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RESPONSE OF HEPATIC MICROSOMAL MIXED-FUNCTION OXIDASES TO VARIOUS TYPES OF INSECTICIDE CHEMICAL SYNERGISTS ADMINISTERED TO MICE

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Abstract—For several hours after a single intraperitoneal (i.p.) dose to mice, piperonyl butoxide, 2-methylpropyl 2-propynyl phenylphosphonate (NIA 16824), and 5,6-dichloro-1,2,3-benzothiadiazole (WL 19255) inhibit hepatic microsomal mixed-function oxidase (mfo) activity and the apparent level of cytochrome P-450 but, after 24–72 hr, they induce increased mfo activity and P-450 content. Other types of insecticide chemical synergists studied are generally less active or inactive in this respect. The active compounds enhance the toxicity of several insecticide chemicals and markedly prolong hexobarbital sleeping time but not hexobarbital toxicity. The action of WL 19255 differs from that of piperonyl butoxide and NIA 16824 in that it is relatively non-specific in inhibiting enzymatic activity on several substrates, gives a more prolonged effect on P-450, and the magnitude of induction is greater.

PIPERONYL butoxide and other methylenedioxyphenyl (MDP) compounds are used as synergists for insecticide chemicals because they inhibit the activity of the microsomal mixed-function oxidase (mfo) enzymes of insects. These MDP compounds have a similar effect on the hepatic mfo enzymes of mammals, but they are generally more effective and persist longer in houseflies than in mice or rats and the housefly mfo enzymes are more sensitive to *in vitro* inhibition by insecticide synergists and metabolize them more slowly.[1] Inhibition of mfo activity by MDP compounds in treated mice is sometimes greater for one reaction as compared with another reaction occurring on a particular substrate,[2] or for one substrate as compared with another substrate.[3] The consequences of mfo inhibition frequently are increased toxicity, potency or persistence of drugs or insecticides acting in mammals or insecticides acting in insects.[1]

Certain compounds produce an initial, transient inhibition of mfo activity in mammals followed by an induction phase of variable magnitude and duration; these include, for example, piperonyl butoxide and other MDP insecticide synergists[2–4] and SKF 525-A (2-diethylaminoethyl 2,2-diphenylvalerate HCl).[5,6] Most of these compounds are also substrates for the mfo system[7] and so the persistence of their inhibitory effect in mammals may be related to the rate at which they are metabolized.

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However, it is likely that the induction of mfo enzyme activity and cytochrome P-450 content is a distinct phenomenon involving porphyrins and heme.[8,9] Cytochrome P-450 responds to different compounds in various ways; treatment with insecticide synergists results in a reduced level of P-450 in houseflies,[10,11] and mice[12] followed by a phase of induced P-450 in mice. On the other hand, treatment with phenobarbital or 3-methylcholanthrene results only in P-450 induction, but the P-450 induced by these two compounds differs in spectral properties, indicating that they may be different hemoproteins or possibly interconvertible forms of the same hemoprotein.[13]

Chemicals other than MDP compounds are active as synergists for insecticide chemicals, and these include *N*-alkyl compounds and 2-propynyl ethers and esters as well as a variety of other materials.[1] Although much is known on the action of piperonyl butoxide and a few other MDP compounds in mammals relative to mfo activity, little is known about the action or potency of other types of insecticide synergists.

The present studies, and an earlier communication,[12] consider the effect of i.p. administration to mice of many types of synergists for insecticide chemicals relative to their potency and specificity for inhibition and induction of mfo activity for several substrates and of P-450. These effects are related, where possible, to potency in prolonging hexobarbital sleeping time and in increasing the toxicity of insecticide chemicals.

MATERIALS AND METHODS

Chemicals. The compounds tested as inhibitors or used as toxicants are listed with their sources in Table 1.

Treatment of mice. Male Swiss–Webster mice (18–22 g) from Bioscience Animal Laboratories (Oakland, Calif.) were maintained on standard laboratory chow and water *ad lib.* prior to and during the experiments. All treatments involved i.p. administration of the chemicals as solutions in water or dimethylsulfoxide (DMSO) or as emulsions or suspensions in 10% (w/v) Tween 80 in water using 0·1 ml injection vehicle per 20 g mouse weight. The controls involved injection of the appropriate carrier vehicle, only. The following series of four experiments were made.

(1) Five synergists dissolved in DMSO were injected at the maximum doses found, in preliminary studies, to produce moderate but not severe signs of acute poisoning or a high percentage mortality within 72 hr. These doses were: piperonyl butoxide (450 mg/kg); NIA 16824 (150 mg/kg); RO 5-8019 (100 mg/kg); WL 19255 (200 mg/kg) and MGK 264 (450 mg/kg). The treated mice were killed at 0·5, 6, 12, 18, 24, 36, 48 or 72 hr after synergist administration and then sacrificed for hepatic mfo enzyme assays or determination of microsomal P-450 content.

(2) Mice were injected with 13 test compounds at 0·1 m-mole/kg using aqueous Tween 80 and 30 min later the livers were removed from a portion of the group for enzyme and P-450 assays while the remaining portion received 62·5 mg/kg sodium hexobarbital in water to determine the affect of synergist pretreatment on sleeping time. The sleeping time studies involved 12 mice for each compound.

(3) Thirty min after treatment with 0·5 m-mole/kg insecticide synergist in aqueous Tween 80, variable doses of coumaphos, dimetilan or rotenone were administered in DMSO or of sodium hexobarbital in water to determine the 24-hr LD_{50} values for these toxicants. Each value reported is based on replicated experiments involving 150–175 mice.

TABLE 1. CHEMICALS TESTED AS MIXED-FUNCTION OXIDASE INHIBITORS OR INDUCERS OR AS TOXICANTS

Chemical*	Name	Source
	Chemicals tested as enzyme inhibitors or inducers	
Methylenedioxyphenyl compounds		
Piperonyl butoxide	α-[2-(2-butoxyethoxy)ethoxy]-4,5-methylenedioxy-2-propyltoluene	Organic Chem. Div., FMC Corp. New York, N.Y.
Propyl isome	dipropyl-5,6,7,8-tetrahydro-7-methylnaphtho[2,3-d]-1,3-dioxole-5,6-dicarboxylate	S. B. Penick and Co., New York, N.Y.
Sesamex	2-(2-ethoxyethoxy)ethyl-3,4-(methylenedioxy)phenyl acetal of acetaldehyde	Shulton Inc., Clifton, N.J.
Sulfoxide	1,2-methylenedioxy-4-[2-(octylsulfinyl)propyl]benzene	S. B. Penick and Co., New York, N.Y.
Tropital	piperonal *bis*[2-(2-butoxyethoxy)-ethyl]acetal	McLaughlin Gormley King Co., Minneapolis, Minn.
N-Alkyl compounds		
MGK 264	*N*-(2-ethylhexyl)-5-norbornene-2,3-dicarboximide	McLaughlin Gormley King Co., Minneapolis, Minn.
SKF 525-A	2-diethylaminoethyl 2,2-diphenylvalerate HCl	Smith, Kline and French Labs., Phila., Pa.
WARF-antiresistant	*N,N*-di-*n*-butyl-*p*-chloro-benzenesulfonamide	S. B. Penick and Co., New York, N.Y.
2-*Propynyl ethers and esters*		
NIA 16824	2-methylpropyl 2-propynyl phenylphosphonate	Niagara Chem. Div., FMC Corp., Middleport, N.Y.
RO 5-8019	2-(2,4,5-trichlorophenyl)-propynyl ether	Hoffman-La Roche Inc., Nutley, N.J.
Other compounds		
DEF	*S,S,S*-tributyl phosphorotrithioate	Chemagro Corp., New York, N.Y.
Metyrapone	2-methyl-1,2-*bis*(3-pyridyl)-propanone	CIBA Pharmaceutical Company Division CIBA Corporation Summit, New Jersey 07901
TOCP	tri-*o*-tolyl phosphate	Eastman Organic Chemicals, Rochester, N.Y.
WL 19255	5,6-dichloro-1,2,3-benzothiadiazole	Shell Research Ltd., Sittingbourne, England
	Insecticide chemicals used as toxicants	
Coumaphos	*O*-(3-chloro-4-methyl-2-oxo-2*H*-1-benzopyran-7-yl) *O,O*-diethyl phosphorothioate	Chemagro Corp., New York, N.Y.
Dimetilan	1-(dimethylcarbamoyl)-5-methyl-3-pyrazolyl dimethylcarbamate	J. R. Geigy S.A., Basle, Switzerland
Rotenone	botanical insecticide chemical	Aldrich Chemical Co., Milwaukee, Wis.

* Structures for most of these chemicals are given in a recent review.[1]

(4) The insecticide synergists were administered at varying doses in DMSO 30 min before administration of sodium hexobarbital at 62·5 mg/kg in water to determine the dose necessary to prolong the sleeping time from approximately 25 min, in the absence of insecticide synergist, to a value of 100 min using about 60 mice for sleeping time determination with each compound.

Preparation of liver fractions and enzyme assays. Livers removed from decapitated mice were weighed, chilled on ice and homogenized in 0·02 M tris–0·15 M KCl buffer (pH 7·4) or 0·2 M sodium phosphate–0·05 M sucrose buffer (pH 7·4) using a glass-Teflon homogenizer of the Potter–Elvehjem type to give a 25% (w/v) homogenate. The supernatant from centrifugation of the homogenate at 9000 *g* for 20 min, referred to as the microsome-plus-soluble fraction, was used directly for enzyme assay or it was subjected to further centrifugation at 105,000 *g* for 30 min to obtain the microsomal fraction which was washed once with tris–KCl buffer by resuspension on homogenization and recentrifugation. The microsomal fraction was finally suspended in the buffer used originally for tissue homogenization with the exception that sucrose was not present in the phosphate–sucrose buffer and KCl in the tris–KCl buffer. All manipulations of tissues and enzyme preparations were carried out at 0–4°.

Assays of mfo activity using four substrates were made on liver preparations from mice at varying times after injection with insecticide synergist with duplicate determinations for each assay and repetition of the entire time-sequence experiment two or three times for each insecticide synergist and substrate used. In each case, a determination was made both with and without NADPH fortification using the latter value, where almost no metabolism was evident, as a correction for the former value. All incubations were at 37° under air in a Dubnoff shaking incubator, and all enzyme concentrations are expressed in relation to the equivalent amount for fresh liver weight. The enzyme concentration was selected so that under the stated conditions of assay there was direct proportionality between enzyme concentration and substrate loss or product formed. The standard assay conditions described below resulted in the following percentages of substrate metabolism: dimethyl *p*-nitrophenyl carbamate, 4·3%; *p*-nitroanisole, 8·6%; hexobarbital, 65%; and aniline, 2·5%.

N-Methyl hydroxylation of dimethyl *p*-nitrophenyl carbamate (DpNC) was determined by coating the substrate (1·5 μmole) uniformly on the bottom of a 25-ml Erlenmeyer flask (by evaporation of an acetone solution), addition of 2·8 μmoles NADPH and 12·5 mg microsomes in 3·0 ml 0·2 M sodium phosphate buffer (pH 7·4), incubation for 60 min, addition of 2·0 ml acetone to precipitate proteins and 0·2 ml 5 N NaOH to hydrolyze the product, and centrifugation to recover the supernatant fraction for colorimetric determination of *p*-nitrophenol at 410 mμ.[14]

O-Demethylation of *p*-nitroanisole yielding formaldehyde and *p*-nitrophenol was assayed by coating 1·0 μmole substrate on the bottom of an Erlenmeyer flask, as with DpNC, addition of 2·8 μmoles NADPH and 10 mg microsome-plus-soluble fraction in 2·0 ml 0·14 M tris–0·03 M KCl buffer (pH 7·4), incubation for 60 min, and determination of *p*-nitrophenol by the procedure used with DpNC.

Hydroxylation of the cyclohexenyl group of hexobarbital was determined by the general procedure of Kato *et al.*[15] involving incubation for 30 min of 1·0 μmole sodium hexobarbital, 1·0 μmole NADPH, 20 μmoles glucose-6-phosphate (G-6-P), 50 μmoles nicotinamide, 50 μmoles $MgCl_2$, and 200 mg microsome-plus-soluble fraction in 5·0 ml 0·11 M sodium phosphate–0·0032 M tris–0·024 M KCl buffer (pH 7·4). The incubations were carried out in 50-ml Erlenmeyer flasks which were also used for subsequent extraction of unmetabolized substrate for determination by the procedure of Gilbert and Golberg[16] modified (to increase the absorbance on measuring the remaining substrate) by using 4 ml instead of the reported 2 ml of the heptane-isoamyl alcohol extract for back extraction with sodium phosphate (pH 11).

Aromatic hydroxylation of aniline to yield *p*-aminophenol was studied by the procedure of Holtzman and Gillette[17] involving 40 min incubation of 4·0 μmoles aniline hydrochloride with 1·0 μmole NADPH, 30 μmoles G-6-P, and 25 mg microsome-plus-soluble fraction in 3·0 ml 0·057 M tris–0·05 M KCl buffer (pH 7·4).

Determination of microsomal cytochromes. Cytochrome P-450 measurements[18] involved the addition of a few mg of sodium dithionite to approximately 1 ml of microsomal suspension, adding 0·5 ml of the reduced microsomal suspension to each of two 0·5-ml microcuvettes (1 cm path), bubbling CO from a fine capillary for 20 sec through the contents of the sample cell, and determining the absorbance at 450 mμ minus the baseline absorbance at 490 mμ for the difference spectrum obtained with a Bausch and Lomb Spectronic 505 recording spectrophotometer. The ethyl isocyanide (C_2H_5NC) difference spectrum was measured in a similar manner but instead of bubbling with CO, 3 μmoles C_2H_5NC (provided by Don Shoeman, Department of Pharmacology, University of Minnesota Medical School, Minneapolis) were added in 10 μl water to the sample cell; the baseline absorbance at 490 mμ was subtracted from the absorbance values for the peaks at 430 and 455 mμ.[18] In every experiment, determinations were made of both enzyme activity and microsomal P-450 content using the same buffer, in each case, involved in resuspending the original microsomal preparation; the two buffers used in P-450 determinations gave the same results for the P-450 content measured. Protein concentrations were determined colorimetrically versus a bovine serum albumin standard.[19] The cytochrome content of microsomes from untreated mice, expressed as absorbance X 1000/mg protein, was: P-450.CO 114 ± 19, P-450.C_2H_5NC at 430 mμ 107 ± 23, P-450.C_2H_5NC at 455 mμ 60 ± 9. Using the molar extinction coefficient of 91 $mM^{-1}cm^{-1}$,[18] the P-450.CO content was 1·25 ± 0·21 mμmoles/mg protein. All percentage values are presented on the basis of cytochrome content per mg microsomal protein in comparing cytochrome content in treated mice with that in untreated mice.

RESULTS

Effect of insecticide synergist injection on liver mixed-function oxidase activity. The patterns of inhibition and induction of mfo activity as a function of time (Figs. 1–4) are dependent both on the insecticide synergist injected and the substrate used for assay. Maximum inhibition occurs during the first 18 hr, frequently at 0·5 hr, while induction is greatest at 36–48 hr after treatment. When substrate differences are noted, the inhibition is greatest with hexobarbital and the induction is greatest with DpNC.

Piperonyl butoxide and NIA 16824 give somewhat similar inhibition patterns, shown in Figs. 1 and 2, respectively, although the persistence of inhibition is greater with NIA 16824. Both synergists quickly inhibit hexobarbital metabolism and maintain this inhibition for 12–24 hr. Inhibition of aniline metabolism is more persistent with NIA 16824 than with piperonyl butoxide.

WL 19255 inhibition is much less substrate-dependent than is the case with piperonyl butoxide and NIA 16824 and it gives no marked difference in the induction except with DpNC as the substrate. The lack of inhibition difference with substrate demonstrates that the insecticide synergist effect is equally profound whether the microsome fraction, as was the case with DpNC, or the microsome-plus-soluble fraction, as with the other substrates, was used as an enzyme source.

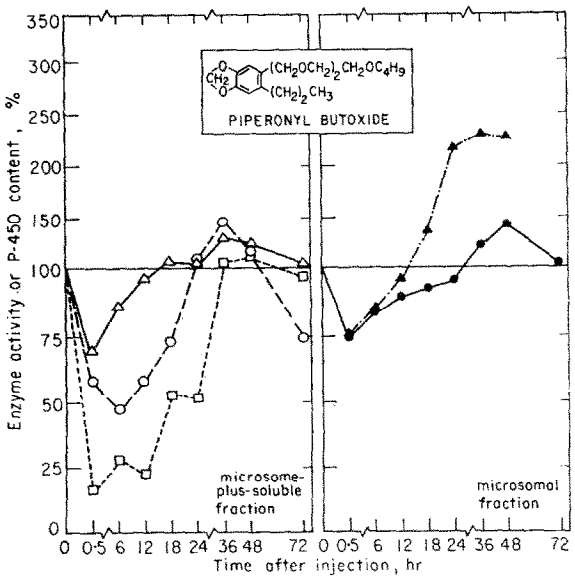

FIG. 1. Effect of piperonyl butoxide injection on mixed-function oxidase activity and microsomal P-450 content in mouse liver. □ - - - - □ Hexobarbital metabolism; ○ - - - - - ○ *p*-Nitroanisole metabolism; △——△ Aniline metabolism; ▲ — · — · ▲ Dimethyl *p*-nitrophenyl carbamate metabolism; ●——● Microsomal P-450 content.

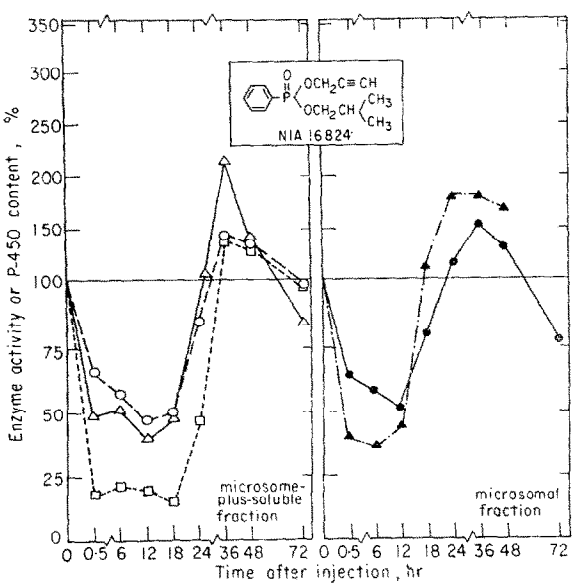

FIG. 2. Effect of NIA 16824 injection on mixed-function oxidase activity and microsomal P-450 content in mouse liver. For symbols, see Fig. 1.

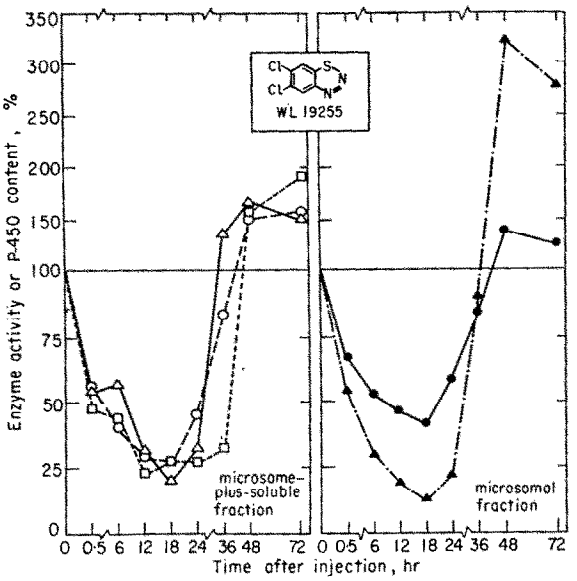

FIG. 3. Effect of WL 19255 injection on mixed-function oxidase activity and microsomal P-450 content in mouse liver. For symbols, see Fig. 1.

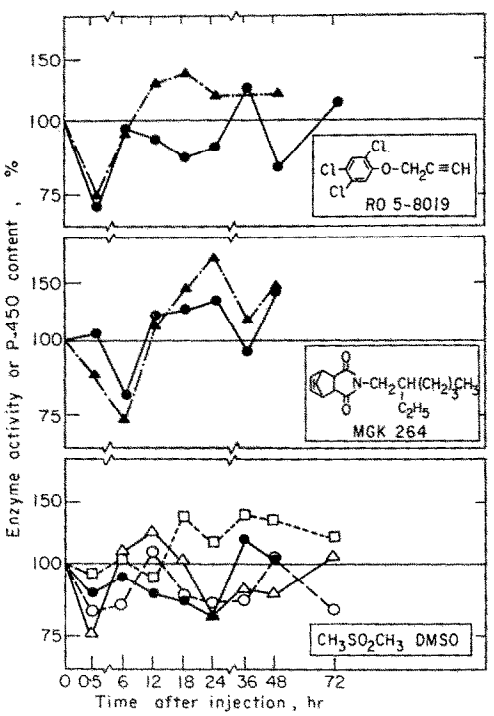

FIG. 4. Effect of RO 5-8019, MGK 264 and dimethylsulfoxide injection on mixed-function oxidase activity and microsomal P-450 content in mouse liver. For symbols, see Fig. 1. The replication is an exception to the general statement in the text because with RO 5-8019 and MGK 264 only two to four replicates are involved.

RO 5-8019, MGK 264 and DMSO, the carrier vehicle, are in general less effective inhibitors and inducers than the three synergists discussed before when assayed for effect on DpNC metabolism (Fig. 4). The control mice always received DMSO 30 min before assay with the single exception of this study evaluating the effect of DMSO on P-450 and enzyme activity.

In a comparison of 13 compounds with a 30-min pretreatment interval at a standard dose using aqueous Tween 80, relatively few compounds are effective in inhibiting aniline hydroxylation and these are MDP compounds, 2-propynyl derivatives, and the dichlorobenzothiadiazole, only one of which, RO 5-8019, is a more potent inhibitor of aniline hydroxylation than of hexobarbital metabolism (Table 2). Metabolism of hexobarbital is generally more sensitive to inhibition, particularly potent inhibitors being piperonyl butoxide, sesamex, SKF 525-A and NIA 16824. It is likely that several compounds, such as WL 19255, would be more effective inhibitors at a longer interval after treatment (Fig. 3).

Effect of insecticide synergist injection on microsomal cytochrome P-450. Cytochrome P-450 levels measured with carbon monoxide by the conventional procedure also show

TABLE 2. THE EFFECT OF INSECTICIDE CHEMICAL SYNERGISTS AND RELATED COMPOUNDS ON ENZYME ACTIVITY, MICROSOMAL P-450 CONTENT AND HEXOBARBITAL SLEEPING TIME DETERMINED 30 min AFTER THEIR ADMINISTRATION TO MICE AT 0·1 m-mole/kg

Compound administered	Enzyme activity or microsomal cytochrome content, treated/control × 100 (%)					
	Enzyme activity		Cytochrome content			
	Hexobarbital	Aniline	P-450. CO 450 mμ	P450. C_2H_5NC 430 mμ	P-450. C_2H_5NC 455 mμ	Hexobarbital sleeping time (min)*
Methylenedioxyphenyl compounds						
Piperonyl butoxide	31 ± 6	76 ± 20	94 ± 9	89 ± 10	77 ± 9	115 ± 6
Propyl isome	76 ± 17	76 ± 18	75 ± 9	78 ± 7	95 ± 3	51 ± 8
Sesamex	54 ± 10	66 ± 9	81 ± 12	83 ± 14	88 ± 3	68 ± 21
Sulfoxide	62 ± 31	83 ± 30	101 ± 16	96 ± 20	100 ± 18	77 ± 15
Tropital	82 ± 25	84 ± 4	82 ± 14	83 ± 16	89 ± 8	56 ± 17
N-Alkyl compounds						
SKF 525-A	50 ± 12	85 ± 31	124 ± 30	111 ± 29	106 ± 18	248 ± 19
WARF-antiresistant	93 ± 40	90 ± 7	94 ± 8	92 ± 13	107 ± 3	35 ± 6
2-Propynyl ethers and esters						
NIA 16824	30 ± 17	66 ± 27	63 ± 16	59 ± 21	63 ± 17	226 ± 36
RO 5-8019	75 ± 14	49 ± 8	68 ± 5	65 ± 7	83 ± 7	67 ± 10
Other compounds						
DEF	68 ± 5	91 ± 35	113 ± 25	117 ± 31	104 ± 23	45 ± 14
Metyrapone	124 ± 18	98 ± 29	109 ± 20	106 ± 27	103 ± 19	41 ± 9
TOCP	127 ± 20	103 ± 11	97 ± 8	95 ± 10	102 ± 10	31 ± 8
WL 19255	73 ± 18	68 ± 26	92 ± 28	98 ± 34	82 ± 26	66 ± 21

* The sleeping time for mice administered sodium hexobarbital at 62·5 mg/kg 30 min after the carrier vehicle only is 27 ± 10 min.

apparent inhibition and induction paralleling, in time but not magnitude, the enzyme inhibition and induction (Figs. 1–3). Maximum induction, of 37–55 per cent, occurs at 36–48 hr with piperonyl butoxide, NIA 16824 and WL 19255; this induction of P-450 is expressed on a protein basis and would be even greater if the increase in microsomal protein on induction was taken into account. The moderate inhibition in P-450 level with MDP compounds at 30 min (Table 2) may not increase in magnitude at longer intervals based on the indication from the time-sequence study with piperonyl butoxide (Fig. 1). Two 2-propynyl compounds give considerable early inhibition (Table 2) that persists for several hr with NIA 16824 (Fig. 2). Dichlorobenzothiadiazole acts slowly on P-450 but the effect continues longer than with the other synergists used for the time-sequence studies (Fig. 3). The findings are generally the same using either CO or C_2H_5NC as the ligand except with RO 5-8019 and propyl isome which inhibit the P-450.C_2H_5NC absorbance at 455 mμ less than the absorbance due to P-450.C_2H_5NC at 430 mμ or P-450.CO (Table 2).

TABLE 3. THE EFFECT OF INSECTICIDE CHEMICAL SYNERGISTS ON TOXICITY OF INSECTICIDES AND ON TOXICITY AND SLEEPING TIME PRODUCED BY HEXOBARBITAL DETERMINED 30 min AFTER SYNERGIST ADMINISTRATION TO MICE

		Synergist				
	Dose, mg/kg	Piperonyl butoxide	NIA 16824	WL 19255	RO 5-8019	MGK 264
Insecticide or drug	LD_{50}	Synergism factor (magnitude of reduction in LD_{50})				
Coumaphos	23·5	7·1	7·6	3·4	2·6	1·2
Dimetilan	16·5	3·9	3·9	1·8	2·5	1·4
Rotenone	3·0	4·7	5·9	1·5	2·0	1·4
Sodium hexobarbital	275	1·2	1·3	1·0	1·1	0·9
	Dose used	Synergist dose producing 100 min sleeping time, m-mole/kg				
Sodium hexobarbital	62·5*	0·065	0·018	0·23	0·13	0·60

* The sleeping time for mice administered sodium hexobarbital 30 min after the carrier vehicle only is 28 ± 3 min.

Relative potency of synergists for prolonging hexobarbital sleeping time and increasing insecticide chemical toxicity in mice. The most potent compounds of those tested for prolonging hexobarbital sleeping time are SKF 525-A, NIA 16824 and, as expected,[20–22] piperonyl butoxide; however, significant activity is evident with some other materials known to synergize insecticide chemical toxicity in insects (Table 2). The minimum effective dose to prolong hexobarbital sleeping time is least with NIA 16824 and piperonyl butoxide as representatives of the different classes of insecticide synergists (Table 3). It is interesting that, in confirmation of earlier findings with SKF 525-A,[23] the synergists generally do not increase hexobarbital toxicity (Table 3) despite their dramatic effect on the duration of narcosis.

Preliminary tests with more than 50 insecticide chemicals were made in order to select three compounds that respond to the greatest degree with increased toxicity in piperonyl butoxide-treated mice. Two of these, coumaphos[24] and dimetilan,* have been previously noted to respond in this way but rotenone has not. NIA 16824 and piperonyl butoxide are similar in effectiveness for increasing the toxicity of the test compounds, whereas WL 19255, RO 5-8019 and MGK 264 have low activity, only.

DISCUSSION

Thirty years of testing for compounds active as synergists for insecticide chemicals appears, in retrospect, to have been in the most part a search for potent and selective inhibitors of insect mfo enzymes. As might be expected, the inhibitory activity extends in many cases to mammals as well as insects. Injection of large doses of certain insecticide synergists into mice results in inhibition of hepatic mfo enzyme activities and apparent cytochrome P-450 levels usually followed by their induction within 24–48 hr.

Synergists interact with microsomal P-450 under both *in vitro* conditions, based on determination of difference spectra,[12] and *in vivo* conditions, based on the apparent P-450 content following synergist administration. Difference spectra of synergists interacting with oxidized P-450 vary in type and magnitude with the synergist used: type I spectra are obtained with piperonyl butoxide, NIA 16824, RO 5-8019 and MGK 264 at 0·1 or 1·0 mM; type II spectra are obtained with WL 19255 at 0·01 mM and metyrapone.[12,25] It is evident from these and other findings[12] that the synergists do not all bind at the same site and that selectivity is involved with different synergists for either type I or type II binding sites; also, affinity differences exist for various synergists at their respective binding site(s). Inhibition of apparent P-450 content occurs not only in synergist-treated mice but also in houseflies treated topically with piperonyl butoxide or sesamex[10] and rats treated subcutaneously with 2-allyl-2-isopropylacetamide.[8] This apparent inhibition possibly results from destruction of P-450 by the synergist or a metabolite or, less likely, binding of these compounds at a site(s) that precludes the usual liganding of the reduced hemoprotein with CO or C_2H_5NC during subsequent P-450 assays. The prolonged duration of effect of WL 19255 in diminishing apparent P-450 levels has many possible explanations, one of which is a slow rate of metabolic breakdown; piperonyl butoxide which has a less persistent effect is known to be rapidly metabolized by mouse liver mfo enzymes and in living mice.[7,26]

Mixed-function oxidase inhibition varies in magnitude and duration with the synergist administered but with any one synergist the inhibition of metabolism of different substrates varies in magnitude but possibly not in duration. This specificity, when present, may result from the relative affinities of the insecticide synergists and substrates for type I, type II, and other sites of binding or interaction. Hexobarbital and aniline are the classical type I and II substrates, respectively.[27,28] Piperonyl butoxide and NIA 16824, which give type I difference spectra, preferentially inhibit hexobarbital metabolism as compared with aniline hydroxylation. WL 19255 inhibits activity on each substrate almost to the same extent indicating an action that is non-

* D. J. Hennessy, *U.S./Japan Seminar on Biochemical Toxicology of Insecticide Action*, Tokyo, 16–20 June (1969).

specific for any type or form of P-450. The inhibiting effect of insecticide synergists on P-450 most closely parallels the inhibition of aniline hydroxylation, in each case, indicating that the interaction occurs on microsomal components examined in both of these types of measurements; there are also other types of evidence for the parallel relationship of aniline oxidation and P-450 content.[29–31] RO 5-8019 and propyl isome of the compounds tested are somewhat unusual in their selectivity following injection for inhibiting aniline metabolism as compared with hexobarbital metabolism and for altering the microsomal P-450.C_2H_5NC difference spectrum. It is not clear whether P-450 has many binding sites, with varying affinity for different insecticide synergists, or whether the selectivity in inhibition results from specificity in binding with one or more of a group of similar enzymes. Another possibility is that the insecticide synergist results in isozymic transformation or conformational change in the substrate-binding site to alter the specificity of mfo enzymes.[2]

The synergist effects on hexobarbital narcosis and insecticide chemical toxicity in mice probably result from inhibition of drug or toxicant metabolism by the microsomal mfo system with higher sensitivity of the enzymes involved in hexobarbital metabolism than of those involved in detoxifying the insecticide chemicals used. There is evidence with compounds other than those studied here that mfo substrates with high affinity and low turnover number can act *in vivo* by binding preferentially at the active mfo site precluding other substrates from binding for metabolism.[32,33]

Increased P-450 levels one or two days following injection of mice with piperolyn butoxide, NIA 16824 and WL 19255 indicate that they may be porphyrogenic agents possibly acting, as appears to be the case with 2-allyl-2-isopropylacetamide,[8] by enhancing the activity of -aminolevulinic acid synthetase which normally is the rate-limiting enzyme in the biosynthesis of porphyrins and heme and is essential to the induction response of the hepatic mfo system.[8,9] The enzyme activity in insecticide synergist-induced mice compared with noninduced mice varies with the substrate used for assay. Metabolism of the classical type I and type II substrates, hexobarbital and aniline, may be induced nonselectively by each of these three compounds when measured from the point of maximum inhibition at 12–18 hr; this type of comparison is only appropriate if the P-450 loss is due to P-450 destruction and not due to reversible enzyme–substrate combination. However, because of its magnitude, DpNC induction appears to be selective, possibly indicating that, in fact, different types or forms of P-450 can respond differently to induction by an insecticide synergist or its metabolites as formed *in vivo*.

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REFERENCES

1. J. E. Casida, *J. Agric. Fd Chem.* **18**, 753 (1970).
2. H. Jaffe, K. Fujii, H. Guerin, M. Sengupta and S. S. Epstein, *Biochem. Pharmac.* **18**, 1045 (1969).
3. H. Jaffe, K. Fujii, M. Sengupta, H. Guerin and S. S. Epstein, *Life Sci.* **7**, 1051 (1968)
4. H. L. Falk and P. Kotin, *Ann. N.Y. acad. Sci.* **160**, 299 (1969).
5. M. W. Anders and G. J. Mannering, *Molec. Pharmac.* **2**, 341 (1966).
6. R. Kato, E. Chiesara and P. Vassanelli, *Medna exp.* **6**, 254 (1962).

7. J. E. CASIDA, J. L. ENGEL, E. G. ESAAC, F. X. KAMIENSKI and S. KUWATSUKA, *Science, N.Y.* **153**, 1130 (1966).
8. F. DE MATTEIS, *FEBS Lett.* **6**, 343 (1970).
9. H. S. MARVER, in *Microsomes and Drug Oxidations* (Eds. J. R. GILLETTE, A. H. CONNEY, G. J. COSMIDES, R. W. ESTABROOK, J. R. FOUTS and G. J. MANNERING), p. 495, Academic Press, New York (1969).
10. A. S. PERRY and A. J. BUCKNER, *Life Sci.* **9**, 335 (1970).
11. H. B. MATTHEWS and J. E. CASIDA, *Life Sci.* **9**, 989 (1970).
12. H. B. MATTHEWS, M. ŠKRINJARIĆ-ŠPOLJAR and J. E. CASIDA, *Life Sci.* **9**, 1039 (1970).
13. A. HILDEBRANDT, H. REMMER and R. W. ESTABROOK, *Biochem. biophys. Res. Commun.* **30**, 607 (1968).
14. E. HODGSON and J. E. CASIDA, *Biochem. Pharmac.* **8**, 179 (1961).
15. R. KATO, M. TAKAYANAGI and T. OSHIMA, *Jap. J. Pharmac.* **19**, 53 (1969).
16. D. GILBERT and L. GOLBERG, *Food Cosmet. Toxicol.* **3**, 417 (1965).
17. J. L. HOLTZMAN and J. R. GILLETTE, *Biochem. Pharmac.* **18**, 1927 (1969).
18. T. OMURA and R. SATO, *J. biol. Chem.* **293**, 2370 (1964).
19. O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR and R. J. RANDALL, *J. biol. Chem.* **193**, 265 (1951).
20. M. W. ANDERS, *Biochem. Pharmac.* **17**, 2367 (1968).
21. B. C. FINE and J. O. MOLLOY, *Nature, Lond.* **204**, 789 (1964).
22. K. FUJII, H. JAFFE, Y. BISHOP, E. ARNOLD, D. MACKINTOSCH and S. S. EPSTEIN, *Toxic. appl. Pharmac.* **16**, 482 (1970).
23. L. COOK, J. J. TONER and E. J. FELLOWS, *J. Pharmac. exp. Ther.* **111**, 131 (1954).
24. W. E. ROBBINS, T. L. HOPKINS and D. I. DARROW, *J. econ. Ent.* **52**, 660 (1959).
25. A. G. HILDEBRANDT, K. C. LEIBMAN and R. W. ESTABROOK, *Biochem. biophys. res. Commun.* **37**, 477 (1969).
26. F. X. KAMIENSKI and J. E. CASIDA, *Biochem. Pharmac.* **19**, 91 (1970).
27. H. REMMER, J. SCHENKMAN, R. W. ESTABROOK, H. SASAME, J. GILLETTE, S. NARASIMHULU, D. Y. COOPER and O. ROSENTHAL, *Molec. Pharmac.* **2**, 187 (1966).
28. Y. IMAI and R. SATO, *Biochem. biophys. Res. Commun.* **22**, 620 (1966).
29. G. LANGE, *Naunyn-Schmiedebergs Arch. exp. Path. Pharmak.* **259**, 221 (1968).
30. H. REMMER, R. W. ESTABROOK, J. SCHENKMAN and H. GREIM, *Naunyn-Schmiedebergs Arch. exp. Path. Pharmak.* **259**, 98 (1968).
31. J. B. SCHENKMAN, I. FREY, H. REMMER and R. W. ESTABROOK, *Molec. Pharmac.* **3**, 516 (1967).
32. A. RUBIN, T. R. TEPHLY and G. J. MANNERING, *Biochem. Pharmac.* **13**, 1007 (1964).
33. A. RUBIN, T. R. TEPHLY and G. J. MANNERING, *Biochem. Pharmac.* **13**, 1053 (1964).