SYMPOSIUM ON METABOLISM

Introduction

Currently, a controversy rages in regard to the wisdom of the continued use of pesticide chemicals, especially the persistent ones being used for insect control. Many people urge that the persistent ones be minimized by replacing them with nonpersistent ones, by using nonchemical means of control, or by banning their use.

The persistence of pesticide chemicals is related to their volatility, their solubility, and their chemical stability on surfaces to which they are applied, in or on organisms which encounter them and in the environment. Also, the use of certain of these chemicals, in combination, often changes the degradation characteristics of one or more components of such mixtures. In recognition of these attributes and for certain other reasons, regulatory agencies now demand more and more complete information on the chemical fate of pesticide chemicals in or on plants and animals, and in the environment. Such data importantly relate to the acceptance and use of chemicals for pest control.

All organic pesticide chemicals, to a varying degree, are metabolized in living organisms and/or are photodecomposed in sunlight. The extent and nature of the transformations vary with the agent causing them and with the chemical in question, time and structure being important factors. The transformation of some of these chemicals takes place in a matter of minutes, while that of others requires months or years. The chemical reactions involved include hydrolysis, hydroxylation, oxidation, reduction, dehalogenation and desulfurization, ring opening, isomerization, and/or conjugation.

In the past two decades, much effort has been expended in a number of laboratories on the metabolism of insecticide chemicals and, especially in recent years, on the elucidation of the role played by enzymes in the metabolism reactions. An important part of this effort has been the work of John Casida and coworkers in regard to organophosphorus compounds, carbamates, and, more recently, rotenone, pyrethroids, and methylenedioxyphenyl synergists. This symposium recognizes this fact by the involvement of several former coworkers of Dr. Casida and by the inclusion of papers in the areas of research associated with him.

The papers presented at this symposium will be published in two parts. The first part—the address by John E. Casida after receiving the International Award for Research in Pesticide Chemistry at the Joint CIC-ACS Meeting in Toronto—appears in this issue. The other papers will appear in the November-December issue.

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Mixed-Function Oxidase Involvement in the Biochemistry of Insecticide Synergists

John E. Casida

Methylenedioxyphenyl (1,3-benzodioxole) compounds enhance insecticide chemical toxicity by inhibiting the mixed-function oxidase (mfo) system of microsomes. This synergist action is very important because it minimizes the amount of insecticide chemical necessary for insect control. The toxicity of pyrethrum, other pyrethroids, and certain methylcarbamates, organophosphates, and chlorinated hydrocarbons is synergized by such compounds, particularly when resistant housefly strains are involved. 2-Propynyl ethers and esters, benzothiadiazoles, and other new compounds of high synergistic activity probably also act as mfo inhibitors. Most of these compounds are mfo substrates as well as inhibitors, and it appears that they act by either serving as alternative substrates, sparing the insecticide chemical from detoxification, or by reacting with another site in the mfo system, preventing oxidative insecticide detoxification. Interactions with other toxicants or drugs metabolized by the mfo system sometimes occur in mammals treated with piperonyl butoxide or other insecticide synergists; however, these interactions in mammals are evident only at high synergist doses and the effect is of short duration. There is a need for synergists of increased effectiveness and safety, and it is important to include tests of mfo inhibition in mammals, along with the usual toxicological parameters, in evaluating the safety of such compounds.

Pesticide chemistry is a relatively new scientific discipline which has, in the main, been developed in the past three decades. Nevertheless, this field of chemistry is now a mature science and is contributing importantly to man's welfare. Not all of the studies in this area pertain to chemicals that are, by themselves, inherently toxic to the pest(s) being controlled; many deal with pesticide synergists which are compounds that are nontoxic or negligibly toxic alone, but which serve to enhance the toxicity of a pesticide chemical when they are combined. The development of synergists for use in pest control stems from the premise, common to all research in pesticide chemistry, that individual chemicals or combinations can be found that selectively and preferentially control pests without harm to man and useful species.

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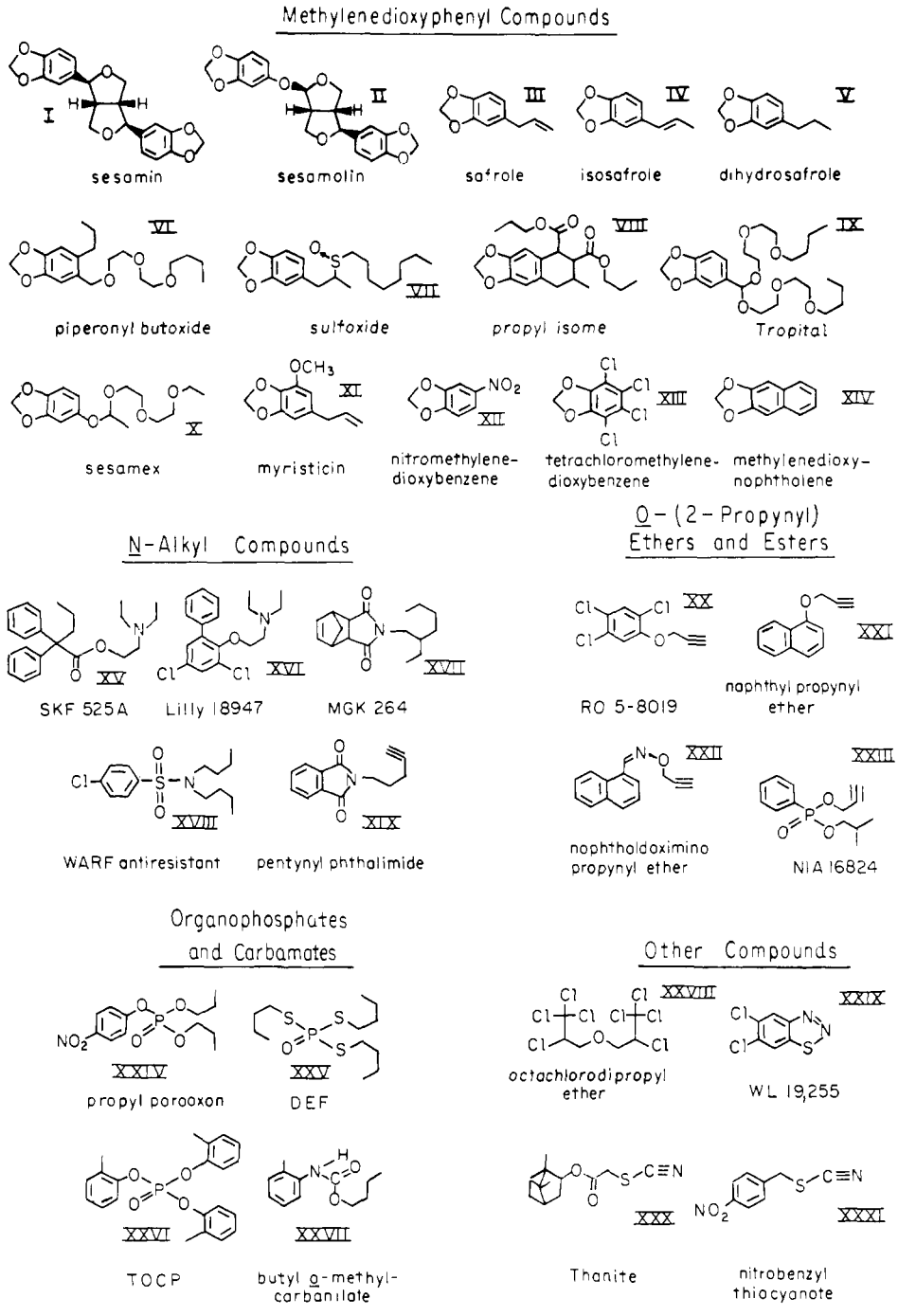

Figure 1. Structures of selected synergists for insecticide chemicals

The practical use of synergists is closely associated with pyrethrum because they greatly enhance the effectiveness of this expensive natural product. Only a relatively few compounds are used as synergists even though three decades of screening have revealed hundreds of candidate compounds of this type, not only for pyrethrum or other pyrethroids, but also for certain other insecticide chemicals (especially methylcarbamates).

This paper reviews methylenedioxyphenyl (1,3-benzodioxole) derivatives and other compounds that act as synergists for various insecticide chemicals and summarizes the known information on the metabolism of synergists by insects and mammals and on their mammalian toxicology. Also, it explores the mode of action of synergists, especially those that inhibit mixed-function oxidases and/or serve as substrates for these enzymes, and considers the effect they have on xenobiotic metabolism.

SYNERGIST CHEMICALS ENHANCING INSECTICIDE TOXICITY TO INSECTS

Structural formulae of selected synergists for various insecticide chemicals are given in Figure 1, along with the Roman numerals used to designate them in the text and tables. This figure also classifies the synergists in five categories. Of these, the methylenedioxyphenyl (MDP) compounds are the most important synergists from the standpoint of historical development, current use, and number of active compounds.

Sesame oil, one of the first synergists used commercially, is active because it contains sesamin (I) and the even more potent sesamolin (II) (Beroza, 1954a,b, 1955; Budowski and

Markley, 1951; Gersdorff *et al.*, 1954; Haller *et al.*, 1942a,b). It is interesting that sesamin is a natural component of pyrethrum extract, coexisting with the insecticidal pyrethrins and cinerins (Doskotch and El-Feraly, 1969). The synthetic MDP synergists currently in use are prepared from the natural product, safrole (III), by converting it initially to isosafrole (IV), dihydrosafrole (V), and other intermediates useful in preparation of the desired MDP product. Progress is being made in the development of alternative synthesis routes for the MDP moiety (Bonthrone and Cornforth, 1969) which are needed to overcome the supply limitation of safrole (less than 1.4 million lb annually; Hennessy, 1969). There are only four MDP synergists used commercially in the United States: piperonyl butoxide (pb) (VI) (Wachs, 1947, 1951; Wachs *et al.*, 1950), sulfoxide (VII) (Synerholm *et al.*, 1947), propyl isome (VIII) (Synerholm and Hartzell, 1945), and Tropital (IX) (Hopkins and Maciver, 1965; Maciver, 1966). Piperonyl butoxide, introduced in 1947, dominates the synergist field. Even though the patent rights on pb (Wachs, 1951) have expired in the United States, commercial use continues at an estimated level of 0.8 million lb per yr (Mrak, 1969). The methylenedioxyphenoxy compound, sesamex (X), is more active than the aforementioned synergists (Beroza, 1956; Fales *et al.*, 1957), but it is not being used commercially because, in the presence of sunlight and moisture, it decomposes to yield objectionable colored products. Among the other synthetic MDP compounds, piperonyl cyclonene was used in the U.S. until the early 1950's and bucarpolate and safroxan have had some commercial use elsewhere (Hewlett, 1968).

Much is known about the structure-synergistic activity correlations for MDP compounds. These data primarily involve houseflies as the test organism and either pyrethrum (Beroza and Barthel, 1957; Hewlett, 1960, 1968; Hopkins and Maciver, 1966; Metcalf, 1955; Moore and Hewlett, 1958) or carbaryl (Metcalf, 1967, 1968; Metcalf *et al.*, 1966; Moorefield, 1958; Moorefield and Weiden, 1964; Weiden and Moorefield, 1965; Wilkinson, 1965, 1967; Wilkinson *et al.*, 1966) as the insecticide chemical. It is unfortunate that the studies on pyrethroid and carbamate synergism are, in general, not appropriate for critical intercomparison because the studies were usually made by different investigators and varied with respect to methods of synergist and insecticide administration. It appears that a long, polyether- or oxygen-containing side chain is ideal for pyrethroid synergism, but this feature is less important or not critical for carbamate synergism. Myristicin (XI), which occurs in the edible portion of parsnips (Lichtenstein and Casida, 1963) and in cigarette smoke (Schmeltz *et al.*, 1966) as well as in nutmeg, mace, and parsley, is very active with carbaryl but not with pyrethrum. Other simple compounds, such as safrole, isosafrole, dihydrosafrole, methylenedioxynitrobenzene (XII), tetrachloromethylenedioxybenzene (XIII), and 2,3-methylenedioxynaphthalene (XIV) are very effective with carbaryl (Barnes and Fellig, 1969; Kuwatsuka and Casida, 1965; Metcalf *et al.*, 1966; Moorefield and Weiden, 1964; Weiden and Moorefield, 1965; Wilkinson, 1967; Wilkinson *et al.*, 1966).

Synergistic activity with insecticide chemicals is not restricted to MDP compounds because other materials are sometimes as active or even more active. These include, for example, compounds of varying synergistic potency with *N*-alkyl groupings such as: SKF 525A, as the free base (XV), and related compounds (Bates *et al.*, 1965; Hewlett *et al.*, 1961; Metcalf and Fukuto, 1965; O'Brien, 1961); Lilly 18947 (XVI) and related compounds (Fahmy and Gordon, 1965; Metcalf and Fukuto, 1965; Metcalf *et al.*, 1967; Moorfield, 1960; Moorefield and Tefft, 1959); MGK 264 (XVII, a commercial synergist) (Hartzell, 1949) and a related commerical compound, synepyrin 500 (Buéi *et al.*, 1963); WARF antiresistant (XVIII), which is particularly active as a DDT synergist (Fales and Bodenstein, 1961); and a *N*-pentynyl phthalimide (XIX) (Neumeyer and Incho, 1966). At present, there is interest in the synergistic activity of *O*-(2-propynyl) ethers and esters, including aryl ethers (XX and XXI) (Barnes and Fellig, 1969; Fellig *et al.*, 1970; Hennessy, 1969; Metcalf, 1968; Sacher *et al.*, 1968), oxime ethers (XXII) (Hennessy, 1969), and phosphonate esters (XXIII, NIA 16824) (Niagara Chem. Div., 1968); the —C≡C function need not be in a terminal position, at least with the aryl ethers (Hennessy, 1969; Sacher *et al.*, 1968). Some organophosphates, including dipropyl paraoxon (XXIV) (Oppenoorth and Van Asperen, 1961), DEF (XXV) (Plapp, 1969; Plapp and Tong, 1966; Plapp and Valega, 1967), which is normally used as a cotton defoliant, and TOCP (XXVI) (Metcalf and Fukuto, 1965), and carbamates such as compound XXVII (Plapp, 1969, 1970; Plapp and Valega, 1967) are synergists for selected insecticide chemicals. Octachlorodipropyl ether (XXVIII), a commercial synergist (Adolphi, 1958; Georghiou and Metcalf, 1961a; Shorey, 1961), and certain 1,2,3-benzothiadiazoles (XXIX) (Felton *et al.*, 1968) are synergists for pyrethroids and carbamates, while certain thiocyanates (XXX and XXXI) are carbamate synergists (Bakry *et al.*, 1968; El-Sebae *et al.*, 1964; Hewlett, 1969). Alkyl and aryl boronic acids are also effective carbamate synergists (Weiden and Moorefield, 1965).

INSECTICIDE CHEMICALS RESPONDING TO SYNERGISTS

Almost all types of organic insecticide chemicals are represented among the compounds synergized by pb (VI) or sesamex (X), at least with houseflies. Selected examples are given in Figure 2, along with the capital letters used to designate them in the text, and many others are mentioned by Brooks (1968a), Metcalf (1955, 1967, 1968), O'Brien (1967), and Wilkinson (1968a,b). However, a relatively few combinations of synergists and insecticide chemicals appear to lend themselves to practical use, that is, show a marked increase in insecticide toxicity with low synergist level.

The dramatic synergism of pyrethrum toxicity by pb is a result of varying degrees of synergism of the individual ester components; for example, the degree of synergism is higher in houseflies for the chrysanthemic esters than for the pyrethric esters (Sawicki, 1962a,b) and for the cinerolone esters than for the pyrethrolone esters (Incho and Greenberg, 1952; Sawicki, 1962a,b). The same general relationships are evident for sesamex and the individual pyrethrum constituents (Chang and Kearns, 1962). With allethrin, the degree of synergism, which is generally less than that with pyrethrin I, varies only to a small extent with the isomer involved (Gersdorff *et al.*, 1957; Gersdorff and Piquett, 1958), the most insecticidal isomer being that shown as A in Figure 2. Compounds formed by replacing the allyl group of allethrin with other substituents are synergized to a varying degree (Barthel, 1961; Hewlett, 1960). These relationships indicate that the alcohol side chain and the *trans*-methyl group of the isobutenyl moiety, but not the stereochemistry of the acid or alcohol moiety, are of great importance in this synergism phenomenon with pyrethrin analogs. The chrysanthemate, NRDC 104 (B), formed by substituting the 5-benzyl-3-furylmethyl group for the allethronyl moiety

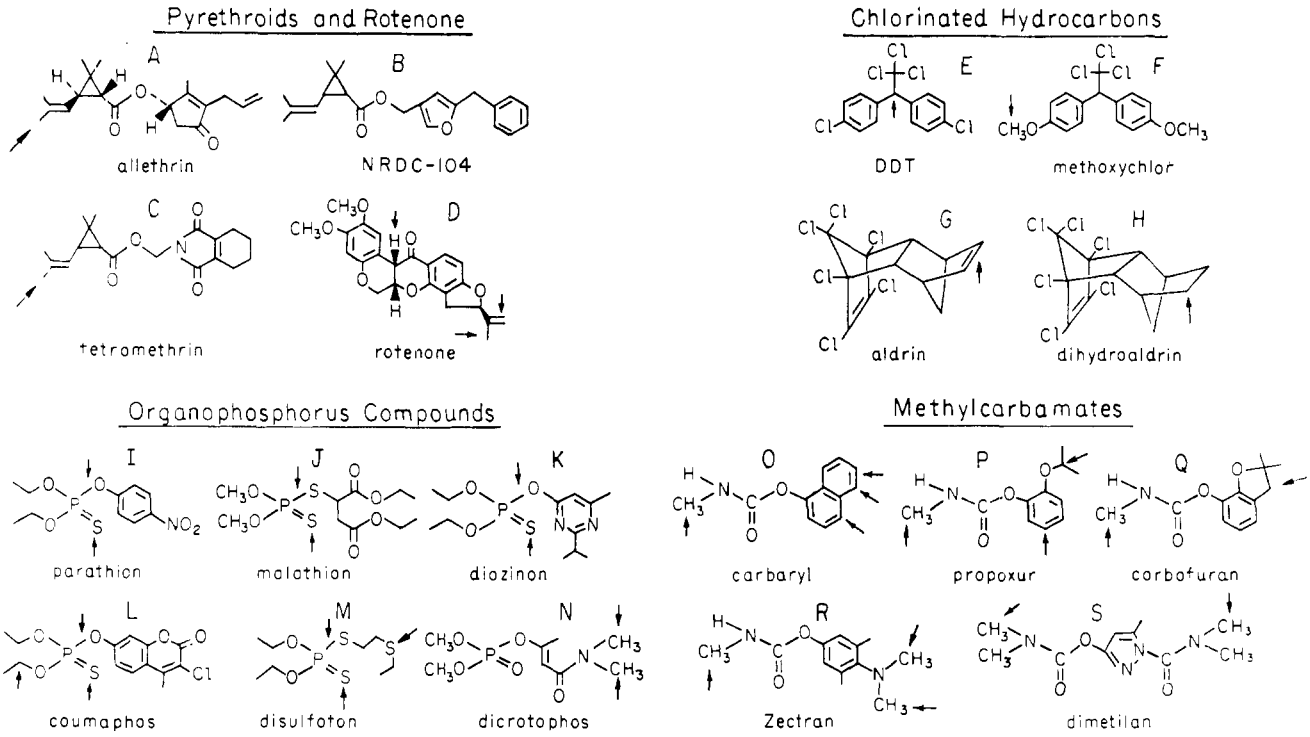

Figure 2. Structures of selected insecticide chemicals synergized in their toxicity to insects. The arrows indicate the oxidative (synergist-sensitive) sites of metabolic attack

of allethrin (A), is highly insecticidal, and little synergism occurs with the usual synergist levels (Elliott, 1967, 1969; Elliott *et al.*, 1967), even though high levels of pb increase the insecticidal potency of this compound (Berteau and Casida, 1969; Elliott, 1969). The high insecticidal activity of the tetramethylcyclopropanecarboxylates is also increased by high levels of pb, as is the lower activity of the amide and ketone analogs of these "pyrethroids" (Berteau *et al.*, 1968; Berteau and Casida, 1969). Tetramethrin (Neopynamin or phthalthrin) (C) is strongly synergized by an *O*-(2-propynyl) phosphonate (XXIII) (Niagara Chem. Div., 1968); so this appears to be a useful combination. There is also a small degree of synergism between tetramethrin (C) and NRDC 104 (B) (Hamuro, 1969). Rotenone (D) is a botanical insecticide which is synergizable in some insects (Brannon, 1947; Matsubara, 1953).

The toxicity of several chlorinated hydrocarbon insecticide chemicals is synergized by certain MDP compounds and other synergists. DDT (E) is synergized by (a) piperonylcyclonene in several DDT-resistant housefly strains (Perry and Hoskins, 1951); (b) pb, sesamex, piperonylcyclonene and DEF in the Fc strain of houseflies (Oppenoorth, 1965b, 1967; Oppenoorth and Houx, 1968; Plapp, 1969); (c) pb, sesamex, SKF 525A, and WARF antiresistant in *Triatoma* nymphs (Fine *et al.*, 1966; Morello, 1964); and (d) pb, sulfoxide, Tropital, sesamex, SKF 525A, and WARF antiresistant in a DDT- (and pyrethrum-) resistant but not in a susceptible strain of grain weevil and in a DDT-resistant but not (or less so) in a DDT-susceptible strain of flour beetle (Dyte *et al.*, 1965, 1966; Lloyd, 1969). Methoxychlor (F) is synergized by pb, sesamex, piperonyl cyclonene, and DEF in various housefly strains (Perry and Hoskins, 1951; Plapp, 1969; Sun *et al.*, 1967). The DDT analogs, TDE, Dilan and Perthane, are also synergized in toxicity to houseflies by sesamex and piperonyl cyclonene (Perry and Hoskins, 1951; Sun *et al.*, 1967). Methanobridged cyclodienes related to aldrin (G), such as dihydroaldrin (H) and many others, are strongly synergized in toxicity to houseflies by sesamex (Brooks, 1966, 1968a; Brooks and Harrison, 1963, 1964a,b, 1967; Khan *et al.*, 1970; Sun and Johnson, 1960; Sun *et al.*, 1967). It may be possible to minimize environmental contamination from residues of the currently-used persistent chlorinated hydrocarbons and still achieve insect control by using in their place a more rapidly degradable analog in combination with an appropriate synergist to enhance its insecticidal activity (Hennessy, 1969).

The toxicity of the organophosphorus compounds, parathion (I), methyl parathion, and malathion (J), to a susceptible housefly strain is either unaffected or antagonized by MDP synergists (Hadaway *et al.*, 1963; Sun and Johnson, 1960); however, pb synergizes malathion toxicity to a malathion-resistant housefly strain (Plapp, 1969). In contrast, the following organophosphorus compounds and many related materials exhibit a high degree of synergism with some MDP compounds, particularly with sesamex when tested on certain insecticide-resistant strains: diazinon (K), coumaphos (L), disulfoton (M), and dicrotophos (N) (Bull *et al.*, 1967; Hoffman *et al.*, 1954; Lewis, 1969; Lewis and Lord, 1969; Menzer and Casida, 1965; Metcalf, 1967; Oppenoorth, 1965b, 1967; Sun and Johnson, 1960, 1969; Sun *et al.*, 1967; Wilkinson, 1968a, b; Yasutomi and Keiding, 1969).

Methyl- and dimethylcarbamates are particularly interesting for synergist studies because many of them respond dramatically to synergists (Casida, 1963; Eldefrawi *et al.*, 1959; Fukuto *et al.*, 1962; Metcalf, 1967, 1968; Metcalf *et al.*, 1960; Moorefield, 1958, 1960; Wilkinson, 1968a,b). The enthusiasm for using a synergist with carbamates results partially from the low synergist-to-insecticide ratios that are effective (Barnes and Fellig, 1969; Eldefrawi *et al.*, 1959; Hennessy, 1969; Hewlett and Wilkinson, 1967; Metcalf *et al.*, 1966; Wilkinson, 1967). The toxicity of both

carbaryl (O) and propoxur (P), the carbamates most extensively studied in relation to synergist action, is markedly increased to houseflies, cockroaches, lepidopterous larvae, aphids, and to other species by pb and aryl 2-propynyl ethers (Barnes and Fellig, 1969; Brattsten and Metcalf, 1970; Fukuto *et al.*, 1962, Metcalf *et al.*, 1966; Moorefield, 1958; Shorey, 1961; Shrivastava *et al.*, 1969). There is considerable selectivity in the synergism phenomenon; so, the optimum synergist for one methylcarbamate is sometimes different than that for another (Fahmy and Gordon, 1965; Metcalf and Fukuto, 1965; Wilkinson *et al.*, 1966). The degree of carbamate synergism by pb in houseflies appears to be inversely correlated with the innate toxicity of the compound, providing it is a sufficiently potent cholinesterase inhibitor (Fukuto *et al.*, 1962; Kolbezen *et al.*, 1954; Metcalf and Fukuto, 1965; Metcalf *et al.*, 1960); thus, a highly potent cholinesterase inhibitor and toxic compound such as carbofuran (Q) is poorly synergized (Metcalf *et al.*, 1966, 1968). However, there are some marked exceptions where nontoxic, potent cholinesterase inhibitors that should penetrate the organism do not respond to synergism (Weiden and Moorefield, 1965). Synergism is also strain dependent such that the degree of synergism is inversely correlated with the unsynergized susceptibility of the strain, as shown with a variety of compounds including carbaryl (O), propoxur (P), and Zectran (R) (Eldefrawi and Hoskins, 1961; Georghiou, 1962; Georghiou *et al.*, 1961b; Metcalf and Fukuto, 1965; Shrivastava *et al.*, 1969). Some combinations of carbamate insecticide chemicals [such as carbaryl (O) and dimetilan (S)] show greater-than-additive toxicity to houseflies, and there is considerable specificity in this "analog synergism" (Gordon and Eldefrawi, 1960).

There have been many attempts to incorporate a synergistically active grouping (synergophore) into a molecule containing an insecticidally active grouping (toxophore) in order that it might serve as its own synergist. In addition, Fine and Molloy (1964) and Fishbein and Falk (1969) have proposed incorporation of the MDP moiety into drug molecules as a possible means of prolonging their action in mammals. Such attempts with insecticide chemicals have generally been unsuccessful, probably because incorporation of a synergophore also alters other conformational and potential binding properties of the molecule as well, but they possibly have been successful with the pyrethroids and methylcarbamates shown in Figure 3: the substituted-piperonyl esters of chrysanthemic acid such as Barthrin (a) (reviewed by Barthel, 1961); the pyrethroid (b) formed by replacing the isobutenyl moiety in allethrin with a MDP group (Takei and Takei, 1960; Takei *et al.*, 1962); molecules incorporating the 3,4-methylenedioxy group (c) or the 3-propynyloxy (d) or 2-propynyloxy group into phenyl methylcarbamate (Fukuto *et al.*, 1962; Metcalf, 1968; Metcalf *et al.*, 1960). There are related studies on compounds with juvenile hormone-like activity, two of which are shown in Figure 3. The terpenoid ether (e) containing the MDP group has very high morphogenetic activity (Bowers, 1969); this is of interest because some MDP synergists, particularly sesamex (X), show activity in the *Tenebrio* juvenile hormone assay and because this suggests that the juvenile hormones and the synergists act by inhibiting hydroxylation or oxidation (or both) to influence the metabolism of the molting hormone(s) (Bowers, 1968). Thus, the hormonemimetic substances may give enhanced hormone activity by acting as analog synergists rather than as compounds with intrinsic hormone activity (Dyte, 1969). The juvenile hormone-like activity of an *O*-(2-propynyl) phosphonate (f)

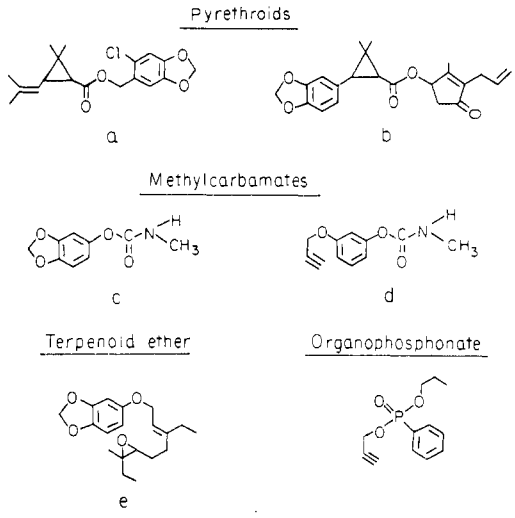

Figure 3. Structures of compounds with synergophoric and toxophoric or morphogenetic groupings

(Bowers, 1968) which has synergistic activity also (Niagara Chem. Div., 1967) and of many other synergists at higher dosages seems to support this suggestion. However, sesamex analogs lacking the MDP moiety (Redfern *et al.*, 1970) and aromatic terpenoid ethers closely related to the Bower's compound (e) but without the MDP group (Stauffer Chem. Co., 1969) are also potent morphogenetic agents, findings that weaken this line of reasoning. Other possible mechanisms for the morphogenetic activity of synergists are that they inhibit metabolism of the natural juvenile hormone(s), prolonging its action, or that they inhibit a critical hormone-induced protein- or enzyme synthesis; the latter property of synergists in blocking enzyme induction is considered later in a different context.

It is interesting to speculate on the possibility of achieving the combined action of synergists or toxicants naturally occurring in plants with the application of appropriate exogenous toxicants or synergists, respectively. For example, would insects feeding on the few plant species which contain high levels of synergistic MDP compounds, such as myristicin (XI) (see Lichtenstein and Casida, 1963), be more susceptible to insecticides than those feeding on plant species having low synergist levels? Also, would naturally occurring toxicants in plants which normally are not lethal to an insect feeding on the plant be made lethal by application of a synergist (Dyte, 1967a,b, 1969)? Although of theoretical interest, this latter possibility does not appear to be useful in insect control, based on tests with southern armyworm larvae (Wilkinson, 1970).

METABOLISM OF SYNERGISTS

The metabolic fate of MDP synergists is partially understood as a result of radiotracer studies involving compounds prepared with C^{14} in the methylene position of the MDP moiety (M-C^{14}-DP compounds) (Kuwatsuka and Casida, 1965; Sacher *et al.*, 1969), C^{14} in the α-position of the side chain (Schmidt and Dahm, 1956), and H^2 in the aromatic ring (Sacher *et al.*, 1969). The investigations involved colorimetric analysis, gas-liquid chromatography (glc), and thin-layer chromatography (tlc) for metabolite detection and characterization, and utilized model compounds as well as commercial synergists. Much of the available information on the metabolism of MDP synergists is reviewed in two recent publications

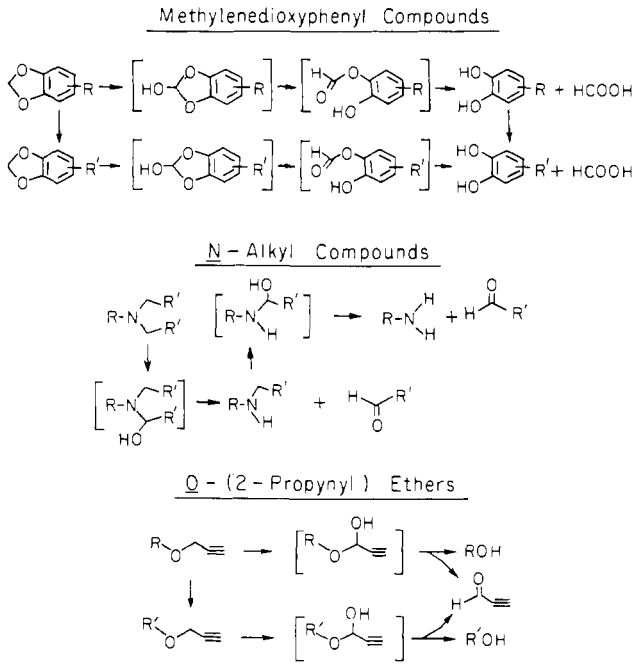

Figure 4. Generalized pathways of synergist metabolism

with emphasis on that occurring in mammals (Fishbein and Falk, 1969; Fishbein *et al.*, 1968).

Piperonyl butoxide-α-C^{14} (VI) is excreted by Madeira cockroaches in unidentified, water-soluble metabolites (Schmidt and Dahm, 1956). Houseflies metabolize pb (α-C^{14} and M-C^{14}-DP) by several alternative pathways involving cleavage at a methylene group adjacent to each oxygen, yielding a variety of alcohol-, catechol-, and carboxylic acid metabolites which are excreted either without conjugation or as glycosides, but the glycine conjugate of 6-propylpiperonylic acid is not formed (Esaac and Casida, 1969). When a large pb dose is fed orally by capsule to dogs, it is excreted unchanged to the extent of 78–88% in the feces within 48 hr, the urine containing little more than trace amounts of pb on the basis of colorimetric analysis (Sarles and Vandegrift, 1952). Mice and rats metabolize orally administered pb by extensive demethylenation and oxidation (70%) of the M-C^{14}-DP preparation to $C^{14}O_2$ and by formation of small amounts of 6-propylpiperonyl glycine; there are many unidentified metabolites, most of which lack the MDP grouping (Casida *et al.*, 1966; Kamienski and Casida, 1970). Intravenous administration of pb to rats yields a large number of unidentified metabolites in bile and urine as determined by tlc and selective chromogenic reagents (Fishbein *et al.*, 1967a) or by radioactivity from the α-C^{14} and M-C^{14}-DP preparations (Fishbein and Falk, 1969; Fishbein *et al.*, 1968, 1969). The lungs and fat are major storage depots for C^{14} from labeled pb (α-C^{14} and M-C^{14}-DP), the lungs containing up to 15% of the dose as unmetabolized material 8 hr after intravenous injection (Fishbein and Falk, 1969; Fishbein *et al.*, 1969). These findings and the commercial use patterns for pb indicate that the inhalation route needs to be used in further metabolic fate and storage studies.

Sulfoxide (VII) metabolism is not well understood but it is known that M-C^{14}-DP preparations of the two diastereoisomeric components (Kuwatsuka and Casida, 1965) are extensively converted to $C^{14}O_2$ in mice but less so in houseflies, and that oxidation of the sulfoxide group to the sulfone occurs, in part, before excretion of the metabolites by flies (Casida *et al.*, 1966, 1968; Esaac and Casida, 1969; Kamienski and Casida, 1970).

Tropital (IX) metabolism in houseflies, mice, rats, and hamsters involves less extensive conversion of the M-C^{14}-DP preparation to $C^{14}O_2$ than with pb or sulfoxide; this is so because the acetal group is rapidly cleaved by hydrolysis and/or oxidation to yield piperonylic acid, which is excreted as the glycine and glucuronide conjugates in mammals (Kamienski and Casida, 1970) and as conjugates with alanine, glutamate, glutamine, glycine, and serine in houseflies (Esaac and Casida, 1968, 1969). Following intravenous administration to rats, a large number of metabolites of labeled or unlabeled Tropital appear in bile and urine but, for the most part, their amounts and composition are not known (Fishbein *et al.*, 1967a,c, 1968). Butyl carbitol, the probable cleavage product from the acetal (and from pb), is converted to many unidentified metabolites after intravenous administration to rats (Fishbein *et al.*, 1967a, 1968).

The metabolic fate of other MDP compounds in houseflies, mice, rats, and humans involves cleavage of the MDP moiety (III, V, XI, XIV, and oxolinic acid), oxidation of the ring (XIV), and modification of the aliphatic side chain (III–V, XI, piperonal, piperonyl alcohol, piperonylic acid, and oxolinic acid) prior to excretion (Casida *et al.*, 1966, 1968; DiCarlo *et al.*, 1968a,b; Esaac and Casida, 1968, 1969; Fishbein *et al.*, 1967b, 1968; Kamienski and Casida, 1970; Sacher *et al.*, 1969).

Synergists other than the MDP compounds are metabolized in either mammals or insects as follows: 1-naphthyl 2-propynyl ether (XXI) suffers ether cleavage, naphthol conjugation, and ring oxidation in houseflies and mice (Sacher *et al.*, 1968); methyl oxidation, followed by cyclization to a saligenin cyclic phosphate, occurs with TOCP (XXVI) in houseflies and rats (Casida *et al.*, 1961; Eto, 1969; Eto *et al.*, 1963); octachlorodipropyl ether (XXVIII) is converted to a large number of unidentified metabolites in bile and urine following intravenous injection in rats (Fishbein *et al.*, 1968); benzyl thiocyanates (R-SC^{14}N) (XXXI and a related compound) are metabolized to $C^{14}O_2$ and unidentified products in houseflies (Bakry *et al.*, 1968).

Generalized metabolic pathways in living insects and mammals for synergistic MDP, *N*-alkyl, and *O*-(2-propynyl) compounds are given in Figure 4. For the most part, the same metabolites are also produced by appropriate enzyme systems *in vitro*. The microsomal mixed-function oxidase (mfo) system (discussed in some detail later) probably carries out the initial oxidative attack in the metabolism of most synergists. This is known to be true based on tlc, glc, and colorimetric studies with respect to: demethylenation of several MDP compounds (III, V–VII, IX, and XI–XIII) (Casida *et al.*, 1966; Esaac and Casida, 1969; Kamienski and Casida, 1970; Kuwatsuka, 1969; Wilkinson and Hicks, 1969); *N*-deethylation of SKF 525A (XV), Lilly 18947 (XVI) and related *N*-alkyl compounds (Anders and Mannering, 1966; Anders *et al.*, 1966); cleavage of an aryl 2-propynyl ether (XX) (Matthews and Casida, 1970a); cleavage of dipropyl paraoxon (XXIV) to *p*-nitrophenol (Nakatsugawa *et al.*, 1968); and hydroxylation of TOCP (XXVI) (Eto, 1969). The mouse liver mfo system has little or no activity in cleaving thiocyanates such as XXX and XXXI; the major metabolic pathway for many organic thiocyanates involves reaction with glutathione *S*-transferases to liberate hydrogen cyanide (Ohkawa and Casida, 1970). Thus, with only a few exceptions, all of the synergists listed in Figure 1 probably are substrates for the mfo system.

EFFECT OF SYNERGISTS ON XENOBIOTIC METABOLISM

Advances in biochemistry and in knowledge of detoxification mechanisms have led to the discarding of earlier theories on the mode of action of synergists (reviewed by Metcalf, 1955, and Hewlett, 1960) in favor of an action, at least for the MDP compounds in Figure 1, based on their ability to inhibit the mfo system in insects responsible for the oxidative detoxification of many insecticides. Synergist action of this type is not restricted to insecticides or insects because other foreign compounds (xenobiotics), including drugs, are sometimes involved, and, often, the synergists are active with mammals, too. These conclusions are based on findings from two types of experiments. In the first type, Table I, which involves studies with living organisms, the synergist is found to reduce the rate of xenobiotic detoxification (as determined by analyzing for the xenobiotic or its metabolites) or to enhance or prolong the xenobiotic action (toxicity, carcinogenicity, sleeping or paralysis time, etc.). The second type of experiment, Table II, involves enzyme assays for inhibition of insecticide or xenobiotic metabolism either (a) *in vivo*, in which the synergist is administered to the organism at prescribed intervals prior to removal of tissues or parts of the organism for mfo assay, or (b) *in vitro*, in which the synergist is added directly into the mfo system or a model enzymatic or nonenzymatic system. Related studies are reviewed by Mannering (1968a,b), with particular reference to drug action.

Table I summarizes the available information on the effects of insecticide synergists on xenobiotic metabolism in living insects and mammals; it also includes some studies with mammals but not with insects where prolongation or enhancement of the xenobiotic action is interpreted as resulting from inhibition of detoxification by the mfo system. No attempt is made to fully tabulate the effects of SKF 525 A (XV) on oxidative metabolism of drugs or toxicants other than insecticide chemicals, even though the early studies with SKF 525A (Axelrod *et al.*, 1954; Brodie, 1956; Cook *et al.*, 1954; Mannering, 1968a,b) laid the background for the subsequent studies with insecticide synergists.

The first indications that MDP synergists act in living organisms by blocking the oxidative metabolism of insecticides or other xenobiotics came from the experiments of Winteringham *et al.* (1955) and Sun and Johnson (1960) with houseflies, and Robbins *et al.* (1959) and Fine and Molloy (1964) with mice. The general nature of the phenomenon is now so well accepted that MDP compounds are commonly used in tests with insects and SKF 525A or MDP compounds in tests with mammals to determine if the test compound is metabolically unstable as a result of detoxification by oxidative mechanisms. For example, the increase in toxicity of propoxur (P) to houseflies in the presence of different concentrations of various synergists is directly related to a reduction in its rate of oxidative metabolism (Shrivastava *et al.*, 1969).

Much useful information on the toxicology of an insecticide chemical has been and can be gained by appropriate investigations with synergists. The potential potency of the toxicant, when the oxidative detoxification mechanisms are completely inhibited, can be determined by appropriate plotting of the LD_{50} values of the toxicant when combined with different amounts of synergists (Hewlett and Wilkinson, 1967; Wilkinson, 1967), a type of data which is available with many insecticide-synergist combinations (Barnes and Fellig, 1969; Hennessy, 1969; Hewlett and Wilkinson, 1967; Metcalf *et al.*, 1966; Wilkinson, 1967). Studies on the degree of synergism of a series of related insecticides may indicate the primary sites at which oxidative attack can occur (Berteau and Casida, 1969; Berteau *et al.*, 1968; Fahmy *et al.*, 1966; Fukuto *et al.*, 1962; Metcalf and Fukuto, 1965; Metcalf *et al.*, 1966; Sun and Johnson, 1969; Sun *et al.*, 1967). Topical administration, injection, and infusion experiments, in the presence and absence of synergists combined with graphical treatment of the data, can help to differentiate the relative significance of penetration and detoxification in structure-insecticidal activity relationships (Sun, 1968; Sun and Johnson, 1969). The involvement of oxidative detoxification in the species, sex, and age differences in susceptibility to toxicants can be determined by minimizing or overcoming these differences by using synergists in those cases where the variability relates largely to the rates at which insecticides or drugs are metabolized (Brattsten and Metcalf, 1970; Mannering, 1968a,b; Metcalf, 1968).

Synergist inhibition of oxidative xenobiotic metabolism can reduce or enhance toxicity, depending on the xenobiotic involved. Most insecticides and drugs are metabolized not only by oxidation or hydroxylation, but also by hydrolysis, conjugation, alkyl transfer, dehydrochlorination and/or other reactions; so, although synergist inhibition generally does not change the pathways of oxidative metabolism, it does alter the reaction rate and, at times, it appears to shift the significant detoxification reactions to nonoxidative mechanisms. Also, oxidation or hydroxylation reactions can form either products of reduced potency (detoxification) or enhanced potency (activation), the latter particularly with respect to desulfuration of the phosphorothionates; thus, the synergist can increase or decrease the toxicity of a chemical, depending on the shift in the balance of the competing activation or detoxification reactions caused by the presence of the synergist. For example, pretreatment of mice with pb or SKF 525A shortly before administration of selected organophosphates generally gives the following effects: decreased toxicity of dimethylphosphorothionates and *N*-alkylphosphoramidates; no effect on potency or increased toxicity with diethylphosphorothionates, dimethylphosphates, and diethylphosphates; an unexpected increase in toxicity of a diethylphosphorothiolate, amiton (for references, see Table I).

Table II gives the available information from enzyme studies on inhibition of the mfo system by synergists, under *in vivo* and *in vitro* conditions, in insects and mammals. It has now been established that most of the oxidative reactions occurring in insecticide chemical or xenobiotic detoxification under *in vivo* conditions are reproduced, at least in part, by the *in vitro* mfo system (Casida, 1969; Dahm and Nakatsugawa, 1968; Metcalf, 1967, 1968; O'Brien, 1967; Terriere, 1968a,b; Wilkinson, 1968a,b). This was first observed with the mammalian liver mfo system because of problems encountered in the preparation and *in vitro* manipulation of the corresponding enzymes from insects. Subsequently, these difficulties have been partially overcome by removing or minimizing the action of natural inhibitors in the insect preparations. As a result, it has now been possible to demonstrate that the reactions effected by the housefly and other insect mfo systems reproduce, in the most part, those caused by the liver mfo system and by the living organism (Benke and Wilkinson, 1970; Casida, 1969; Chakraborty *et al.*, 1967; Fukami *et al.*, 1969; Hook *et al.*, 1968; Krieger and Wilkinson, 1970a; Matthews and Hodgson, 1966; Nakatsugawa and Dahm, 1965; Price and Kuhr, 1969; Schonbrod *et al.*, 1965; Shrivastava *et al.*, 1969; Tsukamoto and Casida, 1967a,b; Wilkinson, 1968a,b).

Table I. Stabilization of Xenobiotics *in vivo* by Insecticide Chemical Synergists in Insects and Mammals

Xenobiotic	Organism	Synergist Used	Type of Assay[a]	Reference
		Studies Involving Insects		
Pyrethroid Insecticides				
Allethrin	housefly	VI–VIII, XVII, XXVIII, piperonyl cyclonene, safroxan	Chem. (H^3 and C^{14})	Bridges (1957); Hayashi *et al.* (1968); Winteringham *et al.* (1955)
Cinerin I	housefly	X	Chem. (C^{14})	Chang and Kearns (1964)
Pyrethrin I	housefly	X	Chem. (C^{14})	Chang and Kearns (1964)
Pyrethrins	housefly	piperonyl cyclonene	Chem. (C^{14})	Winteringham *et al.* (1955)
Tetramethrin	housefly	VI–VIII, XVII, XXVIII, piperonyl cyclonene, safroxan	Chem. (H^3)	Hayashi *et al.* (1968)
Chlorinated Hydrocarbon Insecticides				
DDT	housefly, Fc strain	X	Chem. (glc)	Oppenoorth (1967)
	Triatoma nymphs	VI, X, XV, XVIII	Chem. (C^{14})	Fine *et al.* (1966); Morello (1964)
	German cockroach	X	Chem. (C^{14})	Hoskins *et al.* (1958)
Aldrin	housefly	X	Chem. (glc and colorimetric)	Khan *et al.* (1970); Sun and Johnson (1960)
Chlorinated cyclodienes	housefly	X and others	Chem. (glc and C^{14})	Brooks (1966, 1968a,b); Brooks and Harrison (1963, 1964a,b); Khan *et al.* (1970)
Dihydroaldrin	housefly	X	Chem. (glc)	Brooks and Harrison (1966, 1969)
Organophosphorus Compounds				
Dicrotophos	housefly	X	Chem. (C^{14})	Hall and Sun (1965); Menzer and Casida (1965)
Hexamethylphosphoric triamide	housefly	IX	Chem. (C^{14})	Chang and Bŏrkovec (1969)
Malathion	flour beetle	VI, X, XV, XXVI, XXVIII, XXX, and others	Chem. (glc)	Dyte and Rowlands (1967)
Methyl parathion and two vinyl phosphates	housefly	X	Biochem. (ChE)	Sun and Johnson (1960)
Parathion	housefly	X	Chem. (S^{35})	Nakatsugawa *et al.* (1969)
	cockroach and cockroach gut	XV	Chem. (P^{32})	O'Brien (1961)
Phosphamidon analogs	boll weevil and homogenate	X	Biochem. (ChE)	Bull *et al.* (1967)
Schradan	cockroach gut	XV	Biochem. (ChE)	O'Brien (1961)
Methylcarbamate Insecticides				
Carbaryl	housefly	X	Chem. (C^{14})	Eldefrawi and Hoskins (1961)
m-Isopropylphenyl methylcarbamate	housefly	VI	Biochem. (ChE)	Georghiou and Metcalf (1961b)
Phenyl methylcarbamate	housefly	XXX	Chem. (C^{14})	El-Sebae *et al.* (1964)
Propoxur	housefly	VI, X, XI, XIV, XVI, XVII, XXI, XXX	Chem. and Physiol. (C^{14} and toxicity)	Metcalf *et al.* (1967); Sacher *et al.* (1968); Shrivastava *et al.* (1969)
Zectran	housefly	VI, XXX	Chem. (C^{14})	El-Sebae *et al.* (1964); Metcalf and Fukuto (1965)
Methylenedioxyphenyl Synergists				
Piperonyl butoxide	housefly	XVII, *p*-nitrophenyl dimethylcarbamate	Chem. (C^{14})	Esaac and Casida (1969)
Tropital	housefly	XVII, carbaryl, *p*-nitrophenyl dimethylcarbamate	Chem. (C^{14})	Esaac and Casida (1969)
		Studies Involving Mammals		
Botanicals				
Rotenone	mouse	VI, XVII, XX, XXIII, XXIX	Physiol. (toxicity)	Škrinjarić-Špoljar *et al.* (1970)
Organophosphorus Compounds				
N-Alkylphosphoramidates	mouse	XV, XVI	Physiol. (toxicity)	O'Brien (1961); O'Brien and Davison (1958)
Coumaphos	mouse	VI, XVII, XX, XXIII, XXIX	Physiol., Biochem. and Chem. (toxicity, ChE, P^{32})	Robbins *et al.* (1959); Škrinjarić-Špoljar *et al.* (1970)
Coumaphoxon	mouse	VI	Physiol. and Biochem. (toxicity, ChE)	Robbins *et al.* (1959)

Table I. (Continued)

Xenobiotic	Organism	Synergist Used	Type of Assay[a]	Reference
		Studies Involving Mammals		
Organophosphorus Compounds				
Diethylphosphorothionates	mouse	VI, XV	Physiol. (toxicity)	Kamienski and Murphy (1970); O'Brien (1961); O'Brien and Davison (1958); Welch and Coon (1964)
Dimethylphosphorothionates	mouse	VI, XV	Physiol. and Biochem. (toxicity, ChE)	Kamienski and Murphy (1970); Murphy and DuBois (1958); O'Brien (1961); O'Brien and Davison (1958); Welch and Coon (1964)
Paraoxon	mouse	XV	Chem. (P^{32})	O'Brien (1961)
Parathion	mouse and mouse liver slice	XV	Chem. (P^{32})	O'Brien (1961)
Methylcarbamate Insecticides				
Carbaryl	mouse	XV	Physiol. (toxicity)	Meksongsee *et al.* (1967)
Dimetilan	mouse	VI, X, XVII, XX, XXIII, XXIX	Physiol. (toxicity)	Hennessy, (1969); Škrinjarić-Špoljar *et al.* (1970)
Propoxur	rat	VI, XV	Chem. (C^{14})	Krishna and Casida (1966)
Drugs and Other Toxicants				
Hexobarbital	mouse	43 MDP cmpds., including I, III–X, XII; 18 related compds.; XV–XX, XXIII, XXV, XXVI, XXIX	Physiol. (sleeping time)	Anders (1968); Csillag *et al.* (1969); Epstein *et al.* (1969b); Fine and Molloy (1964); Fujii *et al.* (1968, 1970); Hennessy (1969); Kamienski and Murphy (1970); Škrinjarić-Špoljar *et al.* (1970)
	rat	VI, IX, X, XV	Chem. (glc)	Anders (1968)
Pentobarbital	mouse	VI, XV	Physiol. (sleeping time)	Fine and Molloy (1964); O'Brien and Davison (1958)
Zoxazolamine	mouse	43 MDP cmpds., including I, III–X, XII; 18 related compds.; XV and XIX	Physiol. (paralysis time)	Epstein *et al.* (1969b); Fujii *et al.* (1968, 1970)
Benzo[*a*]pyrene	mouse	VI	Physiol. (toxicity)	Epstein *et al.* (1967a); Falk and Kotin (1969)
	rat	III–VII, IX, XI, XXVIII	Chem. (C^{14})	Falk and Kotin (1969); Falk *et al.* (1965)
Freons 112 and 113	mouse	VI	Physiol. (toxicity and carcinogenicity)	Epstein *et al.* (1967a, b); Falk and Kotin (1969)
Griseofulvin	mouse	VI	Physiol. (toxicity)	Epstein *et al.* (1967a); Falk and Kotin (1969)

[a] Three types are considered: chemical (gas-liquid chromatography, colorimetric, or radiochemical), biochemical (enzymatic), and physiological (toxicity, sleeping or paralysis time, and carcinogenicity).

Mfo systems are present in many insects and vertebrates, and it is possible that they will be found to be ubiquitous in these organisms as improvements are made in the method of assay used to seek their presence in new species (Casida, 1969).

Almost all of the insecticide synergists shown in Figure 1 are now known to inhibit the mfo systems in insects and mammals as they act, sometimes selectively, on a variety of substrates. The arrows in Figure 2 point to the oxidative (synergist-sensitive) sites of attack on the insecticide chemicals depicted there, as they occur in living organisms (Table I) or in the mfo system (Table II). These two tables also give specific references to studies pertaining to the synergist-sensitive metabolic attack of *in vivo* and *in vitro* systems on various individual compounds; further information of a general nature is also given in recent reviews (Casida, 1969; Lykken and Casida, 1969; Menzie, 1969).

MECHANISMS OF MIXED-FUNCTION OXIDASE INHIBITION

There is sufficient information to speculate on the mode of action of MDP compounds and other synergists at the molecular level, but there are not enough data on which to base firm conclusions in regard to such action. Part of the difficulty arises from the limited understanding of the components of the electron transport chain of the mfo system. The scheme shown in Figure 5 (based on Estabrook and Cohen, 1969) indicates the importance, in the mfo system, of (a) two cytochromes, b_5 and P-450 (especially the latter), (b) two flavoproteins (fp), and (c) two reduced pyridine nucleotides (NADPH, primarily, and NADH, secondarily). The nature of the enzyme activation of molecular oxygen to an electrophilic and energy-rich species with six electrons, which is the substrate-attacking species, is not resolved (Ullrich and Staudinger, 1969), nor are the various stages of the oxygenation reactions and the configuration of the substrate binding site(s) on the enzyme surface. While these will undoubtedly be resolved in future studies, any speculations on synergist mode of action at a molecular level, at the present time, must necessarily be tentative. Also, there are difficulties in performing and interpreting kinetic studies with liposoluble substrates and lipid-containing enzyme preparations because of the complex nature of the partitioning and binding characteristics at specific and nonspecific sites. There are many potential rate-limiting steps

Table II. Inhibition of Microsomal Mixed-Function Oxidases Acting on a Variety of Substrates by Insecticide Chemical Synergists Administered Either to Living Organisms (*in vivo*) for Subsequent Enzyme Preparation and Assay or Directly to Enzyme or Model Systems (*in vitro*)

Substrate	Enzyme Source	Synergist Used	Details of Inhibition	Reference
		Mixed-Function Oxidases Inhibited *in vivo*		
Pyrethroid Insecticides				
Allethrin	fly abd.	VI (contact with deposit or topical)	maximum inhibition 2–4 hr after topical VI with recovery by 72–96 hr	Plapp and Casida (1969); Yamamoto *et al.* (1969)
Chlorinated Hydrocarbon Insecticides				
DDT	fly abd.	VI (contact with deposit)		Plapp and Casida (1969)
Aldrin	fly abd.	VI (contact with deposit)		Plapp and Casida (1969)
Organophosphorus Compounds				
Diazinon	fly abd.	VI (contact with deposit)		Plapp and Casida (1969)
Malathion	fly abd.	VI (contact with deposit)		Plapp and Casida (1969)
Methyl parathion	mouse liver	VI, IX	inhibition at 1 hr with VI and IX; stimulation at 48 hr with VI	Kamienski and Murphy (1970)
Parathion	mouse liver	VI, IX	inhibition at 1 hr; stimulation at 48 hr	Kamienski and Murphy (1970)
Methylcarbamate Compounds				
p-Nitrophenyl dimethylcarbamate	mouse liver	VI, XVII, XX, XXIII, XXIX		Škrinjarić-Špoljar *et al.* (1970)
Propoxur	fly abd.	VI (contact with deposit)		Plapp and Casida (1969)
Drugs and Other Compounds				
Aminopyrine	mouse liver	20 MDP cmpds., including III–X; 7 related cmpds.; XIX		Epstein *et al.* (1969b); Jaffe *et al.* (1968)
Aniline	mouse liver	VI–X, XV, XVII, XVIII, XX, XXIII, XXV, XXVI, XXIX		Škrinjarić-Špoljar *et al.* (1970)
Biphenyl	mouse liver	VI–VIII, X, XIX	transient stimulation of *o*- and inhibition of *p*-hydroxylation	Epstein *et al.* (1969b); Jaffe *et al.* (1969a, b)
Hexobarbital	mouse liver	20 MDP cmpds., including III–X; 7 related cmpds.; XV, XVII–XX, XXIII, XXV, XXVI, XXIX		Csillag *et al.* (1969); Epstein *et al.* (1969b); Jaffe *et al.* (1968); Škrinjarić-Špoljar *et al.* (1970)
p-Nitroanisole	mouse liver	VI, XXIII, XXIX		Škrinjarić-Špoljar *et al.* (1970)
		Mixed-Function Oxidases Inhibited *in vitro*		
Botanicals				
Rotenone	carp and rat liver, cockroach fat body	V–VII, XV, XVII, synepirin 500		Fukami *et al.* (1969); Kuwatsuka (1969)
Chlorinated Hydrocarbon Insecticides				
Aldrin	pig and rat liver	17 MDP cmpds., including X, XII and XIV; XV, XXVIII and 5 other mfo inhibitors; dieldrin, dihydroaldrin, heptachlor epoxide, γ-hexachlorocyclohexane (γ-BHC)	curvilinear with X and related cmpd. (may be 2 enz., one inhibited competitively the other noncompetitively); competitive with dihydroaldrin and γ-BHC	Brooks (1968b); Brooks and Harrison (1966, 1969); Lewis *et al.* (1967); Nakatsugawa *et al.* (1965); Wong and Terriere (1965)
	rabbit liver	VI, XV, parathion, γ-BHC, dieldrin, heptachlor, heptachlor epoxide		Nakatsugawa *et al.* (1965)
	mouse liver	X, XIV, XV		Wilkinson and Hicks (1969)
	whole housefly	19 MDP cmpds., including X, XII and XIV; XV, XXVIII and 5 other mfo inhibitors; dieldrin, dihydroaldrin and γ-BHC	curvilinear with X (may be 2 enz., one inhibited competitively, the other noncompetitively); competitive with dieldrin, dihydroaldrin and γ-BHC	Brooks (1968b); Brooks and Harrison (1969); Khan *et al.* (1970); Lewis *et al.* (1967); Ray (1967)
	cricket Malpighian tubules	XV, XX, XXIX; 5,6-Cl_2-MDP		Benke and Wilkinson (1970)
Chlorinated cyclodienes	pig and rat liver, whole housefly	MDP and other mfo inhibitors		Brooks (1966, 1968a,b); Brooks and Harrison (1967)
Dihydroaldrin	pig liver, whole housefly	X, XV, XXVIII and 5 other mfo inhibitors; dieldrin, γ-BHC	competitive in housefly (dieldrin and γ-BHC)	Brooks (1968b); Brooks and Harrison (1966, 1969)

		Mixed-Function Oxidases Inhibited *in vitro*		
Chlorinated Hydrocarbon Insecticides				
Dihydroisodrin	cricket Malpighian tubules	XV, XX, XXIX; 5,6-Cl_2-MDP		Benke and Wilkinson (1970)
Heptachlor	rabbit liver	VI, XV, parathion, aldrin, dieldrin, heptachlor epoxide, γ-BHC		Nakatsugawa *et al.* (1965)
Organophosphorus Compounds				
Amiton	rat liver	XV		Scaife and Campbell (1959)
Coumaphos	rat liver	VI, XV, XVII, 1 other mfo inhibitor		Dahm *et al.* (1962)
Diazinon	whole housefly	X		Lewis (1969); Lewis and Lord (1969)
Dimefox	rat liver slice	XV		Fenwick *et al.* (1957)
EPN	rat liver slice	XV		Murphy and DuBois (1956)
Guthion	rat liver	VI, XV, XVII, 5 other mfo inhibitors		Dahm *et al.* (1962); Murphy and DuBois, (1957a, b)
Hexamethylphosphoric triamide	housefly	VI, IX, X, XV		Akov *et al.* (1968)
Methyl parathion	rat liver	VI–VIII, X, XV, XVII, 4 other mfo inhibitors		Dahm *et al.* (1962)
Parathion	rabbit and rat liver	VI–VIII, X, XV, XVII, XVIII		Davison (1955); Murphy and DuBois (1956); Nakatsugawa and Dahm (1967)
	cockroach fat body	VI–VIII, X, XVII, XVIII	competitive with XVIII; partly competitive and partly progressive with VI and X	Nakatsugawa and Dahm (1965)
Schradan	rat liver	XV		Davison (1955)
Methylcarbamate Compounds				
Banol	cockroach fat body	VI, X		Gemrich (1967)
Carbaryl	rat liver	VI, XV, XVI and related cmpd., XVII		Leeling and Casida (1966)
	blowfly larval fat body	5-Me_2N-6-NO_2-MDP		Kuhr (1968); Price and Kuhr (1969)
p-Nitrophenyl dimethyl- and diethylcarbamates	rat liver	VI, X, XV, XVII		Hodgson and Casida (1960, 1961)
Propoxur	housefly abd.	VI		Shrivastava *et al.* (1969)
Methylenedioxyphenyl Synergists				
Tetrachloromethylenedioxybenzene	mouse liver	X, XIV, XV		Wilkinson and Hicks (1969)
Drugs and Other Compounds				
Aniline	mouse, rabbit, and rat liver	IV–VII, IX, X, XII, XIV, XV; allethrin, carbaryl, rotenone		Anders (1968); Kuwatsuka (1969); Wilkinson and Hicks (1969)
Biphenyl	mouse liver	VI–VIII	stimulation of *o*- and inhibition of *p*-hydroxylation	Epstein *et al.* (1969a); Jaffe *et al.* (1969a)
Ethylmorphine	rat liver	VI, IX, X, XV	competitive with VI; variable with IX and X	Anders (1968)
Naphthalene	whole housefly	14 MDP cmpds., including III, IV, VI–VIII, and X; XV	competitive with X	Philleo *et al.* (1965)
p-Nitroanisole	pig liver	5,6-Br_2-MDP	competitive	Lewis *et al.* (1967)
	rat liver	VI, IX, X, XV	competitive with VI; variable with IX and X	Anders (1968)
p-Nitrotoluene	rabbit and rat liver, housefly abd.	VI–VIII, X, XV		Chakraborty and Smith (1967)
		Model Systems Inhibited *in vitro*		
Insecticide Chemicals				
Aldrin	soil and soil microorganisms	VI–VIII, piperonylcyclonene		Lichtenstein *et al.* (1963)
	Fe^{++}-EDTA-H_2O_2-bovine serum albumin	XII and 8 other MDP cmpds.	competitive	Marshall and Wilkinson (1970)
Parathion	Fe^{++}-EDTA-ascorbate	VI–VIII, X, XVII, XVIII		Nakatsugawa and Dahm (1965)
Other Substrates				
Catechol	fly micr.	6 MDP cmpds., 16 thiocyanates	noncompetitive with XXXI and *p*-nitrophenylthiocyanate	Bakry *et al.* (1968); Metcalf *et al.* (1966)
	mushroom tyrosinase	31 MDP and related cmpds., 16 thiocyanates; XXIX and related cmpd.	noncompetitive with methylenedioxybenzene, XXXI, and *p*-nitrophenylthiocyanate	Bakry *et al.* (1968); Felton *et al.* (1968); Metcalf *et al.* (1966)
Cresol	fly abd. homog., sol. frac., purified	8 MDP cmpds.		Metcalf *et al.* (1966)
Endogenous lipids	pig liver micr.	6 MDP cmpds.		Lewis *et al.* (1967)

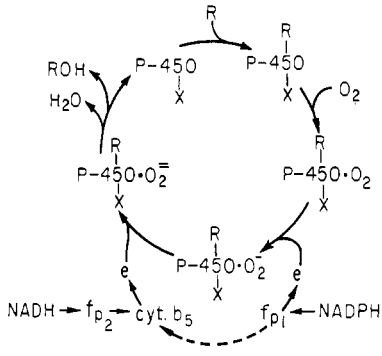

Figure 5. Microsomal electron transfer reactions occurring during drug or insecticide chemical (R) metabolism. [Based on Estabrook and Cohen (1969)]

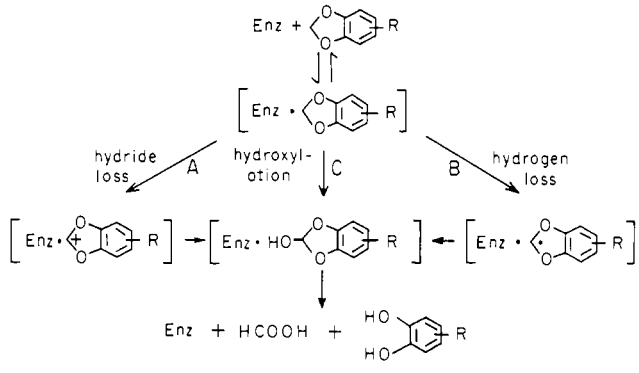

Figure 6. Possible reaction mechanisms for methylenedioxyphenyl compounds at the active site of the mixed-function oxidase system

between the electron donor and the substrate in these complex and unpurified systems. Thus, kinetic studies do not clearly define whether the synergists and related compounds act competitively or noncompetitively in inhibiting the mfo system; rather, the result depends on the type of measurement involved and the enzyme source and concentration, substrate, and inhibitor (Table II) (see Gillette, 1966). For example, SKF 525A (XV) acts as a competitive, noncompetitive or quasi-competitive inhibitor, depending on the hepatic microsome source (rats, mice, and rabbits) and the substrate used for assay (Kato and Takayanagi, 1966; Kato *et al.*, 1969).

The use of model systems for substrate degradation and inhibition studies (see Table II for some examples) minimizes some of the complications noted above but introduces the difficulty of interpreting the findings to the microsomal mfo system; no nonenzymatic model system developed to date adequately reproduces the unique characteristics of the microsomal mfo system (Ullrich and Staudinger, 1969). MDP compounds inhibit mushroom tyrosinase noncompetitively, possibly by interfering with the oxygen-activating step that normally leads to a reversible percupryl complex with molecular oxygen (Brooks, 1968b; Wilkinson, 1965). The soluble and microsomal tyrosinases from houseflies have been used in studies of methylcarbamate metabolism and its inhibition by synergists (Metcalf *et al.*, 1966) but this is not the important enzyme(s) in metabolism of these insecticide chemicals, the mfo system appearing to be of greater significance (Kuhr, 1968, 1969). Another type of model enzyme system may be available from microbial sources because it is known that MDP synergists inhibit the oxidation of aldrin to dieldrin by soil microorganisms (Lichtenstein *et al.*, 1963).

Despite the paucity of direct information on the enzymatic sites involved in the mfo system, there are useful structure-activity studies and physicochemical considerations which help to define the nature of the structural features on which synergistic action depends. Data on the relation of structure to synergistic activity and, particularly, the finding that replacement of the methylenic hydrogen atoms by deuterium or other substituents reduces potency indicate the importance of the methylenedioxy moiety as the site of binding or reaction (Hennessy and Whalen, 1966; Hewlett, 1960; Metcalf *et al.*, 1966; Moore and Hewlett, 1958; Moorefield and Weiden, 1964; Wilkinson, 1965; Wilkinson *et al.*, 1966).

Figure 6 shows three possible mechanisms by which the methylene group of the MDP moiety might interact with the microsomal mfo system. Mechanism A involves oxidative metabolism of the benzodioxole by hydride removal to form an enzyme-attacking electrophilic benzodioxolium ion which, as an acylating agent, may react with a nucleophilic group in the enzymatically-active site. This hydride-transfer mechanism, proposed by Hennessy (1965), is supported by a molecular orbital study (Cloney and Scherr, 1968). The benzodioxolium ion as an aromatic system may form a π-bonded complex with iron (or copper), or in other ways promote superior binding capacity with the microsomal complex (Hennessy, 1969). Mechanism B involves the homolytic cleavage of a hydrogen atom from the methylene group of the benzodioxole by a microsomal enzyme to generate a relatively stable free radical that acts as the inhibitor. Hansch (1968) proposes this mechanism based on a study of structure-activity relationships using substituent constants and regression analysis. Mechanism C involves enzymatic hydroxylation of the MDP compound at the methylene group to yield a hydroxymethylenedioxyphenyl intermediate, which decomposes to formic acid and the catechol which are identified as the products from synergist metabolism (Casida *et al.*, 1966; Kamienski and Casida, 1970; Wilkinson and Hicks, 1969). These pathways can go through a common intermediate because the hydroxymethylenedioxyphenyl compound is the pseudo base of the benzodiheterolium ion (mechanism A) and, on the other hand, the free radical enzymatic reaction to form the MDP·free radical (mechanism B) might act as a rate-limiting step in production of the hydroxymethylenedioxyphenyl compound (Hennessy, 1969). There is not sufficient information at present to further define which mechanism is involved in the inhibition, or if any one or all are operative.

Another explanation for MDP synergist action states that catechol derivatives, released on reaction of the MDP compound with the enzyme, compete with the insecticide at some limiting stage of metabolism (Hennessy, 1965), but this proposal is not supported by studies on the potency of the catechols compared with the MDP compounds as inhibitors of the rabbit liver microsomal mfo system (Kuwatsuka, 1969) and on a variety of catechols and benzoquinones as inhibitors of other mfo systems (Wilkinson, 1970). A modification of this proposal suggests that tyrosinases in housefly microsomes generate products (*i.e.*, benzoquinones) that inhibit mfo activity (Ray, 1967), but this proposal is also lacking in experimental support.

The MDP synergists may act as inhibitors by serving as alternative substrates for the mfo system, sparing the insecticide chemical from detoxification (Casida *et al.*, 1966; Kamienski and Casida, 1970; Wilkinson and Hicks, 1969). To do so, the synergist must, for optimal activity, have a higher affinity (lower Km) than the insecticide chemical

at the active site of the mfo enzyme, with the result that binding of the synergist precludes the binding and subsequent metabolism of the insecticide. Another requirement is that the relative turnover of the synergist must be lower than that of the substrate, or its presence at the site of metabolism will not be maintained long enough for a substantial effect. These conclusions, with supporting data, are considered in more detail by Rubin *et al.* (1964a), Wilkinson (1968b), and Wilkinson and Hicks (1969). This proposal does not necessarily require that the synergist and insecticide chemical bind at one and the same site in the mfo system inasmuch as different substrates could competitively inhibit each other's metabolism, even though metabolized by different enzyme components, because each of the enzymes involved, while reacting with a given substrate, would compete for the relatively limited supply of cytochrome P-450 (Mannering, 1968a,b).

The speculation that MDP synergists inhibit insecticide metabolism by acting as alternative substrates may be applicable to some but not all of the other types of synergists. Although the benzothiadiazoles could undergo oxidation to yield a stabilized cation similar to the benzodioxolium ion possibly resulting from MDP compounds, it seems unlikely that stable cation formation is the basis for the synergistic activity of the benzothiadiazoles (Felton *et al.*, 1968). The active benzothiadiazoles have several sites in their structure which may be susceptible to attack by the mfo system, but this remains to be proven experimentally. The aromatic propynyl ether (Sacher *et al.*, 1968) and thiocyanate (Bakry *et al.*, 1968) synergists possibly serve as chelators for or form coordination complexes with the iron (or copper) component of the metabolizing enzymes. However, as pointed out above, they are potential substrates for the mfo system. Alternative substrate inhibition has also been noted with the rat liver mfo system acting *in vitro* to *N*-demethylate ethylmorphine, sulfoxidize chlorpromazine, and metabolize hexobarbital and zoxazolamine (Rubin *et al.*, 1964a); also, under *in vivo* conditions with rats, ethylmorphine and codeine retard hexobarbital metabolism (Rubin *et al.*, 1964b). Many compounds related to SKF 525A (XV) and Lilly 18947 (XVI) competitively inhibit the *N*-demethylation of ethylmorphine and also undergo dealkylation; the fact that the Michaelis constants for the *N*-dealkylation of these compounds are similar to their inhibition constants towards ethylmorphine supports the view that they are acting by the alternative substrate mechanism (Anders and Mannering, 1966). Finally, several cases are known in which one carbamate synergizes the toxicity to insects of another carbamate or in which one pyrethroid enhances the toxicity of a second pyrethroid. Among the possible explanations for these cases of "analog synergism" is the involvement of a single enzyme or system in destroying two substrates, in such a manner that one serves to protect the other from detoxification as an alternative substrate.

Cytochrome P-450 is the oxygen-activating enzyme and the site of substrate interaction, and so it is important to consider this hemoprotein in relation to synergist action. Kuwatsuka (1969) interprets his studies of the difference spectra of the P-450 hemoprotein and of the kinetics of inhibition with various substrates, including MDP compounds, to indicate that the enzyme probably has different binding sites for different substrates and that the enzyme and MDP inhibitor interact in some relationship other than that of enzyme and substrate, *i.e.*, allosteric effects are involved. Several MDP synergists (VI–VIII and X) and an *N*-alkyl synergist (XIX) administered to mice or added to mouse liver microsomes possibly cause conformational interconversions or isozymic transformations in one or more mfo biphenyl-metabolizing sites leading to a stimulation of *o*- and an inhibition of *p*-hydroxylation (Epstein *et al.*, 1969a,b; Jaffe *et al.*, 1969a,b). Gillette (1966) finds that the unsubstituted amino analog of Lilly 18947 (XVI) combines with P-450 in a manner that prevents its reduction by NADPH, a type of reaction which should yield noncompetitive inhibition (Brooks, 1968b). A type I binding spectrum similar to that produced by hexobarbital is obtained with oxidized P-450 from mouse microsomes and several synergists (VI, XVII, XX, XXIII) but at low concentration WL 19255 (XXIX) produces an aniline-like type II spectrum (Matthews *et al.*, 1970). In interacting with reduced P-450, only WL 19255 of several synergists gives a 444 mμ peak; this peak is diminished on combination with type I substrates but not with aniline, and is replaced by the usual 450 mμ peak on treatment with carbon monoxide (Matthews *et al.*, 1970). Treatment of mice with several synergists (VI–X, XV, XVII, XX, XXIII, XXV, XXIX) results in a decrease in enzyme activity frequently accompanied by a decrease in the absorbance properties of the carbon monoxide and ethyl isocyanide ligands of the reduced microsomal P-450 (Škrinjarić-Špoljar *et al.*, 1970). When administered to mice, propyl isome (VIII) and RO 5-8019 (XX) but not several other synergists result in a shift in the ratio of the 430 and 455 mμ peaks produced on treating reduced P-450 with ethyl isocyanide (Škrinjarić-Špoljar *et al.*, 1970). There is also other evidence, with rabbits, that a single cytochrome is capable of interacting with two different types of substrates, causing a modification in the spectral properties of the hemoprotein (Hildebrandt *et al.*, 1968). In houseflies, topical treatment with pb or sesamex markedly decreases the P-450 peak of microsomes as determined with carbon monoxide and dithionite; the inhibition is transitory, correlating with the time at which the synergists are effective in increasing propoxur toxicity, leading to speculation on a cause-and-effect relationship and that the recovery phase is due to synergist metabolism and removal or *de novo* synthesis of P-450, or both (Perry and Buckner, 1970). SKF 525A and WARF antiresistant (XVIII) which are less synergistic or are inactive give less or no effect on housefly P-450 (Perry and Buckner, 1970). It is clear that many synergists as well as substrates for the mfo system combine with P-450 directly or at least with sites which alter the structure around the heme group. Further progress in defining these interactions may be possible when procedures are developed for isolating P-450 in an enzymatically-active form from microsomes.

The field of interactions goes well beyond the scope of the inhibition of mfo systems and, even, of detoxification mechanisms; however, it is of interest that so much of the information on interactions accumulated in recent years with insecticide chemicals can be explained solely on the basis of the mfo system.

SYNERGIST SPECIFICITY

Synergist potency varies markedly with the synergist, insecticide chemical (or xenobiotic), and the species involved, even when the synergist action results primarily from mfo inhibition. To have maximum effect, the synergist must (a) penetrate or enter the organism, and be transported to and accumulate at the mfo site (or other active site) as rapidly or more rapidly than the insecticide chemical, and, at the

active site, (b) must have a higher affinity and a lower metabolism rate than the insecticide chemical. Differences in any of these steps affects the selectivity of the synergists.

The apolar and fat-soluble nature of the known synergists favors their penetration through insect cuticle as compared with mammalian skin. With houseflies, the rate of penetration of MDP compounds, following topical application, differs markedly with variation in the ring substituents present (Wilkinson, 1967). Consequently, it is anticipated that the structure of MDP compounds will affect their rate of penetration in mammals as well. Of course, the dermal protective mechanism for mammals is no longer effective when the synergists are ingested, inhaled, or injected, although in these cases the synergist must still penetrate numerous internal membranes to reach its site of action in the liver. The acetal, Tropital (IX), is very acid-labile and, as such, it is probably decomposed, at least in part, to butyl carbitol and piperonal in the mammalian stomach prior to absorption; as expected, Tropital is a better inhibitor of benzo[*a*]-pyrene metabolism in rats following injection than after oral administration (Falk and Kotin, 1969). Distribution and localization differences remain largely unexplored because most of the available *in vivo* data does not adequately differentiate between localization of the synergist or insecticide and of their metabolites. An exception is pb, which is known to be long-lived in the lungs and fat following intravenous administration to rats (Fishbein and Falk, 1969; Fishbein *et al.*, 1969). Thus, penetration and distribution of the synergist in various organisms are important in determining its specificity but, unfortunately, there is a paucity of data in regard to these factors.

Persistence of the synergists is usually greater in insects than in mammals. The MDP compounds (III, V–VII, IX, XI, and others) are more stable in houseflies following injection at a high dosage level (Esaac and Casida, 1969) than in mice and some other mammals following oral administration (Kamienski and Casida, 1970). The naphthalene synergists with MDP (XIV) and *O*-(2-propynyl) (XXI) groupings are more readily metabolized in mice following oral administration than in topically treated houseflies (Sacher *et al.*, 1968, 1969). In general, the MDP synergists are considerably more resistant to enzymatic attack by insect microsomes than by those from mouse liver (Casida *et al.*, 1966; Esaac and Casida, 1969; Kamienski and Casida, 1970; Wilkinson and Hicks, 1969), but this is not the case with liver microsomes from some other mammalian species (Wilkinson and Hicks, 1969).

The mfo system is of low activity in some strains of houseflies (Schonbrod *et al.*, 1965, 1968; Shrivastava *et al.*, 1969; Tsukamoto, 1969; Tsukamoto and Casida, 1967a; Yamamoto *et al.*, 1969) and the synergists are relatively ineffective in enhancing carbamate toxicity to these strains. The magnitude of synergism of carbamate insecticide toxicity varies widely with different insect species, suggesting but not necessarily establishing a similar variation with species in the significance of the mfo system in detoxification (Brattsten and Metcalf, 1970). Several examples are as follows: little or no synergism by sesamex occurs for many carbamates in milkweed bugs (Jao and Gordon, 1969) although pb is effective with carbaryl in this species (Brattsten and Metcalf, 1970); MDP synergists are generally ineffective or of low activity in synergizing carbamate toxicity to honeybees (Brattsten and Metcalf, 1970; Metcalf, 1968); in a study involving 54 insect species, the Coccinellidae and some other beetles are unusual in that very little carbaryl synergism is observed with pb (Brattsten and Metcalf, 1970). Synergists are not effective in enhancing pyrethroid toxicity to milkweed bugs (Jao and Gordon, 1969) and mustard beetles (Chrysomelidae) (Elliott, 1969), in agreement with the findings on methylcarbamates. Isopropyl parathion is converted to the oxygen analog more efficiently by housefly slices than by honeybee slices, again indicating the low mfo activity of honeybees (Metcalf and Frederickson, 1965). Thus, although the activity of the mfo system appears to vary significantly with different insect species and strains, much needed information is lacking in regard to the effect of this variation on the activity of synergists.

There are differences between the mfo systems of insects and mammals that may contribute to synergist specificity. The mfo system of insect microsomes is frequently more sensitive than that of mammalian microsomes to inhibition by MDP and some other synergistic compounds (Brooks, 1968b; Brooks and Harrison, 1969; Chakraborty and Smith, 1967; Lewis *et al.*, 1967; Wilkinson and Hicks, 1969), but this is not the case with all synergists or insect microsome preparations (Fukami *et al.*, 1969). There are also differences in pH optima, and in the carbon monoxide-sensitivity and its light reversal of microsomal preparations prepared from the housefly and the mammalian liver (Kuhr, 1969; Ray, 1967). Housefly microsomes are high in tyrosinase and low in lipid peroxidase activity, as compared with pig liver microsomes, and these enzymes can either compete for cofactor, generate inhibitors, or, in other ways, alter the properties of the mfo activity (Lewis *et al.*, 1967). An endogenous inhibitor of lipid peroxidase is present in housefly but not in pig liver microsomes (Lewis *et al.*, 1967). The cytochrome pigments, particularly P-450, of housefly microsomes are generally similar to those of liver microsomes, based on assays with carbon monoxide and ethyl isocyanide (Matthews and Casida, 1970b; Perry, 1968; Perry and Buckner, 1970; Perry *et al.*, 1969; Ray, 1967). The concentration of cytochrome P-450 is generally lower in insect microsomes than in mammalian liver microsomes (Fukami *et al.*, 1969; Kuhr, 1969; Matthews and Casida, 1970b; Ray, 1967) but there are marked exceptions (Wilkinson, 1970). Comparison of various species and strains shows that the enzymatic activity of insect microsomes is not directly related with the P-450 content (Kuhr, 1968, 1969; Matthews and Casida, 1970b; Perry, 1968; Perry and Buckner, 1970; Perry *et al.*, 1969; Wilkinson, 1970) and that large losses in mfo activity on storage of certain insect mfo preparations take place without loss of P-450 content (Benke and Wilkinson, 1970; Krieger and Wilkinson, 1970b); however, it is possible that, in some cases, endogenous inhibitors prevent the potential enzymatic activity from being measured. In addition, these endogenous inhibitors combine in such a way with housefly microsomal P-450 that they reduce the absorbance of or inhibit the cytochrome (Perry and Buckner, 1970). Sex and strain differences in the P-450 content exist in houseflies (Matthews and Casida, 1970b; Perry and Buckner, 1970; Perry *et al.*, 1969) as well as in mammals. Piperonyl butoxide-treated houseflies have a higher apparent microsomal cytochrome b_5 level, when reduced with NADPH, and this is also evident on direct addition of pb and several other synergists directly to the microsomal preparations; this and other evidence indicate that one action of the synergists may be at the link between the interconnected P-450 and b_5 electron transport chains (Matthews and Casida, 1970b). It is obvious that further studies are needed, with MDP and other synergistic compounds, to elucidate the mechanism involved in those cases where the

insect mfo system is more sensitive than the mammalian mfo system to inhibition by synergists.

Even though the mfo systems of various organisms may be similar in their electron transport chain and utilization of cytochrome P-450 as the oxygen-activating enzyme, there is considerable opportunity for the synergist to exhibit specificity in reacting with the various components of this system. This is possible whether or not a single enzyme is involved in each species because there may be substrate-specificity differences between species resulting from subtle variations in the mfo system that do not alter the action on some substrates but do on others (*e.g.*, multiple binding sites, conformational changes, and allosteric effects). Such conditions may confer rate differences, between species, in the reaction of synergists and enzymes. There is considerable evidence that the liver mfo system consists either of a mixture of enzymes, each of which has P-450 as its prosthetic group, or of a common P-450 serving a number of enzymes or multiple binding sites (Gillette, 1966; Kuntzman, 1969; Kuwatsuka, 1969; Remmer *et al.*, 1968). The housefly mfo system also appears to involve more than one enzyme or site of substrate binding for metabolism based on kinetic studies (Lewis *et al.*, 1967; Ray, 1967) of a type which are not always conclusive with the mfo system, substrate-specificity investigations (Plapp and Casida, 1969; Schonbrod *et al.*, 1968) and demonstration of genetic control by two autosomes which can be isolated in separate strains or substrains (Khan, 1969; Plapp and Casida, 1969; Schonbrod *et al.*, 1968; Tsukamoto *et al.*, 1968). Further studies are needed, in relation to synergist specificity for acting with pyrethroids or carbamates, to determine whether or not the enzyme that detoxifies pyrethroids by ω-oxidation of the *trans*-methyl group of the isobutenyl moiety is the same as that detoxifying carbamates by *N*-methyl and ring hydroxylation (Esaac and Casida, 1969). There is some basis for speculating that the different enzyme components or binding sites of the mfo system react differently with the synergists but, to date, there is little direct support for this proposal.

Another factor in specificity may be differences in the ease of induction, as this relates to both synergists and insecticide chemicals. Generally, the magnitude of induction by synergists is low relative to that by other chemicals, particularly chlorinated hydrocarbon insecticides (Conney, 1967; Kuntzman, 1969). However, the liver mfo system is induced *in vivo* by MDP synergists after an initial inhibition phase (Falk and Kotin, 1969; Friedman and Epstein, 1970; Jaffe *et al.*, 1969b; Kamienski and Murphy, 1970; Škrinjarić-Špoljar *et al.*, 1970). In mice treated intraperitoneally, the magnitude of induction, of both P-450 and mfo activity is higher with compounds VI, XXIII, and XXIX than with XVII or XX (Škrinjarić-Špoljar *et al.*, 1970). It is likely that this induction of hepatic P-450 content is the result of δ-aminolevulinic acid synthetase induction because this is the rate-limiting enzyme in porphyrin and heme synthesis (De Matteis, 1970; Škrinjarić-Špoljar *et al.*, 1970). Metabolism of hexobarbital and aniline is induced nonselectively in mice by synergists VI, XXIII, and XXIX when measured from the point of maximum inhibition, but that of *p*-nitrophenyl dimethylcarbamate appears to be selective because of the strong magnitude of induction noted with the latter substrate (Škrinjarić-Špoljar *et al.*, 1970). Also, the induction phenomenon is complicated by the finding that phenobarbital selectively increases cytochrome P-450 in rabbits, whereas 3-methylcholanthrene selectively increases P-446; these cytochromes (P-446 and P-450) possibly are one and the same but give different spectral characteristics because of different types of interactions with substrates (Hildebrandt *et al.*, 1968). Piperonyl butoxide is a less effective inhibitor in phenobarbital- or 3-methylcholanthrene-induced rats than in noninduced rats, possibly due to increased rates of pb metabolism to products of minimal inhibitory capacity in the induced rats (Anders, 1968). In the reverse-type of situation, pb inhibits the activity of 3-methylcholanthrene in inducing mfo activity (Falk and Kotin, 1969). Similarly, in mice, pb inhibits the induction caused by phenobarbital (Wilkinson, 1968b), and benzo[*a*]pyrene (Friedman and Epstein, 1970). There are indications that the metabolism of xenobiotics may be intrinsically involved in their ability to induce mfo activity and so pb acting initially as an inhibitor blocks enzyme induction as a delayed effect (Friedman and Epstein, 1970). The induction phenomenon is in general more prominent in mammals than in insects, based on studies with chlorinated hydrocarbon insecticides, 3-methylcholanthrene, barbiturates, and other known inducers (Agosin, 1963; Agosin *et al.*, 1969; Ahmad and Brindley, 1969; Chakraborty and Smith, 1967; Gil *et al.*, 1968; Khan *et al.*, 1970; Meksongsee *et al.*, 1967; Moorefield, 1960; Oppenoorth and Houx, 1968; Perry and Buckner, 1970; Plapp and Casida, 1970; Walker and Terriere, 1969). In contrast to the adult mammal, the adult insect is characterized by minimal mitoses, these being largely in reproductive or regenerating cells. The fact that the mammalian mfo system is more easily induced by a chemical may allow it to more quickly overcome or reverse the inhibition phase, whereas the less-easily induced insect mfo system may add little if any new enzyme to that already inhibited to overcome intoxication.

EFFECT OF SYNERGISTS ON RESISTANCE MECHANISMS

Resistance mechanisms involving oxidative detoxification can sometimes be overcome by the action of synergists, with the result that the presence of the synergist increases the susceptibility of a resistant strain to a level approaching that of the original susceptible strain. In fact, the rate of resistance development in houseflies on selection with carbaryl or *m*-isopropylphenyl methylcarbamate is greatly reduced when pb is included in the diet (Georghiou, 1962; Georghiou *et al.*, 1961; Moorefield, 1960). These and other findings have led to the speculation that synergists are a partial answer to the resistance problem, at least with carbamates (Metcalf, 1968; Wilkinson, 1968a); however, this hypothesis has not been reduced to practice.

The diversity of resistance mechanisms is best understood in the case of houseflies (Georghiou, 1965; Oppenoorth, 1965a; Plapp, 1969; Tsukamoto, 1969) and, in this species, they can be generalized as involving either semidominant genes conferring increased detoxifying activity, which can be blocked by synergists, or recessive genes that are involved with mechanisms other than detoxification (Plapp, 1969). The synergist-sensitive mfo system represents only a portion of the detoxifying mechanisms; among the other enzymes involved are DDT-dehydrochlorinase with DDT analogs and various types of esterases with organophosphates. Even with the methylcarbamates, certain thiocyanatophenyl compounds appear to be degraded by a mechanism which is not a pb-sensitive system (Weiden and Moorefield, 1965) but rather probably involves glutathione *S*-transferases (Ohkawa and Casida, 1970). The metabolism of several chlorinated cyclodienes, including aldrin and dihydroaldrin but not dieldrin, varies greatly with strain and is inhibited by sesamex; however, detoxification by the mfo system is not the mechanism of cyclodiene resistance in dieldrin-resistant housefly

strains (Brooks, 1966, 1968a; Brooks and Harrison, 1964b; Schonbrod *et al.*, 1968).

Metabolism by the mfo system contributes to the tolerance of housefly strains resistant to carbamates (Plapp and Casida, 1969; Shrivastava *et al.*, 1969; Tsukamoto, 1969; Tsukamoto and Casida, 1967a; Tsukamoto *et al.*, 1968), organophosphates (Lewis, 1969; Lewis and Lord, 1969; Oppenoorth, 1967; Plapp and Casida, 1969), pyrethroids (Plapp and Casida, 1969), naphthalene (Schonbrod *et al.*, 1965, 1968), and DDT (Gil *et al.*, 1968; Oppenoorth, 1965b, 1967; Oppenoorth and Houx, 1968; Plapp and Casida, 1969). Apparently, a small number of mutant genes in the housefly controls the level of oxidative metabolism of many insecticide chemicals and controls a portion of the resistance to them (Casida, 1969; Oppenoorth, 1967; Plapp and Casida, 1969; Tsukamoto and Casida, 1967a). These genes are on autosome 2 or 5, or both, depending on the strain (Khan, 1969; Plapp and Casida, 1969; Schonbrod *et al.*, 1968; Tsukamoto *et al.*, 1968). The level of housefly microsomal P-450 also varies with strain in amount (Matthews and Casida, 1970b; Perry and Buckner, 1970; Perry *et al.*, 1969), nature of the difference spectra obtained with ethyl isocyanide (Matthews and Casida, 1970b), and substrate-specificity (Matthews and Casida, 1970b; Plapp and Casida, 1969). The nature and amount of P-450 possibly is governed by the same type of genetic control involved with mfo activity.

Special comment is appropriate on one aspect of possible mfo involvement in organophosphate resistance in houseflies. One theory attributes resistance to more rapid hydrolysis of organophosphates in resistant than in susceptible housefly strains by the action of "mutant, modified, or altered aliesterases" (Bigley and Plapp, 1960; Oppenoorth, 1959; Oppenoorth and Van Asperen, 1960, 1961; Van Asperen and Oppenoorth, 1959). Resistance in these strains is partially overcome by dipropyl paraoxon (XXIV), DEF (XXV) and certain carbamates, including compound XXVII (Lewis, 1969; Lewis and Lord, 1969; Oppenoorth and Van Asperen, 1961; Plapp, 1969, 1970; Plapp and Tong, 1966; Plapp and Valega, 1967). These findings are interpretable by inhibition of the "mutant aliesterase" but it can also be speculated that they involve inhibition of the mfo system. For example, each of the compounds referred to in these studies as "esterase inhibitors" can also act, as noted before, as mfo substrates or inhibitors. Thus, there is a possibility that the low-activity, organophosphate-detoxifying "mutant aliesterase" is in reality the mfo system assayed *in vitro* under suboptimal conditions. Even though there are some cases in which esterases are definitely involved, the organophosphate resistance mechanisms are certainly more diverse and complex than originally envisaged and may result partially from oxidative mechanisms.

Less complete information on resistant strains of other species (for example, resistant flour beetles and weevils; Dyte *et al.*, 1965, 1966; Dyte and Rowlands, 1967) indicate synergist reversibility and, therefore, the likelihood of the involvement of the mfo system. MDP synergists are effective in increasing the toxicity of carbamates and some other insecticides to many insect species and, particularly, in resistant strains of these species, but the involvement of the mfo system is clearly established in only a few cases. It is tempting to continue emphasizing houseflies in delving further into resistance mechanisms, but it is important to deal with other economic species as well, particularly agricultural pests.

Of intriguing interest is the possibility of finding a compound that is metabolized to a more active toxicant at a greater rate by the mfo system in resistant than in susceptible strains, with the result that a greater kill occurs with the resistant than with the susceptible strain. This negatively correlated cross resistance, if found, could possibly be subject to manipulation by synergists as an additional control factor. Unfortunately, the carbamates and organophosphates selected to test the idea have not, to date, shown the appropriate properties, possibly because the more active toxicant formed in the resistant strain is more rapidly detoxified in the resistant than in the susceptible strain and consequently never accumulates to a toxic level.

MAMMALIAN TOXICOLOGY

In general, the synergists have a low acute- and subacute toxicity to mammals (Table III). The nature and extent of synergist use is such that the intake by man, although unknown, is small. Thus, there is little or no likelihood of acute poisoning from the synergists, when used as directed, and the potential hazard, if any, associated with their use is therefore a function of other aspects of their toxicology (Mrak, 1969).

There is no evidence of mutagenicity with any of the synergists for insecticide chemicals (Mrak, 1969). Tests for possible teratogenicity of pb (VI) in two mice strains show no significant increase in anomalies; also, pb does not show an overall increase in nonspecific anomalies produced by carbaryl, although it results in significantly more cystic kidneys (Bionetics Report referred to by Mrak, 1969). At high levels in rats, pb and sulfoxide (VII) increase bile flow (Falk *et al.*, 1965) while safrole (III) and Tropital (IX) sometimes alter the cholic acid and cholesterol level in bile (Fishbein *et al.*, 1967a,b, 1968). The observation that pb increases the severity in living rats of pathological changes in the liver induced by pyrethrins leads to the conclusion (Kimbrough *et al.*, 1968) that variation in the extent of using synergized pyrethrins (aerosol sprays) at different times has an effect on the variation of the extent of liver cell changes observed with various compounds by other investigators. Each of these effects occurs either at very high doses or is not definitely attributable to the synergist *per se*.

The tumorigenic properties, if any, of MDP synergists merit consideration because it is known that some MDP compounds are carcinogenic. Heated sesame oil is a weak carcinogen on subcutaneous injection into mice (Steiner *et al.*, 1943) and so is sesamol when fed to rats (Ambrose *et al.*, 1958). At high dietary levels, safrole is a rat hepatocarcinogen (Hagan *et al.*, 1965; Homburger *et al.*, 1961, 1962; Long *et al.*, 1963) and dihydrosafrole (V) initiates cancer of the esophagus in rats (Hagan *et al.*, 1965; Long and Jenner, 1963). When mice are treated at very high dosages by stomach tube or feeding for 18 mo, safrole and dihydrosafrole, as expected, produce liver tumors, but isosafrole (IV) is not very potent in this respect (Innes *et al.*, 1969). Also, safrole produces pulmonary adenocarcinomas in addition to liver tumors in mice (Fujii *et al.*, 1970). Several MDP compounds, including pb, sulfoxide, and possibly propyl isome (VIII), produce malignant tumors of the lymphatic system in mice but these studies are considered to be inconclusive and further evaluation is needed, particularly with pb and sulfoxide (Falk, 1969; Innes *et al.*, 1969; Mrak, 1969). It is important to note, however, that liver tumors are not observed in rats fed pb for 18 mo at levels up to 20,000 ppm in the diet (Falk, 1969).

MDP compounds can inhibit carcinogen metabolism and increase carcinogen potency (sometimes referred to as co-carcinogenic activity). These findings, based on initial ob-

Table III. Acute and Subacute Toxicity to Mammals and FDA Tolerances for Various Synergists Used with Insecticide Chemicals

Synergist	Acute LD_{50}, mg/kg			No effect level in rat diet for 4–24 months, ppm	Tolerance category[a]
	Rat, oral	Mouse, ip	Rabbit, dermal		
MDP compounds					
Piperonyl butoxide (VI)	7,500–12,800[b]	>640[c]	1,880[d]	>5,000[d]	1, 2, 3
Sulfoxide (VII)	2,000[d]	>640[c]	>9,000[d]	2,000[d]	2
Propyl isome (VIII)	15,000[d]	>640[c]	>375[d]	>5,000[d]	1, 2
Tropital (IX)	>4,000[e]	>640[c]	>10,0000[f]	>300 mg/kg by stomach tube 5 days/week[f]	2
Sesamex (X)		>640[c]			
Piperonyl cyclonene	5,200[d]	>640[c]	>100 (daily)[d]		1
Compounds of other types					
MGK 264 (XVII)	2,800[d]		470[d]	>5,000[d]	1, 2
NIA 16824 (XXIII)	600[g]		2,500[g]		

[a] Frear, D. E. H. (1969). (1) Exempt from the requirement of a tolerance when used on growing crops (pre-harvest) according to good agricultural practice. (2) Registered for use as a synergist under specified conditions. (3) Tolerances of 8 ppm for many nuts and fruits and 20 ppm for many grains as post-harvest application. [b] Sarles *et al.* (1949). [c] Fujii *et al.* (1970). [d] Metcalf (1955). [e] Hopkins and Maciver (1965). [f] Moore (1970). [g] Niagara Chem. Div. (1968).

servations about 30 yr ago with MDP compounds present in sesame oil (Bischoff, 1957; DeOme *et al.*, 1949; Dickens and Weil-Malherbe, 1942; Morton and Mider, 1939) are, in part, applicable to the synthetic MDP synergists. As shown in Table I, the rate of metabolism of the environmental carcinogen, benzo[*a*]pyrene, is reduced in rats treated with pb and sulfoxide. Also, synergized toxicity and/or carcinogenicity are observed in infant mice treated with high pb levels in combination with griseofulvin, benzo[*a*]pyrene, and Freons 112 and 113 (which are not the Freons used in insecticide aerosols).

Synergism of the acute toxicity of insecticide chemicals sometimes extends to mammals as well as insects (Table I); however, the effective synergist dose in mammals, even by the intraperitoneal route, is relatively high for the compounds now in commercial use, indicating that normal use of the synergists presents a negligible hazard, if any, of acute poisonings. The action of barbiturates in mammals is prolonged by many insecticide synergists and related natural MDP compounds administered orally or intraperitoneally; the effects of the synergists are thus superimposed on the background effects of natural MDP compounds, dietary levels of which, individually or collectively, are unknown (Csillag *et al.*, 1969; Fishbein and Falk, 1969). Pentobarbital-sleeping time is significantly prolonged in mice exposed to aerosols containing pb and pyrethrins but not to ones containing Tropital and pyrethrins; the exposure conditions used involved very high synergist doses (Ingle, 1970). However, high synergist levels administered intraperitoneally do not greatly alter the lethal dose of hexobarbital in mice; the dramatic effect is only on sleeping time (Škrinjarić-Špoljar *et al.*, 1970). The duration and intensity of the effect on drug metabolism varies with the synergist and compound synergized, the action of dichlorobenzothiadiazole (XXIX) being particularly long and nonspecific, and that of pb and the *O*-(2-propynyl) phosphonate (XXIII) particularly intense, at least with hexobarbital (Škrinjarić-Špoljar *et al.*, 1970). Effects on drug sensitivity must enter into consideration of the potential safety of synergists for insecticide chemicals because it is now evident that some synergists are active at low dosages in mammals as well as insects. Tests of this type should reflect routes related to normal exposure and be made at various concentrations, including levels substantially higher than those to which the human population is likely to be exposed.

The toxicology of MDP synergists in mammals suggests that their use is contraindicated where satisfactory insect control is practicable with normal use of insecticide chemicals or other means of insect control. MDP synergists are generally more expensive than the agricultural insecticides; thus the desired insect control can usually be accomplished by increasing the level of the insecticide chemical and thereby avoiding the potential hazard of combination with synergists. However, combination of the MDP synergists with low concentrations of nonpersistent insecticide chemicals (as with the pyrethroids) used to achieve insect control does not appear to constitute a real hazard. And, the insect control is obtained with a substantial savings over that resulting with the nonpersistent insecticide chemical alone. Appropriate combinations of pyrethrum and some other pyrethroids with synergists continue to constitute, as they have for the past 30 yr, one of the best and safest means of insect control.

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