Figure 4). The presence of a single unpaired electron in the ferric iron of low spin cyto-



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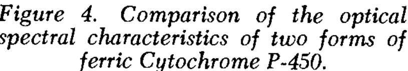

Figure 4. Comparison of the optical spectral characteristics of two forms of ferric Cytochrome P-450.

(Top) *The absolute spectra of soluble Cytochrome P-450 isolated from* Pseudomonas putida *in the presence and absence of the substrate camphor* (23). (Bottom) *The difference spectra obtained when benzphetamine interacts with Cytochrome P-450 of liver microsomes* (24).

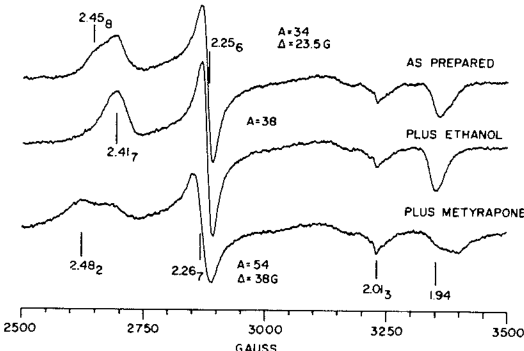

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Figure 5. EPR spectra of Cytochrome P-450 of liver microsomes and the influence of ethanol and the dipyridyl derivative, metyrapone. Rat liver microsomes from phenobarbital treated animals were examined using an E-4 Varian EPR spectrometer (29).

chrome P-450 renders it paramagnetic. This is reflected by an electron paramagnetic resonance spectrum (shown in its first derivative form) of the type illustrated in Figure 5. Considerable interest and debate has centered on the interpretation of this EPR spectrum and the possible presence of unique ligands, such as sulfur, coordinated to the heme of cytochrome P-450 (25,26,27,28).

The addition of an excess of substrate (R) to be metabolized, to cytochrome P-450 results in a marked change in the absorbance spectrum of the ferric hemeprotein (Figure 4) leading to a pigment with its major absorbance band located at about 390 nm. The basis for this hypsochromic effect of the substrate on the spectral properties of oxidized cytochrome P-450 is related to the spin state change of the heme iron. This is confirmed by the appearance of an absorbance with a g value of about 8 when the substrate complexed form of ferric cytochrome P-450 is examined by low temperature EPR spectroscopy methods (30,31). One interpretation of these changes in the physical properties of the heme protein is the binding of the organic substrate molecule in the hydrophilic heme pocket of the hemeprotein, thereby perturbing the electron density distribution of the heme to form the pentacoordinate, high-spin complex (32,33). Such an interpretation (supported by studies using spin-labeled organic compounds (34)) would place the substrate molecule in close proximity to the heme iron and thus in a spatial configuration where it is appropriately located for interaction with the "active oxygen" generated during the cycle of cytochrome P-450 function.

The optical spectral changes associated with substrate binding provide a rather simple technique to measure this first step in cytochrome P-450 action. Since many systems under study involve the membrane bound form of cytochrome P-450, the technique of difference absorbance spectrophotometry has been applied (see discussion in Section II). As illustrated in Figure 6, the addition of increasing concentrations of a substrate (in this example, the steroid androstanedione has been added to a cytochrome P-450 containing preparation of liver microsomes) results in a progressive decrease in absorbance at about 420 nm concomitant with an increase in absorbance at about 385 nm. This form of absorbance change has been termed "Type I" (36) and many substrates metabolized by cytochrome P-450 can be shown to cause this alteration in the spectral properties of the hemeprotein. In a number of instances, the magnitude of spectral change observed can be related to the concentration of substrate added to the reaction mixture and one can then estimate the affinity of cytochrome P-450 for each type of substrate examined. However, a satisfactory interpretation of substrate interaction with ferric cytochrome P-450 (based solely on optical absorbance difference spectrophotometry of the membrane

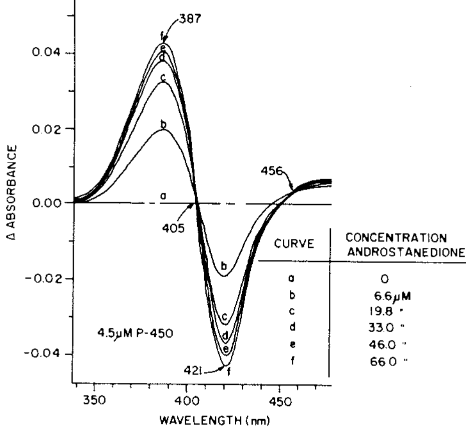

Figure 6. Effect of increasing concentrations of substrate on the optical density changes observed during the spin state transition of ferric Cytochrome P-450.

Liver microsomes from phenobarbital treated rats were suspended to a protein concentration of 2 mg/mL. Various concentrations of androstanedione were added to the contents of the sample cuvette and the resultant difference spectra recorded.

enzyme) is sometimes lacking. This is due in part to complications arising from the presence of "endogenous substrates" associated with the membrane fragments (24, 37-40), extraneous solvent effects which presumably alter the hydrophobic heme environment of the hemeprotein (41,42), as well as temperature dependent changes in the properties of the membrane (31).

The general concept that oxidized cytochrome P-450 reacts with the substrate to be metabolized as the first step in the function of the hemeprotein appears to be generally accepted. The detailed interpretation of the changes in the physical properties of the hemeprotein accompanying the binding of substrate remains largely speculative and serves as an area of research currently under active investigation.

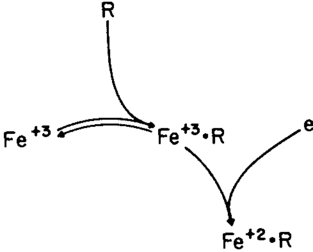

Figure 7

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Reduction of cytochrome P-450. The complex of ferric cytochrome P-450 with substrate undergoes a one electron reduction (Figure 7) to form the ferrous hemeprotein. In reactions involving the Class A type of cytochrome P-450, this electron is donated by a reduced iron-sulfur protein. The initial source of this electron is from reduced pyridine nucleotides (either NADPH or NADH depending on the system under study) which interacts with a specific flavoprotein that serves also as an iron-sulfur protein reductase (6,43, 44). In the case of the Class B type of cytochrome P-450, the flavoprotein, NADPH-cytochrome P-450 reductase, mediates the transfer of the electron from NADPH to the ferric hemeprotein (45). This flavoprotein has attracted a great deal of interest because of its unusual property of containing two flavin prosthetic groups (FMN and FAD) and the ability to generate stable free radical forms of the enzyme during NADPH oxidation (9,10,11). Recent experiments have indicated that the sequence of electron transport by this flavoprotein involves first the reduction of FAD followed by the reduction of FMN (46), i.e., a form of reduced FMN is envisioned as the agent donating an electron to reduce cytochrome P-450. However, the ability to form a number of different states of reduction of this flavoprotein (Figure 8) and the need to transfer only one electron for the reduction of cytochrome P-450 (47,48), leaves unresolved the possible role of other free radical forms of the flavoprotein from participating in this or other reactions.

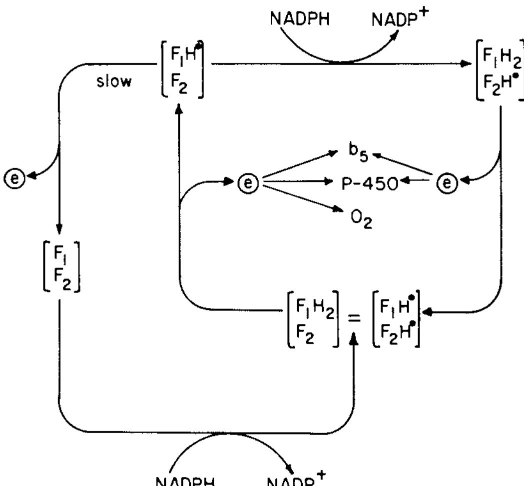

Figure 8. Schematic proposal for the various states of reduction of the flavoprotein, NADPH–Cytochrome P-450 reductase. F_1 is presumed to represent FAD and F_2 to represent FMN. The existence of various free radical species is indicated.

Table I

Concentration of Microsomal Electron Transfer Components

	P-450	FMN	FAD	RATIO
	nmoles/mg protein			P-450/FMN
RATS				
control	0.71	0.075	0.15	9.5
phenobarbital pretreatment	2.1	0.08	0.13	26.3
MICE				
C57BL/6J	0.51	0.021	0.094	24.3
DBA/2J	0.53	0.013	0.090	40.8

Recently interest has developed in the area of membrane structure and the spatial relationship of this flavoprotein to cytochrome P-450 (49). A consideration of the stoichiometry (Table I) of the flavoprotein to cytochrome P-450 reveals a surfeit of hemeprotein molecules relative to its electron donating partner. The problem becomes even more complex when considering the amphipathic properties of the flavoprotein and its physical location on the surface of the membrane (50-54). The potential role of membrane fluidity facilitating the "pinball" like motility of the flavoprotein as it services the dispersed pool of hemeprotein molecules is a hypothesis (55, 56) which stands in contrast to the proposal (49,57-59) of clusters or patches of multienzyme complexes based on hydrophobic interactions in the membrane. Clearly, a more detailed understanding of membrane structure is a prerequisite to the delineation of the process of reduction of the high spin form of oxidized cytochrome P-450 bound by substrate.

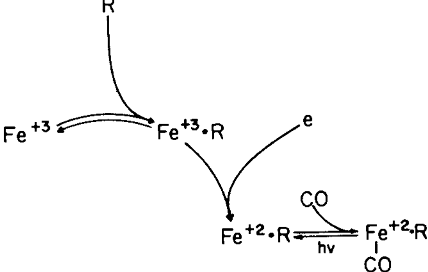

Figure 9

Reaction with Carbon Monoxide. Reduced cytochrome P-450 reacts rapidly with carbon monoxide (Figure 9) to form a complex which serves as the hallmark for this class of cellular pigment (60-62). A characteristic absorbance band (Figure 10)

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at about 450 nm (hence the name P-450) is associated with this complex. The chemical basis for the unique absorbance band at 450 nm for the carbon monoxide adduct of the reduced hemeprotein is unknown although numerous studies have been carried out, in particular using model compound complexes of heme, which suggest that a form of sulfur participates as one of the ligands of the heme of cytochrome P-450 (25-28) and that this "soft ligand" may in part be responsible for this unusual property of this class of hemeproteins. The bathochromic shift in absorbance of reduced cytochrome P-450, when it reacts with carbon monoxide, stands in sharp contrast to other known hemeproteins where a hypsochromic spectral shift is observed (6). The ability of reduced cytochrome P-450 to form complexes with absorbance bands in the vicinity of 450 nm is not restricted to its reaction with carbon monoxide. Interaction of reduced cytochrome P-450 with the dipyridyl derivative, metyrapone, results in the formation of an absorbance at about 445 nm (63). Recently, it was shown that a number of other organic compounds (such as safrole, the class of amphetamines, SKF-525A, etc.) react with reduced cytochrome P-450 to form derivatives, called product adducts or metabolite complexes, with absorbance bands in the spectral region from 450 to 460 nm (64). The details of the chemistry of these types of reactions remains to be further studied. Suffice it to say that all of these studies strongly suggest the existance of an environment in the heme pocket of cytochrome P-450 which is atypical of other known hemeproteins (59).

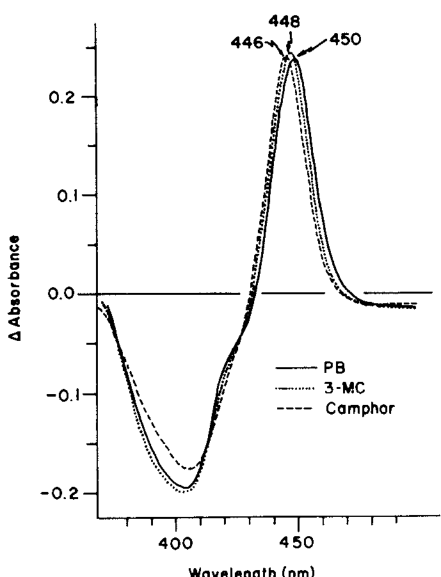

Figure 10. Optical absorbance properties of the carbon monoxide complex of reduced Cytochrome P-450.

Cytochrome P-450 associated with liver microsomes prepared from animals pretreated with phenobarbital (PB) or 3-methylcholanthrene (3-MC) as well as Cytochrome P-450 isolated and purified from Pseudomonas putida *(camphor) were reduced by sodium dithionite and examined by difference spectrophotometry. After gassing the contents of the sample cuvette with carbon monoxide, the various spectra were recorded. Differences in the location of the absorbance band maxima are indicated.*

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Although the most commonly observed property for the reduced cytochrome P-450 complex with carbon monoxide is an absorbance band with a maximum at 450 nm, the exact locus of this absorbance maximum can be subtly shifted depending on the source of the hemeprotein. As shown in Figure 10, the absorbance band maximum is located at about 446 nm for the cytochrome P-450 isolated and purified from the camphor grown bacterium, Pseudomonas putida (43,65). The hemeprotein formed in liver microsomes as a result of exposure of animals to polycyclic hydrocarbons such as 3-methylcholanthrene has an absorbance band at 448 nm (66). These differences undoubtedly result from changes in the environment of the heme for each of these hemeproteins as a consequence of synthesis of proteins with modified amino acid composition and primary structure (67). The result has been a plethera of names, such as P-448, P_1-450, etc., which frequently confuse individuals not familiar with the vagaries of a developing nomenclature which still seeks a common base.

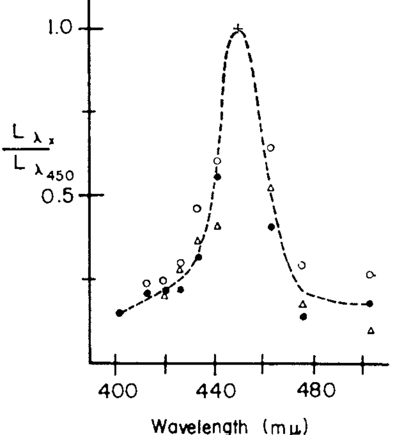

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Figure 11. Photochemical action spectrum for the light reversibility of carbon monoxide inhibition of Cytochrome P-450 catalyzed reactions.

Rat liver microsomes were incubated with (●), codeine; (○), aminopyrine; or (△), acetanilide with various mixtures of carbon monoxide and oxygen and NADPH and irradiated with light of selected wavelengths (69).

The property of reduced cytochrome P-450 reacting with carbon monoxide served as the primary characteristic for delineating the function of this hemeprotein in the oxidative metabolism of many different substrates (68,69). Like other hemeproteins, the reaction of carbon monoxide with the reduced pigment is an equilibrium reaction which is photosensitive (70). The first definition of a biological function for cytochrome P-450 was achieved by applying the photochemical action spectrum methodology of Warburg during a study of the 21 hydroxylation of progesterone as catalyzed by the microsomal fraction isolated from the adrenal cortex (68). Extension of these studies to an examination of many mixed function oxidation reactions catalyzed by liver microsomes (Figure 11)

confirmed the general role of this hemeprotein in the oxygen activation required for the oxidative conversion of a broad spectrum of organic compounds.

The fact that the carbon monoxide complex of reduced cytochrome P-450 has an absorbance band in the Soret region of the spectrum, which is significantly displaced from the absorbance bands of comparable complexes of other reduced hemeproteins, serves as a useful property in evaluating spectrophotometrically changes in the cellular content of this pigment following exposure of animals to a variety of chemicals. As shown in Figure 12, treatment of animals with a barbiturate, phenobarbital, results in a marked increase in the content of cytochrome P-450 in the liver of these animals. This property of "enzyme induction" can be accurately and rapidly monitored spectrophotometrically. It is worth noting that data of the type presented in Figure 12 illustrates the high content of

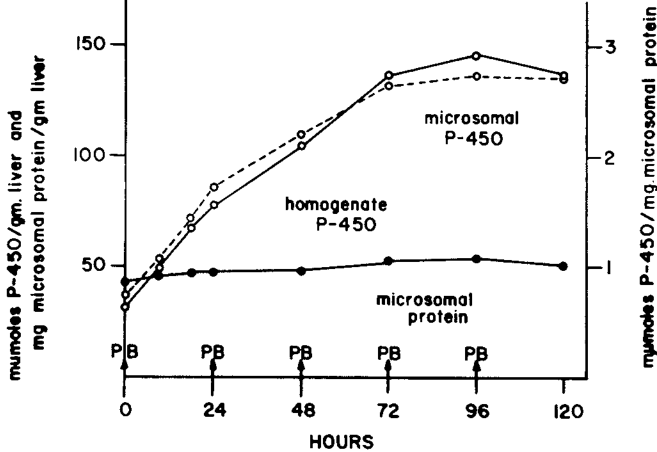

Figure 12. Induction of liver microsomal Cytochrome P-450 following treatment of rats with phenobarbital. Male rats (150–200 gm) were injected intraperitoneally daily with 80 mg of phenobarbital/kg body weight. Animals were sacrificed at the times indicated and the content of Cytochrome P-450 in (O --- O), isolated liver microsomes as well as (O—O), liver homogenates (13).

cytochrome P-450 in an organ such as the liver. Subsequent to treatment of the animals for five days with phenobarbital, approximately 15 percent of the protein of the endoplasmic reticulum of liver is composed of this one class of protein (71). Likewise, as much as 5 percent of the protein of the liver can be cytochrome P-450. Truly, cytochrome P-450 is not a minor constituent in this and other organs - it is the dominant species of hemeprotein which is present at a concentration equivalent to that of myoglobin in some muscle tissue.

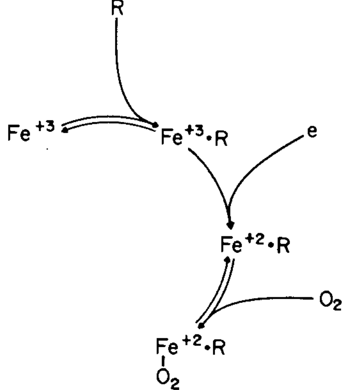

Figure 13

Reaction with Oxygen. In the presence of molecular oxygen reduced cytochrome P-450 reacts rapidly to form an intermediate (Figure 13) termed oxycytochrome P-450. The existence of an oxygenated form of reduced cytochrome P-450 was predicted from the early experiments designed to determine those factors which influence the extent of carbon monoxide inhibition of cytochrome P-450 catalyzed reactions (68). These studies had clearly shown that carbon monoxide was an inhibitor which was competitive with oxygen, i.e., the magnitude of inhibition observed is dictated by the ratio of carbon monoxide to oxygen rather than simply the concentration of carbon monoxide in the reaction system.

Studies by Ishimura *et al.* (72,73) as well as Gunsalus *et al.* (74,75), using the soluble and purified cytochrome P-450 isolated from *Pseudomonas putida*, clearly demonstrated the presence of a reasonably stable derivative of the reduced hemeprotein when exposed to oxygen. This complex was identifiable spectrophotometrically, as shown in Figure 14, and

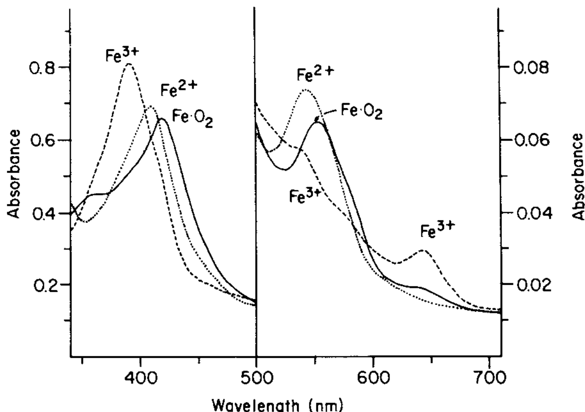

Figure 14. Absorption spectra of oxidized, reduced, and Oxycytochrome P-450. Samples of purified Cytochrome P-450 isolated from Pseudomonas putida *were examined spectrophotometrically in the presence of the substrate camphor (73).*

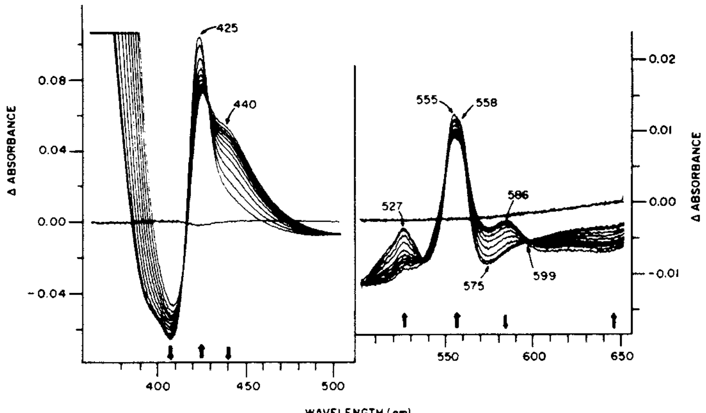

Figure 15. Repetitive scan difference spectral measurements of liver microsomal Cytochrome P-450 during the NADPH supported steady state metabolism of hexobarbital. Liver microsomes from phenobarbital treated male rats were diluted to 1 mg of protein/mL in a buffer mixture containing 50mM TRIS chloride (pH 7.5), 150mM KCl, 10mM $MgCl_2$, and 2mM hexobarbital. NADPH (0.5mM final concentration) was added to initiate the reaction and the difference spectra recorded every 30 sec. The absorbance band at about 440 nm is attributed to Oxycytochrome P-450.

$$\underset{\substack{|\\O_2}}{X\cdot Fe^{+2}} \rightleftharpoons \underset{\substack{|\\O_2^-}}{X\cdot Fe^{+3}} \longrightarrow X\cdot Fe^{+3} + O_2^- \xrightarrow{SOD} H_2O_2$$

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Figure 16. Possible valence states of Oxycytochrome P-450 and its dissociation to form superoxide

was recognized to resemble the oxygenated form of other hemeproteins (76-79). The ability to demonstrate the presence of oxycytochrome P-450 during the functioning of the integrated and membrane bound electron transport system of microsomes (the Class B type of cytochrome P-450) is more complex and subject to many variables. Repetitive scan spectrophotometric measurements during the NADPH supported aerobic steady state oxidation of a variety of substrates by liver microsomes reveals (Figure 15) the presence of an absorbance band with a rather broad maximum at about 440 nm in the difference spectrum (80,81). This absorbance band is similar to the spectral intermediate seen with the purified cytochrome P-450 from Pseudomonas putida. The magnitude of this absorbance band of oxycytochrome P-450, when studied using liver microsomes, is markedly influenced by the type of substrate studied, the associated activity of NADPH-cytochrome P-450 reductase, i.e., the balance of addition of the first electron versus the second electron (see below) for the function of cytochrome P-450, and the presence of excess NADPH and oxygen. As shown in Figure 15, the concentration of oxycytochrome P-450 progressively decreases as the oxygen concentration in the reaction medium decreases.

Oxycytochrome P-450 can be considered to be an equilibrium between two ternary complexes. In one case (Figure 16), the ferrous hemeprotein can be envisioned as complexed with a molecule of superoxide. The latter can be visualized as decaying giving rise to the high spin ferric hemeprotein substrate complex and the superoxide anion (or its protonated form, the perhydroxyl radical). The presence of adventitious superoxide dismutase associated with the microsomal fraction

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would then catalyze the formation of hydrogen peroxide. It is now well established that hydrogen peroxide is formed during NADPH oxidation by liver microsomes (82,83). Although the source of this hydrogen peroxide remains speculative, the decomposition of oxycytochrome P-450 seems a very likely possibility and recent studies (83-85) have supported the hypothesis that superoxide is generated during the function of cytochrome P-450. Thus, one can consider an "oxidase type" cycle for cytochrome P-450 (Figure 17) whereby electrons originating from NADPH are diverted to the reduction of oxygen for the formation of hydrogen peroxide rather than for the mixed function oxidative metabolism of xenobiotics. *In vitro* studies using isolated liver microsomes have revealed the ability of many compounds to stimulate the rate of formation of hydrogen peroxide formation concomitant with their oxidation via cytochrome P-450 (86,87). In the few cases studied using halogenated hydrocarbons (88,89) it is apparent that a significant diversion of electrons occurs resulting in an uncoupling of

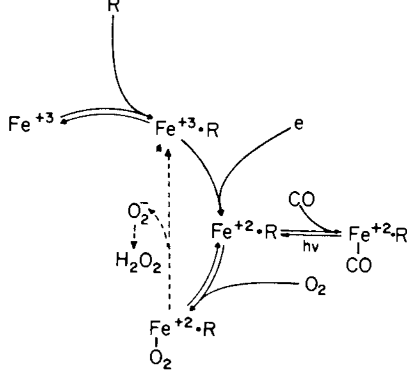

Figure 17. Dissociation of superoxide from Oxycytochrome P-450 for the formation of hydrogen peroxide

oxygenation reactions. This may be of particular interest to those concerned with pesticides because of the frequent use of compounds, such as Lindane.

The Second Electron. Oxycytochrome P-450, to which a molecule of substrate is bound, undergoes further reduction to an intermediate termed peroxycytochrome P-450 (Figure 18). Like oxycytochrome P-450, the proposed intermediate peroxycytochrome P-450 can be written in a variety of equivalent electron valence forms. The inability to isolate and characterize this proposed intermediate has impeded further understanding of its chemistry and physical properties

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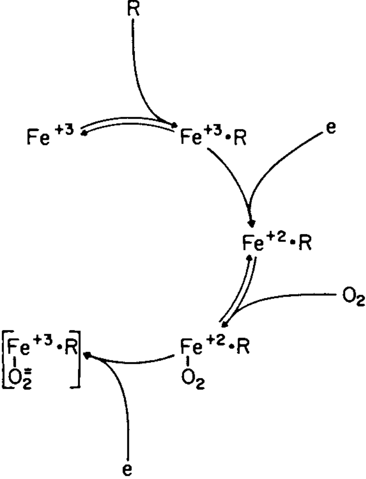

Figure 18

although its existence as a transient intermediate in the function of cytochrome P-450 has been generally assumed. The stoichiometry of mixed function oxidation reactions requires the participation of two electrons; the reduction of the ferric cytochrome P-450 complex has been established (47,48) to be a one electron transfer reaction. Studies with the purified bacterial cytochrome P-450 have shown (7) the transformation of oxycytochrome P-450 by the addition of the reduced iron sulfur protein, putidaredoxin. Further, the ability to observe spectrophotometrically intermediates formed during the reaction of ferric cytochrome P-450 with organic hydroperoxides, such as cumene hydroperoxide, which differ from oxycytochrome P-450 substantiates the existence of additional oxygen containing complexes of cytochrome P-450 (90).

Protonation of peroxycytochrome P-450 and the direct release of hydrogen peroxide concomitant with the formation of the ferric cytochrome P-450 complex with substrate (Figure 19) is an alternative means of explaining the formation of hydrogen peroxide during NADPH oxidation by liver microsomes. Considerable work has centered on distinguishing the two alternatives for the formation of hydrogen peroxide, i.e., the decomposition of oxycytochrome P-450 with the release of superoxide which then undergoes dismutation or the protonation of peroxycytochrome P-450 and the direct formation of hydrogen peroxide. An examination of the reaction scheme shown in Figure 19 reveals a major difference between these two options which should delineate the dominant pathway for hydrogen peroxide formation. As discussed above, two electron transfer steps are required to form peroxycytochrome P-450 while only one electron transfer

step is needed to generate oxycytochrome P-450. Previous studies of drug metabolism by the cytochrome P-450 containing liver microsomes revealed a "synergistic effect" of NADH

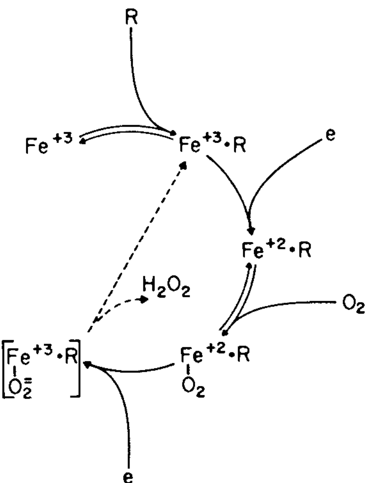

Figure 19. Proposed formation of hydrogen peroxide from Peroxycytochrome P-450

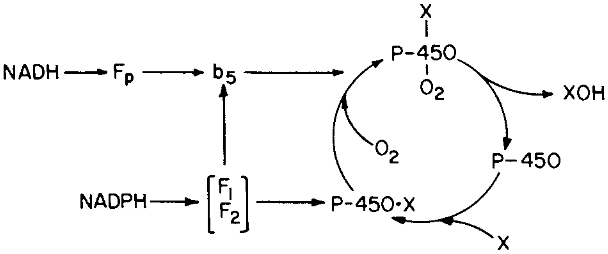

Figure 20. Scheme showing the proposed role of reduced Cytochrome b_5 as the donor of the electron required for the reduction of Oxycytochrome P-450 and the formation of Peroxycytochrome P-450

when supplementing the NADPH dependent mixed function oxidation of various substrates. This "synergistic effect" elicited by NADH (91-94) was attributed to a role for reduced cytochrome b_5 as the donor of the second electron required for the cyclic function of cytochrome P-450. This is illustrated in Figure 20. The presence of NADH would spare reducing equivalents originating from NADPH needed for the reduction of

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cytochrome b_5 and it would also increase the extent of steady state reduction of this hemeprotein. Comparable studies were carried out to determine whether a "synergistic effect" by NADH on the NADPH dependent formation of hydrogen peroxide also occurred. These results were negative (83,85). This suggests that the principal pathway for the formation of

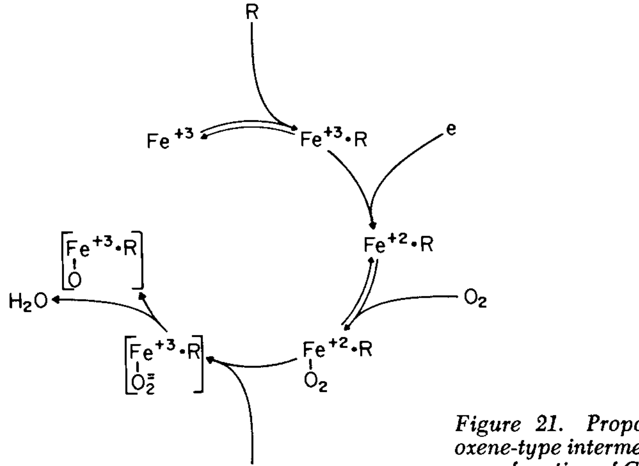

Figure 21. Proposed formation of an oxene-type intermediate during the cyclic function of Cytochrome P-450.

hydrogen peroxide was the decay of oxycytochrome P-450 rather than the protonation of peroxycytochrome P-450. Obviously, more definitive experiments will have to be carried out but the present limitation in methodologies precludes the design of such unequivacol studies.

Other Possible Intermediates. The sequence of reactions involved in the "activation of oxygen" from peroxycytochrome P-450 are unknown. Much has been written and many speculative hypotheses have been proposed but no experimental verification exists to better define possible intermediates. One such hypothesis, which is very attractive, proposes the formation of an oxene complex of cytochrome P-450 resulting from the protonation of peroxycytochrome P-450 and the release of a molecule of water (95,96). This is illustrated in Figure 21. The existence of an oxenoid species of oxygen would fulfill the requirement of a highly electrophilic atom which could participate in hydroxylation reactions. Recently, the presence of free-radical species has also been deduced from deuterium isotope experiments (97). However, the failure to detect any measureable concentration of free radicals during the microsomal catalyzed oxidative metabolism of any substrate yet examined indicates the absence of a steady state concentration of such proposed free radicals. Additional intermediates such as the carbanion form of some substrates, the

existence of a carbene form of the substrate, and the generation of epoxides should all be considered as possibilities until our level of knowledge is sufficiently expanded to permit choices based on experimental rather than theoretical considerations.

The Role of the Membrane

Cytochrome P-450 present in mammalian tissues is recognized to be intimately associated with membranes. Studies of membrane bound enzymes have the disadvantage that special methodologies, such as the use of difference spectrophotometry as described earlier, as well as the presence of high concentrations of lipid can introduce variables that are difficult to fully evaluate. The latter, i.e., the presence of lipid, can perturb the pattern of metabolism because of a non-specific sequestering of the highly lipophilic substrates that are oxidatively transformed during the function of cytochrome P-450 (31,98). Recently considerable interest has centered on the spatial and structural organization of cytochrome P-450 and its associated electron transfer proteins within the mileau of the microsomal membrane. The unique stoichiometry of flavoproteins and cytochromes (cf. Table I) have provided the opportunity to evaluate the role of the membrane in regulating and modifying the types of reactions catalyzed and the influence of restricted mobility of protein within the lipid mosaic of the membrane. It has been proposed (49,58,59) that cytochrome P-450 exists as clusters (Figure 22) which

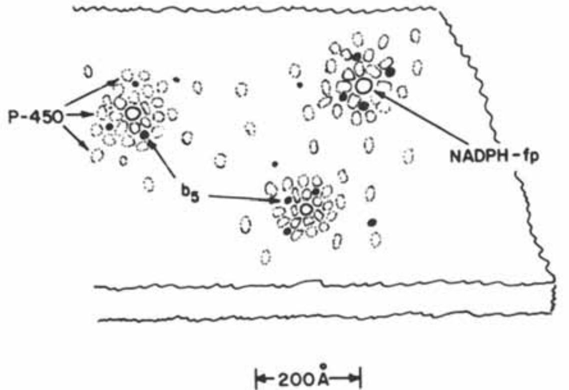

Figure 22. Schematic of the microsomal membrane showing clusters of molecules of Cytochrome P-450 surrounding the flavoprotein reductase

can form patches of electron transport complexes within or withon the membrane. Much controversy surrounds this inter-

pretation (55,56) and the extent of membrane fluidity, as it influences the cytochrome P-450 dependent metabolism of substrates, remains as an area requiring further study. The solution of this intriguing problem will undoubtedly influence our further understanding of the reactions involved and the relationship of studies carried out in vivo to those that have been experimentally ascertained by in vitro studies. The importance of the membrane to studies of the metabolism of xenobiotics cannot be underestimated. Most of the compounds which are oxidatively transformed are highly lipophilic. Further, the lipid composition of the microsomal membrane can be readily modified by the type of diet employed as well as the age and sex of the experimental animal system under study.

Multiple Types of Cytochrome P-450

Any consideration of the factors which influence the role of cytochrome P-450 in the metabolism of xenobiotics must account for the ever increasing body of evidence which shows the multiple types of cytochrome P-450 that exist in a single cell type, such as the hepatocyte. It has been known for many years that various inducing agents can cause a preferential stimulation in the metabolism of drug substrates. The basis for this difference was clearly delineated by the observation (66) that treatment of animals with the inducing agent, 3-methylcholanthrene, resulted in the synthesis of a hemeprotein associated with liver microsomes which had optical absorbance properties distinct from a similar pigment induced upon treatment of animals with phenobarbital (cf. Figure 10). The last decade has seen major advances in the isolation and purification of different types of cytochromes P-450. Differences in response to specific antibodies, protein structure, and molecular weights are all subjects of current research (99-104). A direct demonstration of the change in the pattern of types of cytochrome P-450 associated with liver microsomes isolated from animals subjected to different inducing agents is shown by the polyacrylamide gel electrophoresis results presented in Figure 23. Dramatic alterations in the magnitude as well as mobility of proteins in the molecular weight range between 45,000 and 50,000 can be seen after exposure of animals to the inducing agents, phenobarbital or pregnenolong-16-alpha-carbonitrile. Thus one must consider, when discussing the in vivo enzymatic activity of cytochrome P-450, not only the influence of the membrane and the availability of co-factors such as reduced pyridine nucleotides and oxygen but also the presence of unique types of cytochrome P-450.

A logical extension of these studies is to postulate the presence of unique types of cytochrome P-450 which possess a specificity for various classes of substrates. As of this writing, the demonstration of substrate specificity has not

been proven although substrate preference can be shown, i.e., the cytochrome P-450 isolated from liver microsomes of animals

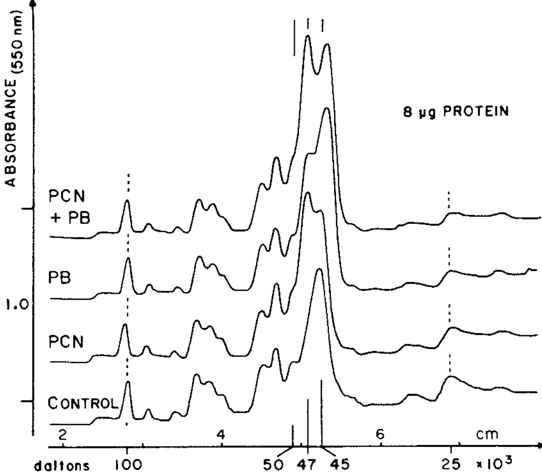

Figure 23. PAGE patterns of proteins present in liver microsomes from untreated animals (control), pregnenolone-16 α-carbonitrile (PCN)-, or phenobarbital (PB)-treated male rats. Microsomes were isolated and treated with SDS prior to electrophoresis and staining with Coomassie blue.

pretreated with a polycyclic hydrocarbon has a high activity in the metabolism of benzo(a)pyrene but also catalyzes the oxidative metabolism of drugs such as benzphetamine. This puzzle becomes even more intriguing when evaluating the recognized competitive effects of various types of substrates when rates of metabolism are measured using the membrane bound form of the enzyme. Clearly, the integration of knowledge gained from studies with various types of purified cytochrome P-450 as well as the electron transport complexes existent in the membrane will be required before a meaningful assessment of the *in vivo* transformation of xenobiotics can be accomplished.

Concluding Remarks

The delineation of the sequence of events which occurs as the hemeprotein, cytochrome P-450, reacts sequentially with a molecule of substrate to be metabolized, an electron derived from NADPH and transferred via the flavoprotein, NADPH-cytochrome P-450 reductase, a molecule of oxygen, and then a second electron has been briefly discussed in the foregoing sections.

The resulting overall scheme shown in Figure 24 attempts to synthesize our present understanding of this cyclic process. One must recognize, however, that much remains to be revealed if we are to gain sufficient knowledge which may permit the development of agents that can predictably perturb this

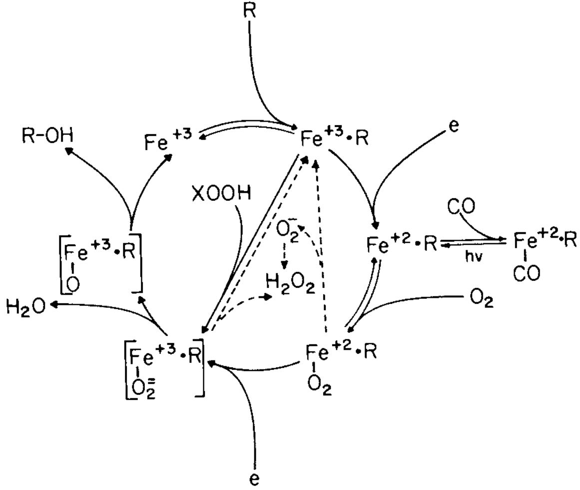

Figure 24. Scheme of intermediates formed during the cyclic reaction of Cytochrome P-450 associated with the metabolism of many xenobiotics. The possible sites of formation of hydrogen peroxide as well as the pathway of metabolism initiated by organic hydroperoxides are illustrated.

reaction system. Indeed, achievement of this knowledge is a necessary prerequisite to attainment of the goal of predicting the oxidative transformation of xenobiotics to highly toxic agents (many with the known potential of serving as carcinogens) or to products that are not detrimental to cellular function. The inventory of questions which remain unanswered is long and includes such items as follows:

a) What is "active oxygen" and how does it perform the necessary chemistry to interact with such a broad spectrum of chemicals as those known to be metabolized by cytochrome P-450?

b) What is the unique chemistry of cytochrome P-450 that permits it to react with oxygen and the substrate molecules in a manner that permits the catalysis of xenobiotic transformation?

c) Do the multiple forms of cytochrome P-450, which are selectively induced by various chemicals, reflect more than a single mechanism for the activation of oxygen?

d) What constraints are imposed by the hydrophobic environs of the membrane and associated problems of protein polarity, fluidity, or existence as clusters?

e) What factors dictate the pattern of electron transport from reduced pyridine nucleotides and how are these reflected in the case of substrate metabolism?

f) Is the concomitant formation of hydrogen peroxide, known to occur during the cytochrome P-450 metabolism of substrates, of any significance to cellular activity?

These and many more questions are under intensive investigation in many laboratories. Undoubtedly, new results will reveal the fragile foundation on which our current hypotheses are developed. The importance of knowning how this enzyme system functions is at the forefront of scientific concern during the present time when a great deal of attention has been focused on the wide spread dissemination in our environment of xenobiotics. The future holds great promise to bring forth new ideas and new concepts. It will be interesting to reflect back on the meager beginning where we stand now.

Abstract

The oxidative conversion of many lipophilic chemicals which are xenobiotics occurs by means of an enzyme system where the hemeprotein, cytochrome P-450, functions as an oxygenase. The importance of these types of reactions is now recognized as the primary step in the metabolic formation of many toxic agents including carcinogens. The details of our current understanding of cytochrome P-450 interaction with the organic substrate molecule, oxygen, and electrons derived from reduced pyridine nucleotides is described in the present paper. Particular attention is directed toward the sequence of reactions occurring and the attendent problems of defining the parameters which regulate the functionality of the membrane bound enzyme complex. The methodologies employed for studies to define the role of cytochrome P-450 in the metabolism of drugs, steroids, and chemicals which can be converted to carcinogens, have been described and typical results illustrated. The extension of these studies to better define the specificity of the reaction system as well as the properties of reactive intermediates poses the direction for future experimentation.

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The Use of Animal Subcellular Fractions to Study Type II Metabolism of Xenobiotics

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Type II biotransformations or conjugation reactions are enzyme-catalysed energy-requiring biosyntheses. The ultimate reaction requires a high-energy donor substrate, an acceptor substrate and an appropriate transferase. The high-energy substrate may contain the endogenous conjugating agent (conjugand) (e.g. glucuronic acid, as in UDPGA) or it may contain the xenobiotic (e.g. 4-chlorobenzoic acid, as in 4-chlorobenzoyl-CoA). If the xenobiotic is to be activated to become a donor substrate, other enzymes and high energy substrates (e.g. ATP) are called into action. If we assume for the moment that the low molecular weight substrates for these reactions have equal access to every component in the cell, then the subcellular location of the transferring enzyme will be the controlling factor in the subcellular distribution of the conjugation reaction.

History of the Technique

The metabolism of foreign compounds has been studied in various subcellular fractions from about 1950. Brodie and coworkers (1) presented the first overview of the enzyme-catalysed metabolism of these compounds in 1958. In the intervening years, the properties of microsomes and other subcellular fractions in relation to the metabolism and toxicity of drugs, pesticides and, more recently, 'environmental' chemicals have received an enormous amount of study. However, it is interesting to note that the best of the earlier reviews of the enzymology of foreign compound metabolism by J. R. Gillette in 1963 (2) contains the essential details of each of the conjugation reactions referred to below. Progress has not been even: it has proved difficult to keep our treatment of glucuronyltransferase within reasonable limits, yet amino acid conjugation, despite some very interesting species differences, has received very little attention at the enzyme level.

For our practical purposes, the animal cell can be regarded as composed of nucleus, endoplasmic reticulum, mitochondria

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and lysosomes suspended together in water containing dissolved proteins and small molecules. One of the values of studying type II reactions at the subcellular level lies in the information gained about the role of these components and the mechanism of the reactions. When the cell is fragmented and the various organelles are separated, the conjugation reaction under investigation may not be observed in any of the fractions because enzymes have been separated from mandatory cofactors. For example, glucuronidation reactions are not observed in the isolated endoplasmic reticulum (which contains the transferase) because the high-energy donor substrate, uridine diphosphoglucuronic acid (UDPGA), is located in the soluble fraction. Thus the effort required to reconstitute the conjugation reactions at the subcellular level has been vital to the full understanding of their mechanisms.

None of this could have happened without the efforts of biochemists who have developed an understanding of intermediary metabolism and subcellular fractionation procedures. Differential centrifugation is still the main method used and therefore the steady development of preparative-scale centrifuges has also been very important.

The Preparation of Subcellular Fractions

Subcellular fractionation is usually carried out by differential centrifugation. However, other methods such as precipitation and chromatography have been investigated for the isolation of specific fractions.

2.1 Differential Centrifugation

Differential centrifugation is by far the most widely used technique. It is effective, clean and gentle and although it could have been displaced at one time by a faster technique, the availability of preparative ultra-centrifuges capable of up to 500,000 g has cut down preparation times to a very competitive level. The machines now also operate at acceptable noise levels. Fresh tissue is homogenised in buffered salt solution and the resultant homogenate is centrifuged at about 600 g to remove unbroken cells and cell debris. The supernatant is then centrifuged at 8000-10,000 g for about 20 min to sediment the mitochondria. Some lysosomes are sedimented at this stage. The supernatant is then centrifuged at about 200,000 g for 20 min to sediment the fragmented endoplasmic reticulum (microsomal fraction) together with some lysosomes. The resulting supernatant is the soluble fraction (cytosol). Each of the particulate fractions may be further purified by washing and recentrifugation. They may be individually further purified by density gradient centrifugation. The isolation of microsomes has received a lot of attention from drug metabolism researchers because of the importance of the microsomal mono-oxygenase system in xenobiotic metabolism (see preceding Chapter). When

the routine procedure outlined above is carried out there is inevitably some contamination of one fraction by another. This is best seen by considering a fractionation of rat liver carried out in our laboratory by Wright and coworkers (3) during studies on the effect of the ingestion of dieldrin on hepatocytes. The fractionation procedure was monitored by measuring the activities of several enzymes in each fraction, including succinic dehydrogenase (mitochondrial), and glucose-6-phosphatase and chlorfenvinphos dealkylase (microsomal). Some of these results are illustrated in Figure 1.

Often we require samples of only the microsomal and cytosolic fractions, in which case we routinely prepare 20% homogenates in 0.1 M potassium phosphate buffer pH 7.4, centrifuge at 10,000 g for 20 min and use this supernatant to prepare microsomes (190,000 g/30 min/pellet) and cytosol (supernatant) (4). Protein concentrations are required in order that enzyme specific activities can be calculated. These are measured using either the sensitive procedure describes by Lowry et al (5) or the simple modification of the biuret reaction described by Robinson and Hodgen (6).

Precipitation

Rapid methods for the preparation of microsomal fractions have been looked for in recent years. Isoelectric precipitation of liver microsomes from post-mitochondrial supernatant at pH 5.4 is a useful such method (7). Mono-oxygenase characteristics in the product compare very well with those of conventional microsomes but the status of the glucuronyl transferase was not reported. These preparations contain about 45% more protein than do conventional preparations and their possible contamination by cytosol enzymes, such as the glutathione transferases, requires further investigation. A calcium ion sedimentation method was accidently discovered during a preparation of plasma membranes which became contaminated with aggregated microsomes. The method was then developed specifically for microsomes (8). Mono-oxygenase characteristics were similar to those in conventionally-prepared microsomes but again glucuronyl transferase was not tested (9)(10). The method has been applied to rat and rabbit kidney and lung tissue. Microsomal yields from the latter differ from those obtained by centrifugation in that there is a higher yield of protein with lower specific activity (11).

Gel Filtration Chromatography

Microsomes and cytosol of rat liver 13,000 g supernatant have been separated by gel filtration through Sepharose 2B; the microsomes were collected in the exclusion volume (Vo) and the cytosol, between Vo and Vt (12). The method has been applied to rat lung with remarkable success giving microsomes with very high specific activities (for oxidative reactions) (13). An example of one of our own separations using this technique is shown in Figure 2 (10 ml of 10,000 g supernatant from 40% rat

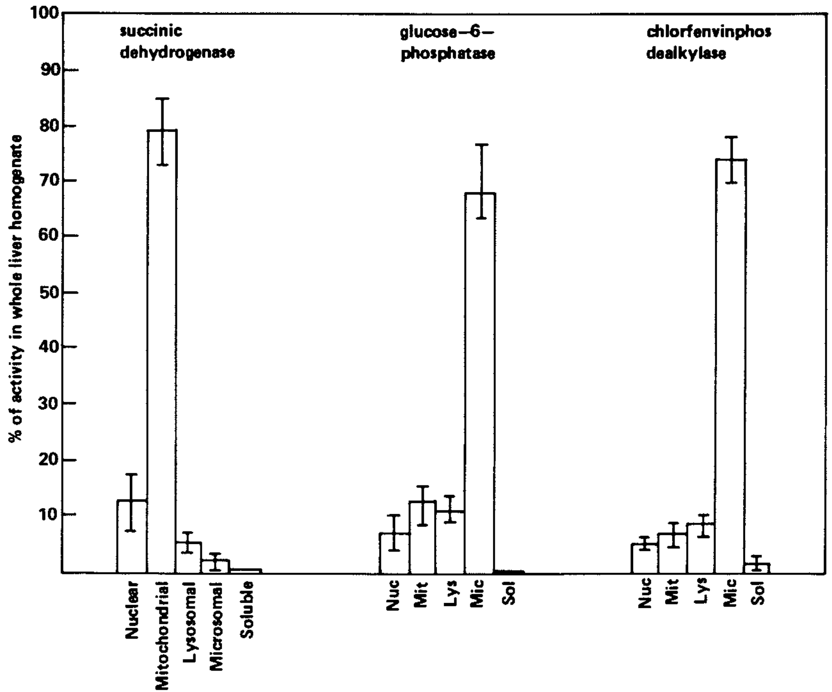

Figure 1. Distribution of marker enzymes between subcellular fractions

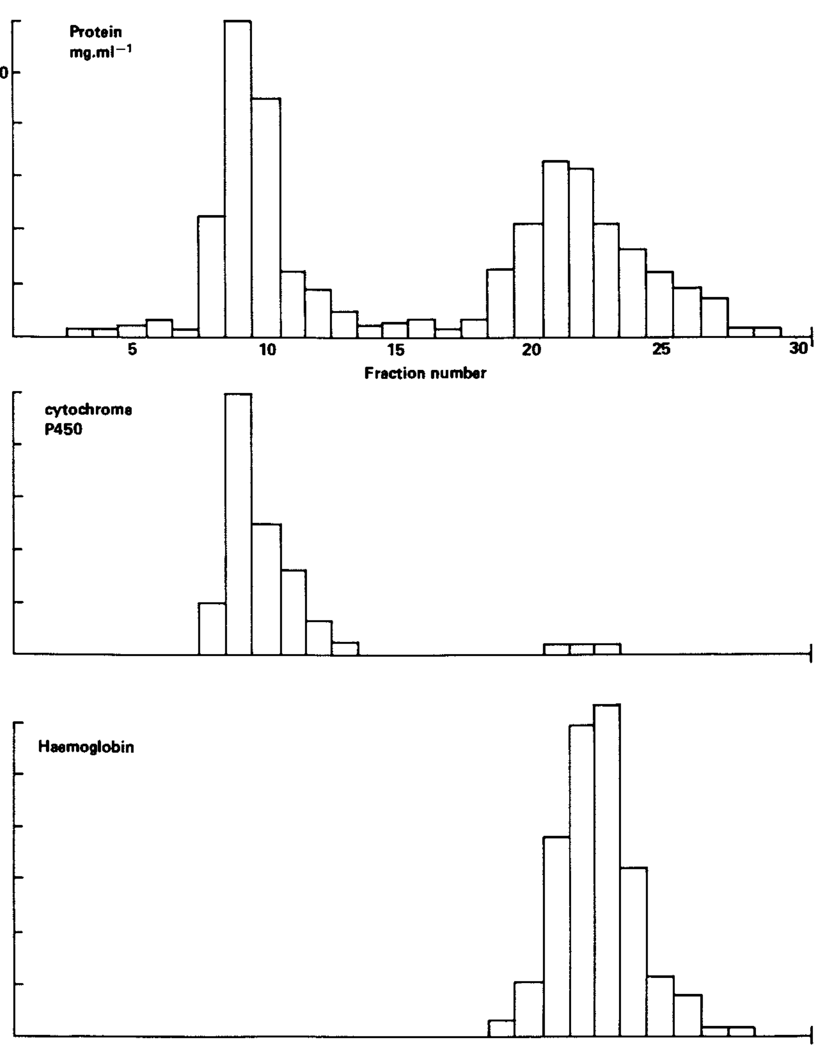

Figure 2. Gel filtration of rat liver 10,000 g supernatant on Sepharose 2B

liver homogenate applied to a 2.5 cm x 25 cm column run at 4-5°, upward flow, 1 ml/min; 5 ml fractions collected).

Advantages of the Subcellular Fractions

Individual subcellular fractions, supplemented with the appropriate cofactors, usually afford information on discrete steps in metabolic pathways in a way that is not possible using animals, organs or cells. The study of cofactor requirements provides information on the mechanism of each reaction, as do studies with isolated purified enzymes. The conditions for the reaction to be studied can be optimised and further metabolism can, if necessary, be blocked so that an intermediate of interest can be isolated.

In a converse sense, now that we know something about the mechanisms of the more common conjugation reactions, a study of the subcellular location and cofactor requirements of the biotransformation of a xenobiotic offers useful information on the metabolism of that compound. It is important to know each discrete step in the biotransformation of a xenobiotic because we then have a chance of identifying intermediates that may prove hazardous under certain circumstances. It is important also to know the effect of toxicants on the function of a particular cell organelle. This requires the study of subcellular fractions.

Another important advantage of subcellular techniques is that the fractions are relatively easy to prepare and they are reasonably robust provided that correct conditions are used. Conditions are described in some detail in the individual sections below.

Disadvantages of the Technique

The difficulties that may be experienced in working with subcellular fractions are detailed below in the sections on the individual conjugation reactions. However, some useful generalisations may be made. The extrapolation back to the situation *in vivo* from subcellular systems is a long one. We tend to base our comparisons on enzyme activity measured under conditions favouring good kinetics (e.g. zero order for cofactors, rate linear with time and protein concentrations). However, *in vivo*, cofactors and/or substrate concentration may be rate-limiting; penetration of substrate to enzyme may be restricted or facilitated; natural modifiers may be present; interaction between metabolic processes may occur and affect reaction rates. The values K_m (a measure of enzyme-substrate affinity) and V_{max} (the capability of the enzyme when saturated with substrate) are valuable, particularly in a comparative sense. Their true relevance to a particular situation *in vivo* however is difficult to assess.

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Another trap, but of our own making, must be considered. A rapid assay system is very important in enzyme purification and is very convenient in tissue and species-comparisons. It also helps, but is less easy to arrange, in structure-activity studies. There has been a tendency in xenobiotic enzymology for convenience to dominate in the selection of substates. The newcomer to the science of xenobiotic metabolism must surely be puzzled at the central role occupied by p-nitrophenol. Unsuitable or restricted substrate selection has led to the perpetration of some quite unwarranted generalisations. In addition certain convenient substrates have proved unsuitable in a physico-chemical sense and their use has led to some very complex kinetics dominated by the properties of the substrate rather than those of the enzyme.

Another difficulty, or rather a potential failure, of approaches using separated subcellular fractions is that one may miss an interaction between two processes. The most common example of this is the production of a metabolite by oxidation (i.e. a type I process) followed by its conjugation (type II process). The two reactions may be more than simply consecutive. Specific examples will be discussed below.

Use in Xenobiotic Metabolism Studies

Subcellular fractions have been used extensively to study the discrete steps in xenobiotic metabolism. They are also very useful in a comparative sense. Conjugation reactions, like other biotransformations, may be affected by a number of factors. These include species, tissue, sex, strain, stress, age, time, chemicals (induction, inhibition, activation) and pregnancy. The effects of these on a particular biotransformation are conveniently studied at the subcellular level. For example, if species comparisons *in vitro* are shown to be valid in terms of *in vivo* results across a range of experimental animals, they can be usefully extended to human biopsy samples, thus furnishing some metabolic data for man. The relationship between chemical structure and metabolism is also very conveniently investigated *in vitro*.

These various aspects are exemplified below for the individual conjugation reactions. The reactions requiring activated conjugand (glucuronide formation, sulphation, phosphorylation, acetylation and methylation) are discussed first followed by those involving activation of the xenobiotic (amino acid conjugation). Glutathione conjugation, which depends upon the mutual intrinsic reactivity of both substrates, is discussed last.

Conjugation with Glucuronic Acid

Mechanism and location. Glucuronic acid conjugation is probably the most quantitatively important of the Type II processes, both in terms of proportion of a particular xenobiotic being so conjugated, and in the variety of compounds taking part

in the reaction. Phenols, alcohols, carboxylic acids and N-hydroxy compounds all form O-glucuronides and a number of S- and N-glucuronides have been detected. The process has recently been reviewed in some detail by Dutton and co-workers (14)(15).

This process is an example of one in which the endogenous conjugand (glucuronic acid) is activated to a high energy donor (uridine-5'-diphospho-α-D-glucuronic acid, UDPGA). The transfer of the α-D-glucopyranuronyl group from UDPGA to the acceptor, forming β-D-glucopyranuronosides, is catalysed by UDPGA-glucuronyl transferase (EC 2.4.1.17). The enzyme is located in the endoplasmic reticulum of mammalian cells and therefore appears, on subcellular fractionation, in the microsomes.

Isolation, properties and use. The components required for an *in vitro* study: microsomes, UDPGA, xenobiotic and buffer, are readily available. UDPGA is also available radiolabelled. This simple system is perfectly adequate for many studies of pesticide metabolism. It is inappropriate to review assay methods in great detail in this chapter because, in xenobiotic metabolism, we are so often interested in a specific compound or series of compounds and the assay procedure will be based on the conversion of that compound. There are, however, some recent methods that may be generally useful. A method involving UDP[^{14}C]GA utilises an Amberlite XAD-2 column to separate unchanged UDPGA, conjugate and unchanged substrate (16). A continuous assay based on the enzymatic assay of the UDP released during the transfer has also been reported (17).

The isolation of the enzyme is achieved by the preparation of microsomes as described above. The enzyme is relatively stable in frozen 10,000 g supernatant and in frozen microsomal pellet. If stored at -196°C (liquid nitrogen), rat and rabbit enzymes can be kept for many days (18). Because the enzyme is closely associated with the lipoprotein microsomal membranes, further purification must be preceded by solubilisation. A recent method (19) involves treatment of the microsomes with 1% Lubrol 12A9 (a condensate of dodecyl alcohol with approx. 9.5 mol of ethylene oxide per mol) for 20 min at 4°C. The enzyme remained in the supernatant after further centrifugation i.e. solubilisation apparently occurred. It was then precipitated with ammonium sulphate and further purified in the presence of 0.05% Lubrol by DEAE cellulose chromatography. The product contained only 3 polypeptides; enzyme activities towards 2-aminophenol and 4-nitrophenol were increased 43- and 46-fold respectively. The purified enzyme had much improved stability in comparison with the microsomally-bound enzyme. Although there is much kinetic evidence for the existence of several glucuronyl transferases (15), very few pairs of aglycone specificites have been separated physically. One such separation is that of the activities towards 4-nitrophenol and morphine by Dell Villar *et al* (20). It is in this area where purification of the enzymes is important. Purification, however desirable in theory and however necessary

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to the study of the enzyme, is rarely carried out in studies of pesticide conjugation. Our main concern is the fate of the pesticide in the whole animal and the role that the conjugation may play in the disposition of the pesticide. Nevertheless we have to be aware of some practical complications consequent on the particulate nature of this enzyme.

UDPGA-glucuronyl transferase is 'latent' in freshly prepared microsomes. Membrane-perturbing processes such as aging, freezing and thawing, and treatment with detergents, chaotropes, organic solvents, alkali, trypsin or phospholipases may activate the enzyme by a factor of 40 or more (15). This property, together with evidence derived from kinetic studies, some involving competitive substrates, indicates that the enzyme is deeply buried, possibly on the inner surface of the microsomal vesicle. For this reason it is advisable to pretreat microsomes with Triton X-100 (overall 0.05% solution) before adding the xenobiotic substrate. This treatment should fully activate the enzyme and remove errors due to unknown amounts of activation caused by age, storage, mechanical effects or even an effect of the xenobiotic substrate itself. The treatment should also allow access of both UDPGA and the xenobiotic to the enzyme. This may be restricted, particularly if an ionic substrate (e.g. a carboxylic acid) is being investigated. There are two further problems associated with its use however. The amount of activation is species-dependent. For example, the glucuronidation of 7-hydroxychlorpromazine (21) is activated twice as much in rat liver microsomes as it is in guinea-pig liver microsomes. Activation is also dependent on the concentration of Triton X-100 (see Figure 3).

Another problem is the destruction of UDPGA by pyrophosphorylase. This is partly inhibited by EDTA but a better solution is apparently the use of citrate buffer which virtually abolishes the pyrophosphorylase action (15).

The enzyme has a pH optimum of about 7.4 though this may vary somewhat with ionisable substrates. Reaction rates are linear for at least an hour at 37°C.

Examples of use. One of the most frequent uses of the glucuronidation system in our laboratory is in the biosynthesis of glucuronides of faecal metabolites. These are then chromatographically compared with suspected glucuronides found in the urine or in the bile of treated animals. Figure 4 illustrates the formation of the glucuronide of *anti*-12-hydroxy-[^{14}C]endrin under the following conditions: volume, 5 ml; hydroxyendrin, 0.004 mM; UDPGA, 0.75 mM; washed rabbit liver microsomes (4.2 mg/ml) in 0.1 M TRIS-HCl buffer (pH 7.4 at 37°C); incubation temperature, 37°C. Portions (1 ml) were withdrawn at intervals and partitioned between benzene and water which were then radioassayed to afford the proportions of unchanged substrate and conjugate respectively (22). Triton X-100 had no effect on this reaction, possibly because the very lipophilic *anti*-12-

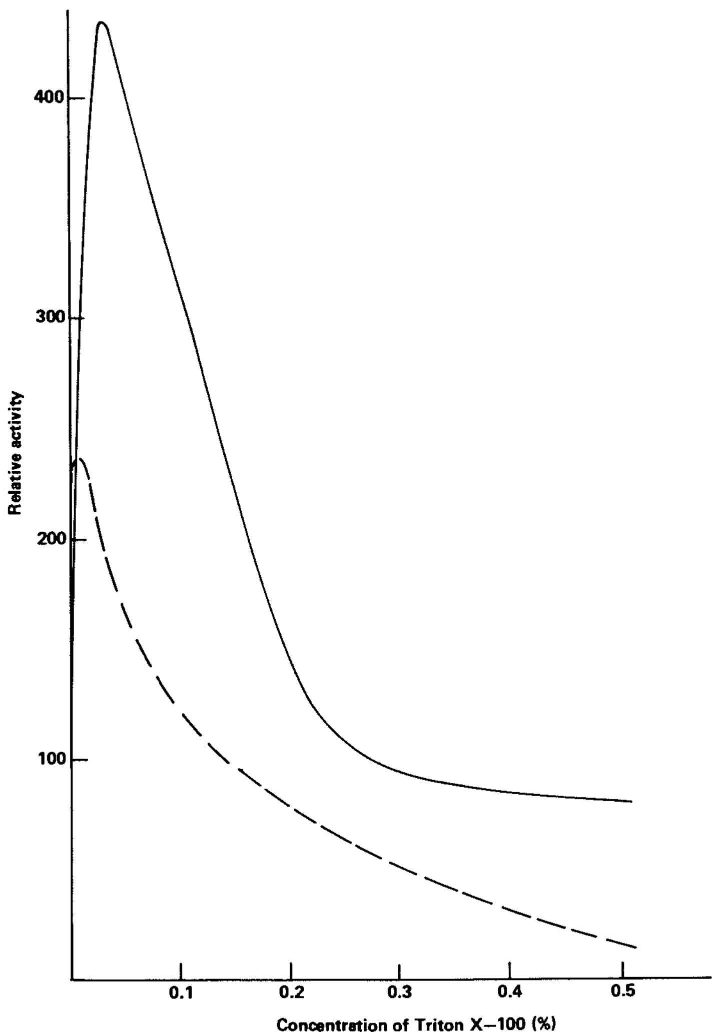

Figure 3. Effect of Triton X-100 on glucuronidation: (——), rat liver microsomes; (— — —), guinea pig liver microsomes.

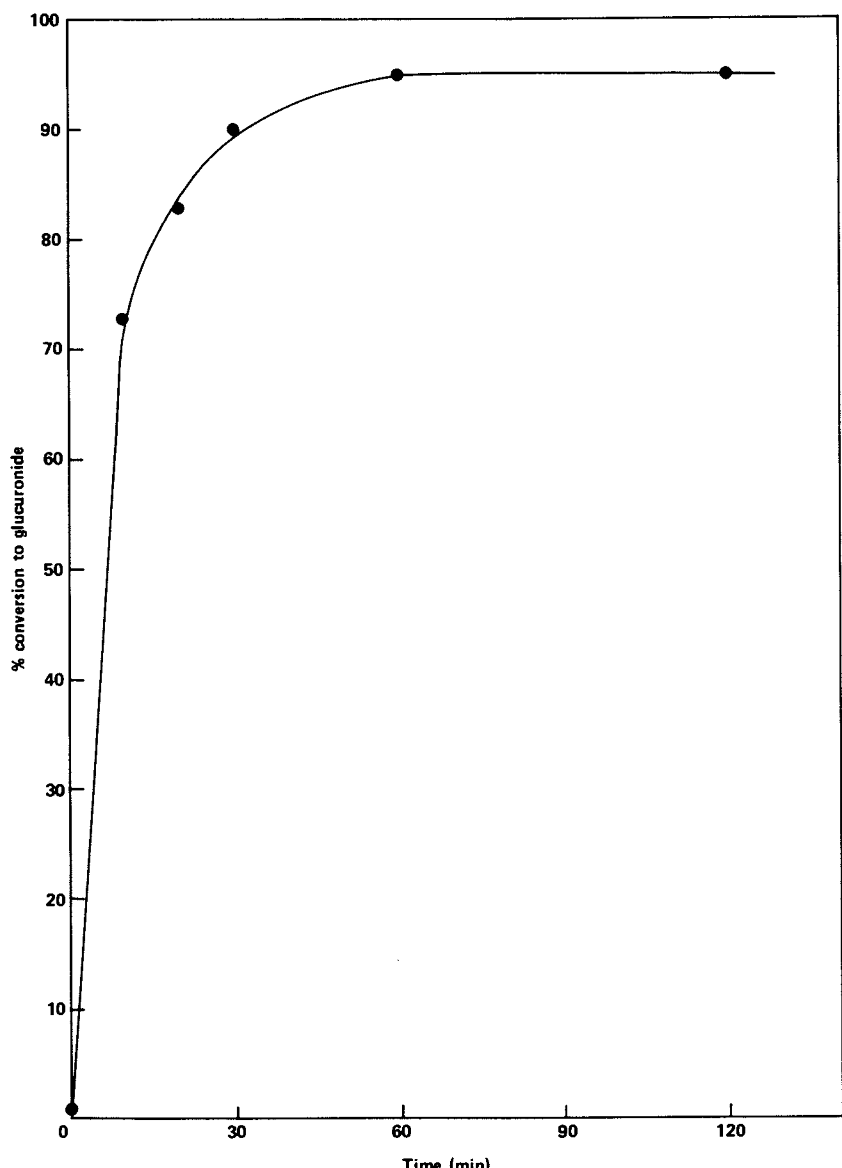

Figure 4. Time course of the glucuronidation of ^{14}C anti-12-hydroxyendrin by rabbit liver microsomes

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hydroxyendrin was readily accessible to the enzyme.

The common experimental animals, e.g. guinea-pig, rat, mouse, hamster and dog, all possess reasonable amounts of hepatic microsomal glucuronyl transferase. Litterst et al (23) have compared the hepatic enzyme in rhesus monkey (currently in short supply) with species which may be used as alternative test animals. Activities towards 4-nitrophenol (nmol per min per mg protein) were: rhesus monkey, 7; squirrel monkey, 8-11 (sex difference); common tree shrew (9-12); miniature pig (6-9); Sprague-Dawley rat (2-3). These measurements were made without the addition of detergent. Conditions were: substrate, 0.2 mM; UDPGA, 3.3 mM; protein, 1 mg/ml; Tris buffer pH 7.4, 1 mM. The cat and other Felidae excrete little or no glucuronide conjugates of xenobiotics (24). The high K_m value for 4-nitrophenol with cat liver glucuronyl transferase compared to that with rat liver transferase (25) is in accord with this observation. Hens similarly do not excrete many glucuronide conjugates and have very low enzyme activities in liver. These are examples of the in vitro subcellular results comparing well with the situation in vivo. The occurrence of glucuronyl transferase in birds generally has not been studied. Fish excrete glucuronides (26) and have been shown to possess hepatic glucuronyl transferase activity towards 2-aminophenol (27) 3-trifluoromethyl-4-nitrophenol (26), and 4-nitrophenol (28). Glucuronide formation is important in man but the hepatic enzyme has not been examined in detail.

Another important use of in vitro techniques is for the comparison of biotransformation in different tissue types. However, after a survey using a sensitive methylumbelliferone assay, Aitio (29) concluded that liver is the most important organ of glucuronide synthesis. It was estimated that the whole gastrointestinal tract possessed only 15-20% of the enzyme activity found in liver. Some results are summarised in Table I.

Low enzyme activity has also been found in the placenta of several species, including man (30). The relative activities found for various tissues are partly a function of the choice of substrate. For example, brain, heart, fat and diaphragm possess very low activities towards methylumbelliferone (30) but quite high activites towards the carbaryl metabolite, 1-naphthol (31). This demonstrates that model substrates serve only as a guide; for relevant information on a specific chemical, only studies on that chemical will really suffice.

Toxicological significance. The value of glucuronidation lies in the dramatic change in polarity that the process confers. The glucuronides are also very readily secreted in bile or via the kidneys and thus removed from the body. Glucuronidation can also prevent certain types of metabolite from being further bioactivated to reactive species such as the quinones which may be formed from aromatic dihydrodiols (32). The process is almost always a detoxification, however in some important

cases the reverse is observed. The glucuronidation of N-hydroxyarylacetamides (e.g. N-hydroxyphenacetin, Figure 5) affords chemically reactive molecules capable of interacting with tissue macromolecules (33). The demonstration of this type of bioactivation is a valuable use of in vitro test systems.

Table I. Glucuronidation of methylumbelliferone in rat tissues

Tissue	Enzyme activity	
	nmol product per g wet wt. liver	nmol product per whole organ
Liver	460	3200
Duodenal mucosa	260	96
Adrenal glands	170	63
Kidneys	150	120
Spleen	30	35
Lungs	28	34
Thymus	19	9
Heart	1.4	1.1
Brain	0.8	1.3

The use of separated subcellular fractions may fail to reveal possible interactive effects or functional relationships between the fractions. For example, the addition of UDPGA to rat liver microsomes increases their rate of 12-hydroxylation of dieldrin (34). The rationale for adding UDPGA to an oxidative system was that syn-12-hydroxydieldrin (Figure 6), although more hydrophilic than dieldrin, is still a very lipophilic molecule, particularly in view of the hydrogen bonding of the hydroxyl group to the epoxide oxygen. Thus, when formed as a metabolite via the action of microsomal mono-oxygenase, it would remain near its site of formation, possibly inhibiting further hydroxylation of dieldrin. This effect was first noted by von Bahr and Bertilsson (35) with demethylimipramine.

The functional relationship between microsomal mono-oxygenase, epoxide hydratase and glucuronyl transferase has recently been investigated in liver microsomes and in isolated hepatocytes (32). The results provide a good illustration of one of the limitations of the subcellular approach. In the hepatocyte and in microsomes fortified with NADPH and UDPGA, naphthalene is oxygenated to its 1,2-oxide which is cleaved to a dihydrodiol (by epoxide hydratase) which, in turn, is conjugated with glucuronic acid (Figure 7). However, microsomes afford the dihydrodiol as the major metabolite; hepatocytes afford the glucuronide. Only by using either more microsomal protein or the

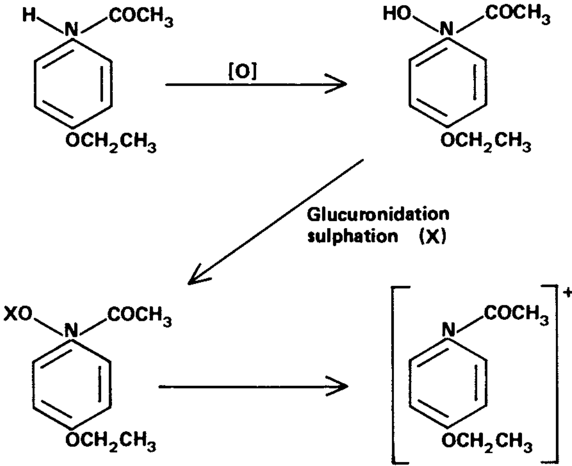

Figure 5. Conjugation in the formation of reactive metabolites from phenacetin

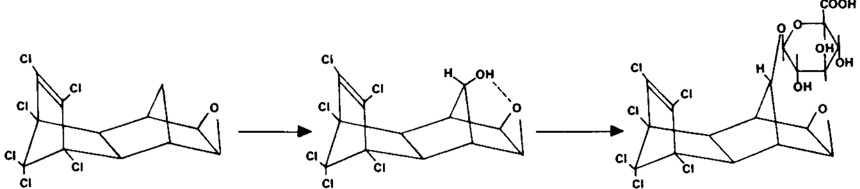

Figure 6. Hydroxylation and glucuronidation of dieldrin

Figure 7. Relationship between the enzymes affecting the metabolism of naphthalene

allosteric effector of glucuronyl transferase, UDP-*N*-acetyl-glucosamine, could microsomes be forced to yield reasonable quantities of glucuronide. This effector may also competitively inhibit the destructive effect of the pyrophosphorylase on UDPGA *in vitro*.

Conjugation with Sulphate

Mechanism and location. Conjugation with sulphate is mediated by the sulphotransferase enzymes (historically sulpho-kinases). The donor substrate, which contains the activated sulphate group, is 3'-phosphoadenosine-5'-phosphosulphate (PAPS). Acceptor groups are principally phenolic but an alcoholic or primary amine function can also be sulphated via the same mechanism. Irrespective of the chemical nature of the final sulphated product, sulphoconjugation follows the same general pathway: activation of inorganic sulphate to yield first adenosine 5'-phosphosulphate (APS) and then 3'-phosphoadenosine 5'-phosphosulphate (PAPS), followed by transfer of the sulphate group from PAPS to suitable acceptors:

$$ATP + SO_4^{2-} \longrightarrow APS + PP_i \quad (1)$$

$$APS + ATP \longrightarrow PAPS + ADP \quad (2)$$

$$PAPS + Acceptor \longrightarrow PAP + \text{sulphated acceptor} \quad (3)$$

The enzymes catalysing these reactions are (1) ATP-sulphate adenylyl transferase (ATP-sulphurylase); (2) ATP-adenylyl sulphate 3'-phosphotransferase (APS-kinase) and (3) an appropriate sulphotransferase (EC 2.8.2). In mammalian cells the enzymes responsible for the production of PAPS from sulphate are localised in the cytosol. Sulphotransferases are located in both the microsomal and the cytosolic fractions of a wide variety of tissues; their general tissue distribution resembles that of the UDP-glucuronyltransferases, except that they are also abundant in placenta. Boundaries of their specificities are more clear than those of the glucuronyl transferases, and distinctive phenol, steroid and arylamine sulphotransferase activities have been detected. Other transferases deal apparently solely with endogenous compounds, e.g. cerebroside and polysaccharide sulphotransferases. Simple endogenous sulphates includes those of steroids, adrenaline, tri-iodothyronine and serotonin. The picture emerges (cf. the glucuronyl transferases) of the sulphotransferases responsible for the sulphation of small molecules being freely soluble in the cytosol, whereas those that are involved in the assembly of larger molecules are arranged, together with other requisite biosynthetic enzymes, in assembly-line fashion on the membranes of the endoplasmic reticulum and the Golgi apparatus. It is extremely doubtful whether any of these bound enzymes play any role in the metabolism of xenobiotics (36).

Isolation, properties and use. The uncertainty in the total number, precise roles and specificities of the sulphotransferases reflects the fact that they are exceedingly difficult to purify and to separate from one another. Moreover, some of them aggregate and/or change conformation under certain conditions; multiple peaks of similar activity, which may or may not be due to the same enzyme, appear on chromatography columns and are a constant hindrance to the experimentalist.

A sulphotransferase has been partially purified from bovine kidney (acetone powder) using 4-nitrophenol as the assay substrate (37). The activity of the enzyme was measured using [^{35}S]PAPS in the assay mixture. Substrate specificity was investigated and it was found to be relatively specific for simple aryl sulphate formation as shown in Table II.

Table II. Relative substrate specificities for bovine kidney sulphotransferase

Substrate	Relative specificity
4-Nitrophenol	100
4-Hydroxybenzaldehyde	75.9
4-Chlorophenol	79.7
1-Naphthol	32.5
Phenol	6.5
o-Cresol	21.7
m-Cresol	8.3
3-Nitrophenol	49.8
3-Hydroxybenzaldehyde	21.0

No activity could be demonstrated towards ethanol, propan-1-ol, butan-1-ol, or towards a range of steroids.

The isolation of N-hydroxy-2-acetylaminofluorene (N-OH-2-AAF) sulphotransferase has recently been achieved from the cytosol fractions of male and female rat livers (the latter possess very low activity) (38). A 2000-fold purification with a yield of over 12% was achieved using the following procedure: ammonium sulphate fractionation, DEAE-cellulose column chromatography, hydroxyapatite column chromatography, sephadex G-200 gel filtration, isoelectric focussing and, finally, more sephadex G-200 gel filtration. The final preparation was homogenous on analytical disc gel electrophoresis. The purified enzyme had activity towards 4-nitrophenol with an approximately 1600-fold increase in specific activity over the crude homogenate, but it had very low activity towards endogenous steroids and serotonin. PAPS was used as the sulphate donor in these assay mixtures and was synthesised enzymatically (39). The pure enzyme was very unstable, especially in dilute solutions. Thiol compounds were found to have a stabilising effect and thiol blocking reagents were potent inhibitors.

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The involved nature of these purifications suggests that the study of phase II sulphoconjugations using purified sulphotransferase from various animal species and animal tissues is impractical at present. An in vitro assay system for studying these reactions using crude subcellular fractions has been developed by Mulder et al (33). It comprises cytosol (600 μg protein/ml), xenobiotic (0.5 mM), Tris-HCl buffer (100 mM, pH 8.0) and a PAPS-generating system (PAPS-GS): 3'5'-adenosine diphosphate (PAP, 10 μM) and 4-nitrophenyl sulphate (10 mM). In this assay mixture 4-nitrophenyl sulphate is used to convert PAP into PAPS, a reaction presumably catalysed by a phenolsulphotransferase in the cytosol. The rate of formation of 4-nitrophenol is spectrophotometrically determined and is calculated by subtracting the amount of 4-nitrophenol released in a control incubation mixture (xenobiotic absent) from that released in the presence of the xenobiotic. The rate of sulphation may thus be indirectly calculated from the amount of 4-nitrophenol released using a molar extinction coefficient of 17,500 M^{-1} cm^{-1} (at pH 8.0). This assay procedure makes an important contribution to the field of xenobiotic metabolism in that it provides a cheap and simple method for studying the mechanisms and rates of phase II sulphate conjugation reactions using a PAPS-GS and cytosol. Hitherto PAPS has been commercially available in the labelled form only and its enzymic synthesis is a tedious process (39)(40). A more traditional assay procedure is that described by Wu and Straub (38) where PAPS is used at a concentration of approximately 0.3 mM when substrate concentration is 0.2 mM.

Examples of use. Harmol (Figure 8) is a good substrate for phenol sulphotransferase in rat liver 600 g fraction (41) but harmalol (Figure 8) is a very poor substrate. The reason for the difference in rate of sulphation of these two substrates is unknown, but the findings agree with the different rates and modes of conjugation (sulphation and glucuronidation) of the two compounds found in vivo.

The metabolism of harmol illustrates a problem that often arises when discussing the phase II conjugation of xenobiotics with sulphate. Sulphation of a xenobiotic or its metabolite is practically always accompanied by conjugation with glucuronic acid. Glucuronic acid conjugation often predominates and this has been assumed to be due to a limited supply of sulphate in vivo. Mulder and coworkers have developed a method for the simultaneous measurement of UDP-glucuronyltransferase and phenolsulphotransferase from rat liver in vitro, using harmol as substrate and 600 g supernatant as the enzyme source (42). Triton X-100 was used to activate glucuronyl transferase (Section 5.1) and was shown to have no effect on the activity of phenolsulphotransferase. The amount of the conjugates formed was measured fluorometrically and the activities of the enzymes were expressed as arbitrary units of fluorescence recovered from a tlc analysis of the incubates. At low substrate concentrations

Figure 8. Harmol and harmolol

glucuronidation
sulphation
phosphorylation (?)

(reactive)

Figure 9. Acetylation and conjugation in the activation of aromatic amines

(<40 μM) harmol was preferentially sulphated but with increasing substrate concentration, glucuronidation became more important. These results agree with earlier in vitro results (41) when the K_m value for harmol of UDP-glucuronyl transferase was found to be 150 μM and of phenolsulphotransferase was <15 uM. Therefore, in some circumstances, the affinities of the enzymes for the substrate may control the relative amounts of conjugation. 2,6-Dichloro-4-nitrophenol may be used as a selective inhibitor of sulphation if glucuronic acid conjugation needs to be studied in the 10,000 g fraction of liver (43). Thus, using in vitro subcellular fractions containing even crude enzyme preparations, a greater understanding of in vivo findings is possible.

A second important facet of in vitro studies is the assessment of the relative importance of various animal organs in xenobiotic metabolism. Using rabbit lung soluble fraction, significant sulphation of 4-nitrophenol and 4-methylumbelliferone has been demonstrated (44). The former was sulphated by lung cytosol at about one third the rate found for liver cytosol (per mg protein). In these experiments PAPS was included in the incubation mixtures. This may account for the apparent disparity between these results and those of Gram et al (45) who could not detect any sulphotransferase activity towards 4-nitrophenol in rabbit lung supernatant. These authors utilised a PAPS-generating system in their experiments.

Sulphation of steroids has been studied in the cytosol of brain and liver from guinea-pigs and rats (46). [^{35}S]PAPS was used as the sulphate donor. 17-β-Oestradiol (E) and diethylstilbestrol (DES) were used as substrates. The following activities (pmol conjugate/mg tissue/20 min) were measured with brain preparations: rat: E, 0.6; DES, 2.0; guinea-pig: E, 0.1; DES, 4.1. Activities with guinea-pig liver were: E, 98; DES 333. Thus, with these substrates, the activity of the transferase in brain is only a small fraction of that of liver.

Toxicological significance. Conjugation of a xenobiotic with a completely ionised and highly hydrophilic moiety such as sulphate markedly alters its physico-chemical properties and this usually leads, like glucuronidation, to a complete loss of pharmacological or pesticidal activity. This and a rapid rate of excretion are the beneficial consequences of sulphoconjugation.

Several xenobiotics exert their toxic effects in mammals though the formation of reactive metabolites that combine with cellular macromolecules. The O-sulphation of N-OH-2-AAF has been postulated as an activation reaction (Figure 9) leading to the carcinogenic action of this compound (47). There is no direct evidence for the formation of these NO-sulphates in vivo; their instability precludes their isolation and characterisation. However, their transient existence has been demonstrated in vitro by the use of indirect methods, such as trapping the conjugates in situ by reaction with nucleophilic compounds (48). There is also indirect evidence that the sulphate conjugate of N-OH-2-AAF

is formed in rat liver in vivo (49). Mulder et al (33) have recently used the PAPS generating system described earlier in this section, to indirectly measure the rate of sulphation of N-OH-2-AAF. The reaction rate was linear with cytosolic protein concentration up to 1.2 mg/ml and was about 2.7 nmoles 4-nitrophenol formed per ml per mg soluble protein (at 31°C). These results suggest that the NO-sulphate of N-OH-2-AAF had been formed, but again isolation of the metabolite was not possible because of its instability. Evidence that an NO-sulphate conjugate was formed in the incubation mixtures was obtained by showing that the covalent binding of N-OH-2-AAF to macromolecules increased dramatically during incubation with 4-nitrophenyl sulphate and PAP. N-Hydroxyphenacetin, N-hydroxyacetanilide, N-hydroxy-4-chloroacetanilide and N-hydroxy-2-acetylaminonaphthalene also formed sulphate derivatives at rates comparable to that of N-OH-2-AAF. N-Hydroxyphenacetin and N-hydroxy-2-acetylaminonaphthalene also became rapidly covalently bound after sulphation of their N-hydroxy groups, but the O-sulphates of N-hydroxy-4-chloroacetanilide and N-hydroxyacetanilide did not react (Table III). DeBaun et al (48) have also extensively studied this effect of sulphation on covalent binding of N-OH-2-AAF, and the work is a good example of the use of subcellular fractions in the investigations of mechanisms of carcinogenesis.

Table III. Conjugation rates and effect of conjugation on covalent binding of some N-hydroxy-N-arylacetamides

Substrate	Sulphation rate (nmoles/min/mg soluble protein)	Amount covalently bound (nmoles/ml)	
		with 4-nitrophenylsulphate	without 4-nitrophenylsulphate
N-hydroxyphenacetin	2.6	25.0	0.2
N-hydroxyacetanilide	1.6	0.2	0.2
N-hydroxy-4-chloroacetanilide	2.4	0.4	0.2
N-hydroxy-2-AAF	2.7	21.8	0.6
N-hydroxy-2-acetylamino naphthalene	2.0	1.9	0.2

This study shows clearly that the NO-sulphate of N-hydroxyphenacetin binds covalently to protein as soon as it is synthesised. Since phenacetin is N-hydroxylated by microsomal enzymes (50)(51), it seems likely that the sulphation and glucuronidation pathways provide a reactive intermediate for covalent binding. At the present time it is not clear whether either reaction plays a role in causing the short-term toxic effects of

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high doses of phenacetin *in vivo* (liver necrosis); however, this work demonstrates the value of subcellular studies in the characterisation of enzymatic determinants of toxicity.

Phosphorylation

The excretion of di(2-amino-1-naphthyl)hydrogen phosphate by dogs dosed with 2-naphthylamine (52) suggests that phosphorylation occurs in xenobiotic metabolism. However, only very few examples have been found in the intervening 17 years. The process has been postulated as a bioactivation step in carcinogenesis by 2-acetylaminofluorene (53). Monophenyl phosphate is excreted in the urine of cats dosed with phenol (54). Rat liver mitochondria contain an enzyme that catalyses the ATP-dependent phosphorylation of 1-aminopropan-2-ol (55). These facts summarise most of our knowledge on the phosphorylation of xenobiotics; they are stated here to emphasise the apparently very minor role of this conjugation. The central role of phosphorylation in energy metabolism is presumably, of necessity, controlled by very substrate-specific processes in which xenobiotics cannot readily participate.

Acetylation

Mechanism and location. Acetylation is of relatively minor importance in pesticide metabolism in comparison with glucuronidation, sulphation and glutathione conjugation. However, the reaction must be expected whenever an aromatic amine is under investigation *per se* or liberated during metabolism. The transfer of acetate occurs from acetyl-coenzyme A and is catalysed by arylamine *N*-acetyltransferase (EC 2.3.1.5). The enzyme is located in the cytosol of mammalian cells. The reaction is general for aromatic amines, sulphonamides, hydrazino compounds and some non-aromatic amines (56). The enzyme operates via a simple ping-pong mechanism of two consecutive steps: acetylation of the enzyme by acetyl-CoA followed by transfer of acetate from acetyl-*N*-acetyl-transferase to the acceptor substrate. This has been confirmed by the isolation of a [^{14}C]acetyl-*N*-acetyltransferase protein capable of donating its acetyl group to isoniazid (57). The reaction is important in the metabolism of endogenous amines, including serotonin, tryptamine, histamine and phenylethylamine. Man and rabbit exhibit a genetic polymorphism in *N*-acetylation (58) (for example, of 4-aminobenzoic acid and isoniazid) and individuals can be classified as 'rapid' or 'slow' acetylators. The location of the *N*-acetyltransferases is not always cytosolic. Those involved in the last stages of mercapturic acid formation are located in the liver microsomal fraction (59).

In principle, *O*-acetylation and *S*-acetylation of foreign compounds may occur (as they do in the formation of the endogenous substrates *O*-acetylcholine and *S*-acetyl-CoA); however, they have not yet been demonstrated.

Isolation, properties and use. *N*-Acetyl transferase (to aromatic amines etc.) is best used and measured in cytosol prepared by ultra-centrifugation. This may be dialysed before use.

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Rabbit cytosol enzyme is stable for at least a month when frozen, and for 48 hours if stored at 4° or 25°. It also withstands repeated freezing and thawing. Its pH optimum varies with substrate but lies between 6 and 8.5. A purification procedure from rabbit liver cytosol has been described by Weber and Cohen (60). This involved centrifugation to afford a 100,000 g supernatant, ammonium sulphate precipitation, Sephadex G-100 and DEAE-cellulose chromatography. Typical conditions for use are as follows: substrate (0.1 μmole), acetyl-CoA (0.5 μmole), 0.3 mM phosphate buffer pH 6.8 (perhaps higher), cytosol (0.5-5 mg protein) in a volume of 1 ml.

As usual, the assay system will depend on the substrate under investigation. However, a useful general method involves [^{14}C-1-acetyl]-acetyl-CoA, the products from which can be extracted into organic solvent and radioassayed either before or after chromatographic separation (61).

Examples of use. *In vitro* measurements (4-aminobenzoic acid) have been used to show a wide species distribution of *N*-acetyltransferase (23). For example, the activity of the hepatic enzymes lies in the order: rhesus monkey = pig > squirrel monkey = tree shrew > rat. The industrial intermediate 2,4-toluenediamine is *N*-acetylated preferentially at the 4-amino group. The enzyme activity (in cytosol) is found in liver > kidney > intestinal mucosa > lung. The activity of the liver enzyme varies with species: hamster > guinea-pig > rabbit > mouse > rat > human (trace) > dog (zero) (62). The genetic polymorphism noted above was discovered at the *in vivo* level in man and rabbits. It is of the same type in both species (controlled as a simple autosomal Mendelian character with rapid acetylation dominant to slow (56)). It has been understood via subcellular studies. It is probably due to varying amounts of the same enzyme rather than to different enzymes (56). At least two *N*-acetyltransferases exist in the rabbit. They differ in their tissue distributions, substrate specificities and pH-activity profiles. The one responsible for the genetic polymorphism has a wide substrate specificity and is found mainly in the liver and gut (63).

Toxicological significance. *N*-Acetylation is very important in the inactivation of the pharmacological and biocidal properties of many amine drugs; however, this property is of minor importance in the pesticide field. Of greater interest and potential importance is the role that *N*-acetylation plays in the bioactivation of carcinogenic aromatic amines. 2-Aminofluorene, 4-aminobiphenyl and 2-aminonaphthalene (Figure 9), for example, are all thought to be *N*-acetylated in the first step on their metabolic routes to ultimate carcinogens. The enzyme activity is of course readily available in the liver cytosol of various species. It is of considerable interest that dog liver cytosol contains no detectable activity (Figure 10) (nor towards toluenediamine above) and that dog liver is not susceptible to the carcinogenic action of these compounds (64). When a variety of arylamines are

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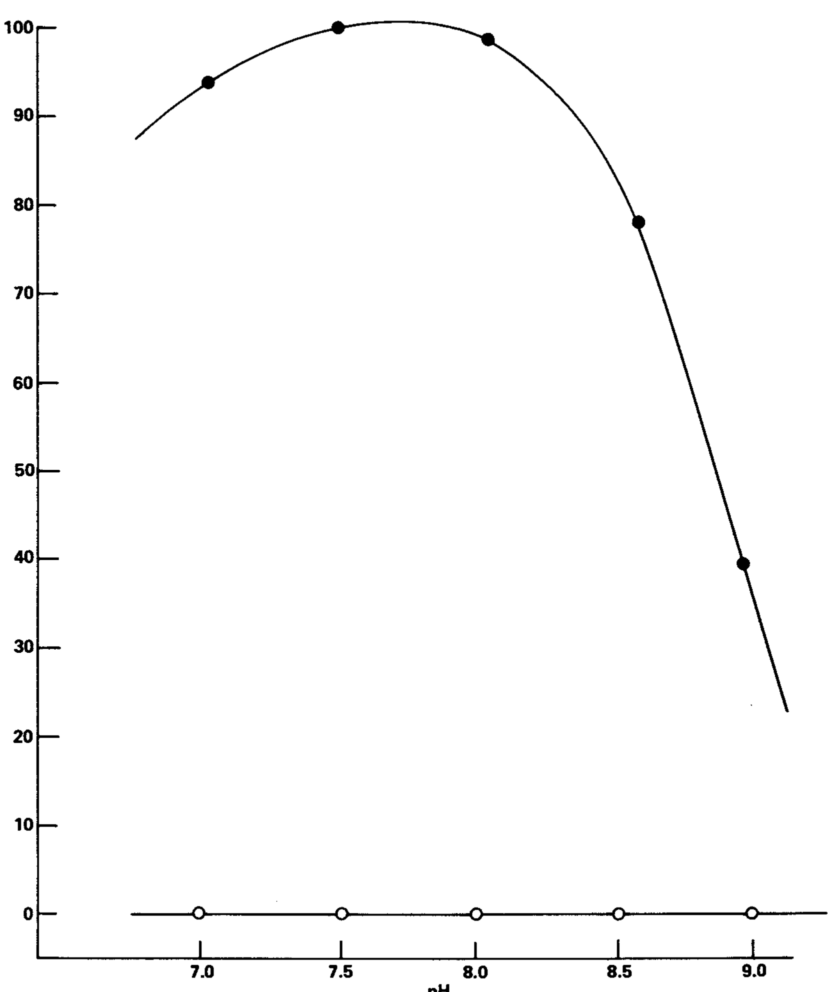

Figure 10. N-*acetylation of 2-aminofluorene by dog and mouse liver cytosol: (●), mouse; (○), dog* (64).

administered to dogs, only urinary bladder tumours are noted. However, the administration of carcinogenic arylacetamides causes both bladder and liver tumours. Clearly hepatic N-acetyl transferase is a determinant of liver carcinogenesis by arylamines.

The N-acetylation of isoniazid may also be a toxicating reaction. An increased incidence of 'isoniazid hepatitis' has been noted in rapidly acetylating humans (65).

Methylation

Mechanism and location. The methylation of phenols, thiols and amines involves a methyl donor (S-adenosylmethionine) and methyltransferases. Thus the mechanism is typical of type II reactions although the result, in terms of increase in polarity, is much less or even the reverse of the other type II processes.

O-Methylation. Catechol O-methyl transferase (66) (EC 2.1.1.6) is the most common O-methylating enzyme in xenobiochemistry. It is highly specific for the catechol (i.e. 1,2-dihydroxybenzene) configuration, but given that, it is undemanding in its other requirements (67). It is probably encountered most commonly in xenobiotic metabolism at the and of the sequence: arene → arene oxide → dihydrodiol → catechol → monomethylcatechol. Most of the enzyme activity is present in the cytosol of various tissues. Phenol O-methyltransferase, a microsomal enzyme, has been detected in vitro (68) but its significance in vivo is unknown because the O-methylation of monohydric phenols apparently does not occur in vivo. A potentially useful general assay method involving the use of the tritiated cosubstrate S-adenosyl-L-[methyl-^{3}H]methionine has been described (69). The radioactive methylated product is extracted into organic solvent for radioassay.

S-Methylation. S-Methylation is a general pathway of metabolism of the thiopyrimidone antithyroid drugs such as 6-propyl-2-thiouracil. Tetrahydrofurfuryl mercaptan is also S-methylated in an S-adenosylmethionine-dependent enzymatic reaction. The enzyme was found in the microsomal fraction of liver, kidney and small intestine (70). Thiophenol liberated during the metabolism of dyfonate (Figure 11) is methylated (71). This reaction has not been studied in vitro.

N-Methylation. Pyridine derivatives are N-methylated but the reactions are quantitatively of minor importance. The most widely studied N-methylation reactions are those involving the biogenic amines. For example, phenylethanolamine N-methyltransferase (EC 2.1.1) (involved in the biosynthesis of epinephrine) has been studied recently and found to methylate non-aromatic substrates as well as aromatic ones (e.g. Figure 12, R = propyl, cyclohexyl, cyclohex-3-enyl,cyclo-octyl). The enzyme has an absolute requirement for a hydroxyl group at the β-position on an ethyl side chain (72).

Methylation reactions have not featured widely in pesticide metabolism; however, there is enough information derived from endogenous biochemistry anddrug metabolism to be useful should

Figure 11. Methylation of thiophenol in the metabolism of dyfonate

Figure 12. Substrate for phenylethanolamine N*-methyltransferase*

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it be necessary to study these reactions with particular pesticides.

Conjugation with Amino Acids

- Mechanism and location. Amino acid conjugation in pesticide metabolism has recently been reviewed (73). Substrates for amino acid conjugation are usually either aromatic carboxylic acids or arylacetic acids. The most important conjugating amino acid is glycine and, together with glutamine, accounts for the majority of α-amino acid conjugates formed in mammals.

α-Amino acid conjugation occurs with the formation of an amide bond. Thus, both the substrate and the metabolite possess an ionisable carboxyl group. The first step of the reaction occurs with the interactions of the xenobiotic with coenzyme A (I). Conjugation of the activated xenobiotic with glycine occurs in the second step and is catalysed by glycine N-acylase (EC 2.3.1.13) (II) (Figure 13). Thus, the formation of glycine conjugates may be contrasted with the mechanism of the other conjugation reactions discussed so far. It is the xenobiotic rather than the endogenous conjugating moiety that is activated prior to the transferase action.

Activation of benzoate into benzoyl-CoA is thought to be catalysed by butyryl-CoA synthetase (EC 6.2.1.2) (74), which is found in the mitochondrial matrix (75). Glycine N-acylase has been prepared from mitochondria (76)(77) and has recently also been found by Gatley et al (78) to be located in the mitochondrial matrix. These authors have also shown that hydrolysis of benzoyl-CoA occurs in situ and they postulate that the function of the simultaneous activity of benzoyl-CoA synthetase and benzoyl-CoA hydrolase is to prevent the accumulation of too much benzoyl-CoA. This would occur if the supply of glycine, or its rate of conjugation, were limiting for hippurate synthesis and could thus impair other CoA-requiring reactions (79). It is thought that undissociated benzoic acid and hippuric acid cross the inner membrane because of their lipid solubility. Further, Halling et al (80) conclude that glycine crosses the inner membrane as a zwitterion. Therefore the location of the enzyme does not apparently impair the access of these substrates.

In addition to the conjugation of xenobiotics with amino acids, endogenous compounds, notably the bile acids, conjugate with glycine and taurine. Recently, arginine conjugates of bile acids have also been identified (81).

Choloyl-CoA synthetase (EC 6.2.1.7), the enzyme responsible for the formation of the activated bile acid intermediate, has been located in the microsomal fraction of guinea-pig liver (82). The intracellular location of the amino acid N-acyltransferase enzymes responsible for the conjugation of the activated substrate with taurine or glycine has been in dispute for some time. Siperstein and Murray (83) localised the enzyme(s) in the soluble fraction of liver cells. Bremer (84) and Elliott (85) concluded that they were located in the microsomal fraction and Schersten

C_6H_5–COOH + ATP + CoASH ⟶ C_6H_5–CO–SCoA + AMP + PP (I)

C_6H_5–CO–SCoA + COOH–CH_2–NH_2 ⟶ C_6H_5–CO–NH–CH_2–COOH + CoASH (II)

Figure 13. Amino acid conjugation

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(86), in the lysosomal fraction. Vessey et al (87) have recently found enzyme activity in the soluble fraction of bovine liver cells and they have presented evidence for two distinct enzymes: glycine N-acyltransferase and taurine N-acyltransferase. Killenberg and Jordan (88) on the other hand have isolated a single acyltransferase from rat liver which is capable of conjugating chemically synthesised bile acid - CoA derivatives with glycine or taurine.

Isolation, properties and use. There have been no recent publications on the isolation of mitochondrial acyl-CoA synthetases responsible for the activation of xenobiotics or their metabolites. We have to turn to 'pure' biochemistry for information. Choloyl-CoA synthetase, as studied by Vessey and Zakim (82), was stable for at least one month when the microsomal fraction of guinea-pig liver was stored at -50°C. The standard assay procedure contained 16 μM [^{14}C]cholate, 100 μM CoA, 5 mM Mg^{2+}, 5 mM ATP, 0.8 mg of microsomal protein/ml and 100 mM Tris/HCl buffer, pH 7.3 in an assay volume of 0.1 ml. The identification of [^{14}C]choloyl-CoA was based on its comparison with chemically synthesised choloyl-CoA on paper and thin-layer chromatography. The pH optimum for its reaction was pH 7.2-7.3 and the reaction had an absolute requirement for bivalent cations, Mg^{2+} and Mn^{2+} being the most effective. High concentrations of ATP appeared to cause substrate inhibition. The enzyme could be solubilised from the microsomal membrane in an active form by treatment with Triton N-101. Inhibition studies were consistent with the hypothesis that one enzyme is responsible for the synthesis of the CoA-derivatives of all the major bile acids, but that this enzyme is different from that metabolising fatty acids to their CoA derivatives. These conclusions are supported by the findings of Siperstein and Murray (83) that a preparation of palmitoyl-CoA synthetase does not catalyse the synthesis of choloyl-CoA.

The enzyme responsible for the second step in the formation of amino acid conjugates, is amino acid N-acyltransferase. It is in this reaction that amino acid specificity would be expected to influence the spectrum of conjugates seen in vivo. Glycine N-acylase has been isolated from an acetone powder of beef liver mitochondria (76). When tested with benzoyl-CoA as the acyl donor, the enzyme was specific for glycine. After purification, a 53-fold increase in the specific activity of the enzyme was realised with an 11% recovery of the original activity. No loss of activity was found when the enzyme was stored at -25°C for 5 months. Dilute solutions of the enzyme were stable at 0°C for several hours. Enzyme activity was measured spectrophotometrically, based on the decrease in absorbance at 208 nm due to the utilisation of benzoyl-CoA during the reaction (89).

James and Bend (90) have shown from subcellular fractionation studies that glycine N-acyltransferase activity was mainly located in the mitochondrial fractions of rat and rabbit tissues.

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Cytosol also contained significant levels of the enzyme but this fraction may have contained protein from the matrix of the mitochondrion. The enzyme is believed to be located in the mitochondrial matrix, but is probably not membrane bound.

As previously mentioned, Vessey et al (87) have concluded from their data that separate enzymes are responsible for the formation of taurocholic and glycocholic acids. Assays contained 50 mM phosphate buffer (pH 8.0) 0.8 mg of soluble fraction protein, ^{14}C-labelled amino acid and 25 µM choloyl-CoA in a total volume of 0.1 ml. At this concentration of choloyl-CoA, the reaction with [^{14}C]taurine had a K_m for taurine of 0.75 mM and a V_{max} of 5.9 mol of conjugate synthesised/min/mg of cytosol protein. The reaction with [^{14}C]glycine had a K_m for glycine of 0.4 mM and V_{max} of 2.0 nmol/min/mg protein. Thus, in the bovine liver the maximum potential for taurine conjugation is greater than that for glycine, although glycine has greater affinity for the enzyme. Using the technique of alternative-substrate inhibition, the authors concluded that there were separate enzymes for the conjugation of cholic acid with glycine and taurine.

In a different approach to the problem, Killenberg and Jordan (88) have purified an enzyme from rat liver 20,000 g supernatant exhibiting amino acid N-acyltransferase activity. A 200-fold purification of the enzyme was achieved with a 6.5% recovery of the original activity. Polyacrylamide gel electrophoresis localised the glycine and taurine activities of the enzyme to a single band. Both activities were optimal at pH 7.8. Killenberg and Dukes (91) have purified chemically synthesised coenzyme A thioesters of cholic, chenodeoxycholic, deoxycholic and lithocholic acids. The biological activity of these thioesters was greater than 94%. In the presence of these substrates and at physiological concentration of taurine and glycine, taurine was shown to be the favoured substrate. This is consistent with the almost exclusive production of taurine conjugates seen in the rat in vivo.

A specific radiochemical method for the in vitro assay of the glycine conjugation of carboxylic acids has been developed using phenylacetyl-CoA-[carboxy-^{14}C] as the activated carboxylic acid acceptor (90). This compound is a commercially available radiochemical. The paper is noteworthy in that it describes the first attempt to really study glycine N-acyltransferase free from the complications of other (cofactor-synthesising) enzymes, e.g. an acyl-CoA synthetase.

Examples of use. In rabbit the specific activity of glycine N-acyltransferase (90) was found to be ten times higher in kidney mitochondria than in liver mitochondria. The relative quantitative roles of the two tissues in vivo is uncertain. In kidney mitochondria less than 1% of the adult activity was detected in the foetus at 20 days gestation and about 10% of the adult activity at 28 and 30 days gestation. After birth, activity rose very slowly to adult levels (10-14 weeks) (92). Using a similar

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sensitive radiochemical procedure, enzyme activity was found to be undetectable in rabbit lung (44).

Caldwell *et al* (93) have reported a rapid and sensitive *in vitro* semi-micro method for the determination of hippuric acid formation by human and rat cadaver tissue samples. Typical incubation mixtures (1 ml) contained [^{14}C]benzoic acid (200 nmol; 0.5 μCi), CoA (100 nmol), ATP (for kidney 2.5 μmol; for other organs, 5 μmol), glutathione (20 μmol), $MgCl_2$ (3 μmol) and glycine (60 μmol) with tissue homogenate (equivalent to 20 mg tissue) in 0.2 M Tris buffer, pH 8.0. Human cadaver liver and kidney samples formed hippuric acid conjugates, whereas brain, intestine, heart and lung did not. Tissues from human and rat corpses stored for 72 h at 4°C were still capable of forming hippuric acid. Hippuric acid formation in liver and kidney samples of a 31 week old premature foetus was one half to one third of that seen in comparable adult tissue, a finding similar to that reported for liver by Irjala (94).

The most striking features of amino acid conjugation are the variety of amino acids used and how these vary with species and with structure of the xenobiotic. Hirom *et al* (95) have recently reviewed amino acid conjugations in mammals; these and other authors have noted the involvement of glycine, glutamine, glutamic acid, serine, alanine and taurine in mammals, of ornithine in birds and of several others in plants. Two examples will serve to illustrate the effects of substrate and species. 1- and 2-Naphthylacetic acids are conjugated with glycine to equal extents in the rat, but in the ferret a greater proportion of the 2-isomer is conjugated with glycine. The 1-isomer is 63% conjugated with taurine by the ferret whereas the rat excretes only a trace of this metabolite. The 2-isomer is conjugated with taurine equally in the two species (96) (Figure 14). 3-Phenoxybenzoic acid, liberated during the metabolism of the pyrethroid insecticides permethrin and cypermethrin, is excreted in different forms by four species so far studied (97)(98)(99). Its major metabolite in cows is the glutamic acid conjugate, in mice, the taurine conjugate and in dogs, the glycine conjugate; in rats it is *p*-hydroxylated and sulphated prior to excretion (Figure 15). These interspecies variations may be a consequence of amino acid availability or of *N*-acylase specificity.

The situation *in vivo* is complicated by the fact that carboxylic acids are also conjugated with glucuronic acid. Dixon *et al* (100) have used a similar *in vitro* assay system to that previously described (93) to study the physico-chemical, structural and biological factors influencing the pattern of conjugation reactions of arylacetic acids. Using phenylacetic acid, 1-naphthylacetic acid, diphenylacetic acid and hydratropic acid (methylphenylacetic acid) as substrates, they concluded that the main factor influencing conjugation with glycine is the chemical structure of the substrate. For example, α-substitution of the methylene group of phenylacetic acids results in a

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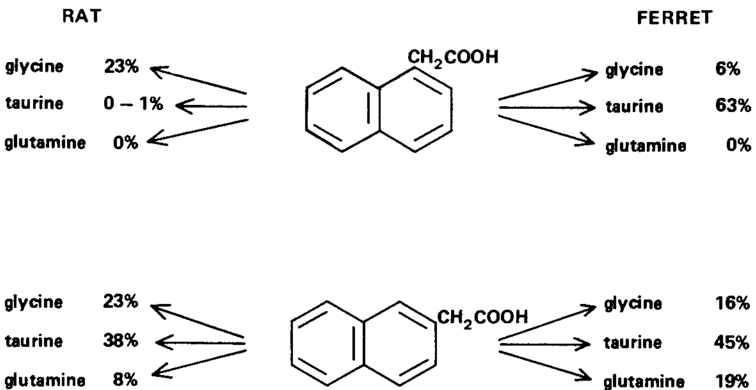

Figure 14. Amino acid conjugation of naphthylacetic acids

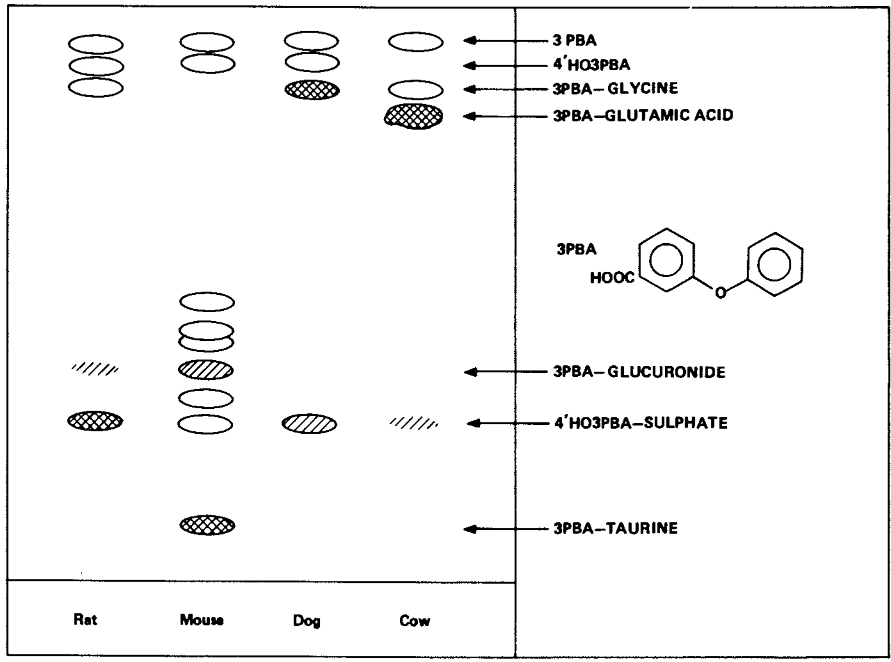

Figure 15. Species differences in the conjugation of 3-phenoxybenzoic acid

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blocking of amino acid conjugation *in vitro*. By contrast, glucuronic acid conjugation of arylacetic acid seems to be favoured by extensive non-specific entrapment by the endoplasmic reticulum, a process related more to lipid solubility than to chemical structure. These findings support *in vivo* metabolic profiles obtained when these acids are orally dosed to rats (101-106).

The explanation of these various differences will be found via the use of subcellular fractions, yet to date virtually no work directed to this end has been reported.

Toxicological significance. The change in physical properties effected by the amino acid conjugation of a carboxylic acid is not dramatic (although there is an obvious difference in the extreme case of, for example, 3-phenoxybenzoic acid to 3-phenoxybenzoyltaurine). However, one may expect a somewhat more efficient excretion of a conjugate compared with that of its parent acid. In the case of a bioactive acid (comparatively rare with respect to mammals in pesticide chemistry) conjugation is also likely to alter the molecular structure to diminish the activity. The species differences noted above are unlikely to have great consequences because amino acid conjugation is a relatively minor molecular change and it occurs usually near the end of a metabolic pathway.

Conjugation with Glutathione

Mechanism and location. Glutathione conjugation is the initiating step in mercapturic acid biosynthesis in mammals. The first step is the most important in detoxification because it confers hydrophilic character on the (usually) lipophilic xenobiotic and, in addition, the glutathione conjugates are readily secreted in bile and are thus readily eliminated from the animal. The second stage (γ-glutamyltransferase action) and third stage (peptidase action) are neglected areas of study in mercapturic acid biosynthesis, though some recent work has been published (107). They seem to occur in both liver and kidney. Both enzymes, for example, have been found in kidney microsomal fraction (108). The fourth stage is described in the Acetylation section above.

Glutathione conjugation is unique among the Type II processes in that a discrete high-energy substrate is not required. The chemical energy for the reaction is derived from the mutual reactivity of the nucleophilic sulphur of glutathione for an electrophilic centre in the xenobiotic substrate. The electrophilic centre may be present in the molecule *per se* or it may be generated by type I metabolism (see preceeding chapter). The conjugation step is catalysed by one or more of a number of glutathione transferases (EC 2.5.1.18) which are located in the cytosol of mammalian cells.

Isolation, properties and use. A very simple method of preparation from liver therefore consists of: homogenisation, centrifugation at 200,000 g for 30 min and dialysis against pH 7.4 buffer. These enzymes can be stored at $-25^{o}C$ for months. The demethylation of phosphoric acid triesters (with which we have

most experience) is retained after freeze-drying and storage at 4°C for months. However, the purified enzyme is, as usual, much less stable. Whether fresh samples are dialysed or not, they must be fortified with glutathione (to about 5 mM) for full activity. The endogenous glutathione is lost during liver processing in three ways: catabolism, oxidation and dilution. The acidity of glutathione is sometimes overlooked by newcomers in this field. It is essential to adjust the pH back up to 7.4 when preparing the stock solution, even in 0.1 M phosphate buffer.

The history of the glutathione transferases is a classic example of the dominant effect of substrate selection on research, nomenclature and thinking. The electrophilic centres that are subject to attack by glutathione include those in alkyl halides, aralkyl halides, aryl halides, epoxides, alkenes, chlorotriazines, nitrocompounds and compounds containing electrophilic nitrogen or sulphur atoms. The situation in the early 1970s, reviewed by Chasseaud (109)(110) and Hutson (111), was one in which the number of enzymes was increasing with the increasing number of substrates investigated. These enzymes (Figure 16) were not shown to be discrete proteins but efforts were being made to separate the various activities. Jakoby and coworkers then entered the field and used the approach of separating rat liver cytosol proteins by column chromatography and subsequently defining the activities of the separated proteins. Their classification system (112)(113) is based on the reverse order of elution from a carboxymethyl-cellulose column which was the major step in the separation of the transferases from one another. Seven proteins were isolated: transferases AA, A, B, C, D, E, and M. Enzymes A, B. C and E have molecular weights of 45,000 and are dissociable into two approximately equal sub-units. There is not much information available on transferases AA (114), D or M. Whilst clarifying the situation with respect to the protein, this work has not allowed a neat correlation between protein and substrate type. There is considerable overlap in substrate specificity (112)(115) which is illustrated in general terms in Figure 16. An earlier purification, aimed specifically at 'dimethyl phosphoric acid triester - glutathione methyl transferase' from rabbit liver, used a combination of ammonium sulphate precipitation, zinc salt formation, calcium phosphate gel treatment and gel filtration on Sephadex G150. A 45-fold purification was achieved (116) but the final product, which also retained its activity towards methyl iodide, was probably a mixture of all of the transferases (which form about 10% of the liver cytosol). Four organophosphorus insecticide -degrading enzymes have recently been separated from rat liver cytosol by chromatography on hydroxyapatite (117). Transferase I catalysed the demethylation of methylparathion; transferases III and IV catalysed the demethylation and the de-arylation of methylparathion and the depyrimidinylation of diazinon. When different groups of workers use different purification methods and assay substrates it is difficult to correlate the results of the various

'Enzymes' classified by substrate	Jakoby's protein classification (AA,A,B,C,D,E,M)
Glutathione –	
S–alkyl transferase	E ≫ AA > B
S–aralkyl transferase	A = C > E > M > B = AA
S–aryl transferase	A > AA > B = C ≫ E
S–epoxide (alkyl) transferase	E ≫ A
S–epoxide(aryl) transferase	A = C = E > B > AA
S–alkene transferase	C ≫ A ≫ B
S–triazinyl transferase	?
nitro transferase	C > AA > B > A
mercaptoalkyl transferase	C > A = AA > B

Figure 16. Glutathione transferases

studies. Recently a study of the activity of transferases A, B, C and E (115) in the demethylation of dimethyl 1-naphthylphosphate revealed that this was an activity of transferase E (118). Therefore it is possible that transferase I (117) and transferase E (115) are identical. Some encouraging results have been gained recently by affinity chromatography on agarose to which glutathione was attached by 1,6-diaminohexane spacer molecules. Transferases AA, A and C were retained while transferase B appeared in the void volume. The bound transferases could be recovered by affinity elution with KCl/glutathione gradients (119). Bromosulphophthalein -sepharose has also been used successfully for the pig cytosol enzyme (120).

The selection of assay method depends, as usual, on whether the priority for study is the enzyme activity or the reaction it catalyses. Jakoby et al (112) have mostly used spectrophotometric methods. Clarke et al (121) have worked out such a method for the methylparathion methyl transferase. A reasonably convenient method applicable to radioactive substrates (4) involves partition of the reaction mixture between water and toluene and radioanalysis for product and substrate respectively. A potentially useful partition procedure employing [^{35}S]glutathione has been described by Hayakawa et al (122). It is rather cumbersome but it is very sensitive and, of course, applicable to non-radioactive xenobiotic substrates, thereby allowing much wider substrate selection for structure-activity studies.

It is likely that high-pressure liquid chromatography will be used increasingly in assays of glutathione conjugation. When operated in the reverse-phase mode it is very suitable for the analysis of polar conjugates, as has recently been demonstrated (123). This method could operate with ultra-violet, fluorescence or radioactivity detection, depending on circumstances.

A technical problem, particularly prevelant in glutathione conjugation, is the tendency of the xenobiotic to react spontaneously with glutathione. This feature may give very high blank rates which sometimes alter dramatically as the pH is increased above 8. Therefore great care is needed in making allowances for this in quantitative work.

Examples of use. A combination of methods, usually *in vivo* studies supported by studies with subcellular fractions, has been used to demonstrate the involvement of glutathione conjugation in the metabolism of several classes of pesticide. These were reviewed in some detail recently in this series (111) and only more recent key references are given in the list of such reactions shown in Table IV. Most of these are important detoxification modes because they effect the alteration of the bioactive molecule. An exception is the metabolism of the alkyl thiocyanates; in the reaction between glutathione and the electrophilic sulphur atom, cyanide ion is liberated and is probably responsible for the bioactivity of this class of compound. It should be noted that this reaction is an example of glutathione reacting with an

electrophilic atom other than carbon. Glutathione conjugation also occurs frequently in the further metabolism of the primary biotransformation products of various pesticides. Some examples are given below.

Table IV. Glutathione conjugation in pesticide metabolism

Pesticide class	Reaction	Reference
Organophosphorus triesters	de-alkylation	(117)
		(116)
		(126)
	de-arylation	(117)
	de-pyrimidinylation	(127)
DDT	?	
Hexachlorocyclohexanes	dehydrochlorination	(128)
Diphenyl ethers	de-arylation	(129)
Alkylthiocyanates	de-thiocyanylation	(130) (131)
Propachlor	de-chlorination	(129)
Haloacetamides	de-chlorination	(129)
Chloro-*s*-triazines	de-chlorination	(132)
Alkylmercapto-*s*-triazines	de-alkylsulphoxylation	(124)
Thiocarbamates	de-alkylsulphoxylation	(125)

Two of the compound classes shown in Table IV, the alkylmercapto-*s*-triazines herbicides and the thiocarbamate herbicides, are good examples where the metabolism was studied at the subcellular level in order to explain the biotransformation pathways observed *in vivo*. Moreover, two reaction classes, in different subcellular fractions, used in a specific sequence, were needed to reconstruct and explain the overall reactions. A herbicidal methylmercapto-*s*-triazine was eliminated in rat urine as the *s*-triazinyl mercapturic acid and in rat bile as the *s*-triazinylglutathione conjugate. As the methylmercapto-*s*-triazine itself was not a substrate for cytosolic glutathione transferase, clearly an extra step was occurring *in vivo*. Incubation of the herbicide with rat liver microsomes (+ NADPH) afforded a new product, the herbicide *S*-oxide. When incubated with glutathione, the *S*-oxide reacted spontaneously to afford the same *s*-triazine conjugate as that observed in the bile (124). A similar sequence was discovered for the herbicides EPTC and butylate (*N*,*N*-dialkyl-*S*-alkyl thiocarbamates). The thiocarbamates were oxidised by microsomes to thiocarbamate sulphoxides. These compounds (which are reasonably stable) were substrates for a glutathione transferase-catalysed formation of *S*-(*N*,*N*-dialkylcarbamoyl) glutathiones (125). These sequences, illustrated in Figure 17, could not have been demonstrated easily be the use of a higher level of organisation such as whole cells.

Glutathione has been shown to participate in

Figure 17. Glutathione in the metabolism of thiocarbamate and thiotriazine herbicides

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biotransformation without being obvious in the final products. Even with the well-known glutathione-dependent demethylation of dimethylphosphate triester insecticides, most of the *S*-methylglutathione formed is metabolised to CO_2 and very little methylmercapturic acid is excreted. Clearly the use of subcellular fractions is indicated in discoveries of this type. The fate of the vinyl phosphate insecticide, dimethylvinphos, may be used to illustrate this point further. The main radioactive metabolites derived from ^{14}C-phenyl labelling *in vivo* were de-methyldimethylvinphos and a metabolite derived from the phenylvinyloxy group, 1-(2,4-dichlorophenyl)ethanol (as glucuronide) (4). The demethylation, predictably, was effected by (dialysed) cytosol and glutathione.

2,4-Dichlorophenylethanol was assumed to be derived from 2,4-dichloroacetophenone which in turn was thought to be formed by the reductive dechlorination of the hydrolysis product of dimethylvinphos (2,4-dichlorophenacyl chloride). The mechanism of the reductive dechlorination was unknown until we studied the metabolism of the phenacyl chloride in rat liver fractions. The following sequence of reactions was discovered using cytosol: (i) spontaneous reaction of phenacyl halide with glutathione to afford 2,4-dichlorophenacyl-glutathione, (ii) enzyme-catalysed reaction of the latter with another molecule of glutathione to form oxidised glutathione and the phenacyl anion which rearranged to 2,4-dichloroacetophenone (133). Microsomes and NADPH simply reduced the keto group of 2,4-dichlorophenacyl chloride to give the chlorohydrin (not observed in the *in vivo* metabolism). Thus glutathione enters the metabolic pathways of dimethylvinphos at three points (Figure 18) but it is scarcely observed in the excreted metabolites. In this series of experiments we also used the *in vitro* technique to demonstrate that the administration of phenobarbital to rats (which protected them 10-fold against the acute oral toxicity of dimethylvinphos) induced the activity of the cytosol demethylating enzyme 2-fold. Dimethylvinphos exhibits a large difference in acute toxicity to rat and dog (rat > dog); dog liver cytosol was shown to demethylate dimethylvinphos at about twice the rate of rat liver cytosol (4). Thus, the enzyme could have some role in the selective toxicity of dimethylvinphos.

This example illustrates several important features:

(i) direct glutathione conjugation of parent molecule (effecting detoxification)

(ii) glutathione conjugation after bioactivation by primary metabolism (hydrolysis in this case)

(iii) glutathione conjugation by attack on an electrophilic atom other than carbon (sulphur in this case)

(iv) only traces of the glutathione-conjugated products (mercapturic acids) appearing in the excreted metabolites

(v) use of subcellular fractions to investigate a species difference (rat versus dog)

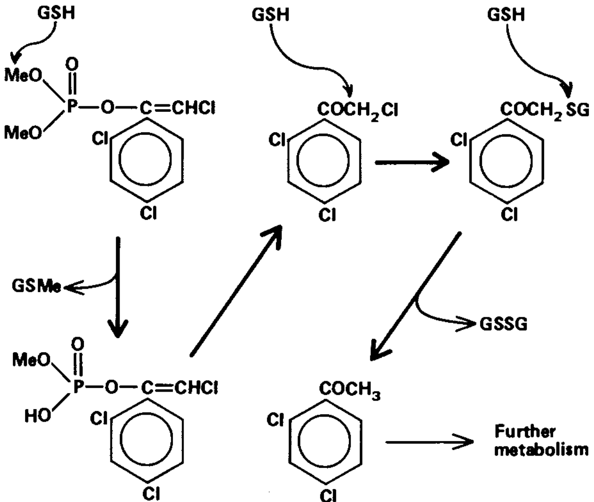

Figure 18. Glutathione in the metabolism of dimethylvinphos

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(vi) use of subcellular fractions to investigate the alteration of drug-metabolising enzymes of an animal.

Studies of the liver enzyme have shown that glutathione transferases occur in many other animals including rabbit (118), pig (118), (23), monkey (23)(134)(135), tree shrew (23), sheep (136), guinea-pig (122), horse (122), cow (122), mouse (122), chicken (126), and man (137). Where detailed studies have been carried out (135)(136) (137) the enzymes, including those of man, have been shown to be very similar in physical properties.

Liver is the richest source of the enzymes. For example, a recent study of Japanese monkey (Macaca fuscata) (134) was typical in showing the following aryltransferase (1,2-dichloro-4-nitrobenzene) activities (μmol/min/mg protein): liver, 18; spleen, 2.6; kidney, 2.1; lung, 1.9; brain, 1.9; muscle, 1.8; placenta, 0.3; pancreas, 0; erythrocytes, 0; blood, 0. Activity has recently been reported in small intestines (138)(139) and in leucocytes (140).

Enzyme measurements have also been used to assess glutathione conjugation during development of the neonate. Alkyl transferase activity in the rat neonate is very low for about 6 days and then rises steadily for about 40 days to the adult level (141). Transferase B (measured as aryltransferase) is present at birth at about one fifth of the adult level and rises steadily for about 40 days (142).

However, in all general statements about the presence of glutathione transferase in various species, tissues or altered states, the substrate used for assay must be noted before the relevance to one's own work is assessed. The importance of glutathione conjugation in limiting the cytotoxic, mutagenic and carcinogenic action of electrophilic compounds (143) is generating much research into the species and tissue distribution of the enzyme(s). However, too much reliance on results derived from tests using substrates (e.g. polycyclic aromatic hydrocarbon epoxides) unrelated to one's own problem may well prove to be only of limited value.

Toxicological significance. Glutathione conjugation results in a dramatic change in the physical properties of a molecule, usually leading to a loss of bioactivity. The conjugate is ideally structured for biliary secretion and therefore it is efficiently removed from the liver. Other enzymes efficiently convert the conjugate into a mercapturic acid that is readily excreted via the urine. However, perhaps the most important function of this conjugation process is the protection it affords against electrophilic compounds, be they ingested as such or generated within the organism via metabolism. Without this protection mammals would be much more susceptible than they are to low doses of teratogens, mutagens, carcinogens and cytotoxic compounds.

Other Uses of Subcellular Fractions in Xenobiotic Metabolism Studies

A relatively recent use of subcellular fractions is in bacterial test systems for mutagenicity of the type developed by Ames and coworkers (144). The fraction commonly used is a 9000 g fraction (S-9 fraction) from the livers of rats treated with an Arachlor (to induce microsomal enzymes). Its role in the test system is the provision of a mammalian metabolism capability for the (possible) activation of intrinsically inactive compounds. This may not commonly be seen as a use in metabolism studies; but if the bacteria are regarded as a bioassay technique for the detection of mutagens, the system is a useful addition to the techniques used for the study of metabolism *in vitro*. The main component of the bioactivation system is regarded as being the microsomal mono-oxygenase (hence the use of inducers to prepare a more 'potent' S-9 fraction). However, it will be clear from the various reactions discussed above that some of the type II reactions effect the bioactivation of certain metabolites. They also effect the deactivation of many compounds and their metabolites. The potentially great predictive value of these test systems has led to their widespread, but often uncritical use. The importance of a standardised preparation containing active microsomal mono-oxygenase is appreciated but the role of the many other enzymes in the S-9 fraction has been largely ignored.

The widespread use and enthusiastic reception gained by this simple, quick and potentially very useful system has led to a reaction from certain quarters and a heated debate is currently being conducted. There is clearly a need to define the reactions occurring in the test system. The balance between activation and deactivation is critical to its relevance to the *in vivo* situation. The state of the various enzymes in the S-9 fraction and the concentrations of the various cofactors (many of which are described above) requires measurement and control. Species variations are important. For example it is possible that 2-aminoanthracene (mutagenic in the presence of S-9 from rat liver) would not give a positive response if dog liver S-9 fractions were used. The first step in its bioactivation (*N*-acetylation, see Figures 9 and 10) is inoperative. Glutathione-dependent deactivation is largely inoperative in standard S-9 fraction because, although the glutathione *S*-transferases are present, glutathione itself is largely destroyed by catabolism, dilution and oxidation (145). A study of the various enzyme activities and cofactor concentrations in human liver fraction (prepared as S-9) would also prove very useful in the interpretation of results of the Ames test.

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Methods for the Study of Metabolism of Xenobiotics in Insect Cell and Organ Cultures

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Although insect organ and cell culture methodologies have been used for several years in the study of insect endocrinology and development (1,2,3), little use has been made of these techniques in studying the metabolism of xenobiotics in insect tissues. However, as our knowledge of insect biochemistry and the necessity for environmentally compatible pesticides increase, the use of such methodologies will grow.

The physiological studies that have been done with insect organ cultures so far have shown us that when experiments are carried out using appropriate techniques and carefully defined conditions, cultured tissues can be induced to behave much the same way as they do in whole insects (1). To date, studies have included such diverse processes as the biosynthesis and secretion of hormones (4,5), the production and deposition of cuticle (6,7), the biosynthesis of yolk materials (8,9), the regeneration of nerve tissues (10), and the differentiation of epidermal cells into setae (11). In all these studies, acceptable approximations of what is known to occur *in vivo* have also been demonstrated *in vitro*. Thus, it seems reasonable that cell and organ culture systems could be used to advantage for studying the metabolism of xenobiotics in insects, particularly as it relates to mode of action. The questions, then, are what can be gained from the use of tissue culture methodology and whether the advantages justify the additional effort and expense.

Organ Culture Systems

Most of the physiological and biochemical studies on insect cell and organ cultures to date have been done with organ culture systems. Organ culture differs from other *in vitro* techniques in that an organ must be cultured for more than 24 hours under aseptic conditions and in medium that contains an adequate energy source. In such a culture, the

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architecture of the organ remains intact, and some degree of organotypic activity can be anticipated (12). Under these conditions the tissues are often viable for several weeks or even months. For example, processes such as nerve regeneration or cuticle deposition may begin weeks after the tissue is explanted and may continue for as long as 90 days (10,11). In organ cultures the problems encountered with short-term incubations, such as surgical trauma and microbial contamination, are no longer a factor in interpreting the results.

Advantages of Organ Culture Techniques

One of the main advantages of organ culture is the experimental flexibility. The investigator can add or remove tissues, substrates, and products at any time during the incubation period; can select or exclude any tissues or substrates; and can include tissues from different insects in the same culture. Also, the medium can be sampled at intervals, and substrate levels can be controlled and by-products removed without seriously disturbing the system. This experimental flexibility is demonstrated by the use of a hanging drop preparation to show that the release of peptide neurohormones synthesized by cultured brains could be induced by adding cultured cardiaca, which, in turn, sequestered and stored the neurohormones released into the medium (13).

A second advantage of the organ culture methodology is that a larger quantity of metabolites can be obtained from a given amount of tissue because of the prolonged incubation. Also, feedback inhibition can be prevented by frequent renewal of the medium. Isolation and purification of metabolites are frequently easier from the relatively clean medium than from whole body extracts. These advantages have been discussed previously (14).

Additional advantages include the exclusion of unwanted tissue from the cultures and the preservation of intact cell membranes in the living cells. Thus, when one is working with whole animals, it has been virtually impossible to determine whether or not α-ecdysone in itself has any activity since it is readily converted by adjacent tissues to 20-hydroxyecdysone in quantities sufficient to produce an endocrine response (15). With homogenates, although tissue specificity is maintained, the cell membranes are destroyed and endogenous inhibitors and other intracellular products that are normally retained by the cells are released, and these in turn induce a variety of uncontrolled reactions. Thus, organ cultures, in which the integrity of the cells is maintained, provide a biochemical specificity that is difficult to obtain in other kinds of *in vitro* systems.

Finally, a most important advantage of the organ culture approach is that the investigator can include both the metabolizing and the target tissues in the same system. This

permits study of the interrelationship or interaction between the metabolism of the test compound and the mode or site of action. This is illustrated by our work on the metabolism of 22,25-dideoxyecdysone (16,17).

Disadvantages of Organ Culture Techniques

The disadvantages of organ culture are as numerous and as apparent as the advantages. For example, isolated tissues may lack substrates or cofactors normally present in the intact animal, which may, in turn, cause minor pathways to assume a dominant role, and thereby confuse the true picture (18). Thus, *in vitro* results must be compared carefully with what is known from *in vivo* studies.

Some tissues do not survive well *in vitro* and thus cannot be used successfully in organ culture studies; for example mature muscle frequently dies shortly after explantation. Another difficulty lies in obtaining uncontaminated tissues for culture. With holometabolous pupae or eggs, this is not a problem (19) but in some other stages of the life cycle, it may be extremely difficult (20). The problem of contaminated tissue also puts a practical limit on the number of explants that can be placed in a single culture. For example, if one explant out of four is contaminated, 75% success may be expected in cultures containing one explant per culture, less than 45% success would be expected in cultures containing 3 explants and there is only a 17% chance of success in cultures containing six explants and contamination is a virtual certainty. Thus, when large amounts of tissue are needed, the explants must be cultured separately or in small groups, an arduous task. Even with large numbers of cultures, the amounts of metabolites are often vanishingly small, so microchemical methods must be used (4).

Finally, organ culture is expensive, both in time and materials, and should be used only when less laborious methods are not available.

Metabolism of 22,25-Dideoxyecdysone (Triol) in Organ Culture

Having discussed the use of organ culture in the abstract, we would now like to discuss in some detail a study of the metabolism of the ecdysteroid 22,25-dideoxyecdysone (triol, Fig. 1). In different species of insects, or at different times or stages during the development of the same species of insect, this interesting ecdysteroid may either enter the major pathway(s) of molting hormone metabolism and serve as a precursor for the biosynthesis of the insect molting hormones (21,22) or it may be treated as a xenobiotic and metabolized to a complex mixture of hydroxylated steroids (22,23). The same dual role is reflected in the biological or physiological activity and the action of 22,25-dideoxyecdysone: In certain insects or biological test systems, it has molting hormone

22,25-Dideoxyecdysone
(Triol)

Figure 1

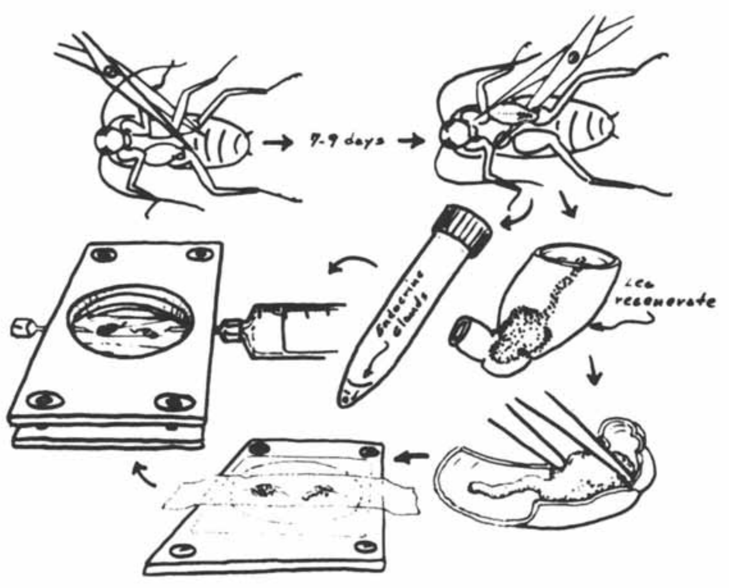

Figure 2. Schematic of leg regenerate–Rose chamber technique.

The mesothoracic legs are removed from freshly molted, late instar cockroach nymphs. After 28 days the coxal stump is removed and the leg regenerate is dissected out. The test tissues are placed under the dialysis strip and the chamber is assembled and filled with medium. The test compound(s) are placed under the dialysis strip with a microsyringe.

activity (21,24,25); in a number of other insect species it is a potent in vivo inhibitor of development, metamorphosis, and reproduction (24,25,26).

Our initial studies with the triol were carried out in Rose multipurpose tissue chambers. These chambers, which we have found to be extremely useful for many kinds of experiments, consist of a silicone gasket that is held between two coverslips by a pair of metal plates. With a strip of dialysis membrane to hold the leg regenerate tissue in place, (Fig. 2) the Rose chamber permits experimental flexibility combined with a high quality optical image for visual evaluation of the tissue with a compound microscope (27). Also, the soft silicone gaskets permit the use of a microsyringe to place the experimental compound in direct contact with the tissue. In chambers containing a threshold concentration of 20-hydroxy-ecdysone (0.05 μg/ml), cuticle appeared on explanted cockroach leg regenerates between 10 and 14 days after the molting hormone is added (10).

When the triol was tested against cultured leg regenerates of the cockroach Leucophaea maderae (F.), it produced cuticle formation in only 27% of the regenerates and gave erratic results even at high doses (28) (Table I). The experimental results obtained were not readily understood, especially since the responses that did occur were not concentration-dependent. Only when the triol was looked upon as a substrate that could be converted to biologically active ecdysteroids by peripheral tissues such as blood or fat body, did the results become meaningful. When we tested this hypothesis by co-culturing fat body and leg regenerates, the incidence of

Table I. Induction of cuticle formation in cockroach leg regenerates by certain ecdysteroids when tested in the presence or absence of fat body.

	Cuticle Formation			
Ecdysteroids[a]	Leg Regenerates		Leg Regenerates + Fatbody	
	N	%	N	%
Control	10	10	10	0
20-Hydroxyecdysone	14	93	-	-
α-Ecdysone	17	82	-	-
22,25-Dideoxyecdysone	22	27	22	93
22-Isoecdysone	12	8	10	0

[a] All ecdysteroids tested at 10 μg per chamber.

To confirm these findings, we made a second series of experiments in which we separately cultured cockroach fat bodies and leg regenerates with ^{14}C-labeled triol for 6 days in 1 ml of M20S medium in clean, sterile glass scintillation vials. Two volumes of methanol were added, and the cultures were worked up and then analyzed for radiolabeled steroids by thin-layer radiochromatography. The cultures containing only leg regenerate tissue and triol produced no additional steroidal compounds. They contained only the unmetabolized triol. However, the fat body cultures showed chromatographic peaks for tetraols, pentaols, and conjugates as well as the triol (Fig. 3), evidence that the metabolism of the triol had proceeded via hydroxylation and conjugation (17). Thus, our initial hypotheses were confirmed: the triol acts as a precursor rather than as an active hormone, and the leg regenerate and fat body have quite different biochemical capabilities.

Table II. Effect of certain azasteroids and nonsteroidal amines and an amide in inhibiting the activity of 22,25-dideoxyecdysone (triol) in cockroach leg regenerate-fat body cultures.

Compound[a]	Cuticle Formation %	Inhibition %
Control	93	0
I	0	100
II	16	83
III	50	46
IV	50	46
V	70	25
VI	80	14

[a] All coumpounds tested at 10 μg per chamber.

From this point, the work proceeded in two directions. One thrust was concerned with a group of compounds consisting of certain 25-azasteroids and nonsteroidal amines and amides (Fig. 4) that disrupt steroid metabolism and molting and metamorphosis in insects (29,30); these were tested for inhibition of the activity of the triol in inducing cuticle deposition in cockroach leg regenerate-fat body cultures (16). The results obtained with several of these compounds indicated significant inhibition of metabolism of the triol to an active ecdysteroid by the cultured tissue (Table II). Subsequent metabolic studies with three of the more active

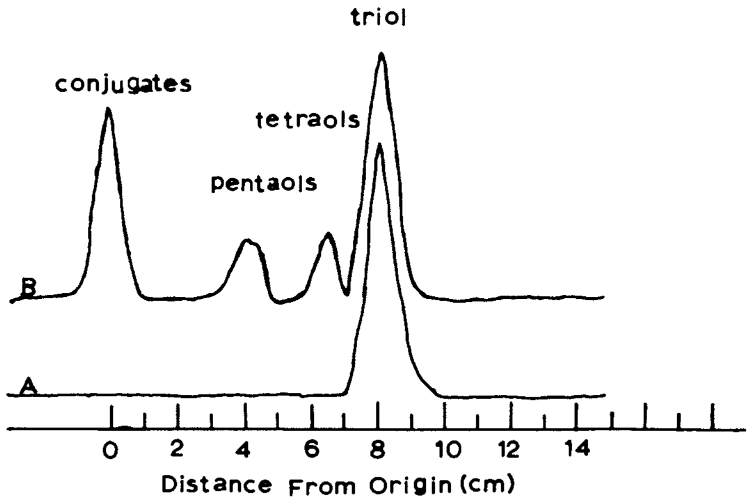

Figure 3. Radiochromatograms of 4-C^{14}-ecdysteroids extracted from the medium of cockroach tissue cultures incubated with 4-^{14}C-22,25-dideoxyecdysone (triol): (A) from cultures containing leg regenerates; (B) from cultures containing leg regenerates plus fat body.

I II

III IV

$CH_3(CH_2)_{10}CON(CH_3)_2$ $CH_3(CH_2)_{11}N(CH_3)_2$

V VI

Figure 4. Azasteroids, nonsteroidal amines, and an amide

inhibitors (17) demonstrated that they have a pronounced effect on the metabolism of the triol in cockroach fat body cultures (Table III). Taken together, these two studies provided the first *in vitro* physiological data and first biochemical evidence that certain of the inhibitory azasteroids and nonsteroidal amines represent a new class of insect hormonal chemicals with a novel mode of action--they interfere with the metabolism of the endogenous molting hormones of insects.

Table III. The effects of an azasteroid and nonsteroidal amines on the metabolism of 4-^{14}C-22,25-dideoxyecdysone (triol) in cockroach fat body cultures during an incubation period of 6 days.

Inhibitory Compounds[a]	% Unmetabolized 22,25-Dideoxyecdysone[b]	% Metabolites[b]		
		Conjugates	Pentaols	Tetraols
Control	45.5	35.6	11.3	7.6
II	6.0	55.0	0.0	39.0
III	15.9	40.8	5.6	37.8
IV	18.7	34.7	5.2	41.5

[a] The concentrations of the inhibitory compounds and of 4-^{14}C-22,25-dideoxyecdysone were 10 μg each per culture flask.

[b] Determined by radio TLC analyses.

The second thrust, which is perhaps more germane to the metabolism of xenobiotics, involved over 100 cultures of fat body that were incubated with triol. The principal pathways of metabolism of triol in the fat body cultures turned out to be hydroxylation and conjugation, as they are *in vivo*. Interestingly, enzymic hydrolysis of the conjugate fraction showed no unmetabolized triol. The major tetraol metabolite was 22-deoxyecdysone, and other tetraol and pentaol metabolites were hydroxylated at the 25, 26, or 20 positions (17)(Fig. 5). Most of the metabolites found had been previously isolated from the frass of triol-fed larvae of the tobacco hornworm *Manduca sexta* (L.) (23). In this respect, the metabolic pathways for triol in cultured cockroach fat body resembled the pathways that occur in hornworm larvae. Also, in both cases the mechanism for hydroxylation of the triol at C-22 appears to be lacking. When samples of the tetraol and pentaol fractions were tested against cultured leg regenerates,

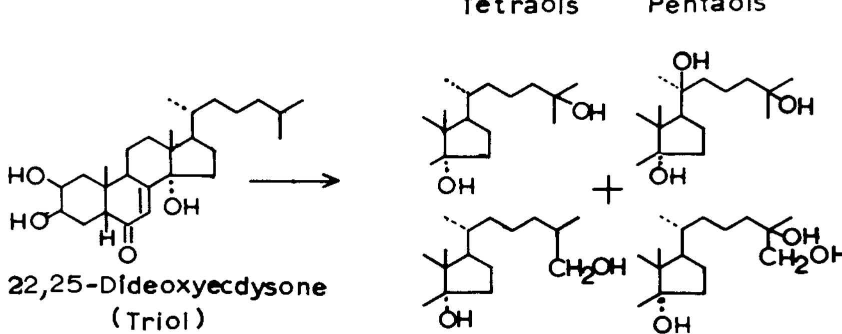

Figure 5. Hydroxylation of 22,25-dideoxyecdysone (triol) by cockroach fat body cultures

both fractions induced cuticle formation (Table IV). Since the leg regenerates cannot metabolically activate the triol directly, they are probably unable to efficiently hydroxylate the C-25 position. In addition, assuming that 22-deoxyecdysone, like α-ecdysone, is effective in the leg regenerate system only after metabolic conversion (31), then further metabolism of the tetraol and pentaol metabolites by the leg regenerates may also occur. Thus, both fat body and leg regenerate tissue may be necessary to traverse the entire pathway from triol to an ecdysteroid(s) with molting hormone activity in the leg regenerate system.

Table IV. Effect of metabolite fractions isolated from cockroach fat body tissue cultures incubated with 22,25-dideoxyecdysone (triol) on cuticle formation by cultured cockroach leg regenerates.

Metabolite Fractions[a]	N	Cuticle Formation	
		%	$\bar{X}$ days
Control	4	0	-
Tetraol	5	62	10.8
Pentaol	4	80	11.3

[a] Tested at 10 μg equivalents as determined by UV analyses.

This study is still in progress, and a number of questions remain unanswered. One of these concerns the biological activity of the various tetraol and pentaol metabolites of the triol that are produced in fat body cultures. Other questions involve the site and mode of action of certain of the inhibitory azasteroids and nonsteroidal amines.

During the course of this work, we learned certain things that may be of use to investigators who use similar techniques: One of these is that the use of antibiotics to control contamination must be approached with extreme caution. We found that the presence of gentamicin, an antibiotic commonly used in tissue cultures, interfered with the metabolism of the triol in fat body cultures. It was also observed that the amount of substrate and/or the length of incubation produce changes in the ratios of the metabolic products formed (17).

The purpose of discussing the work on the metabolism of 22,25-dideoxyecdysone has been to show how organ culture methodology has been applied to the study of this ecdysteroid that often behaves as a xenobiotic. Our results obtained by using organ culture techniques so far agree quite well with

what has been learned by using in vivo methods. However, the usefulness of this methodology is limited somewhat by the expense in both time and materials compared with the use of live insects. In general, these in vitro methods would appear to be more useful for the in-depth investigation of critical problem areas rather than for general usage.

Cell Culture Systems for Metabolic Studies

The use of insect cell culture systems for the study of the metabolism of xenobiotics has not, to our knowledge, been attempted to date. The peripheral literature consists of only a few papers on the effects of pesticides (32,33) and insect growth regulators (34,35) on insect cell lines and a series of studies on the effects of various ecdysteroids on the morphology of cultured cells (36,37,38). However, we would like to discuss briefly some of the possibilities for using insect cell lines for the study of the metabolism of xenobiotics.

Cell cultures differ from organ cultures primarily in that they consist of populations of dividing individual cells rather than highly organized tissues from a single organism. As a result, the life of an organ culture is finite, while the life of cell cultures is potentially infinite (12). Primary cultures that are derived directly from the donor may or may not develop into self replicating established cell lines. Monolayer cultures of fat body cells from pupae of large insects such as the tobacco hornworm and embryonic cells from the oothecae of grasshoppers and cockroaches have been successfully prepared in sufficient quantities for experimental work (19) (Fig. 6). However, because such small amounts of tissue are available from most insects and the contamination problems arising from the use of large numbers of tissue donors are so severe, the use of primary cultures of insect cells for experimental purposes offers few advantages over organ cultures. The number of established lines of insect cells is rapidly increasing, and many of these are available for experimental purposes (39).

Advantages of Cell Culture Systems

The use of cell lines for metabolic studies has a number of inherent advantages and disadvantages (40). Perhaps the most obvious advantage is that large amounts of uniform material are available for experimentation. Large-scale replication is possible and there is good sample uniformity when spinner flasks (41) and roller bottles are used (42). The problems of surgical shock and microbiological contamination can be virtually eliminated; and automatic devices for handling and counting cells are available to keep labor at a minimum. Cell lines are available from a number of major dipteran, lepidopteran, and homopteran pests, although to

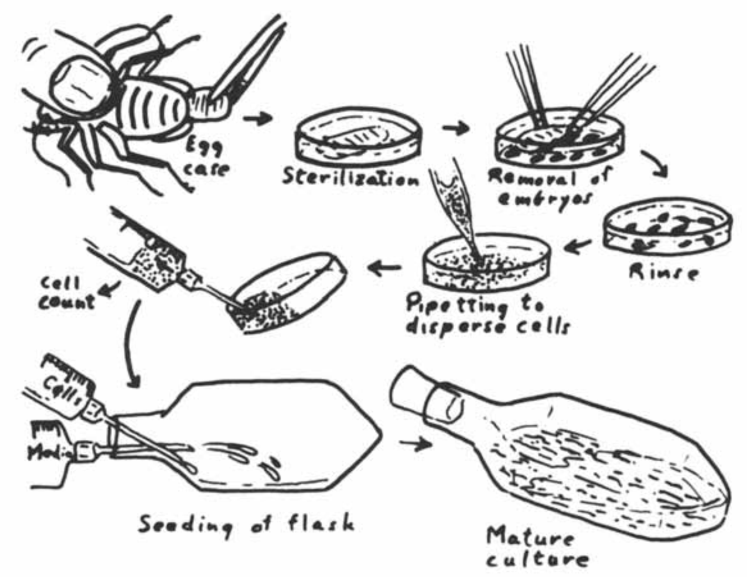

Figure 6. Schematic of technique for preparing primary cell cultures from pre-dorsal closure cockroach embryos. In preparing primary cultures of fat body cells, trypsinization is required to disperse the cells.

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date, none are available from coleopteran or hymenopteran species (39).

Disadvantages of Cell Culture Systems

At the present time, only a limited number of cell lines for which the tissue of origin can be identified is available. Even for these lines, the evidence for identity is weak (3). Skillful and determined efforts will be necessary to develop new lines from known tissues such as fat body or to identify the tissue of origin for present lines. Another problem is that while organ cultures can be related back to individual insects, cell lines reflect the dynamics of large populations of individuals kept under constant selection pressure. The cells must therefore be frequently cloned and kept as frozen stocks to maintain stability and prevent shifts in the biochemical characteristics of the population (40,43). As yet, very little is known about the metabolic capabilities of existing insect cell lines and much remains to be done before such cell lines can be considered useful tools for the study of metabolism.

At the present time we are working with a cell line (MRRL-CH-2) derived from embryos of the tobacco hornworm (44). These cells respond with a change in morphology to the presence of 20-hydroxyecdysone at physiological concentrations and are capable of hydroxylating the C-20 position of α-ecdysone (43) (Table V). Other biochemical parameters of this cell line are currently under investigation.

Table V. α-Ecdysone and 20-hydroxyecdysone present in cell cultures during incubation of tobacco hornworm embryonic cell line with 30 μg of α-ecdysone.

Incubation Days	α-Ecdysone μg	20-Hydroxyecdysone μg
0	26.2	0.0
1	25.9	0.0
3	15.5	0.1
5	20.0	0.1
7	18.0	0.2

Cell Cultures as a Source of Subcellular Components

We have recently been using another tobacco hornworm embryonic cell line (MRRL-CH-1) as a source of cell membrane for studies of membrane transport systems. As a result of this work it has become apparent to us that cell lines have

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some unique advantages as a source of subcellular components for various types of metabolism studies. We found that cell cultures can be subjected to bioassay and indeed to some biochemical procedures in the same medium in which they were grown (45). Thus, several of the hazards involved in tissue preparation such as osmotic shock and contamination with microorganisms are eliminated. Furthermore, large amounts of highly uniform material are available. Mammalian and plant tissue cultures are being used for such purposes (46,47), but few examples of such work with insect tissue cultures have been reported. (48).

Concluding Remarks

Insect organ cultures have proved to be extremely useful tools for studies of the metabolism of the ecdysteroid 22,25-dideoxyecdysone. The experimental flexibility of this methodology and its use for the confirmation and extension of information obtained from *in vivo* studies make organ culture the method of choice for certain types of studies. The use of insect cell cultures for studying the metabolism of xenobiotics is still only in the early stages of development. Much work will have to be done before cell cultures can be considered useful tools for such studies. However, insect cell cultures are presently being used successfully to provide a highly uniform source of subcellular components for metabolic studies.

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The Use of Insect Subcellular Components for Studying the Metabolism of Xenobiotics

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Our ability to use to maximum advantage and in relative safety the vast number of drugs, pesticides and other lipophilic xenobiotics currently at our disposal is dependent to a large extent on our ability to establish their metabolic fate in living organisms. Metabolic studies in mammals are essential in assessing the efficacy and safety of new drugs and data on the nature and toxicological properties of pesticide metabolites are mandatory in evaluating the potential hazard to man of residues of these materials in food or in the environment. Comparative studies with fish, birds and other species are important in determining the hazards posed to these animals by a large variety of environmental pollutants.

In addition to their direct importance in safety/hazard evaluation metabolic studies are basic to our understanding of the mode of action of biologically active materials. They often yield important information on enzymatic activation and detoxication processes and frequently provide a mechanistic explanation of cases of selective toxicity. It is in the quest to obtain a better understanding of these processes and to utilize this information in the design of more effective and safer insecticides that has stimulated interest in metabolic studies in insects.

As is obvious from the presentations in this symposium, metabolic studies may be carried out *in vivo* in the living organism or *in vitro* in a variety of preparations consisting of isolated organs, tissues, cells or subcellular components. *In vivo* investigations provide quantitative information on the overall rate of metabolism and on the nature of the terminal metabolites; they seldom provide data on the nature of the metabolic intermediates or on the enzymatic mechanisms by which they are formed. *In vitro* experiments on the other hand permit the identification and study of individual reaction mechanisms and products but are usually of only limited use in explaining the rate and pattern of metabolism in the intact organism. Clearly any complete metabolic study has to include both *in vivo* and *in vitro* components; the situation is analogous to a puzzle where the

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in vivo studies provide the pieces to the puzzle and the *in vitro* studies yield the clues which enable the pieces to be put together in a meaningful way.

This chapter will address itself to the use of insect subcellular components in *in vitro* studies of the metabolism of xenobiotics. Since most of the advantages and disadvantages of *in vitro* studies are of a general nature and largely independent of the species employed, emphasis will be given to discussing some of the major problems encountered in developing suitable *in vitro* systems and techniques with insect species. It is not the objective of this chapter to conduct a comprehensive survey of metabolic reactions with individual compounds or to discuss in detail the characteristics of the various enzymes involved in xenobiotic metabolism. Those interested in these areas are referred to some of the recent books or review articles which are available (1-10), and the many references contained therein.

Historical development

The capacity of insects to metabolize synthetic organic chemicals *in vivo* was first recognized during the late 1940s when it was discovered that metabolic dehydrochlorination of DDT to the relatively nontoxic DDE was a major causative factor in the development of insect resistance to this insecticide. In the years immediately following this discovery *in vivo* investigations with other insecticides including the cyclodienes, the organophosphorus compounds and the carbamates revealed that almost all groups of compounds were susceptible to metabolic attack by both insects and a variety of non-target organisms and that this was often the dominant factor in determining the degree and duration of their toxic action. Recognition of the critical role of metabolism in relation to selective toxicity and insect resistance to insecticides gave further impetus to metabolic studies and the intense activity of agro chemical industry during the 1950s provided a steady flow of new compounds with which to work. As a result, *in vivo* metabolic studies with insects, mammals and other organisms assumed an ever important position in insecticide research and were aided considerably by improved technology, particularly the use of radioactive tracers and the introduction of more sophisticated instrumentation for the resolution, detection and identification of small amounts of metabolites.

Since almost all of the early studies with insects were conducted *in vivo* only the end products of metabolism were observed. In most cases these were water soluble secondary conjugates which were difficult to identify and which provided little or no information on the structures of the primary or intermediate metabolites from which they were derived or on the nature of the enzyme systems effecting the initial attack on the parent compound. Indeed in most of the early work these conjugates were simply classified as "water solubles" and no further attempt was

made to identify them. In spite of these problems the importance of several primary metabolic reactions was well established and it was known that these could catalyze both the activation and detoxication of the parent compound. Dehydrochlorination was known to constitute a major metabolic pathway for DDT and its derivatives and hydrolysis and desulfuration were well recognized reactions with a variety of organophosphorus compounds. In addition the probable involvement of enzymatic oxidation was strongly indicated by the identification of metabolites such as epoxides, sulfoxides and sulfones from several compounds although the full extent of this involvement was not obvious. This then was the general situation which pertained in insect metabolic studies up to and during the early 1950s.

Somewhere around this time there occurred a rather sudden realization that the nature of the metabolites produced by insects and mammals were essentially similar and that presumably this reflected a basic similarity in the enzymatic mechanisms involved. This tended to open up new and improved lines of communication between individuals conducting metabolic studies in insects and those working with mammals. The use of a variety of *in vitro* systems such as liver slices, homogenates and subcellular fractions had already proved useful in mammalian studies and it was not long before the first attempts were made to develop similar systems from insects.

Early *in vitro* studies in insects usually employed intact tissues or crude minces and homogenates. Although several organophosphorus esters were found to be hydrolyzed by the "aromatic esterase" of the bee (*Apis mellifera*) abdomen (11) and malaoxon and acethion were shown to be degraded slowly by carboxylesterase action in cockroach (*Periplaneta americana*) minces and whole guts (12), homogenates of other insects proved generally inactive towards a variety of organophosphorus insecticides (13). Somewhat earlier than this, intact insect tissues especially the gut had been found capable of activating schradan (14) and catalyzing the oxidative activation (desulfuration) of phosphorothionates such as parathion (15). Fenwick (16,17) was the first to demonstrate phosphoramidate activation in a subcellular preparation from locust (*Schistocerca gregaria*) fat body. He reported that 84% of the schradan oxidizing activity of a fat body homogenate was in the upper layer of a heterogeneous 14,000g centrifugal sediment (referred to somewhat questionably as the microsomal fraction) and that activity required the addition of either the supernatant or exogenous NADPH. It is interesting that as a result of these and related studies Fenwick (17) as late as 1958 found it worthwhile to note that the locust fat body homogenate contained ". . . (at least) two distinct types of particles which resemble mammalian liver mitochondria and microsomes."

By the late 1950s it was well established that in mammals the primary metabolic attack on a large number of lipophilic drugs and xenobiotics was effected by a series of oxidative

reactions associated with hepatic microsomes (18). The first *in vitro* demonstration of microsomal enzyme activity in insects was that of Agosin *et al.* (19) who showed that NADPH-fortified microsomes prepared from whole german cockroaches (*Blattella germanica*) and other species catalyzed the hydroxylation of DDT. This represented a milestone of sorts since it provided the first real indication that insects contained an active microsomal oxidase system similar to that in mammalian liver and also that *in vitro* metabolic systems could be developed to reproduce the reactions occurring in the living insect.

Since this time *in vitro* studies employing subcellular fractions derived from whole insects or insect tissues have become almost routine practice in many laboratories. As a result a great deal of information has been obtained on the types of metabolic reactions which take place in insect tissues and on the general biochemical characteristics of the enzymes catalyzing these reactions (1,6-10). It has become clear that as in mammals, xenobiotic metabolism in insects is accomplished by relatively few general types of reactions. The importance of ester cleavage by a series of hydrolases (phosphatases, carboxylesterases, amidases, etc.) has been demonstrated with various substrates (20) and glutathione transferases are known to play important roles in primary reactions such as dealkylation and dearylation (21,22), dechlorination (22,23,24) and thiocyanate metabolism (22,25). Epoxide hydratase activity is known to be widely distributed in insects (20,26) and conjugating enzymes are known to catalyze numerous secondary reactions such as glucosidation and sulfation (22). But perhaps most important of all is clear recognition of the dominance of the cytochrome P-450 mediated system in insects and its ability to catalyze the primary metabolic attack on a large number of lipophilic xenobiotics through reactions such as epoxidation, hydroxylation, *N*- and *O*-dealkylation thioether oxidation and desulfuration (1,8,9,10,27). What is now emerging from comparative studies is a picture of remarkable functional unity with respect to the reactions of xenobiotic metabolism. There are of course some differences between species as will be discussed later in this symposium by Dr. Terriere but in spite of the dramatic variations in size, morphology, nutrition, ecological habitat and general life style between say mammals and insects, one cannot help but be impressed by the basic similarities which exist at the subcellular level. At the present time, however, the picture is still out of focus and much remains to be done, particularly at the *in vitro* level, before a more complete understanding can be achieved.

General considerations relating to *in vitro* studies

The first major decisions which have to be made in initiating an *in vitro* study are the species and life stage of the insect to be employed. This will be dictated largely by the purpose of the

proposed study.

In vitro metabolic studies are usually directed towards providing information either on the products of a particular reaction(s) or on the biochemical character and mechanism of the enzyme which catalyzes the reaction. In the former case, the *in vitro* system is used as a tool to produce primary or intermediary metabolites of a particular xenobiotic which may be required to confirm the identity of trace metabolites or metabolic pathways suggested from corresponding *in vivo* studies; in the latter case selected xenobiotics are used as tools to characterize the system which can then be employed as a source of more quantitative metabolic data or in comparative studies.

If the proposed investigation is concerned with establishing the primary metabolites of some insecticide in a specific insect pest then clearly the species to be employed is already determined and the life stages of most interest (egg, larva, nymph or adult) are presumably those at which the insecticide is directed in the field, i.e. those causing the most economic damage. The major requirement here is to develop an *in vitro* system that will reproduce metabolites observed *in vivo*. In this type of study a detailed knowledge of the system is not of primary importance and, indeed, in many cases the use of intact tissues or crude homogenates may be more useful than individual subcellular components.

If on the other hand the objective of the study is to characterize the enzymatic system responsible for a certain type of metabolic reaction, it is usually desirable to work with more homogeneous, purified fractions and to give greater emphasis to obtaining quantitative information. For studies of this type there is considerably more leeway in the selection of the species to be employed and in view of the estimated existence of 2-10 million species of insects, the theoretical possibilities are enormous.

In practice, however, the choice of species is usually determined by a series of convenience factors which relate mainly to the amount of biomass available for study. This is probably the major limiting factor in most studies on insect biochemistry. Consequently, wherever possible a relatively large insect species should be selected. In many cases the life cycles of large insects are quite long so that it is often necessary to reach a compromise between size and turnover time (i.e. length of generation). The species selected for study should be readily amenable to mass rearing under laboratory conditions (the availability of a satisfactory artificial diet is advantageous) and should be available on a year-round basis. Clearly a species with only one generation per year or one with an obligative period of diapause would not be convenient for continuous study. It is as a result of requirements of this type that to date *in vitro* studies have been limited to perhaps 30-40 insect species very few of which can be considered serious pests of agriculture or public health.

Tissue sources

Having selected an appropriate insect species and life stage with which to work the next step is to decide on the tissue source to be employed as a starting point for subcellular fractionation.

Because of obvious limitations on the amount of tissue usually available, most of the early metabolic studies were conducted with homogenates or subcellular fractions derived from whole insects. The use of whole-insect preparations is still common in several laboratories and can frequently provide useful qualitative information on xenobiotic metabolism. More often than not, however, enzyme activity in such preparations, particularly that associated with the microsomal fraction, is very low or non-existent and usually bears little resemblance to the true enzymatic capability of the insect under investigation. Indeed, in view of the heterogeneous mixture of materials they contain it is really quite surprizing that such whole-insect preparations exhibit any enzyme activity at all. Clearly, the homogenization of whole insects causes a total disruption of tissue and cellular organization and results in the release of a large number of endogenous materials with potential inhibitory effects on the enzyme under investigation.

Several different types of endogenous inhibitors have been encountered and identified in the course of studies with insect microsomes (8,10) and there is no doubt that they often represent a serious practical problem in *in vitro* investigations.

The insect eye pigment, xanthommatin has been established as an important inhibitory factor in preparations from whole house flies (28,29), fruit flies (*Drosophila melanogaster*) and honey bees (*Apis mellifera*) (30). It causes substantial inhibition of house fly epoxidase activity at concentrations as low as 5×10^{-7}M and inhibitory activity is accompanied by a marked increase in NADPH oxidation (28,29). Studies on the mode of action of xanthommatin have shown that it accepts electrons from the flavin, NADPH cytochrome *c* reductase, of the microsomal electron transport chain thereby acting as an electron sink to impede the flow of reducing equivalents to cytochrome P-450 (Figure 1) (28,29). The ability of dihydroxanthommatin to undergo autoxidation and to be oxidized in the presence of cytochrome *c* or tyrosinase suggests the rapid regeneration of xanthommatin and a consequent enhancement in its inhibitory potential. Since xanthommmatin is widely distributed as an insect eye pigment this type of inhibition is of potential importance in almost all preparations from whole insects.

What may be a similar type of inhibition has also been encountered in attempts to measure microsomal oxidation in preparations from whole last-instar lepidopterous larvae just prior to pupation (31,32). In this case, inhibition is associated with soluble products (a variety of quinones) of the melanization or darkening process which involves the tyrosinase-mediated oxidation

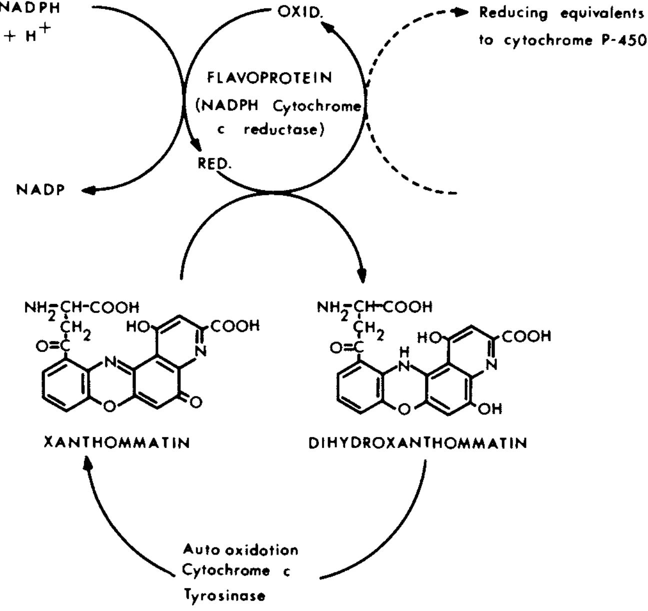

Figure 1. Inhibition of microsomal oxidation by xanthommatin (8)

of a variety of *ortho*-dihydroxy compounds (e.g. DOPA) and their subsequent incorporation into the insect cuticle. In lepidopterous larvae, this type of inhibition appears to be important only during late larval development when the tyrosinase system is activated in preparation for formation of the pupal coat. It can be counteracted by the addition of 1-phenyl-2-thiourea to the preparations to inhibit tyrosinase activity (31,32). A similar type of inhibition has also been implicated in the instability of mixed-function oxidase activity in house fly microsomes where the addition of cyanide (also a tyrosinase inhibitor) has a marked stabilizing effect (33). In theory, quinones may be formed whenever catechols are brought into contact with tyrosinase and since both are common in insects, workers should beware of preparations which become progressively darker on exposure to air.

Another major group of endogenous inhibitors which can seriously impede *in vitro* studies in whole-insect preparations are those associated with the insect gut contents. Potent inhibitors of microsomal oxidations have been reported in the gut contents of several insect species including several lepidopterous larvae (34,35), a caddisfly larva (*Limnephilus* sp.) (37), a sawfly larva (*Macremphytus varianus*) (38) and the house cricket (*Acheta domesticus*) (38,39). The inhibitory factors in the gut contents of the southern armyworm (*Spodoptera eridania*) (40) and the house cricket (39) have been partially purified and characterized as proteolytic enzymes with molecular weights of 26,000 and 16,500 respectively. They are both undoubtedly naturally occurring digestive proteinases quite similar to trypsin and like trypsin their inhibitory action results from a direct proteolytic attack on the microsomal protein (39,41). The effect of these proteases on the microsomes is quite specific in that they cause the solubilization of the flavoprotein, NADPH cytochrome *c* reductase, and consequently disrupt electron flow to cytochrome P-450 (39,41) (Figure 2). There appears to be no effect on cytochrome b_5 of P-450 and no effect on substrate binding to the latter (41) so it appears that the flavoprotein is the major target, possibly due to its vulnerable location on the outer surface of the membrane. Although similar in their overall effect on the microsomes, the armyworm and cricket gut content materials exhibit properties indicating they are not identical (Table 1). Thus in contrast to the armyworm material which shows a similar inhibitory effect on insect and mammalian liver microsomes the cricket material is much less active towards the latter and while both appear to be serine proteinases susceptible to inhibition by phenylmethanesulfonyl fluoride (PMSF) the cricket but not the armyworm material is sensitive to soy trypsin inhibitor. Bovine serum albumin (BSA) has a marked protective effect on microsomes in the presence of the armyworm gut inhibitor whereas this is observed with the cricket material only at high levels of BSA (39). The protective action of BSA against at least some of these proteolytic enzymes undoubtedly accounts for the enhanced microsomal enzyme activity

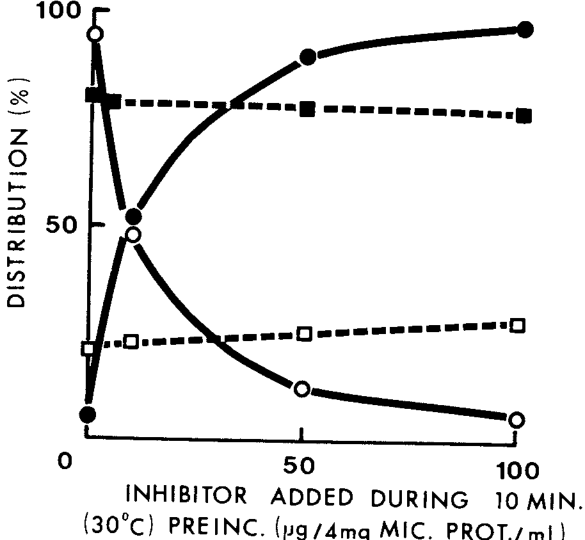

Figure 2. Solubilization of NADPH–Cytochrome c reductase by gut contents inhibitor of southern armyworm (S. eridania) *(41).*

TABLE I

Properties of gut content inhibitors from *S. eridania* and *A. domesticus*[a]

Property	Source of inhibitor *S. eridania*	*A. domesticus*
Molecular weight	26,000	16,500
Proteolytic activity (casein)	Yes	Yes
Mg^{2+}, Ca^{2+} (mM)	Stimulates (F_2)	No effect (F_1)
Soy trypsin inhibitor (1mg/inc.)	No effect (F_2)	Inhibition (F_1)
PMSF	Inhibition	Inhibition
Inhibition epoxidase (I_{50}, mg/inc.)		
Armyworm midgut	0.97 (F_1)	0.17 (F_1)
Mammalian liver	∿2.0 (F_1) (mouse)	7.0 (F_1) (rat)
Reversal of microsomal inhibition		
BSA	Yes	No (slight)
PMSF (0.5-1.0mM)	Yes	Yes
Soy trypsin inhibitor (1mg/inc.)	---	Yes

[a] Data from Krieger and Wilkinson (40) and Brattsten and Wilkinson (39). F_1 is crude freeze-dried gut contents soluble fraction; F_2 is freeze-dried material after passage through Sephadex G-50 or G-100.

observed following addition of this material to homogenates of whole house flies (42). Caseinolytic activity has been reported in whole insect homogenates of several species (40) and it is likely that proteolytic enzymes of this type are of broad general significance in *in vitro* studies in insects.

It should be obvious from this discussion that a variety of endogenous inhibitors liberated during homogenization make whole insects a generally poor tissue source for *in vitro* studies particularly those concerned with the biochemical characterization

of the enzymes involved in metabolism.

In some cases, however, due to factors such as very small size (e.g. mosquitoes or aphids) or limited availability of the species in question the use of whole insect preparations is unavoidable and can often be useful in gross metabolic studies. In this case compromises must be made. The chances of encountering endogenous inhibitors are, of course, considerably decreased when specific body regions of the insect are employed. Thus the use of house fly abdomens has proved advantageous over whole house flies (8,9,43) (since it immediately avoids the problems associated with xanthommatin) and ingenious methods have been developed for the mass separation of insect body segments. Further improvement may be achieved by the addition of BSA (39), cyanide (33) or other protective agents to the preparations although care must be taken to ascertain that such materials are not having other effects on the system.

In general, the clear message is that wherever the size of the insect permits, individual tissues or organs should be employed as a tissue source for homogenization and subcellular fractionation. Unfortunately, tissue dissection is often a tedious process and there is usually no way to avoid the individual handling of large numbers of insects. The most convenient procedures for this vary considerably from one species to another.

As a result of considerable work in recent years, it has been established that the patterns of tissue distribution of microsomal oxidase activity vary considerably both between different orders of insects and between species in a single order. In general, however, the tissues found to be most active are various portions of the alimentary tract, the fat body or the Malpighian tubules (Figure 3) (8).

In lepidopterous larvae maximum oxidase activity is associated with the gut tissues, particularly those of the midgut (Figure 4) (34). This distribution pattern has been demonstrated with approximately 40 different species (44,45) the only exception to date being the cabbage looper (*Trichoplusia ni*) where preparations from the fat body are more active than those from the gut (35). High titers of microsomal oxidase activity have also been reported in the gut tissues of various other insects (8) although in many cases this is associated with a much broader pattern of tissue distribution as in various orthopteran species. In several species of cockroach epoxidation and hydroxylation activity occur in various tissues including the gut, fat body and Malpighian tubules the relative activity of each varying with the species (Figure 4B,C) (8). In the house cricket (*Acheta domesticus*) maximum activity is found in the Malpighian tubules (Figure 4D) (38).

As in mammals, therefore, microsomal oxidase activity in insects is found in several different tissues and the observed patterns may reflect a strategic localization linked with the major portals of entry of xenobiotics into the organism (8,27).

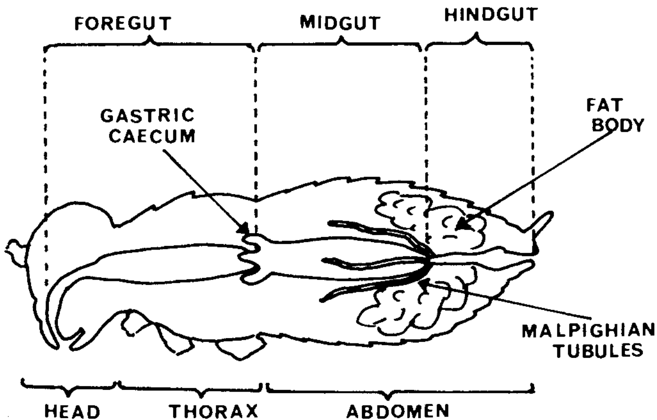

Figure 3. Insect tissues involved in microsomal oxidation

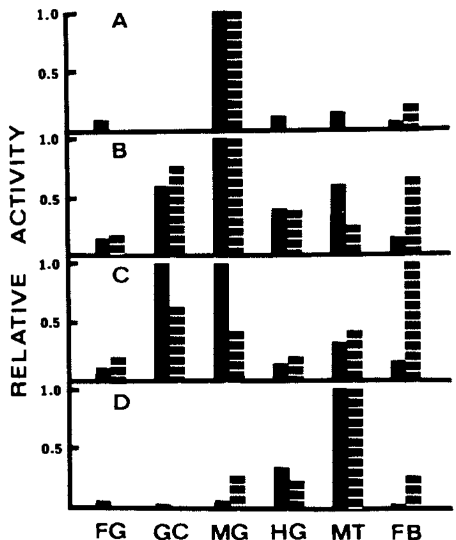

*Figure 4. Distribution of microsomal epoxidase activity in insect tissues: (A) southern armyworm (*S. eridania*) (34); (B) Madagascar cockroach (*G. portentosa*) (C) American cockroach (*P. americanum*) (48); (D) house cricket (*A. domesticus*) (38). Solid bars represent specific activity (per mg protein); striped bars represent activity per insect.*

Thus the gut tissues may constitute the major site for metabolism of xenobiotics ingested in the food whereas the fat body and/or Malpighian tubules possibly play a greater role in the metabolism of materials entering the insect by direct integumental penetration. This can be viewed as analogous to the distribution of microsomal oxidation in the skin, lung, gut and liver of mammals where they appear to be strategically located to act as the first line of defense against xenobiotics.

In assessing the relative metabolic importance of a given tissue to the intact insect, it is important to consider not only the specific activity of the enzyme in a homogenate or subcellular fraction (i.e. activity/mg protein) but also the relative biomass of the various tissues. Consequently, a tissue of large biomass but low specific oxidase activity may be more important in its overall metabolic capacity than one of small biomass and high specific activity. This can be clearly seen by comparing the patterns of distribution of oxidase activity based on measurements of specific activity with those calculated on a per insect basis (Figure 4).

Even when working with individual tissues, it is important to be aware of the possibility of encountering endogenous enzyme inhibitors. These can often be detected by combining homogenates of different tissues and examining the activity data for inhibitory effects. As shown in Table 2, the total activities of combinations of homogenates of various armyworm tissues are equal to the sum of their individual activities and, therefore, can be assumed to be free of inhibitory factors. In other cases, the

TABLE 2

Epoxidase activities of armyworm midgut homogenate alone and in presence of homogenates of other tissues

Tissue	Protein (mg/incubation)	Epoxidase activity (nmoles/10 min)	
		Observed	Calculated
Midgut (MG)	2.1	14.8	
Foregut	1.0	0.5	
Foregut + MG	3.1	15.1	15.3
Hindgut	1.2	0.6	
Hindgut + MG	3.3	15.6	15.4
Fat body	6.6	3.3	
Fat body + MG	8.7	17.8	18.1
Malpighian tubules	1.0	2.2	
Malpighian tubules + MG	3.1	16.8	17.0

In Xenobiotic Metabolism: In Vitro Methods; Paulson, G., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 1979.

activities of homogenates or subcellular fractions of tissues can be compared with that of the intact tissue. In the case of adult worker honey bees, epoxidase activity of intact midguts was decreased approximately 90% simply by opening the gut by longitudinal incision and was totally lost following homogenization of the tissue (Table 3) (46). Subsequently a potent intracellular inhibitory factor was isolated and partially purified from the soluble fraction of the gut (47). Inhibition was associated with the nucleic acid (RNA) moiety of a macromolecule (possibly a nucleoprotein) with a molecular weight of approximately 19,000 and could be reversed by digestion with ribonuclease (RNase T_1 of

TABLE 3

Epoxidase activity in intact and homogenized honey bee (adult worker) midgut tissues

Midgut treatment	Epoxidase activity pmole/min/mg wet. wt.
Intact	1.31
Opened	0.14
Homogenized	Not detectable

Data from Gilbert and Wilkinson (46)

pancreatic RNase) (Table 4). Following this finding, it was discovered that several commercially available nucleic acids including core and transfer RNA from baker's yeast and transfer RNA from *Torula* yeast also inhibited insect (armyworm gut) microsomal oxidases but had little or no effect on those from mammalian liver (47). Although to date this is the only inhibitor of this type reported, it is possible that others will be found in various insect preparations.

Another important parameter which has to be considered in the selection of a suitable source of tissue for *in vitro* investigation is the age or life stage of the insect to be used. It is now well established that the activity of the enzymes involved in xenobiotic metabolism change dramatically with age and stage of development in various insect species (8). This is clearly illustrated by the patterns of microsomal oxidase activity shown in Figure 5. In general, microsomal oxidase activity is only found in actively feeding life stages of the insect. Thus maximum activity is usually associated with the larval or nymphal stages of development and in the adults where these feed; insect eggs and pupae are usually devoid of oxidase activity. In southern armyworm larvae, microsomal oxidation activity (specific activity) in

In Xenobiotic Metabolism: In Vitro Methods; Paulson, G., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 1979.

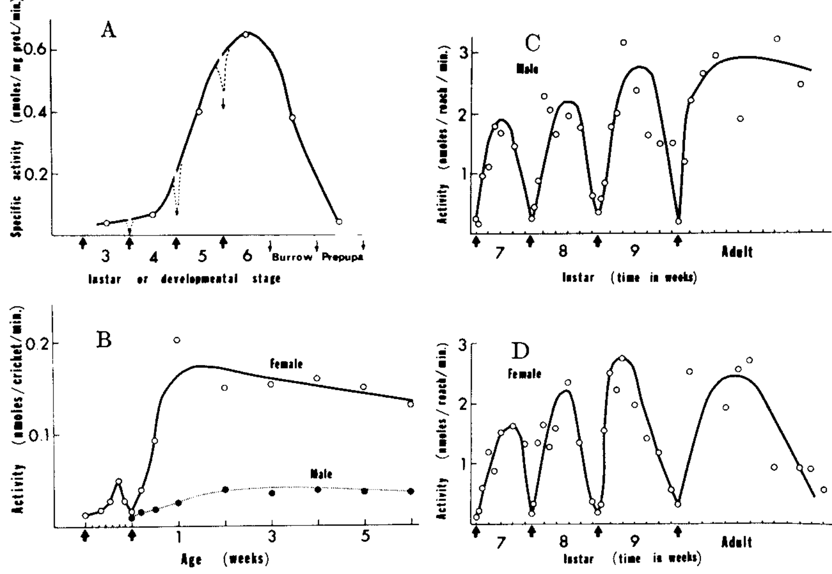

*Figure 5. Age–activity profiles of microsomal epoxidation in (A) southern armyworm (*S. eridania*) (34); (B) house cricket (*A. domesticus*) (38); and (C and D) Madagascar cockroach (*G. portentosa*) (48).*

TABLE 4

Effect of RNase T_1 and pancreatic RNase on activity of the honey bee midgut inhibitor

Enzyme (unit)		Reversal of inhibition (%)
RNase T_1	(5)	0
	(260)	24
	(2800)	100
Pancreatic RNase	(0.072)	14
	(0.144)	22
	(0.216)	28
	(0.360)	50

Data from Gilbert and Wilkinson (47)

the gut increases about 30-fold during development from the fourth to sixth larval instar (Figure 5A) and shows an equally dramatic decline as the larvae terminate feeding and prepare for pupation (34); a similar pattern has been observed with several other species (44). The increase in activity does not occur in a continuous manner, however, but shows a marked decrease during each larval molt (dotted lines Figure 5A).

Studies with other insect species have emphasized the remarkable patterns of oxidase activity which occur during insect development particularly those occurring during the larval or nymphal molts. Thus aldrin epoxidation activity in the Malpighian tubules of the house cricket is low during the molts from 8th to 9th nymphal instar and from the 9th instar to the adult and then increases as the adult matures (Figure 5B) (38). This rhythmic developmental cycle becomes much clearer in the midgut-caeca preparation from the Madagascar cockroach (Figure 5C and D) where it is quite evident that oxidase activity is low during the molt and passes through a maximum about midway through each instar (48). The results of these and other investigations strongly suggest that microsomal oxidase activity is under strict metabolic control and that this is closely linked with the process of metamorphosis.

From a practical viewpoint these rapid and dramatic changes in enzyme activity can provide a real headache in *in vitro* studies. Researchers in this field would be well advised to either establish the activity patterns in the insect species with which they are working or to avoid working with insects close to the molt. In some types of investigations (e.g. enzyme induction studies) where comparisons between treated and control groups are required, it is essential to use groups of insects closely matched

(±2h) with respect to age (49).

Procedures for obtaining insect subcellular components

As with other tissues, the preparation of subcellular components from insects is effected by two successive steps: homogenization and fractionation. In general, the methods which have been employed are based on procedures which have proved successful for mammalian tissues. However, due to large variations in the nature and morphological heterogeneity of the insect tissues employed, numerous modifications have been made in attempts to optimize the procedures. These have been discussed at some length in previous reviews (8,9,10).

Various procedures have been described for the homogenization of whole insects, insect body regions or individual insect tissues and usually the method of choice can only be determined by a process of trial and error for any given tissue source. Homogenization is usually achieved with either a mechanical blender, a hand-operated or motor-driven tissue grinder or a mortar and pestle (8,9,10). There is a general concensus of opinion that the procedure should be as gentle as possible and yet sufficiently drastic to satisfactorily disrupt cells in heterogeneous tissues often containing sclerotized cuticle, muscle fibers, etc. All operations should be carried out at 0-2°C.

Mechanical blenders (Waring blender, Omni-mixer, etc.) have been used for large batches of whole insects or insect body regions (e.g. house flies or house fly abdomens) but in general, oxidase activity in microsomal fractions derived from such homogenates is considerably lower than in those from homogenates prepared with a Potter Elvehjem tissue grinder (43,50) or by a gentle pounding action in a mortar (10,51). This loss of enzyme activity may result in part from denaturation through excessive abrasive action of hard chitinous portions of the insect or by the more efficient release of endogenous inhibitors. Violent mechanical blending undoubtedly aids in the release of the sparingly-soluble xanthominatin in preparations containing insect heads and there are reports that microsomes from blended whole house flies contain a b-type cytochrome (probably cytochrome oxidase) which is associated with thoracic sarcosomes or sarcosomal fragments and which interferes with spectral determination of microsomal cytochrome P-450 (10,51,52). The latter is not observed in house fly homogenates prepared by the so-called "mortar" procedure (10,51). The degree or duration of homogenization of whole insects or body regions is also important even when a tissue grinder is employed. Usually only a few passes of the plunger are required and better results are achieved by use of a relatively loose-fitting pestle of Teflon or smooth glass.

The homogenization of individual tissues usually presents fewer problems and here a variety of hand-operated or motor-driven tissue grinders provide the best results. The small

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amounts of tissue available as well as the heterogenous often "stringy" nature of the tissue (e.g. armyworm gut) usually make mechanical blenders quite ineffective for homogenization purposes. Homogenization with a tissue grinder should be as gentle and brief as possible and again Teflon or smooth glass pestles are preferable to those of ground glass.

Several different types of homogenization media have been employed by various workers and obviously can have an effect on the final enzymatic activity of the preparation. Although there are reports that the ionic strength of the medium may be important (43) the most commonly used media are 0.15 M KCl or 0.25 M sucrose which may or may not be buffered at various pH levels (8,9,10). Occasionally BSA (1-2% w/v) or other materials such as cyanide, phenylmethanesulfonyl fluoride, etc. may be added to at least partially counteract the effects of endogenous inhibitors (8,42,43).

Subcellular fractionation of homogenates of whole insects or insect tissues is usually achieved by the technique of differential centrifugation, first pioneered by Albert Claude with mammalian liver cells and subsequently widely applied to many other tissues (53). Briefly the technique involves centrifuging the initial homogenate at about 1,000xg for 10 minutes to remove nuclei and larger cell debris followed by a 9,000-12,000xg centrifugation for 10-30 minutes to sediment mitochondria and other intermediate sized particles and cell organelles. Further centrifugation of the postmitochondrial supernatant for 60 minutes at 100,000-200,000xg yields the so-called microsomal fraction and a "soluble" supernatant. With insect tissues this general procedure is often modified and the actual centrifugal conditions (force, time) usually vary somewhat between different laboratories. The procedure may also include filtration of the original brei through cheese cloth, cotton or glass wool to remove the often substantial amounts of chitinous debris and lipids released during homogenization.

The application of this technique to the subcellular fractionation of insect tissue homogenates is based on the largely unproven assumption that the subcellular organelles and membrane particles derived from insect tissues exhibit similar sedimentation characteristics to those from mammalian liver cells. In view of the highly heterogeneous nature of most of the insect tissue courses used for studies of xenobiotic metabolism this is a dangerous assumption which should be only made with extreme care. Moreover the procedures found satisfactory for one tissue may not be applicable to another.

Subcellular fractions can be classified morphologically by means of light or electron microscopy as containing nuclei, mitochondria, microsomal particles (from the membranous endoplasmic reticulum) etc. In addition, the discovery that certain types of enzymes are usually associated with a single class of particles has led to the development of a series of "marker" enzymes which

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can be used to classify the various subcellular fractions in biochemical terms. Few attempts have yet been made to characterize insect subcellular fractions either morphologically or biochemically and most workers in this field still seem content to associate their enzyme activities with subcellular fractions defined only by values obtained from the ultracentrifuge. Little attention is also given to calculating a complete balance sheet of the total enzyme activity and protein concentration in each fraction and comparing these with values for the original homogenate. Thus in the absence of total protein concentration the high specific activity of an enzyme in some subcellular fraction yields little information with regard to its overall distribution and a meaningful discussion of subcellular fractionation is difficult.

From the studies which have been conducted, it is reasonable to conclude that as in mammals, the enzymes involved in xenobiotic metabolism in insects are almost all located in either the soluble or the microsomal fractions of the cell. Although it is possible that problems could be encountered by the adsorbance of soluble enzymes onto larger cell organelles, the preparation of satisfactory soluble enzyme fractions does not appear to have been a serious problem to date. Most attention has been focussed on subcellular fractionation procedures to obtain satisfactory microsomal fractions from insect tissues.

In several cases, microsomal fractions exhibiting high levels of drug and insecticide oxidizing activity can be prepared by a differential centrifugation procedure similar to that used for mammalian liver. Thus in the case of the armyworm (*Spodoptera eridania*) larval gut preparation, the microsomal fraction (100,000xg, 60 minutes) is clearly the major intracellular location of the enzymes catalyzing epoxidation and *N*-demethylation (Figure 6A and B respectively) and Figure 6B shows that the distribution pattern for *N*-demethylase activity is essentially identical to that for NADPH-cytochrome *c* reductase a recognized microsomal marker enzyme (54); that this fraction is derived mainly from the endoplasmic reticulum has been obtained by electron microscopy (55).

In other cases, however, the preparation of insect microsomes by differential centrifugation has proved much more difficult. One of the most common problems which is encountered is that most of the xenobiotic oxidizing activity associated with the microsomal fraction is sedimented at unexpectedly low g-forces (8). This has been reported for fat body preparations from the American cockroach (*Periplaneta americana*) (56,57), and blowfly larva (*Calliphora erythrocephala*) (58) and homogenates of cricket (*Acheta domesticus*) Malpighian tubules (38) and Madagascar cockroach (*Gromphadorhina portentosa*) gut-caeca tissues (48). Thus in the latter example approximately 48% of the aldrin epoxidase activity was sedimented during a 15 minute spin at 12,000xg (Figure 6C) using the typical differential centrifugation procedure. If, instead the crude homogenate (in 0.25M sucrose

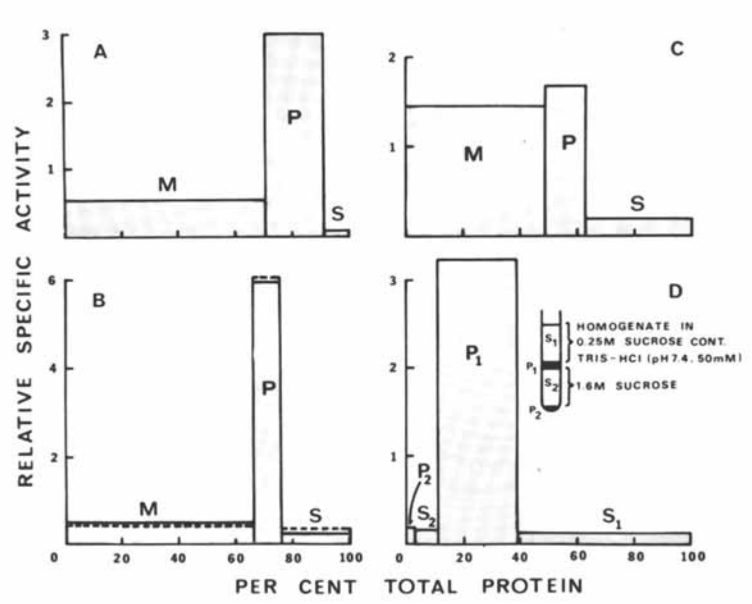

Figure 6. Subcellular fractionation of microsomal oxidase activity from insect tissues.

A and B are epoxidation (34) and N-demethylation (54) activities, respectively, in southern armyworm midgut tissues; dotted line in B is NADPH–cytochrome c reductase activity. M is 10,000–12,000 g × 10–15 min pellet, P is 105,000 g × 60–90 min pellet, S is supernatant. C and D are epoxidation activity from midgut caeca tissue of Madagascar cockroach (48).

containing 50mM Tris-HCl, pH 7.4) is layered over 1.6M sucrose and centrifuged for 45 minutes at 150,000xg in a swinging bucket rotor two major particulate fractions are obtained a dense pellet (P_2) which sediments to the bottom of the tube and a light protein band (P_1) at the sucrose interface. The latter which represents about 28% of the total protein contains 90.4% of the total epoxidase activity (Figure 6D) has been shown by electron microscopy to consist largely of membranous vesicles from the endoplasmic reticulum. A similar procedure involving sucrose density gradient centrifugation has been found useful in preparing microsomal fractions from cricket Malpighian tubules (38) and honey bee larval gut tissues (46).

It therefore appears that under certain conditions, the microsomal particles in homogenates of some insect tissues either aggregate or become adsorbed to the surfaces of larger particles such as mitochondria. Workers in the field should be aware of this possibility and where necessary modify their procedures accordingly. The suspension medium may have some effect on this behavior since it can modify the physicochemical forces involved. It is interesting to note that in the case of microsomes from the southern armyworm midgut, the morphological appearance of the membrane vesicles prepared in 0.25M sucrose and 0.15M KCl are quite distinct; thus although both fractions exhibit essentially identical levels of microsomal oxidase activity the vesicles prepared in sucrose are relatively smooth and rounded compared with the angular appearance of those prepared in KCl.

As a result of the sometimes broad distribution of microsomal oxidase activity in several subcellular fractions, the activity measured in the microsomal fraction *per se* may be a poor indicator of the total enzyme capability of a given insect tissue. In comparative studies, therefore, where a measure of total metabolic capacity is often required, it may be more appropriate to measure the activity in less homogeneous fractions such as the 10,000xg supernatant or even in crude homogenates. Indeed, such preparations are probably most convenient in many metabolic studies since their use significantly decreases the time of preparation.

The stability of enzyme activity in homogenates and subcellular fractions from insect tissues varies considerably depending on the tissue source, the method of preparation and the storage conditions employed (8,9,10). As a general rule they should be used without delay after preparation. Oxidase activity in preparations from whole house flies or house fly abdomens usually is rapidly lost on storage at 0-2°C (8,10,31,43) but may be retained for several weeks under certain conditions (8,10,31). Freezing of these microsomes has been reported to be deleterious (8,10,51). However, microsomes from armyworm gut may be stored for up to a month as well-drained pellets at -15°C (59) (Table 5) and microsomal suspensions from cricket Malpighian tubules lost only about 15% of their initial epoxidation activity after 3 weeks at the same temperature (38).

TABLE 5

Effect of freezing on oxidase activity and cytochrome P-450 content in armyworm midgut microsomal preparations

Storage (weeks)	Oxidase activity (nmoles/mg protein/min) ($x10^3$) Epoxidation	Hydroxylation	Cytochrome P-450 (nmoles/mg protein)
0	92.5	46.5	1.37
1	92.5	54.2	1.30
2	88.0	46.0	1.37
3	94.6	48.4	1.48
4	98.2	51.4	1.42

Data from Krieger and Wilkinson (59)
Microsomes were stored as well-drained pellets at -15°C

Measurement of enzymatic activity

Following preparation of a suitable subcellular fraction with which to work the next step is to measure its enzymatic activity towards an appropriate substrate. The incubation mixture and conditions to be used will clearly depend on the nature of the enzyme, subcellular fraction and substrate under investigation and, of course, the purpose of the study. The optimization of incubation and assay conditions is extremely important and can in itself provide a great deal of information on the nature and mechanism of the enzyme concerned. Unfortunately it is often overlooked or given minimal attention where the subcellular fractions are being used primarily as biochemical tools to generate metabolites.

The procedures employed to optimize conditions for measuring enzyme reactions in insect subcellular fractions are essentially those pertaining to mammalian systems. Major parameters which should be evaluated are the type, strength and pH of the buffer, the temperature under which the incubations are conducted and the addition of appropriate cofactors or other materials.

Phosphate or Tris buffers of varying pH (7.0-8.5) and ionic strength usually prove adequate for most *in vitro* studies on xenobiotic metabolism (8,9,10). Although most of the early *in vitro* studies were carried out at 37°C it is now generally agreed that insect enzymes seem to function more satisfactorily at a temperature of about 30°C (8) and in many cases optimum values of 20-25°C have been reported (35,36). Since enzyme activity in many insect preparations is less stable than that in corresponding mammalian fractions, linearity of the reaction with respect to both time and protein concentration should be ascertained.

The addition of cofactors is usually necessary to obtain maximal *in vitro* activity and the nature of these clearly depends

on the type of enzyme under study. For the enzymes involved in xenobiotic metabolism there are only a few major types of cofactors.

Microsomal oxidation activity is dependent primarily on the addition of NADPH or an appropriate generating system, the latter often proving preferable by extending the time linearity of the reaction (8). Incubations must be conducted aerobically as oxygen is a requirement for oxidase activity. Although materials such as EDTA and/or nicotinamide are often added to enhance microsomal activity in mammalian microsomes where they stabilize NADPH through blocking lipid peroxidation and pyridine nucleotidase activity respectively, their inclusion in insect microsomes appears to have little or no beneficial effect on oxidase activity (8). This is also generally true of most metal ions (8). BSA (1-2% w/v) is sometimes added to the incubation medium to counteract the actions of residual proteinase inhibitors but care must be taken that this or other additions do not have other deleterious effects on enzyme activity.

Soluble enzymes of importance in xenobiotic metabolism include a variety of hydrolases (phosphatases, carboxyesterases, amidases and pyrethroid hydrolases) (20), several glutathione (GSH) requiring enzymes (alkyl and aryl transferases, DDT-dehydrochlorinase and organothiocyanate metabolizing enzymes) (21,22) and numerous other conjugating enzymes (glucosyl transferase, sulphotransferase, etc.) (22). The properties and *in vitro* requirements, many of these enzymes have already been discussed in this symposium.

Perhaps a few words should be added at this point with respect to the substrate employed in *in vitro* studies. Unfortunately most of the substrates which are used in xenobiotic studies are of necessity highly lipophilic materials and their addition to an aqueous incubation medium often presents a problem. Usually this can be achieved by dissolving the substrate in a small volume of ethanol, acetone or other organic solvent (care should be taken to ensure that this does not inhibit the enzyme) but even then it can often be observed to precipitate out of the incubation medium. This raises serious questions about the actual concentration of substrate in the incubation mixture and how this should be expressed. Studies with microsomal preparations from mammalian liver and armyworm midgut have shown that in the presence of a constant amount of microsomal protein, the amount of aldrin epoxidized to dieldrin is independent of the total volume of the reaction mixture and related only to the absolute amount of aldrin added (Table 6) (60,61). This suggests that the true concentration of substrate available for enzyme conversion is not that expressed by the molar concentration calculated from the total incubation volume but is probably the unknown concentration existing in the lipid phase of the microsomes. There are, therefore, problems inherent in expressing the concentrations of lipophilic substrates in molar terms and K_m values and other

TABLE 6

Effect of aldrin solubility on epoxidation in pig liver microsomes

Incubation Medium		Aldrin added	Dieldrin produced	Aldrin Concentration	
Total vol. (ml)	Microsomal suspension (ml)	(μg)	(μg)	(μg/ml)	(μM)[a]
1	0.2	50	9.2	50	137
2	0.2	50	9.3	25	68.5
3	0.2	50	9.4	12.5	45.7
4	0.2	50	10.2	10	34.3

Data from Lewis *et al.* (60)

[a] Based on total volume of incubation medium

kinetic parameters reported in this way should be accepted with reservation. Perhaps the best way of expressing substrate concentration is in terms of the absolute amount added to the reaction medium since the actual concentration is directly related to this value.

Particularly in the case of the microsomal oxidase system, the choice of substrates for *in vitro* studies is large and many excellent and sensitive assays are currently available. Initial studies to optimize the conditions for some reaction or to characterize a given subcellular fraction are often facilitated by using a model substrate which yields a single metabolic product. Having thus established the characteristics of the system these can usually be applied directly to studies with more complex drug or insecticide substrates which may have several sites at which enzyme attack can occur and which consequently produce several different metabolites.

Application of *in vitro* studies

To date, the major application of *in vitro* studies with insect subcellular fractions has been to complement *in vivo* studies on the metabolic fate and pathways of insecticide chemicals. *In vivo* studies are often complicated by the fact that the terminal metabolic products (conjugates) are the ones commonly observed and these often provide little information on the nature of primary or intermediary metabolites which may exist at trace levels or be of a transient nature. In insects the situation is compounded by the fact that with highly toxic materials only very small doses of material can be applied without killing the insect. With *in vitro* systems toxicity does not have to be considered and high concentrations of toxicants can be employed. Furthermore, the use of various subcellular fractions permits the individual

study of isolated components of the overall metabolic machinery in a relatively concentrated form without the complications arising from the presence of other metabolic components. Thus using a suitably fortified microsomal fraction the nature of the primary oxidative metabolites can be studied in the absence of the type II conjugating systems found largely in the soluble fraction.

Early *in vivo* metabolic studies with the carbamates, for example, showed that these insecticides were rapidly metabolized by insects to water soluble products and many of which were conjugates of primary hydroxylated metabolites (5) (Figure 7). Subsequent *in vitro* studies using isolated microsomal and soluble fractions have enabled these primary and secondary products to be more readily identified and the appropriate enzyme systems better characterized.

Of particular significance to the utility of subcellular components in *in vitro* metabolism studies is the potential which exists for manipulating the system by the omission or addition of specific cofactors or inhibitors. Thus the requirement of a certain subcellular fraction for a particular cofactor to produce a certain metabolite often provides a fairly clear picture of the type of enzyme which is involved in the conversion. This type of experiment is especially informative where a compound may be metabolized to the same product by two different enzyme systems. The data shown in Table 7 were obtained during the course of a study of diazinon and diazoxon metabolism in house flies (62).

TABLE 7

Effect of reduced glutathione and NADPH on the degradation of diazinon and diazoxon by house fly subcellular fractions *in vitro*

Enzyme source[a/]	Cofactors	Degradation (mμmoles/hr/g ♀ abdomen)	
		Diazinon	Diazoxon
Microsomes:	None	0	0
	GSH	3.2	0
	NADPH	122.1	23.2
	NADPH + GSH	164.3	27.0
Soluble fraction:	None	52.3	5.2
	GSH	259.0	80.6
	NADPH	47.0	1.5
	NADPH + GSH	251.0	78.4

Data from Yang *et al.* (62)

[a/] Microsomes and soluble fractions equivalent to 35 and 10 ♀ house fly abdomens respectively

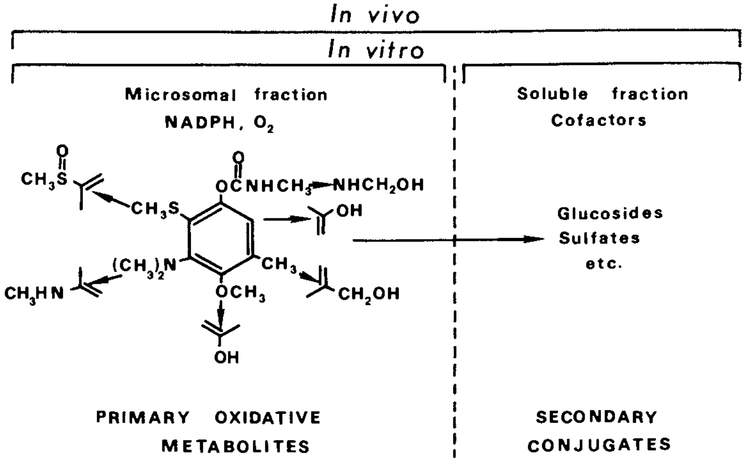

Figure 7. Metabolism of carbamate insecticides by microsomal and soluble fractions

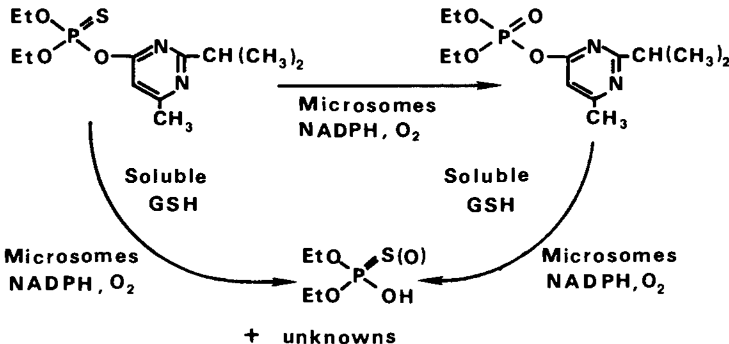

Figure 8. Metabolism of diazinon and diazoxon (62)

These clearly show that because of the requirement for NADPH the microsomal degradation of diazinon and diazoxon is primarily oxidative in nature. In contrast the soluble fraction contains an efficient GSH-dependent enzyme capable of degrading both compounds. The results of these studies combined with an analysis of the metabolites found in the in vitro incubations indicated that diazinon could be either oxidatively activated (desulfuration) to diazoxon or could be degraded by microsomal oxidation or soluble GSH-transferase to diethyl phosphorothioic acid. Diazoxon was likewise susceptible to the two degradation pathways to yield diethyl phosphoric acid (Figure 8).

Several groups of compounds are known to act as inhibitors of the enzymes involved in xenobiotic metabolism and some of these have value as insecticide synergists which enhance the insecticidal potency of various materials in vivo (63). Inhibitors of microsomal oxidation include many methylenedioxyphenyl derivatives, aryl-2-propynyl ethers, 1,2,3-benzothiadiazoles and a large number of substituted imidazoles (63). Many of the soluble hydrolases such as carboxylesterase and the pyrethroid metabolizing esterases are effectively blocked by phosphates such as tetraethylpyrophosphate and tri-o-cresyl phosphate (20,64) or carbamates such as 1-naphthyl N-propylcarbamate (65) and many GSH-transferases are blocked by compounds such as p-chloromercuribenzoate (21). Like the various cofactors previously discussed such inhibitors can be extremely useful in pinpointing the enzymes involved in certain reactions.

For example in some cases a xenobiotic may be metabolized by more than one type of enzyme system in the same subcellular fraction. In this case specific enzyme inhibitors can be employed to discriminate between the two enzymes. To illustrate how this technique can be applied in vitro we will use the results from a mammalian study. It has been known for some time that many of the modern pyrethroids are metabolized in mammals by both esterases and mixed-function oxidases in liver microsomes (66). Recent in vitro studies have established structure-biodegradability relationships with a series of 44 pyrethroids and model compounds and have clearly shown the comparative role of esterases and oxidases in their metabolism. Each of the test compounds was incubated with microsomes alone (esterase activity) with microsomes + NADPH (esterase plus oxidase activity) and with microsomes + NADPH + tetraethylpyrophosphate, an esterase inhibitor (oxidase activity). The results shown in Table 8 for the isomers of phenothrin and cyanophenothrin typify the results obtained with all compounds tested. Thus the primary alcohol esters of IR, trans-substituted cyclopropane carboxylic acids (e.g. phenothrin) were most rapidly metabolized by both esterase and oxidase attack, the corresponding IR, cis isomers were degraded mainly by oxidase action and were quite resistant to esterase attack and the α-cyano compounds were resistant to the degradative action of both types of enzymes.

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TABLE 8

Effect of structure on in vitro metabolism of 3-phenoxybenzyl chrysanthemum esters by mouse liver microsomes

Structure (IR, trans isomer)

Compound and isomer	Percent metabolism rate relative to [IR, trans]-resmethrin[a/]			
	Esterase	Oxidase	Esterase + Oxidase found	Esterase + Oxidase calc.
Phenothrin (R = H)				
IR, trans	59 ± 3	27 ± 2	78 ± 7	86
IR, cis	<4	37 ± 5	37 ± 3	37
Cyanophenothrin (R = CN)				
IR, trans, α-RS	3 ± 1	5 ± 1	11 ± 2	8
IR, cis, α-RS	<3	8 ± 1	12 ± 1	8

[a/] Esterase + oxidase activity with IR, trans resmethrin is 100 and its $t_{1/2}$ is 3.3 ± 0.8 min.

Data from Soderlund and Casida (64)

In addition to showing how specific enzyme inhibitors can be used in vitro to more clearly identify the metabolic role of other enzymes in the same system, the results of this study illustrates another important application of in vitro studies, i.e. to establish relationships between chemical structure and biodegradability in large series of structurally related compounds. This type of information is of considerable importance in the development of new drugs, insecticides and other biologically active compounds.

Other examples of the use of in vitro systems in structure-activity studies are those employed in the search for new and potentially more active insecticide synergists. It is now well established that most known synergists act in vivo by virtue of their ability to block microsomal oxidation (63). Consequently measurement of their ability to inhibit microsomal mixed-function oxidation in vitro provides a rapid and effective way of assessing their synergistic potential in vivo and of establishing the

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structural requirements or physicochemical parameters on which their activity depends. Regression analysis of data on the potencies of twenty 5-, 6- and 5,6-substituted 1,2,3-benzothiadiazoles as inhibitors of epoxidation in armyworm midgut preparations revealed that inhibition could be satisfactorily described by the following equation in terms of hydrophobic (π^2 and π) and electronic (σ) parameters (67). Similar structure-activity studies have been conducted with selected series of substituted imidazoles.

$$pI_{50} = -0.249\pi^2 + 0.761\pi - 0.457\sigma + 3.834$$

[negative log. I_{50}(M)] $n = 20;\ r = 0.942;\ s = 0.212$

In the same way that a given *in vitro* system can provide useful qualitative and quantitative information on the biological activity or ease of degradation of a series of chemicals, a single model compound (or type of reaction) can be employed as an indicator for comparing enzymatic activity in a series of different strains or species of organisms. Comparative studies of this type where the emphasis is on establishing the biochemical similarities or differences between different species or strains of resistant insects are extremely important in the design of compounds which exhibit some degree of selectivity in their biological activity. Since the comparative aspects of this subject will be covered by Dr. Terriere in the next presentation it will not be given further consideration here.

Relationship between *in vitro* and *in vivo* studies

In the process of conducting *in vitro* studies with subcellular fractions we have essentially dismantled the insect in an attempt to simplify the system and understand more clearly the properties and functions of some of its component parts. The study of an isolated enzyme *in vitro* is clearly a highly unphysiological situation since we have totally destroyed the morphological and functional integrity of the system and the complex and delicate interrelationships on which the intact animal depends. In interpreting the results of such studies, we must be aware of the possibility of artifacts. Nonetheless one of the requirements of any *in vitro* study is that it should have some relevance to the intact animal and an attempt should always be made to evaluate this.

Although it is quite clear that the *in vivo* toxicity of many compounds to insects is often related directly to metabolism, correlations between the results of *in vitro* metabolic studies and *in vivo* toxicity are rather few and far between and to date are only of a qualitative nature. We are still a long way from being able to approximate in quantitative terms the *in vivo* metabolic capability of an intact organism from the sum of the individual

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metabolic capacities of its individual organs. Indeed in view of the large number of factors which have to be taken into consideration (penetration barriers, binding to inert tissues, etc.) in the living animal, it is unlikely that this will be accomplished in the near future.

The best qualitative in vitro/in vivo correlations can be made in those cases where relatively large changes in metabolic activity occur within a single species. This situation exists in the dramatic age-related variations in microsomal oxidase activity which occur in several insect species and also where microsomal oxidase activity can be enhanced by treatment of the insects with various inducing agents.

In the house cricket (Acheta domesticus) microsomal enzyme activity is located primarily in the Malpighian tubules; it increases dramatically during the first one to two weeks of adult life and thereafter decreases through weeks two to six. The in vivo importance of the Malpighian tubules in insecticide detoxication is strongly suggested by the fact that these changes in the toxicity of carbaryl and its synergism by piperonyl butoxide to crickets of different age and sex (Figure 9) (68).

A good correlation has also been observed in the toxicity of carbaryl to control and induced armyworm larvae (Table 9) (49). Thus armyworms with microsomal activity in the gut tissues enhanced 3-fold as a result of a 3-day dietary exposure to pentamethylbenzene (2,000 ppm) showed a remarkable 11-fold increase in tolerance to orally administered carbaryl; a good correlation was also observed with the less effective inducing agent hexamethylbenzene.

TABLE 9

Effect of induction on in vitro microsomal oxidase activity and in vivo tolerance of armyworm larvae to orally administered carbaryl

Inducer treatment	Percent control activity		LD_{50} Carbaryl (μg/g)
	Epoxidase	Cytochrome P-450	
Control	100	100	30
Hexamethylbenzene (2,000 ppm in diet)	225	219	67
Pentamethylbenzene (2,000 ppm in diet)	314	299	350

Data from Brattsten and Wilkinson (49)

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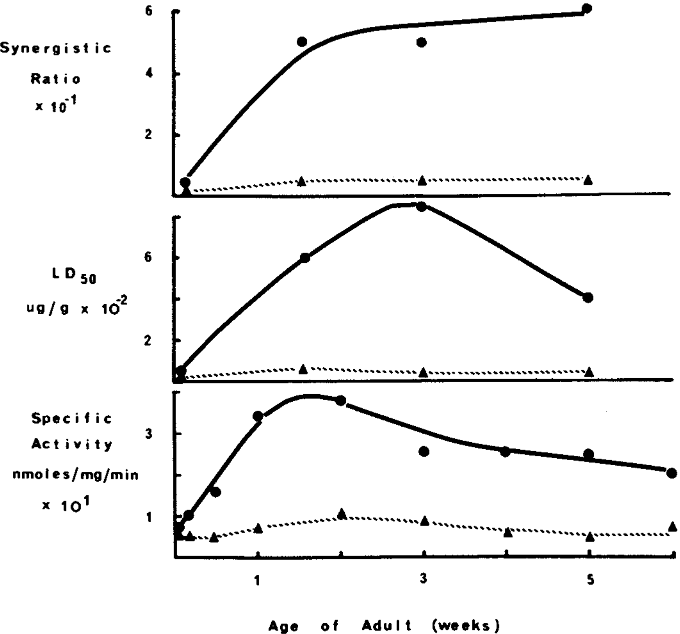

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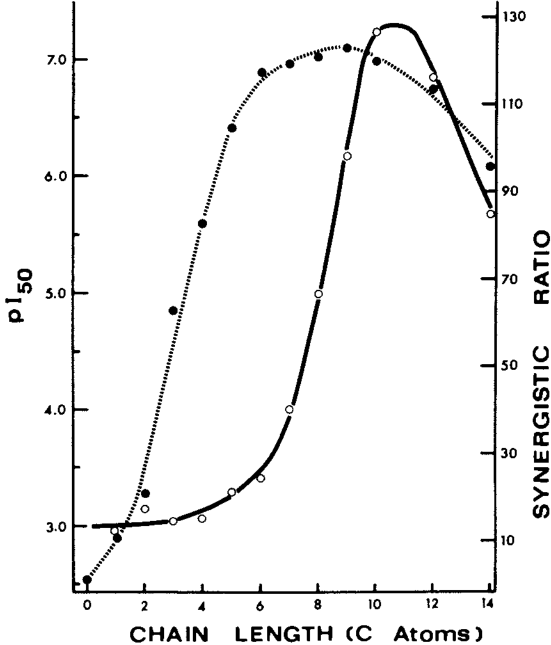
*Figure 9. Relationship between epoxidation activity in Malpighian tubules, susceptibility to carbaryl, and degree of synergism of carbaryl by piperonyl butoxide in adult crickets (*A. domesticus*) of different age and sex: (——), females; (– – –), males (68).*



Biochemical Pharmacology

Figure 10. Activity of 1-alkylimidazoles in insects in vitro and in vivo: (●---●), pI_{50} aldrin epoxidase activity in armyworm gut preparation; (○ — ○), synergistic ratio with carbaryl to house flies (69).

Finally, the *in vitro* potency of many inhibitors of microsomal oxidation in insects is often reflected in their ability to synergize insecticidal activity *in vivo*. This is evident from Figure 10 which shows that the activity of a series of 1-alkylimidazoles as inhibitors of microsomal aldrin epoxidation in armyworm midgut preparations closely parallels their *in vivo* activity as synergists for carbaryl in house flies.

Conclusion

Although numerous problems are encountered in *in vitro* studies with insect subcellular fractions, it is apparent that such studies can and will provide a great deal of valuable information concerning the metabolism of xenobiotics. Substantial progress has already been made in developing satisfactory methodology for the preparation of appropriate subcellular fractions from insects and in establishing the biochemical characteristics of the many of the enzymes concerned. Indeed, within the constraints imposed by the limited availability of insect tissues there is no reason to suppose that insect subcellular fractions and purified enzyme components cannot be used in exactly similar ways to those from mammalian tissues.

Abstract

In vitro studies employing subcellular components from whole insects or insect organs can provide valuable information on the structure of primary metabolites likely to be encountered in *in vivo* studies and the use of specific cofactors or inhibitors can prove useful in pinpointing reaction mechanisms and indicating qualitative differences in metabolite patterns between species or strains. There are, however, several problems which require special attention in conducting *in vitro* studies with insect subcellular components and little can be assumed in moving from one species to another. Particularly in the case of microsomal oxidation, the presence of a variety of endogenous inhibitors in homogenates from whole insects can have serious effects on enzyme activity and the stability of the preparation and the patterns of organ localization change in different species. Even in preparations derived from specific organs, optimal preparatory procedures must be established and anomalous centrifugal sedimentation characteristics of subcellular fractions are often observed. Furthermore, *in vitro* enzyme activity in subcellular components changes dramatically with physiological factors such as age and stage of development and is highly susceptible to the presence of inducing agents in the diet. Data so far obtained emphasize the basic similarities which exist in the enzymatic composition and metabolic function of insect and mammalian subcellular components.

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9

The Use of In Vitro Techniques to Study the Comparative Metabolism of Xenobiotics

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The underlying motive of all biochemical comparisons, in vivo or in vitro, of different species of plants and animals is the search for differences. The discovery of such differences can be useful in many ways--the improvement of drugs and pesticides, the protection of beneficial species and of man, the understanding of evolution, and the development of new principles. Indeed, some reflection about past accomplishments will show that species comparisons have led to some of our most important discoveries in biochemistry.

Gillette (1) has described the many ways in which species may differ in their metabolic disposition of foreign compounds and why it is difficult to interpret such differences on the basis of urinary excretion or the plasma level of these compounds or their metabolites. Of the several major parameters which can cause species differences, he considers the quantity and quality of the enzymes and their interactions with endogenous and exogenous compounds to be the most important. Since the in vitro method is well suited to the study of the kinetics, substrate specificities, and metabolites of single as well as multiple enzyme systems, it would appear that a full understanding of species differences in metabolism cannot be gained without this tool.

Some of the more common uses of the in vitro method to study species differences include: 1) comparisons of enzymes and enzyme systems, 2) correlation of enzyme activity with toxicity or other biological response, 3) study of the selectivity of drugs and pesticides, 4) evolutionary comparisons, i.e. association of detoxication activity with habitat or feeding habits, 5) mode of action studies, 6) environmental studies, i.e. the detoxication capacity of non-target species, and 7) development of the technique to replace in vivo methods. In search of such

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information birds have been compared with mammals, insects have been compared with fish and mammals, fish with crustaceans, and reptiles with amphibians, fish, birds, mammals, and insects, just to mention a few of the more unusual combinations.

In vitro methods offer the same advantages in comparative biochemistry as in other fields but, because of the inherent differences in physiology, morphology, genetics, and behavior of species, their use is more uncertain. The investigator who uses in vitro methods in this way should be aware of the potential for error in the design of experiments and in the interpretation of results. The purpose of this review is to critically examine current practices in the use of these methods in species comparisons, to suggest some procedures for conducting such studies, and to mention problems in need of further study.

Due to their importance in the metabolism of drugs, pesticides, and other xenobiotics by plant and animal species, the enzymes of the microsomal oxidase system have been the subject of most comparisons in vitro. This has resulted in more information about methods and problems and is the reason for their prominence in this review. However, the ideas discussed here should apply equally well to other metabolic systems.

Historical

Quinn et al (2) appear to be the first to use the newly discovered microsomal oxidase assay to explain species differences in response to a drug. They obtained a good correlation between hexabarbital sleeping time and the in vitro metabolism of this drug in the mouse, rabbit, rat, and dog. Similar results were reported by Brodie et al. (3) who, in addition, noted a sex difference in hexabarbital metabolism in rats and were able to explain this on the basis of microsomal oxidase activity.

An interest in the evolution of detoxication mechanisms led Brodie and Maickel (4) to compare the microsomal metabolism of several drugs in mammals, birds, reptiles, amphibians, fishes, and invertebrates (crab, lobster, and cricket). Although some of their conclusions were not supported by later work, they stimulated much interest and many similar comparisons began to appear in the late 1960's and have continued to this time. Apparently the first species comparison, in vitro, involving aquatic species, was that of Potter and O'Brien (5) who studied the conversion of the insecticide, parathion, to its more toxic product, paraoxon.

The *in vitro* method was used to compare the azo and nitroreductase activity of the five major classes of vertebrates (6), showing that the fishes were much less active in such metabolism than the birds and mammals. An important contribution of this work was the demonstration that the species varied in their temperature optima for maximum enzyme activity, aquatic species requiring lower temperatures (21-26°C) while the reductases of the birds were more active at 40°C). In the case of the turtle, nitroreductase activity was 20 times greater at 21°C than at 37°C.

Early evidence that the substrate specificity of the microsomal oxidases might vary with the species was obtained by Creaven et al. (7) who studied the *in vitro* hydroxylation of biphenyl by 11 species. Some species hydroxylated this compound in both the 2 and 4 position while others favored one but not the other of these positions. This was probably one of the earlier indications that cytochrome P-450 exists in more than one form.

An early attempt to use *in vitro* techniques to compare the metabolism of xenobiotics in insects was that of Chakraborty and Smith (8) but their *in vitro* tests were unsuccessful in detecting the expected metabolic activity. This was probably due to the presence of natural inhibitors released during preparation of the homogenates. Another early use of the *in vitro* method in the study of insect species was that of Schonbrod et al. (9). The microsomal hydroxylation of naphthalene was shown to correlate fairly well with naphthalene toxicity to resistant and susceptible house flies and to blow flies.

The Reliability of *in vitro* Methods in Species Comparisons

If *in vitro* methods are to be used in predicting or explaining species differences in the metabolism of xenobiotics, their reliability must be established. This is done most readily by comparing results from appropriate experiments *in vitro* and *in vivo* on the same species. Not many investigators do this, however, so it is necessary to use other criteria such as agreement between laboratories studying the same species. In such cases a reliable method of enzyme assay should result in the same relative activity for the species studied. Another test of reliability is whether the patterns and products of metabolism revealed by *in vitro* techniques agree with those of the intact organism. This is, of course, very necessary in the use of

In Xenobiotic Metabolism: In Vitro Methods; Paulson, G., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 1979.

in vitro methods to relate or extrapolate between man and experimental animals.

A good example of such comparisons is the report of Chipman et al. (10) who studied the in vivo and in vitro metabolism of the dieldrin analog, 1, 2, 3, 4, 9, 9-hexachloro-exo-5, 6-epoxy-1, 4, 4a, 5, 6, 7, 8, 8a-octahydro-1, 4-methano-naphthalene (HCE) in rats, rabbits, pigeons, and Japanese quail. Their in vivo method included the use of a re-entry bile cannula with provisions for the routine collection of samples. The nature and quantity of metabolites collected with the bile, as well as the urine, were compared with those found after a 30 minute incubation of HCE with liver microsomes (in vitro at 37°C for mammals, 42°C for birds). The in vivo metabolites (bile and urine) which were largely conjugates were converted to the primary compounds prior to measurement.

As shown in Figure 1, HCE undergoes two routes of metabolism, epoxide hydration to the trans-diol, a minor product in the species studied, and hydroxylation of the epoxide to HHC, the major metabolites in all four species. This is followed by epimerization and further hydroxylation to the dihydroxy epoxide, DHHC.

The results of Chipman et al. are summarized in Table I and Figure 2. It will be seen that the failure of the pigeon to convert the epoxide, HCE, to its trans-diol was correctly predicted by the microsomal studies, Table I. Also, the in vitro experiments confirmed the relative order of importance of the oxidative metabolites HHC, endo-HHC, and DHHC (Table I) in the pigeon, quail, and rabbit, and reversing only the endo-HHC and DHHC in the rat. The two methods disagree on the relative importance of epoxide hydration in the rabbit.

Figure 2 shows that time is an important variable in such comparisons. Only in the short tests (20 minutes in vitro, 1 day in vivo) do the two methods agree that HHC is the major metabolite. After 2 days in vivo the order, most to least, is DHHC, HHC, and endo-HHC and after 80 minutes in vitro it is endo-HHC, DHHC, and HHC, although the differences here are much less. This disagreement is probably due to the availability, in vivo, of additional enzymes, the conjugases, which alter the levels of HHC and endo-HHC.

Sullivan et al. (11) have developed a tissue maintenance technique for the comparative study of drug metabolism. The method involves the incubation of small pieces (e.g. 2mm cubes) of fresh tissue for up to 18 hrs with the radioactive drug. The incubation medium is then extracted and the metabolic

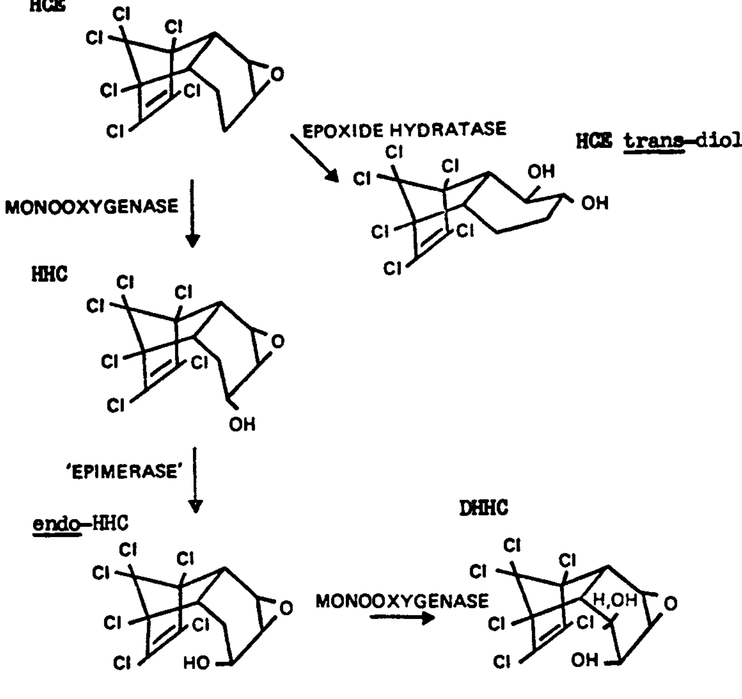


Figure 1. Metabolism of HCE (10)

Table I. HCE Metabolites Found in vitro and in vivo.

(a) in vivo

Species	^{14}C, % extractable	Metabolites, % of Total: HHC	endo-HHC	DHHC	HCE trans diol	X	Others
	in bile[a]						
rat (7)	89	61	7	14	4	8	6
pigeon (4)	91	50	46	4	0	0	
Jap. quail (4)	57	63	19	5	0	13	
	in urine[b]						
rabbit (3)	87	68	10	19	3		
pigeon (4)	95	89	6.4	4.6	0		

(b) in vitro (microsomes)[c]

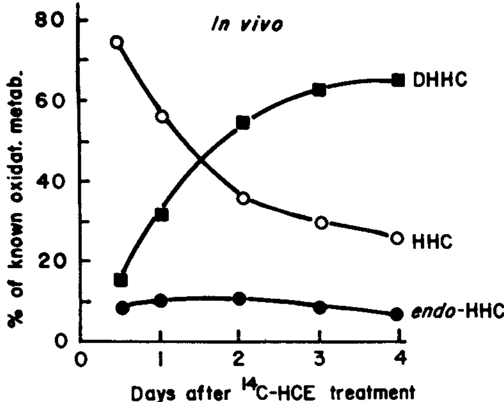
Species	% Substrate converted	g Liver represented/incubation	Individual metabolites as % of total metabolites: HHC	endo-HHC	DHHC	HCE trans diol	Other
rat (6)	60	1.5	41	33	20	5	0
rabbit (3)	75	0.8	41	4	10	30	15
pigeon (3)	55	1.0	45	31	20	0	4
Jap. quail (3)	25	1.0	70	12	8	7	0

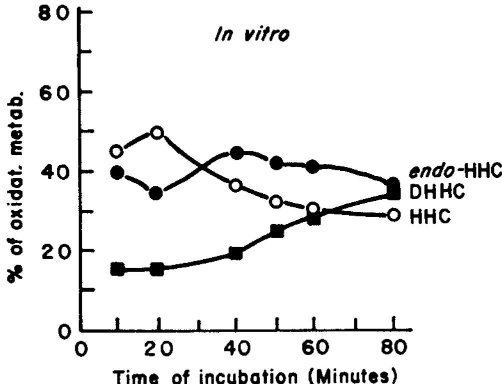
[a] Only traces of radioactivity were found in rat urine and rabbit bile. All urine and bile collections were made during the first 2 hours following dosing (15 mg kg^{-1} ^{14}C-HCE).

[b] Urine samples were adjusted to 2 M HCl before incubating for 30 min. at 75°C to break down conjugates.

[c] Microsomes incubated 30 min. at 37° C or 42° C (birds). Five milliliters of incubation medium contained 40 μg HCE.

Data from Chipman et al. (10).





Biochemical Pharmacology

Figure 2. Pattern of HCE oxidative metabolism observed in the rat with respect to time. In vivo: metabolites found in bile with intermittent collections; in vitro: metabolism by hepatic microsomes reinforced with NADPH and O_2 (10).

products resolved by DEAE cellulose chromatography. Using ^{14}C-carbaryl, ring and N-methyl labelled, as a model, they were able to obtain good agreement in the number and nature of metabolites produced, with those previously found in *in vivo* studies. The comparisons were made with liver explants of rat, dog, guinea pig, and man. Their results also have some quantitative value, indicating major and minor metabolites in the four species. Some typical results from their study are shown in Figures 3 and 4.

In a further demonstration of their method, Chin et al. (12) compared carbaryl metabolism in several human tissues. This study also illustrated another useful aspect of the method, the ability to compare various organs and tissues of the same species with respect to the metabolic capabilities of each.

It is clear from the results of Sullivan et al. and of Chin et al. that the tissue explants exhibit most, if not all, of the primary and secondary metabolic processes expected of the organ *in vivo*. In addition to the oxidase and conjugase systems observed in the metabolism of carbaryl, (N-demethylation, epoxidation, ring hydroxylation, epoxide hydration, glucuronidation, and sulfation), the authors claim to have observed nitroreduction and sulfoxidation. All this was attained without added co-factors.

In seeking an explanation for the 1000-fold greater oral toxicity of the insecticide chlorfenvinphos to rats than to dogs, Hutson and Hathway (13) found several differences in the two species. Rat brain acetylcholinesterase was about 10 times more sensitive to inhibition by chlorfenvinphos, rat erythrocytes absorbed the toxicant more readily from plasma (difference about 3-fold) and there was a greater uptake of chlorfenvinphos by rat brain. There was about 15-fold less toxicant in the blood of the dogs, probably due to a combination of absorption and metabolic differences.

The metabolism of chlorfenvinphos by liver slices from the rat, mouse, rabbit, and dog was studied in a further investigation of species differences in the toxicity of this insecticide (14). The liver slices were incubated with the ^{14}C-labelled compound for 1-2 hrs at 37°C. The metabolism was shown to be due to a microsomal mono-oxygenase, resulting in the elimination of one of the ethyl groups. This had already been shown to be the major *in vivo* reaction in both rats and dogs. Initial reaction rates for the de-ethylation, calculated from the curves shown in Figure 5, place the four species in the order 1, 8, 24, 88 (rat, mouse, rabbit, dog). In comparison, the acute, oral LD_{50}'s (mg/kg) of

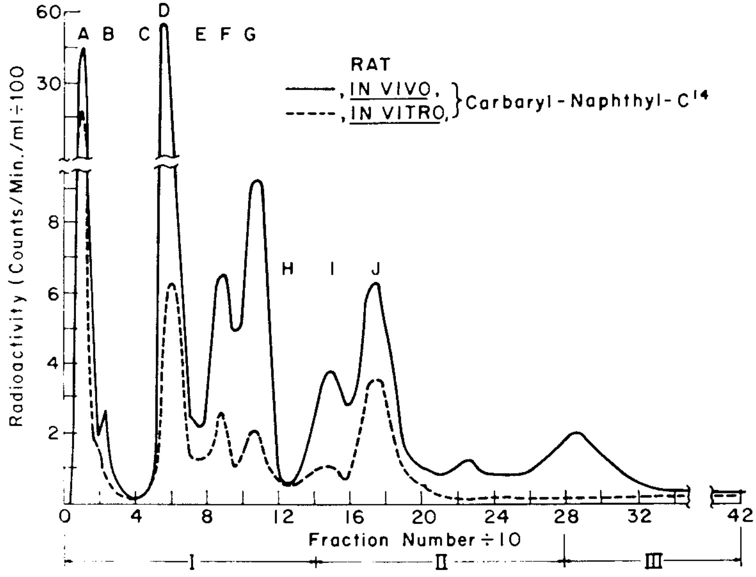


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Figure 3. DEAE–cedlulose chromatogram of in vivo and in vitro rat metabolites of carbaryl-naphthyl-^{14}C. Gradient elution program: (I) 0.01M Tris · HCl buffer, pH 7.5 to 0.05M Tris · HCl buffer, pH 7.5; (II) 0.05M Tris · HCl buffer, pH 7.5 to 0.1M Tris · HCl buffer, pH 7.5; (III) 0.1M Tris · HCl buffer, pH 7.5 to 0.5M Tris · HCl buffer, pH 7.5 (11).

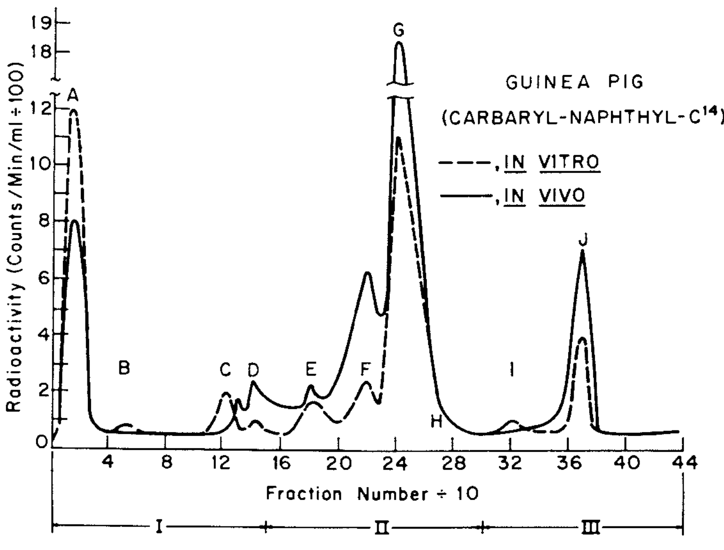

Figure 4. DEAE–cellulose chromatogram of in vivo and in vitro guinea pig metabolites of carbaryl-naphthyl-^{14}C. Gradient elution program as in Figure 3 (11).

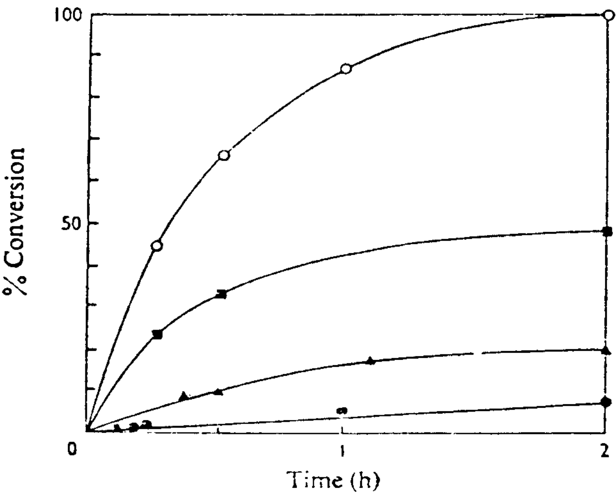

Figure 5. Relative rates of dealkylation of chlorfenvinphos in liver slices: (●), rat; (▲), mouse; (■), rabbit; (○), dog (14).

chlofenvinphos in these species is 10 (rat), 100 (mouse), 500 (rabbit), and >1200 (dog). Thus the in vitro experiments would have correctly predicted the order of acute toxicity in the four species.

Only two in vitro studies could be found in which the same species and the same drugs were compared. These were the reports of Chhabra et al. (15) who compared the microsomal oxidase activities of liver and intestine against four compounds in five species (hamster, guinea pig, rat, mouse, rabbit) and Litterst et al. (16) who used some of the same drugs to compare microsomal metabolism in lung, liver, and kidney of the same species. Table II shows that they found very little difference in cytochrome P-450 content, the rabbit, hamster, and guinea pig having higher P-450 levels but with considerable overlap. Both studies agree in ranking the rat as the lowest of the five species and in the narrow range, less than 2-fold, of cytochrome P-450 concentration.

Table II. A Comparison of Hepatic Cytochrome P-450 in Five Species

Litterst et al. (16)		Chhabra et al. (15)	
Species	Δ A 490-450 /mg protein/ml	Species	nmoles/mg protein
rabbit	0.177 ± 0.038[a]	guinea pig	1.45 ± 0.16[b]
hamster	0.140 ± 0.023	hamster	1.26 ± 0.07
guinea pig	0.125 ± 0.038	rabbit	1.1 ± 0.32
mouse	0.108 ± 0.022	mouse	1.1 ± 0.07
rat	0.098 ± 0.025	rat	0.84 ± 0.07

[a] ± S. D. [b] ± S. E.

There was reasonable agreement in the ranking of the five species according to their microsomal analine hydroxylase activities, Table III, the hamster and mouse being highest in both studies, and both showing the rabbit and guinea pig as the lowest. The activities differed about 4-fold in the Litterst et al. study. In the case of biphenyl hydroxylase, Table IV, the two studies agree on the rat as lowest in liver hydroxylase activity but there is little agreement in the ranking of the other species. The mean activities for the biphenyl hydroxylase varied about 3-fold in both studies.

From this limited review of results from two laboratories, it appears that the technique is reasonably reliable in the ranking

Table III. A Comparison of Hepatic Analine Hydroxylase in Five Species

Litterst et al. (16)		Chhabra et al. (15)	
Species	Activity nmoles/mg protein/min	Species	Activity nmoles/mg protein/15 min
hamster	2.7 ± 0.9[a/]	mouse	24.1 ± 1.9[b/]
mouse	1.5 ± 0.3	hamster	19.1 ± 1.3
rat	0.8 ± 0.4	rat	15.0 ± 0.9
guinea pig	0.8 ± 0.4	guinea pig	10.6 ± 1.0
rabbit	0.6 ± 0.4	rabbit	9.8 ± 2.3

[a/] ± S.D. [b/] ± S.E.

Table IV. A Comparison of Hepatic Biphenyl Hydroxylase in Five Species

Litterst et al. (16)		Chhabra et al. (15)	
Species	Activity nmoles/mg protein/min	Species	Activity nmoles/mg protein/15 min
guinea pig	4.1 ± 3.1[a/]	mouse	95.3 ± 10.0[b/]
hamster	3.4 ± 1.3	guinea pig	77.9 ± 1.8
mouse	2.8 ± 0.6	rabbit	58.1 ± 1.6
rabbit	1.7 ± 0.5	hamster	47.4 ± 3.0
rat	1.6 ± 0.4	rat	34.4 ± 1.4

[a/] ± S.D. [b/] ± S.E.

of microsomal oxidase activity if the differences between species are at least 2-fold.

In comparing the enzyme activities of Table III and IV with the cytochrome P-450 concentrations shown in Table II, it will be seen that there is often little correlation between the two parameters even though cytochrome P-450 is known to be the key enzyme in these oxidase systems. This has also been observed in studies of single species. Indeed, in view of our present understanding of this important hemoprotein, a direct relationship on the basis of cytochrome P-450 content alone would be surprising since this enzyme is known to exist in several forms with different substrate specificities.

A careful study of the liver enzyme (oxidases and esterases) involved in the activation and degradation of parathion in nine species failed to provide an explanation for the differing toxicities of parathion (17). This in spite of the fact that there were large differences, 20-fold in paraoxon formation and 8-fold in paraoxon degradation, among the species studied. The authors concluded that organs or tissues other than the liver must be involved in protecting the animal against such toxicants. Of course it is also possible that the target of the toxicant, the acetylcholinesterase system, also varied in sensitivity among the nine species.

The agreement between *in vitro* metabolism and toxicity in five mammalian and three avian species was also unsatisfactory in a study of the insecticide, diazinon (18). Only with the sheep, which can tolerate 2-50 times more diazinon than pigs, guinea pigs, cows, rats, turkeys, chickens, and ducks, was the toxicity relatively well correlated with *in vitro* metabolism. The authors concluded that extra hepatic metabolism was more important than liver metabolism.

Factors Affecting Comparisons--Sources of Error

There are several biological, experimental, and even environmental factors which can affect the performance of *in vitro* systems. Some examples which are to be found in the current literature include:

1. *Sex and age effects*: DeWaide (19) found that the variation in aminopyrine N-demethylase and aniline hydroxylase activity between individuals in four species of fish obscured any possible sex differences in the activities of these enzymes. Sex differences in microsomal oxidase activity are well known, however. For example, the rat and mouse exhibit sex

differences in kinetic constants (K_m and V_{max}) but the guinea pig, rabbit, and monkey do not (20). Another example is seen in the report of Whitehouse et al. (17) who found a sex difference in the desulfuration of parathion in two of nine species tested, guinea pig and rat, and in its hydrolysis in only one, the rat.

Differences in the rate of enzyme development are well known from studies of the common laboratory animals, hence similar characteristics should be expected in other species. Some investigators fail to take this into account in comparisons of invertebrates or of wild vertebrates, however, perhaps because they have no choice or because they are unaware of the possible effects of age differences. Extreme variations in microsomal oxidase activity can occur in insects of different stages of development as data from the author's laboratory (Figures 6, 7, 8) show. Similar observations have been made with the esterases which metabolize juvenile hormone analogs (21, 22) and with epoxide hydrase which metabolizes the juvenile hormone (23). Age dependent fluctuations in microsomal oxidase activity have also been seen in the house cricket (24) and the cockroach (25).

Species differences in the rate of development have been observed with the glucuronidation system (26). Chick embryos were almost as active in the glucuronidation of O-aminophenol at 12 days as in 6 week-old cockrels whereas the liver of the 16 day-old mouse fetus was negative and even the 10-day infant liver contained only 50% of the adult activity.

2. Source of enzymes: DeWaide (19) measured the apparent V_{max} and K_m for the *p*-hydroxylation of aniline of eight tissues, liver, kidney, heart, lung, gut, muscle, blood, and spleen in pigeon, rat, trout, roach, and crab (substituting hepatopancreas for liver in the latter). In the crab, trout, and roach, he also included the gill in these measurements. The liver was the most active source of enzyme in the four vertebrates, but the crab gill was considerably more active than its hepatopancreas. The kidney was nearly as active as the liver in the case of the trout and both the kidney and lung were important sources of the enzyme in the pigeon.

Pohl et al. (27) also found differences in the distribution of enzyme activity among the organs of different species. In the little skate, for example, the liver and kidney had approximately the same activity, per mg of microsomal protein, while in the winter flounder the liver was approximately 20 times as active as the kidney.

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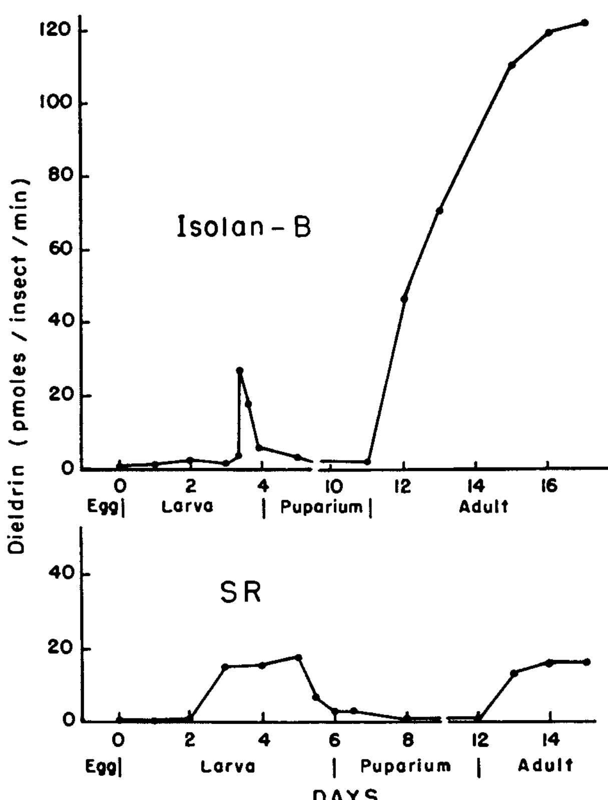
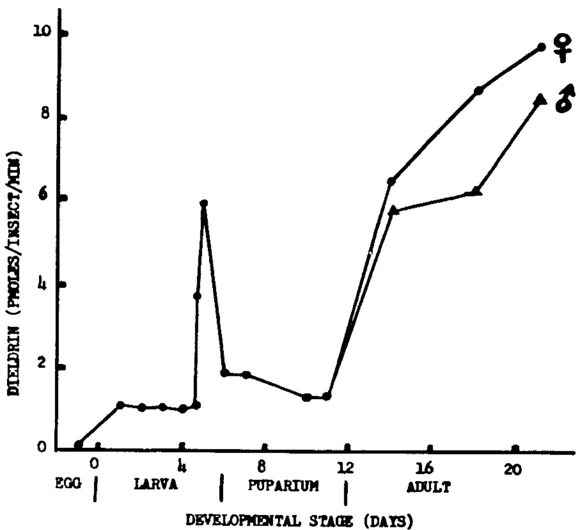


Figure 6. Microsomal aldrin epoxidases activity in the developmental stages of Isolan-B and SR house flies



Pesticide Biochemistry and Physiology

Figure 7. Microsomal aldrin epoxidase activity in developmental stages of the blow fly (39)

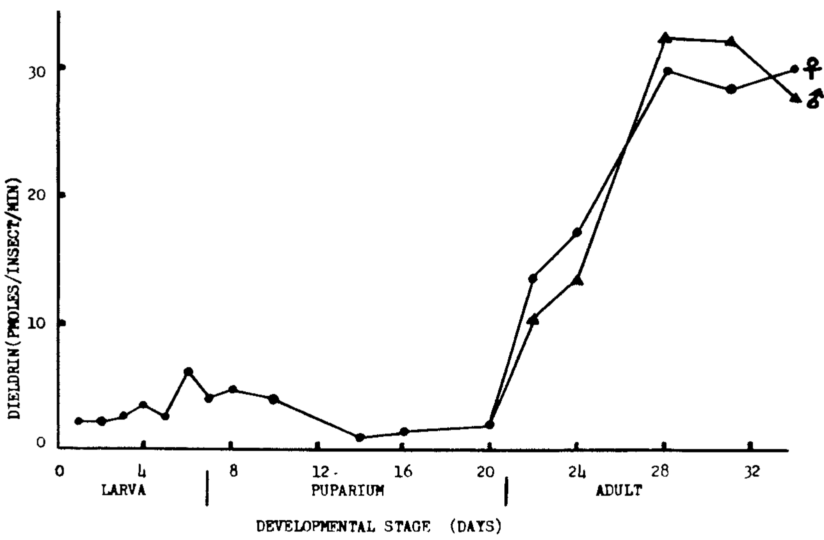


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Figure 8. Microsomal aldrin epoxidase activity in developmental stages of the flesh fly (39)

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There is considerable uncertainty about the site of mfo activity in invertebrate animals. Pohl et al. (27) were unable to detect hydroxylase or dealkylase activity in the hepatopancreas of the lobster or crab while Burns (28) did find some drug metabolizing activity in this tissue in the crab but found more in gill and in claw muscle tissue. DeWaide (19) also found more activity in the gill tissue of the crab than in the hepatopancreas.

Elmamlouk and Gessner (29) were also unable to detect hydroxylase or demethylase activity in the microsomal fraction of the hepatopancreas of the lobster but they did find some aniline hydroxylase and nitroreductase activity in the soluble fraction.

An interesting result in Burns' (28) study of aldrin epoxidase activity in various tissues of the fiddler crab was the high activity of claw muscle. In terms of specific activity, the gill microsomes were considerably more active than those of hepatopancreas or claw muscle but the total capacity of the muscle system was higher than that of gill and hepatopancreas combined, 3.6 nmoles per hour compared to 1.3 and 1.2 nmoles per hour for gill and hepatopancreas.

3. Cell fraction: In comparing the usual centrifugal fractions of liver homogenates of the rat and the trout, DeWaide (19) found that trout microsomes contained only 47% of the N-demethylase activity of the homogenate whereas the rat liver microsomes contained 79% of the activity. Even the process of centrifuging the 9000 xG fraction (containing microsomes and soluble fraction) at 100,000 xG for 1 hour, then remixing with a tissue grinder, resulted in a 31% loss of activity by the trout system compared to a negligible loss by the rat system. These results led DeWaide to use the 9000 xG fraction in his species comparisons. As he points out, this not only reduces errors due to the differential loss of activity in tests involving different species and tissues, but it simplifies the procedures by shortening the time involved and eliminating other opportunities for loss of activity.

Other observations support the use of the more inclusive intermediate fraction of tissue homogenates (e.g. the 9000 xG fraction, etc.). Buhler and Rasmussen (30) found that the nitroreductase activity of several species of fish was almost entirely in the soluble fraction while in mammalian species it is equally distributed between the microsomal and soluble fraction (31). Other examples of this kind are the report by Elmamlouk and Gessner (29) who observed that the aniline hydroxylase activity

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of lobster hepatopancreas homogenates was in the soluble fraction while nitroreductase activity was in both microsomal and soluble fraction.

Eight species were studied in a direct comparison of microsomal and whole homogenate fractions of the liver as the enzyme source for the desulfuration of parathion (17). The two systems agreed in the relative positions of the first five species but disagreed in the positions of the last three. The differences among these three were only about 2-fold, however.

The insect esterases which hydrolyze the juvenile hormone analogs are distributed between soluble fraction (20%) and microsomal fraction (40%) in the house fly (21) and flesh fly (22) while the distribution in the case of the blow fly is soluble fraction (60%) and microsomal fraction (20%) (22).

4. Enzyme stability: In comparisons between the rat and the roach DeWaide (19) subjected the 9000 xG supernatant fraction to various treatments prior to incubation with the substrate. When the enzymes were allowed to stand for 5 hrs at room temperature prior to assay the N-demethylating system of the roach lost 42% of its activity while that of the rat lost 14%. Similar treatment of the whole homogenate (used in the assay of p-nitrophenol glucuronidase) resulted in a 62% increase in the activity of the roach and a 16% increase in that of the rat. Other treatments (storage of the enzymes at $-15^{o}C$ for 15 hrs, repeated freezing and thawing of the enzymes, and ultrasonication) affected the N-demethylase and hydroxylase systems of the two species to approximately the same extent.

A difference in enzyme stability was also observed in a study of the effect of temperature on microsomal aldrin epoxidase in eight strains of the house fly (32). Microsomal oxidases of the World Health Organization Standard Reference Strain of flies could be incubated at $47.5^{o}C$ for 5 min and still retain 81% of their maximum epoxidase activity whereas seven other strains lost 62-90% of their maximum activity after such treatment.

5. Temperature of incubation: The error which can occur by the use of the wrong temperature in incubations of microsomal oxidase enzymes can be appreciated by a study of DeWaide's results in which the temperature-activity curves were determined for eight species, Figure 9. In all cases the region of maximum activity was narrow, about $5^{o}C$, dropping off sharply at temperatures outside this range. DeWaide concluded, in agreement with earlier studies by Adamson (6) and others, that the liver demethylase of birds (based on studies with the pigeon) should be incubated at $42^{o}C$, those of mammals at $37^{o}C$ and

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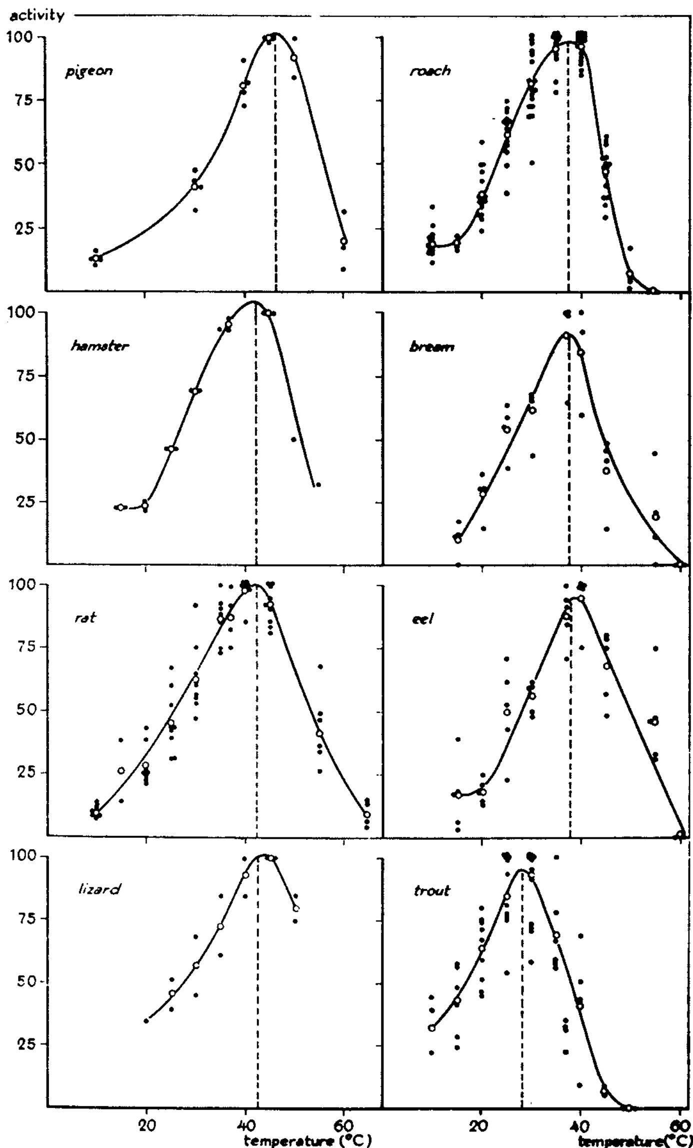

J. H. DeWaide (thesis)

Figure 9. N-demethylation of aminopyrine by 9000 g supernatants prepared from liver homogenates of various animal species. Activity was measured for 10 min at different temperatures. The values of each species were expressed in relation to the maximal value (numerically 100) (19).

those of fish at 25°C. These temperatures would not be optimum in every case, however, as was shown by further comparison of enzyme activities at these and at the specific temperature optima for each species. With some species the N-demethylase activity was as little as 46% that observed at the specific optimum. The differences were less in the p-hydroxylation of aniline where the operative temperature resulted in activities at least 64% of those obtained in the optimum range. Presumably it was not practical to conduct the incubations for each species at its optimum level.

Another example of a temperature effect during enzyme assay is mentioned by Bend (33). The liver O-dealkylase system of the little skate was much less active at both 41°C and 12°C than at 30°C.

Recent reports indicate that the choice of temperature for such studies is still open. Pohl et al. (27) incubated microsomes prepared from the livers of marine species or the hepatopancreas of crab and lobster at 30°C, while Burns (28) used temperatures of 14.5°C and 20°C for microsomes from the fiddler crab and Elmamlouk and Gessner (29, 34) incubated lobster hepatopancreas microsomes at 20°C. None of these authors provide data in support of their use of these temperatures.

DeWaide (19) also found a temperature differential in the stability of the N-demethylase system of rat and trout, the trout system remaining linear for a longer period (20-30 min) at incubation temperatures near its optimum, than that of the rat (5-10 min) when incubated at its optimum.

6. Kinetics: Aside from its value in characterizing enzymes, the kinetic constant K_m is useful in the determination of the optimum level of substrate to be used in measuring enzyme activity. As a general practice substrate concentrations should be at least twice the K_m to achieve maximum activity of the enzyme being measured. Frequently investigators comparing activity of an enzyme in different species use the same substrate concentration, apparently assuming that the K_m for the enzyme is the same in all species or that the substrate concentration chosen will exceed even the highest K_m. DeWaide's study (Table V) shows that this assumption is not always justified. The K_m values for N-demethylase varied 24-fold among the 16 species investigated (from 0.42 mM for the pigeon to 10 mM for the pike) and 200-fold for the hydroxylase system (0.05 mM for the rat to 10 mM for the white bream). The K_m for the glucuronidase system, compared in 11 species, varied much less, from 0.32 mM for the mouse and lizard to 1.3 mM for the eel.

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Table V. Apparent K_m-Values of Substrates for Hepatic Enzymes.[a]

	N-demethylation of aminopyrine[b]	p-Hydroxylation of aniline[c]	Gluduronidation of p-nitrophenol[d]
hamster	0.8	0.8	
mouse	1.3 ± 0.5	0.29 ± 0.04	0.32
rat	0.65 ± 0.20	0.05 ± 0.02	0.72 ± 0.10
hen	0.6		
pigeon	0.42 ± 0.07	1.31 ± 0.34	0.64 ± 0.14
lizard	3.23	1.67	0.33
frog	3.0 ± 0.7	2.4 ± 0.3	
bream	6.1	4.5	0.47
carp	1.5	4.2	0.83
tench		5.3 ± 1.3	0.53
white bream	7.6 ± 2.7	10	
roach	9.3 ± 0.6	2.4 ± 0.4	0.97
rudd	6.5	2.6	
rainbow trout	2.8 ± 0.5	0.48 ± 0.12	0.48
eel	3.5 ± 0.9	1.7 ± 0.3	1.3 ± 0.3
pike	10	4.0	0.67
sea lamprey	8.0 ± 3.5	5.5 ± 2.0	
wool-handed crab[e]		0.3	

[a] K_m-values in mM; means of 2 or more determinations; for animals of which the apparent K_m-values are determined by 4-10 determinations the standard error of the mean is given.
[b] Range of aminopyrine concentration used: 1.5-35 mM.
[c] Range of aniline concentration used: 0.1-22 mM.
[d] Range of p-nitrophenol concentration used: 0.14-1.4 mM.
[e] Assayed with 9000 g supernatant of gill homogenate.
Data from DeWaide (19).

Knowledge of apparent K_m and V_{max} values is important in another way, the interpretation of *in vitro* data in terms of the *in vivo* conditions. This was discussed by Castro and Gillette (20) in their studies of the *N*-demethylation of ethylmorphine by microsomes of rat, mouse, rabbit, guinea pig, and monkey. As they state, the differences in substrate concentrations between the *in vitro* and *in vivo* situation could obscure a difference in enzyme activity in one case or the other if the K_m is not taken into account.

7. Natural inhibitors of enzyme activity: Several of the early failures to detect enzyme activity in the tissues of insects were later found to be due to the presence of natural inhibitors which totally or partially inhibited the reactions. This is not too surprising since the small size of such animals prevents the use of specific organs or tissues. Thus the homogenizing of whole insects or major body segments might be expected to introduce catabolic enzymes into the subcellular fraction being assayed. This was the reason for low mfo activity in lepidopterous larvae (35), the house cricket (36), and the honey bee (37).

The inhibition of mfo activity in microsomes prepared from whole house flies was traced to eye pigments (38) and was corrected by removing the heads before homogenization. Such inhibitors are also present in the flesh fly and blow fly (39). Two natural inhibitors of house fly microsomal oxidases have been characterized by Jordan and Smith (40), one of these probably the eye pigment identified by Schonbrod and Terriere (38).

Another potential source of interference with microsomal oxidase activity in species comparisons is the lipid peroxidase system. A 15-fold difference in the activity of this system between rat liver and rabbit liver microsomes has been reported (41). This was sufficient to change the relative activity of the rat demethylase compared to that of the rabbit from 2.2 times (rat more than rabbit) to 3.4 times when the peroxidation was prevented by the addition of EDTA.

Pohl et al. (27) could not detect mfo activity in the hepatopancreas of lobster or crab, attributing this in part to the presence of digestive juices released during homogenization. These juices were found to be inhibitory of the hydroxylation of aniline and the *N*-demethylation of benzphetamine. However, the inhibitor had only a slight effect on the de-ethylation of 7-ethoxycoumarin. This observation could explain the failure of other workers (28, 29) to detect microsomal oxidase activity in the hepatopancreas of the crab and lobster.

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8. Environmental effects: During his study of the drug metabolizing activity of liver microsomes from fish species obtained from the Rhine River and its tributaries, DeWaide (19, 42) observed a seasonal variation in enzyme activity. Further investigation revealed that the *N*-demethylase activity of the roach (Figure 10) and the rudd (Table VI) collected during the summer months was more than twice that of specimens collected in the winter. The difference was not explained on the basis of liver weight or protein content or on differences in water temperature. It was concluded that the presence of enzyme inducing chemicals in the fishes' environment during the summer months was the main cause of these differences in enzyme activity.

There is now considerable evidence that some marine species are induced by such pollutants as crude oil and the PCB's (43, 44, 45). Increases in aryl hydrocarbon hydroxylase activities (benzo-(α)-pyrene as substrate) up to 4-fold have been noted (46). However, not all species are affected nor are the effects on the xenobiotic metabolizing enzymes the same as noted by Payne (47). For example, the aryl hydrocarbon hydroxylase was induced in the trout but *N*-demethylase was not affected. Neither enzyme was induced in the crab and lobster.

Yawetz (48) also raised this question on finding both DDE and PCB's in the tissues of six species of birds in the *in vitro* study of aldrin epoxidation. Residue concentration in heart tissue (liver was not analyzed) were 0.1-1.2 ppm for the two inducers.

There may also be an effect on enzyme activity by the environmental temperature of the species under study. DeWaide (49) found that trout and roach held at 5°C for 2 weeks had liver hydroxylase and *N*-demethylase activities about twice those of fish held at 18°C. In similar experiments with the hamster and the rat (temperatures 5°C and 23°C) the differences were about 1.5-fold or less.

9. Basis of comparison: Nearly all investigators of xenobiotic metabolism report metabolic activity in terms of the protein content of the fraction being assayed. This is based on the assumption that the preparative methods used will result in constant and reproducible amounts of enzyme protein. In comparative studies a second assumption is required--that the tissues or organs of the different species will yield the same proportions of active protein if the methods used are the same. There are good reasons to doubt this, however, considering the possibilities for variation in tissue constituents, especially non-specific protein and in physical texture (thus altering grinding conditions). These

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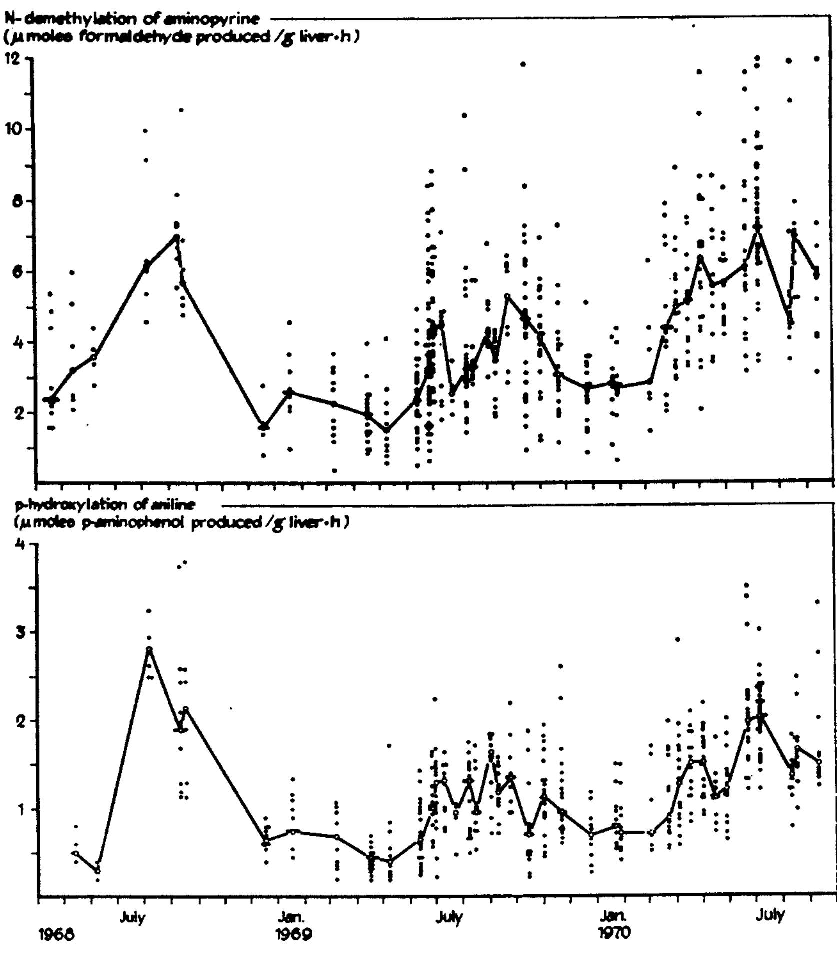

Figure 10. Seasonal variation in hepatic drug-metabolizing capacity in the wild roach. Measurements were performed with 9000 g supernatants derived from liver homogenates. Curves are drawn through the median values of the samples (19).

Table VI. Hepatic drug oxidation in the rudd at different times of the year

	Date of assay	
	March 12, 1970 (n = 25)	June 29, 1970 (n = 11)
N-demethylation of aminopyrine activity per[a/]		
g fresh liver	1.37 ± 0.52[b/]	3.60 ± 1.83
mg liver protein	0.0119 ± 0.0042	0.0379 ± 0.0197
mg liver DNA	0.63 ± 0.28	1.87 ± 0.89
100 g body-weight	2.40 ± 0.86	10.8 ± 6.3
p-Hydroxylation of aniline activity per[c/]		
g fresh liver	0.41 ± 0.17	0.87 ± 0.45
mg liver protein	0.0035 ± 0.0015	0.0093 ± 0.0046
mg liver DNA	0.19 ± 0.09	0.47 ± 0.25
100 g body-weight	0.72 ± 0.28	2.76 ± 1.67

[a] μmoles formaldehyde produced per hour.

[b] Means with standard deviation. The corresponding values of the two dates are significantly different at $P < 0.01$ (Wilcoxon two-sided two-sample test).

[c] μmoles p-aminophenol produced per hour.

Data from DeWaide (19).

factors could lead to differences in the amounts of specific and non-specific protein in the fraction finally assayed. Inasmuch as the methods used for protein determination do not distinguish between active and inactive protein, the chances for error are obvious.

The possible extent of the error can be seen in data from Pohl et al. (27) who found a 6-fold variation in the yield of microsomal protein per gram of liver in 12 vertebrate species. A variation in microsomal protein content is reported in a study of six species of wild birds (7.0 ± 1.3 to 12.6 ± 2.5 mg protein per g liver) (48) and for the rat, rabbit, mouse, hamster, and guinea pig (24.0 ± 3.9 to 35.9 ± 10.3 mg protein per g liver) (16).

DeWaide (19) compared the N-demethylation, hydroxylation, and glucuronidation activity of the 27 species in his study on the basis of liver weight, liver protein, liver DNA, and body weight. As might be expected, the drug metabolizing activities of the liver of the various species ranked differently when expressed in different parameters. In the N-demethylation of aminopyrine, the rat (average of 30 animals) was 14 times more active than the trout (average of 30 animals) when compared on the basis of liver weight, 10 times more active on the basis of liver protein, 12 times more active on the basis of liver DNA, and 35 times more active on the basis of body weight. In the hydroxylation of aniline, the multiples were 4, 3, 3, and 9 for comparisons on the basis of liver weight, liver protein, liver DNA, and body weight.

This problem has been discussed by Harper et al. (50). They prefer to compare activities between species on the basis of V_{max} rather than specific activities and they also show comparisons on the basis of cytochrome P-450 content (catalytic constant, k_{cat}). The benzene metabolism of liver and lung enzymes from rat, rabbit, and hamster is ranked according to these parameters in Table VII, compiled from their data. There is no agreement between relative activities based on cytochrome P-450 content and those based on protein content or V_{max}. The reason for this is now understood as mentioned earlier in this symposium.

Discussion

Three general conclusions emerge from the foregoing review of the use of *in vitro* methodology in species comparisons: 1) most of the problems occur in studies with a quantitative rather than a qualitative aspect; 2) the problems increase as the

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Table VII. Microsomal Benzene Hydroxylase Activity of Rat, Rabbit, and Hamster Compared in Three Ways

Microsomes from	Basis of Comparison		
	V_{max} [a]	Specific Activity [b]	K_{cat} [c]
rat lung	1.13 ± 0.29	0.49 ± 0.18	-
hamster lung	3.16 ± 0.35	2.41 ± 0.35	27
rabbit lung	10.36 ± 1.14	4.65 ± 0.44	18
rat liver	1.35 ± 0.55	1.09 ± 0.27	1.8
hamster liver	9.29 ± 4.03	4.26 ± 0.84	2.2
rabbit liver	3.86 ± 0.20	2.08 ± 0.29	1.8

[a] nmol/min/mg protein

[b] nmol/min/mg protein

[c] nmol/nmol cytochrome P-450/min

Data from Harper et al. (50)

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systems become more highly defined; and 3) the problems are more likely to occur in studies of invertebrates and wild vertebrates. It is also apparent that, in spite of the difficulties which have been identified, the method has produced much useful knowledge not available by other means, about species differences.

Much of the work which has been done so far in this area, especially that involving non-laboratory animals, would have been improved by more attention to the use of optimum conditions. A general recommendation for the future use of *in vitro* methods in comparative studies is that none of the experimental conditions or biological factors be taken for granted. This includes the pH and temperature of incubation, activity of the sub-cell fractions, substrate level, enzyme stability during preparation, use, storage, and enzyme source, the possibility of endogenous inhibitors, and the effect of age and sex. Some suggestions for dealing with some of these problems are as follows:

1. *Choice of in vitro system:* The choice of *in vitro* system to be used in the comparisons will depend on the objectives of the experiment. When metabolic activities are to be compared quantitatively, the more complete systems such as tissue explants and slices should be used. With these there is less danger of losing important enzymes and co-factors and the cellular organization of the constituents is preserved. A new approach to such systems is the intact or whole cell, usually prepared from the liver and thus known as the hepatocyte (51). This technique appears to offer several advantages over the liver slice or explant method, maintaining the organizational integrity of the original tissue without the artifacts introduced by mechanical injury to the cells and by abnormalities in the diffusion of substrate or oxygen. Some recent uses of this technique are described below.

It is not possible to prepare such fractions from small animals, either because methods are not known or because of the size of the tissue or organ. In these cases there seems no choice but to use a homogenate of the entire animal or a major body segment followed by mild centrifugation. Although partially disorganized, this system should contain all of the natural constituents required for the reactions under study. However, the investigators should check the requirement for supplemental co-factors such as NADPH.

More highly defined fractions such as microsomes or microsomal supernatant and subfractions of these are appropriate

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when the objective of the experiments is more qualitative in nature, such as the comparison of enzyme specificities, structure-activity relationships, effect of inhibitors and activators, kinetic studies, etc. In these cases the investigator is willing to sacrifice organization and completeness for better control of experimental conditions.

2. Temperature of incubation: The temperature for optimum metabolic activity should be determined for each of the species being compared. When this is not feasible, it is probably best to incubate enzymes from poikilothermic species at or in the temperature range of their natural habitat.

3. Enzyme kinetics: When the comparisons are being made with specific sub-cellular fractions such as microsomes or soluble fraction, it is important to determine the K_m for the substrate being studied in order that saturating concentrations can be used in each incubation. When tissue slices, tissue explants, hepatocytes, etc., are used, K_m determinations will have little value, since, presumably, the substrate concentration within the cell will be determined by permeability and diffusion properties characteristic of the tissues being studied. In these cases, it is important to maintain the substrate level of the medium at saturating conditions.

4. Age, sex, and source of animals: Whenever possible *in vitro* comparisons of metabolic activity should be made only after the relationship between sex, age, and developmental stage and enzyme activity have been determined. The experiments should then be designed to accommodate these variables. In the case of species obtained from the field, attention should be given to environmental background, especially to such factors as temperature range of the habitat and the possibility of induction of enzymes by environmental pollutants.

5. Endogenous inhibitors: The presence of inhibitors which prevent or reduce metabolic activity during *in vitro* assays should be assumed in lieu of evidence to the contrary whenever new species or new organs and tissues are being examined. This is particularly important in studies of small invertebrates where it is necessary to homogenize all or part of the animal in order to perform the assay. One method of detecting inhibition is to incubate the suspected fraction with one of known activity for evidence of inhibition of the latter.

6. Basis of comparison: The common practice of basing metabolic activity on the protein content of the fraction being assayed is probably the best which can be devised at present. However, sufficient data should be provided to enable others to

make their own evaluation of the activity of the tissues and organs chosen for study. This includes the yield of protein per unit weight of tissue, the total weight of organ, tissue, or animal, and the protein method used. When the species being compared are from different taxonomic groups at least one additional method of expressing activities should be provided for better cross-referencing between laboratories. With vertebrates and large invertebrates, activities should be related to the fresh weight of the tissues being studied and for small invertebrates, to the body weight.

It is regrettable that few investigators of the comparative biochemistry of xenobiotics use the same substrates in their studies. Often, of course, the special demands of the research would not permit this, but there are many cases in which "standard xenobiotics" could be used. Examples include effect of inducers and inhibitors, sex and age dependency of enzyme activity, relative enzyme activity, and the optimization of experimental conditions. The use of such standard substrates would greatly improve communication between laboratories and thus contribute to the value of the *in vitro* approach.

Usefulness of the *in vitro* method in species comparisons.

All of the merits of *in vitro* methods which have been discussed in the previous chapters apply as well to their use in species comparisons. These include improved control over experimental conditions, elimination of variables, isolation and study of specific systems, greater precision in measurements, more flexibility in design of experiments, and economies of time and labor. In addition, and in spite of the greater susceptibility to experimental error mentioned earlier in this chapter, the method has some special advantages in species comparisons. Some examples follow.

1. *Studies of metabolism in wild species*. *In vitro* methods have already been of value in drawing attention to the lower capacity of fish, reptiles, amphibians, and other wild species for metabolizing foreign compounds (3, 6, 19, 27, 30, 33, 43). It is not likely that this information could have been gathered so quickly and on such a large scale by any other means.

The difficulties of collecting or rearing wild animal species in sufficient numbers for *in vivo* studies and of treating such species with chemicals without introducing various stresses, almost eliminates the use of such methods of experimentation. In addition, with aquatic species, there may be problems with

collecting excreted metabolites for identification. Furthermore, these methods permit more use of the same animals since several metabolic systems could be studied with the same or different organs.

2. Metabolism of xenobiotics by humans: The *in vitro* method has obvious advantages in studies of the metabolism of foreign chemicals by humans. Without the direct exposure of human subjects, these methods could provide urgently needed information about metabolic pathways, identity of metabolites, effect of inhibitors, detection of interactions between chemicals, location of sites of metabolism, and the detection of age and sex relationships. Such information could be obtained only with difficulty by other means. In connection with similar knowledge of laboratory animals, the translation of results from animal experiments into decisions about human safety could be done with greater precision than at present.

3. Use in screening programs: The author is unaware of the extent to which *in vitro* methods are being used at present by the drug and pesticide industries. The techniques should be helpful in bridging gaps between synthesis of new compounds and their testing for efficacy with laboratory animals, insects, or plants. It should also be useful in gathering information on environmental safety. *In vitro* studies of metabolism by the experimental organisms might be helpful in determining the reasons for failures in toxicity, indicating whether this is due to inadequate uptake, lack of transport, or too rapid metabolism. The method is also useful in detecting unexpected metabolites or in producing metabolites free of interfering compounds.

Research needs.

The greatest obstacle to the expanded use of *in vitro* methods is the lack of evidence of their reliability in explaining and predicting *in vivo* events. This obstacle can be removed only by additional studies in which *in vivo* and *in vitro* methods are compared as in the experiments reported by Chipman et al. (10) and by Sullivan et al. (11, 12). The greater use of common substrates and toxicants (i.e. standard chemicals) by different laboratories would help in achieving this goal.

More work is needed on enzyme systems other than the microsomal oxidases which are important in the metabolism of xenobiotics. This includes the carboxyesterases which, along with the microsomal oxidases, account for most of the primary metabolism of drugs and pesticides. More information is also

needed on the glutathione-S transferases, epoxide hydrase, and other enzymes which are important in the secondary (Type 2) metabolism of xenobiotics. With knowledge of the distribution and properties of these enzymes in various species, the metabolism of xenobiotics can be conducted on a broader scale and thus improve the predictive value of the method.

Except for the common laboratory animals, there is a serious deficiency in our knowledge about the rate of development of xenobiotic metabolizing enzymes. It is quite likely that age and stage of development are important factors in these metabolic processes and, until the facts are known, it will be difficult to plan good experiments.

Immediate attention should be given to the use of the intact cell technique in comparative metabolism studies. Interesting results using rat liver cells have begun to appear and a recent report describes a system using both liver and kidney cells to reconstruct the entire metabolism of a drug from its oxidation and conjugation with GSH to the production of the corresponding mercapturic acid (52). These cells have been shown to resemble the microsomal oxidase system in enzyme activity and substrate specificity. They also appear to contain the conjugation systems of the liver (53-58), and to demonstrate the effect of inducers (52, 57, 59). Hepatocytes have also been prepared successfully from pig and human liver (60). Methods for the preparation of hepatocytes are described by Mouldes et al. (51).

Abstract

In addition to the usual doubts about the use of *in vitro* methods to replace or support those conducted *in vivo*, their use in comparative biochemistry encounters other uncertainties. These arise from the genetic, behavioral, morphological, and physiological differences among species. There is a need for evidence that, in spite of these special difficulties, *in vitro* methods can be reliable in detecting metabolic differences between species.

The best method of establishing the reliability of data obtained *in vitro* is to compare the results with those from *in vivo* experiments performed on the same species. Another test of reliability is to determine how different laboratories rank the same species in terms of their relative enzyme activity. Up to the present time there are only a few reports which permit the use of either of these methods of evaluation. A critical examination of such data indicates that reliable comparisons of xenobiotic metabolism can be made *in vitro* providing a number of precautions are taken.

In vitro studies of species differences are of two types, those in search of quantitative relationships and those seeking qualitative information. There will be fewer problems in studies of the first type if the investigator uses the more complete *in vitro* systems such as the tissue explant or slice or, in the case of small animals, the low speed centrifugal fraction of the tissue homogenate. When the experiments are qualitative in nature, the more highly defined but less complete sub-cellular fractions are recommended.

Special problems arise in the study of wild species. These include: lack of information regarding the relationship between enzyme activity and age or stage of development; sites of metabolic activity; species differences in temperature of optimum enzyme activity; the presence of endogenous inhibitors of metabolic enzymes; and unpredictable environmental effects on metabolic activity. Another problem encountered in all comparative work but most acute in studies of wild species is the lack of a suitable basis of reference for comparing metabolic activity. The practice of comparing activities on the basis of protein content appears to be the best that can be devised at present, but its limitations should be understood. It is recommended that an additional system of reference be used in most species comparisons.

Carefully planned *in vitro* experiments can be very useful in comparative studies, especially those involving wildlife species which are difficult to rear or manage. The methods should also be useful in screening programs for new drugs and pesticides and in studies of drug metabolism by humans.

Research needed to expand the use of these methods includes studies of the use of hepatocytes and other intact cells as substitutes for tissue explants and slices and for sub-cell fractions. Additional information on the age dependency of metabolic enzymes and on conditions affecting *in vitro* assays for other enzymes such as the carboxyesterases, epoxide hydrase, and the conjugating enzyme systems is also needed.

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