Table III. Total Hydrolyzable Chlorine in Cyanuric Chloride

Conditions	$\bar{x}$	s	n
Direct titration	99.78	±0.32	5
30-minute reflux after acidification	100.08	±0.20	5

chloride content. As a final check on the procedures, a mixture of this Dyrene with cyanuric chloride and sodium chloride was analyzed for all components. The results are shown in Table II. Each of the determinations was carried out five or six times. It can be shown that the differences between the calculated and found figures for the mixture are not significant as based on the standard deviations of the previous table. If each determination were carried out in duplicate, a standard error for the Dyrene content would be ±0.13%, for cyanuric chloride ±0.05%, and for sodium chloride ±0.04%. Both accuracy and reproducibility are satisfactory for control purposes.

The method is subject to interference by pesticides such as DDT or BHC which are capable of undergoing alkaline dehydrohalogenation.

Application to the Analysis of Technical Cyanuric Chloride. Following on from the above, there seemed no reason why the relevant methods should not be applied to the assay of technical cyanuric chloride. The published method (*1*) for this determination involves the reaction of excess sodium methylate with cyanuric chloride, the excess reagent being hydrolyzed and determined acidimetrically in the usual way. The results with aqueous sodium hydroxide suggested a somewhat simpler procedure.

From the hydrolysis curves (Figure 2), one would expect hydrolysis at room temperature to be erratic. This was confirmed by hydrolyzing a series of 0.25-gram samples for 30 minutes in 1.N sodium hydroxide at about 22° C. The liberated chloride was determined by direct titration in acid solution. Five determinations gave a mean of 93.32% with a standard deviation of ±2.46. As hydrolysis at room temperature was obviously unsatisfactory, the reaction was reinvestigated by heating for 30 minutes at reflux. In this study the effect of boiling after acidifying the hydrolyzate was investigated for cyanuric chloride as it had been for Dyrene. The results obtained by the refluxing procedure are shown in Table III. Theoretical values are obtained whether the posthydrolysis boiling step is included or not and this step was omitted in applying the procedure to a mixture of cyanuric chloride and sodium chloride (2.47%). In this case a mean value of 100.0% (standard deviation ±0.19 for seven determinations) was obtained. The calculated value for the sample was 99.92%.

The method for free chloride as described for Dyrene was unsatisfactory for cyanuric chloride because of a small amount of hydrolysis of the latter compound in ice water. Accordingly, a procedure (*1*) involving solution of the sample in chloroform and shaking for 2 minutes with ice-cold water was employed. In this method the inorganic chloride is supposed to be extracted into the water without causing any hydrolysis of cyanuric chloride. When this method was applied to pure cyanuric chloride, five determinations gave a mean value of 0.09% as sodium chloride. This indicated that a slight hydrolysis of cyanuric chloride was occurring even under these conditions. That the value is real and not due to a slight contamination of the cyanuric chloride with inorganic chloride was shown by the fact that a plot for the hydrolysis of cyanuric chloride in water at 0° C. went through the origin. If there had been as much as 0.1% of inorganic chloride present, this would not have been true.

When the method was applied to a sample of cyanuric chloride containing 2% of added sodium chloride, the mean of six determinations gave a value 0.1% higher than the theoretical. However, for control purposes this was not considered serious.

The total hydrolyzable chlorine procedure is suggested as an alternative to the methylate procedure for the analysis of cyanuric chloride. It may be corrected satisfactorily for ionizable chloride by the method described above.

Acknowledgment

The authors acknowledge the assistance of Daniel MacDougall in the preparation of the manuscript.

Literature Cited

(1) American Cyanamid Co., New York, N. Y., "Aero Cyanuric Chloride," Leaflet **658-5M-7-55.**

(2) Burchfield, H. P., Storrs, E. E., *Contribs. Boyce Thompson Inst.* **18,** 319 (1956).

(3) Comp, J. L., Heckle, W. A., Organic Chemistry Division, ACS, 13th Southwest Regional Meeting, Tulsa, Okla., December 1957.

(4) Fierz-David, N. E., Matter, M., *J. Soc. Dyers Colourists* **53,** 424 (1937).

(5) Meagher, W. R., Anderson, C. A., Gonter, C. Ellen, Smith, S. B., MacDougall, D., J. AGR. FOOD CHEM. **7,** 558 (1959).

Received for review May 18, 1959. Accepted August 27, 1959. Division of Agricultural and Food Chemistry, 135th Meeting, ACS, Boston, Mass., April 1959.

ACARICIDE BIOASSAY

Two Organisms Suitable for Bioassaying Specific Acaricides

SUTHARM AREEKUL[1] and ROBERT F. HARWOOD

Department of Entomology, Washington State University, Pullman, Wash.

BIOASSAY with susceptible organisms is a well established method for determining residues of pesticides. However, the specific acaricides—pesticides specifically toxic to mites but virtually nontoxic to insects—cannot be successfully determined by bioassay with insects commonly used for this purpose.

Phytophagous mites, notably the two-spotted mite *Tetranychus telarius* (L.), have been used to screen candidate pesticides for acaricidal activity. Reports on this subject, found in the unpublished files of pesticide manufacturers, indicate a great deal of variability in response to any given level of toxicant. Several reasons may exist for such variability, but undoubtedly difference in host plants is a prominent factor. Thus variation in age and nutrition of plants probably affects the mites, and their almost continual dependence on plant food necessitates careful dipping or spraying practices, which are in turn modified by interactions between pesticide and plant surface. The instability of response to a given level of toxicant accounts for the present lack of bioassay methods for specific acaricides.

A current investigation on the useful-

[1] Present address, Department of Entomology, Kasetsart University, Bangkok, Thailand.

Bioassay of specific acaricides was attempted because no suitable organisms for this type of analysis had been tested. The brine shrimp, *Artemia salina* Leach, and the stored products mite, *Tyrophagus putrescentiae* Schrank, showed promise for assaying this group of toxicants. The former was exposed in shell vials to toxicant in acetone introduced into salt solution, the latter to a dry film of acaricide in a chamber made from opposed microscope depression slides. Toxicity curves with solutions of highly purified toxicant were established for Aramite, Chlorobenzilate, Dimite, Kelthane, Mitox, ovex, and Sulphenone. A bioassay of Kelthane on pears and cherries, using extractives from these fruits to establish a standard curve, was in close agreement with the colorimetric method frequently employed.

ness of four organisms for bioassaying a number of pesticides showed that a stored product mite, *Tyrophagus putrescentiae* Schrank (Syn. *T. castellanii* Hirst, *T. noxius* Zakhvatkin, *T. bravni* Turk), and the brine shrimp, *Artemia salina* Leach, are suitably sensitive to specific acaricides in pure form. Methods of exposure and toxicity curves for seven of these compounds are reported here.

Organisms

The brine shrimp, *Artemia salina*, has been previously utilized to determine residues of insecticides. There are several advantages in its use (*6*). Eggs, which can be stored for long periods, are readily obtainable from tropical fish suppliers. They are easily hatched, and the nauplii are tolerant to a wide range of salt concentration. They tolerate small amounts of acetone, but minute amounts of insecticide are rapidly toxic to all age groups from 24-hour-old nauplii to adults. Sensitivity to methoxychlor, DDT, lindane, chlordan, toxaphene, endrin, Bayer 17147, parathion, and dieldrin has been reported (*6, 11*), but their usefulness in the assay of specific acaricides has not been determined.

A stored products mite, *Tyrophagus putrescentiae*, was tested for susceptibility to specific acaricides. This mite is world-wide in distribution, and has been reported to feed on many kinds of stored products, preferably flour and cheese. They have been reared on blocks of Cheddar cheese at 55° F., and acaricides have been evaluated for their control (*2*). This mite was demonstrated to be two to three times more productive in semolina than in wheat (*8*). It has a very high fecundity, and can seemingly be cultured indefinitely and easily under proper laboratory conditions. The minimal care required to maintain this mite aids in its desirability as an organism for bioassay.

Pesticides Tested

Dosage-mortality curves were established for 29 pesticides using these two test organisms. All test compounds were highly purified, or the technical chemical was used. The results related here are for preliminary tests with purified compounds against the two organisms used, and merely show their suitability as sensitive organisms provided relatively pure toxicant is employed. The following seven specific acaricides were tested:

Aramite [2-(*p-tert*-butylphenoxy)-1-methylethyl 2-chloroethyl sulfite]; Aramite standard A-7499; Naugatuck Chemical.

Chlorobenzilate (Geigy 338; ethyl-4,4′-dichlorobenzilate); pure chlorobenzilate; Geigy Agricultural Chemicals.

Dimite (DMC; 4,4′-dichloro-α-methylbenzhydrol); purified DMC, *p,p*-DMC, content 98.5%, OR-307; Sherwin-Williams Co.

Kelthane [FW-293; 1,1-bis(*p*-chlorophenyl)-2,2,2-trichloroethanol]; purified Kelthane; Rohm & Haas Co.

Mitox (Chlorbenside; *p*-chlorobenzyl *p*-chlorophenyl sulfide); recrystallized Mitox, California Spray-Chemical Corp.

Ovex (Ovotran; *p*-chlorophenyl *p*-chlorobenzenesulfonate); highly purified ovex, Dow 360914; Dow Chemical Co.

Sulphenone (R-242; *p*-chlorophenyl phenylsulfone); technical Sulphenone; 4-chlorophenyl phenylsulfone 80%, 4,4-chlorophenyl phenylsulfone 5%, phenyl phenylsulfone 15%; Stauffer Chemical Co.

Rearing Methods

The salt solution previously recommended (*6*) was used for hatching brine shrimp eggs. Rather than endure the uncertainties of rearing the nauplii to adults, those hatched within 24 hours were used. The eggs were placed in the appropriate salt solution in one side of a crystallizing dish 6 inches in diameter, separated down the middle by a floating wooden divider. A light source illuminated the side lacking eggs and the photopositive nauplii swam to that side. In this manner nauplii were readily separated from unhatched eggs, and presumably only vigorous individuals uniform in their reaction to light were tested. Use of newly hatched nauplii precluded the variability in susceptibility of various growth stages previously noted (*11*).

The stored product mite was raised on wheat in 32-ounce wide-mouthed bottles. Only mechanically damaged grain is attacked (*8*); therefore the kernels were cracked in a grinding machine after a wash in hot water to ensure the elimination of other stored grain pests. The jars were filled almost to the top and about 3 grams of wheat germ and 1 gram of brewers yeast added. To promote fungal growth which is known to favor vigorous mite reproduction (*7*), 10 ml. of distilled water were deposited along the side of each filled jar to soak the bottom layer of the medium. Mites were introduced with grain from an old culture, and were found feeding at the bottom layer in considerable numbers within a week. Usually three or four jars were prepared simultaneously and placed on a metal pan surrounded by a barrier of motor oil. In 3 to 4 weeks tremendous numbers of mites developed, swarming on the filter paper covering each jar. A productive culture supplied sufficient mites for testing over 4 to 5 months. All cultures were maintained in a room at 75° F. and 40% relative humidity.

Method of Exposure

Brine Shrimp. Acaricides being tested were prepared in 1.25-ounce salve jars. The exposure medium consisted of salt mixture diluted with distilled water to one fifth of hatching concentration to which acaricide in acetone had been added. In practice this was accomplished by placing 8 ml. of distilled water and acaricide in each jar with 2 ml. of full-strength hatching medium containing approximately 50 shrimp. Solvents other than acetone in plant extracts of acaricide were evaporated in a fume hood and were replaced by acetone before the test exposure was made. The control (no acaricide) consisted of similarly diluted hatching solution, and diluted hatching solution plus acetone. Concentration of acetone varied within a test, but never exceeded 2% on a volume basis.

Brine shrimp and test solution were transferred to glass shell vials approximately 0.25 inch in diameter and 1.25

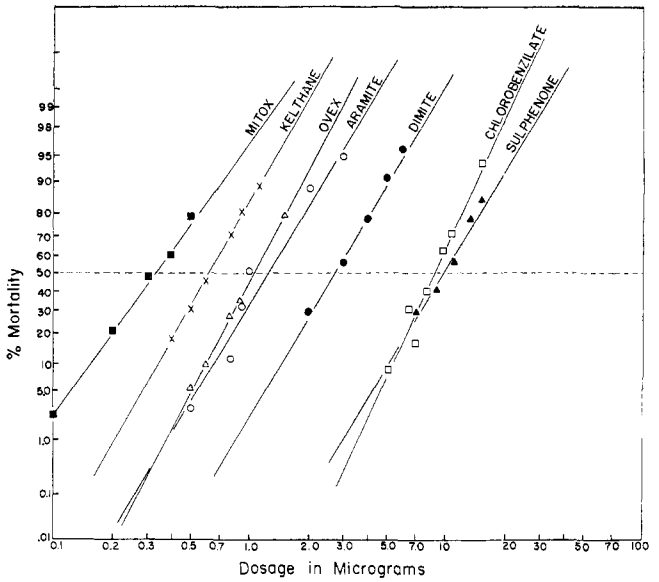

Figure 1. Log dosage–probit mortality regression lines of seven specific acaricides for brine shrimp, 24-hour exposure at 75° F.

Dosage expressed in micrograms per milliliter

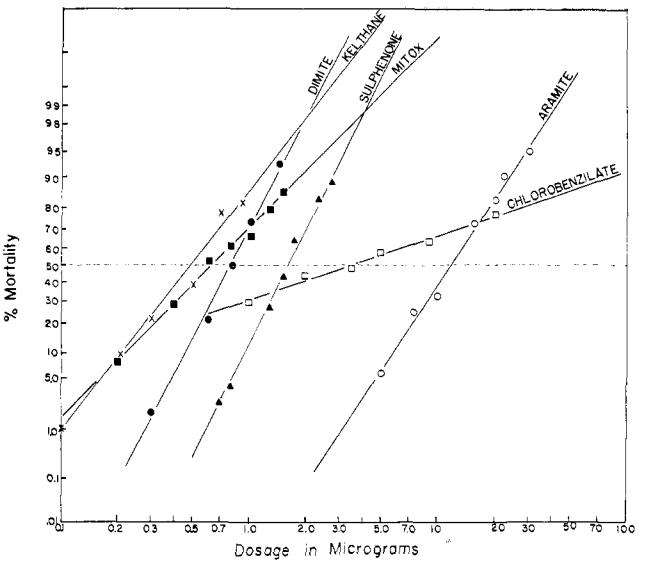

Figure 2. Log dosage–probit mortality regression lines of six specific acarides for the stored products mite, 24-hour exposure at 78° F.

Dosage expressed in micrograms per depression chamber

inches in height. Ten shrimp were placed in each vial, which was filled nearly to the top. Four vials were filled (1.2 ml.) with an identical concentration of salt solution and acaricide. The vials were then placed in a rack designed to permit easy observation of effects when placed before a dark background. Each concentration of toxicant was tested at least twice for a minimum of eight replications at any given level. All tests were conducted at 75° F. and 40% relative humidity.

Mortality, rather than time in relation to cessation of swimming activity (*6*), was the criterion used to determine levels of toxicant. Exposure was for 24 hours, during which time approximately 0.2 ml. of test solution evaporated. However, this was not considered particularly important, as in any case all test containers were subject to equal conditions.

In making a reading the exposure rack was placed in front of the light from two 15-watt fluorescent tubes and the shrimp were allowed approximately 1 minute to exhibit response to light before mortality was determined. Unaffected brine shrimp can cover the distance of the vial depth in less than 20 seconds, and move rapidly up and down the column of solution. In response to toxicants they sink to the bottom due to a loss of muscular coordination. They were counted as "dead" if they failed to travel at least 0.25 inch vertically during approximately 30 seconds of observation.

Stored Product Mite. Microscope slides possessing three shallow depressions were used as exposure chambers for the mites. The toxicant in solvent was placed in the slide depressions by calibrated micrometer syringe or small-volume pipet, a given amount being repeated in each depression for (a total of) three replications. Each depression had a capacity of approximately 0.1 ml. The solvents commonly employed for extraction of pesticides (acetone, benzene, carbon tetrachloride, chloroform, petroleum ether, Skellysolve B) were rapidly evaporated at room temperature, leaving a dry film of toxicant. For plant extracts it was sometimes necessary to evaporate the solvents more rapidly in a fume hood on a slightly warm surface because of wax and other plant residues retarding evaporation.

The mites were transferred to the treated depressions on pieces of dark blue or black paper formed with an insect point punch. In a vigorous culture they swarm over the filter paper covering the wide-mouthed rearing bottle. This was moistened with a few drops of water and the dry paper points were placed on top. In about 10 minutes the mites congregated on the surfaces of the dry points, which were transferred by forceps singly to treated slide depressions. The dark paper made the white mites plainly visible, so that points covered with 30 to 80 mites were readily chosen. The use of paper points for transfer minimized injuries caused by trying to handle the mites by other methods.

An enclosed exposure chamber was formed by opposing the depressions of each treated slide with those of an untreated one. A drop of 1% aqueous methylcellulose solution was placed at each end of the treated slide, causing a seal over all flat surfaces when the clean slide was placed on top. In addition to sealing off the exposure chambers, the methylcellulose solution appeared to maintain a desirable humidity during exposure. Mites that crawled out of the depressions before application of the untreated slide were crushed between the flat surfaces and not counted further in the test. Test chambers were laid horizontally in a light-excluding holding cabinet at 78° F. and 40% relative humidity. The mites were active and walked over both treated and untreated surfaces. Each level of acaricide was replicated at least six times.

Mortality counts were made following 24 hours of exposure. Each exposure chamber was readily observed when placed against a dark background in the field of a dissecting microscope at 15× magnification. The total number of living and dead mites was counted at this time. Living mites were activated by placing the exposure chambers for 15 seconds on a surface warmed to 95° F. Living individuals responded by active locomotion or movement of appendages. Dead individuals were motionless and often contorted and dried.

Results and Discussion

Percentage mortality was calculated by compensating for the mortality in untreated controls by Abbott's formula (*7*). For all tests the mortality of control brine shrimp was zero in diluted saline, less than 1% in saline solution with 2% acetone. Mortality of mites was less than 5% for all controls. Regression lines for specific acaricides against brine shrimp and mites were graphed on a log dosage-probit mortality scale (Figures 1 and 2). The lines were fitted statistically by means of the least square method (*3*), and the LD_{50} level was calculated (Table I). The mites were very insensitive to ovex under these experimental conditions, making a regression line impossible to plot. The LD_{50} level for DDT, parathion, and dieldrin, obtained under identical experimental conditions, is included in Table I.

Results indicate the toxicity of the acaricides to brine shrimp at the LD_{50}

evel to be: Mitox > Kelthane > ovex ≅ Aramite > Dimite > Chlorobenzilate > Sulphenone. A comparison at the LD_{50} level between the specific acaricides and three reference insecticides is as follows: Mitox > parathion > Kelthane > Aramite = DDT > Dimite > dieldrin > Chlorobenzilate. As the brine shrimp has been successfully employed by previous workers for the determination of insecticides, and as it is similarly sensitive to specific acaricides, exhibiting a satisfactorily steep slope for the dosage-mortality regression lines, it is obvious that this organism is suitable for the bioassay of residues of specific acaricides.

For the mites the toxicity of the acaricides at the LD_{50} was: Kelthane ≅ Mitox > Dimite > Sulphenone > Chlorobenzilate > Aramite. Ovex was virtually nontoxic to the postembryonic forms tested. In comparing specific acaricides to reference insecticides dieldrin > Kelthane > parathion > Chlorobenzilate > DDT = ovex.

From the standpoint of sensitivity at the LD_{50} level, and slope of the regression line of the seven specific acaricides tested, brine shrimp is more suitable for bioassaying Aramite and ovex. Under the same criteria *T. putrescentiae* is more suitable for the assay of Dimite and Sulphenone. They are roughly equivalent at the LD_{50} level for Kelthane and Mitox, but the brine shrimp possesses a steeper and therefore more satisfactory regression line for these two compounds. Although the brine shrimp is not as sensitive at the LD_{50} level as the mite to Chlorobenzilate, the steep pitch of the regression line suggests it to be a much more suitable organism for bioassay of this compound. In fact, the slope of the regression line for this compound against the mite suggests that it is unsuitable for determination of different levels. The response of brine shrimp to ovex is interesting, as the mite as well as two insects tested (*Drosophila melanogaster* and *Cryptolestes ferrugineus*) were virtually unaffected. This compound is used as an ovicide against mites, and it may be that brine shrimp are suitable for bioassay of other compounds that are primarily ovicidal in activity.

Assay of Plant Extractives. Tissue extractives, as might be expected, hindered the determination of specific acaricides by interfering with toxicant or by possessing naturally toxic substances. The strong-smelling extracts of turnips and onions, extracting 5 grams of tissue with 1 ml. of Skellysolve B, did not affect the mortality of mites in control exposure chambers. An extract of Kelthane from pears (6.45 grams of tissue per ml. of Skellysolve B) was toxic to brine shrimp, causing a calculated recovery of 163% based on the reference curve developed for acaricide and solvent only. Extract of cherries with Skellysolve B (15 grams per 1 ml.) did not significantly affect mortality of brine shrimp, yielding a 101% recovery. However, these same raw extracts appeared to mask the toxicity of Kelthane for mites, yielding only a 49.5% recovery for the pear extract, and an even smaller recovery for the cherry extract. Although standards prepared under identical conditions can be used to counterbalance such effects (*10*), the removal of plant-derived extractives as suggested (*4*, *5*) is desirable if they markedly modify toxicity.

The apparent agreement of chemical analyses of Kelthane with a limited number of bioassays indicates the validity of using brine shrimp and mites for determining residues of specific acaricides. Extracts of pears and cherries were obtained by tumbling them with Skellysolve B and anhydrous sodium sulfate for 1 hour. The filtered extract was determined colorimetrically (*9*), as 0.30 p.p.m. for pears and 2.3 p.p.m. for cherries. This same extract bioassayed with brine shrimp yielded determinations of 0.34 and 2.1 p.p.m., respectively, when compared to reference curves established with toxicant in appropriate plant extract. Bioassay with *T. putrescentiae* under similar conditions yielded determinations of 0.33 and 1.9 p.p.m., respectively. Apparently the differences in toxicity of pure solutions and plant extracts can be compensated for by establishing a toxicity curve with the plant extract in question.

Perhaps both organisms should be used in determining a residue sample. The results would serve as a cross check and might profitably be averaged to give a more accurate determination. For example, two samples of highly purified Chlorobenzilate and Kelthane were prepared by one investigator and determined by the other lacking previous knowledge of their concentration. For Chlorobenzilate a recovery of 96.2% (30.1 mg.) was obtained with brine shrimp, 116.7% (36.54 mg.) with mites. When averaged the recovery was 106.4%, actual amount being 31.30 mg. For Kelthane a recovery of 107.4% (7.57 mg.) was obtained with brine shrimp, 92.6% (6.53 mg.) with mites. When averaged the recovery was 100%, actual amount being 7.05 mg.

Table I. LD_{50} in Micrograms of Toxicant per Milliliter for Brine Shrimp, and per Exposure Chamber for Mites

	Organisms					
	Brine Shrimp			Mites		
Toxicants	LD_{50}	Dosage limits[a]	Slope	LD_{50}	Dosage limits[a]	Slope
Aramite	1.25	1.12–1.38	2.539	11.72	11.61–11.83	3.570
Chlorobenzilate	9.51	9.37–9.65	3.328	3.97	3.85–4.09	1.321
Dimite	2.56	2.45–2.67	3.207	0.71	0.60–0.82	4.422
Kelthane	0.75	0.63–0.87	3.302	0.42	0.34–0.60	2.538
Mitox	0.40	0.28–0.52	2.272	0.57	0.45–0.69	2.064
Ovex	1.16	1.04–1.28	3.534	100.0+	...	
Sulphenone	12.98	12.79–13.17	2.890	1.74	1.51–1.97	4.267
DDT	1.25	1.12–1.38	0.901	100.0+	...	
Dieldrin	5.45	5.29–5.61	0.589	0.041	0.029–0.053	2.969
Parathion	0.52	0.41–0.63	2.500	3.50	3.32–3.68	0.537

[a] Dosage limits were calculated by transforming the limits of % mortality at LD_{50} level to dosages, using the formula:

$$\text{Lower limit } X' = XLD_{50} - \frac{t05\ S\bar{y}.x}{b}$$

$$\text{Upper limit } X' = XLD_{50} + \frac{t05\ S\bar{y}.x}{b}$$

$$\text{where } S\bar{y}.x = \frac{S\bar{y}.x}{\sqrt{n}}$$

x = log dosage
y = probit mortality
$S\bar{y}.x$ = standard deviation
n = no. of observations
b = slope
$t05$ = value of t at 0.05 level

Acknowledgment

The authors are indebted to David Lindgren, Citrus Experiment Station, Riverside, Calif., for providing the initial specimens for the culture of *Tyrophagus putrescentiae*, subsequently determined by Gerald Krantz, Oregon State College, Corvallis, Ore. The colorimetric analyses of Kelthane referred to were made by the Department of Agricultural Chemistry, Washington State University, Pullman, Wash., under the direction of R. R. Legault. Cooperation of insecticide manufacturers in providing samples of acaricides listed is appreciated. The LD_{50} values and determination of the regression lines were calculated with the assistance of the Computing Center, Washington State University.

Literature Cited

(1) Abbott, W. S., *J. Econ. Entomol.* **18,** 265 (1925).
(2) Dicke, R. J., Ihde, K. D., Price, W. V., *Ibid.*, **46,** 844 (1953).
(3) Finney, D. J., "Probit Analysis," 2nd ed., Cambridge Univ. Press, Cambridge, England, 1952.
(4) Hoskins, W. M., Erwin, W. R., Miskus, R., J. Agr. Food Chem. **6,** 914 (1958).
(5) Hoskins, W. M., Schiller, D., Erwin, W. R., 48th Annual Convention,

Natl. Canners Assoc., February 1955.
(6) Michael, A. S., Thompson, C. G., Abramovitz, M., *Science* **123,** (3194), 464 (1956).
(7) Rivard, I., *Can. Entomologist* **90,** 146 (1958).
(8) Rodionov, Z. S., *Zool. Zhur.* **16,** 511 (1937) (Russian, English summary).
(9) Rosenthal, I., Frisone, G. J., Gunther, F. A., J. AGR. FOOD CHEM. **5,** 515 (1957).
(10) Sun, Y. P., Sun, J. Y. T., *J. Econ. Entomol.* **45,** 26 (1952).
(11) Tarplay, W. A., *Ibid.*, **51,** 780 (1958).

Received for review June 1, 1959. Accepted September 25, 1959. Scientific Paper 1856, Washington Agricultural Experiment Stations. Work conducted under Project 1332.

INSECTICIDE METABOLISM

Metabolism of Malathion by Rat Tissue Preparations and Its Modification by EPN

F. W. SEUME[1] and R. D. O'BRIEN[2]

Department of Entomology, University of Wisconsin, Madison 6, Wis.

Homogenates of 11 rat tissues metabolized malathion at comparable rates and to similar metabolites. The main hydrolysis occurred at the carboxyester linkage. The hydrolysis at this linkage, the over-all hydrolysis, and the formation of malaoxon by various tissues in vitro were all inhibited by EPN in vivo. The synergism of EPN and malathion in vivo is therefore probably not attributable to an increased level of malaoxon in the body, but to a greater persistence of malathion and malaoxon in the tissues.

POTENTIATION" OR "SYNERGISM" as it is called in this paper, with organophosphate insecticides in mammals is of considerable interest, because of possible health hazards (*6*) and of the need for investigating the physiological background of the phenomenon (*3, 4, 10*).

Combinations of malathion and EPN deserve particular interest as after the initial report of Frawley *et al.* (*6*) it has frequently been confirmed that certain combinations of these two insecticides in the dog, rat, and mouse resulted in some remarkable synergistic effects (*5, 13*). It has been shown by Cook *et al.* (*3*) that malathion degradation by liver in vitro is inhibited by numerous organophosphates, and by Murphy and DuBois (*10*) that after EPN administration, the ability of the liver and serum to degrade malaoxon (the anticholinesterase which is a metabolite of malathion) is inhibited. Both groups, after studies on rats, concluded that their findings accounted for the observed synergistic effects.

The present study was initiated by the need for a broad approach to the metabolic background of the phenomenon, especially to locate the point of attack of EPN upon the metabolism of malathion. According to Cook and Yip (*4*), the principal liver metabolite of malathion in vitro is one of the half-esters—*O, O*-dimethyl *S*-(1-carboethoxy-2-carboxyethyl) phosphorodithioate. However, their Table I suggests that, in fact, more than one metabolite was produced; if this were so, it would reopen the problem of where EPN attacks.

This study examines the hypothesis that EPN inhibits degradation of malathion and/or malaoxon, and consequently raises the level of malaoxon in tissues. Such an effect in vivo would explain the potentiation of malathion by EPN.

Materials and Methods

Holtzman albino male rats, 160 to 180 grams, were used as sources of tissues which were pooled from at least two animals for every experiment. The animals were decapitated and the tissues immediately chilled after removal. The heparinized blood was separated into plasma and corpuscles (whole corpuscular fraction) by centrifugation.

The tissue preparations used were:

Natural form (plasma and corpuscles).

Homogenates, made with a Lourdes stainless steel homogenizer. Homogenates were used, because they were expected to yield primarily information on degradation. They were also the only type of preparation which could be used for all tissues.

Slices (thickness 0.2 mm.), cut with a Mickle tissue slicer, were used, because it was anticipated that they would yield information primarily on the balance between malathion oxidation and degradation (the procedure of slicing helps preserve cofactor-sensitive oxidative enzyme systems).

Acetone powders. One part of chilled tissue was homogenized in 20 parts of acetone at −50° C. and filtered, and the residue was rehomogenized twice in fresh, cold acetone portions. Acetone was removed by a Labline evaporator, and the powder was dried overnight under room conditions. Incubation suspensions were made up by homogenizing the acetone powders in isotonic solution. Acetone powders were used for comparison with the findings of Cook *et al.* (*3*) which were partially based upon this type of preparation.

Liver cell component fractions. Liver was homogenized in a Potter-Elvehjem type glass homogenizer and differentially centrifuged essentially according to Schneider (*14*); the preparation and incubation medium was isotonic sucrose solution (A) as specified below.

In experiments where only liver was used, not comparatively with other tissues, the liver was perfused before preparation with cold 0.9% sodium chloride solution in situ via the portal and hepatic veins in order to remove blood.

The incubation conditions were, unless stated otherwise: fresh tissue preparation (or the equivalent of fresh whole tissue as acetone powder or live cell component fractions) incubated at a concentration of 5% in isotonic solution A (0.018*M* calcium chloride in 0.25*M* sucrose) containing $6.8 \times 10^{-4}M$ P^{32} malathion for 30 minutes at 37.5° C. while shaken at 120 cycles per minute.

In experiments especially indicated below, a buffered isotonic solution, referred to as B, was used as incubation medium: 0.15*M* sodium chloride, 0.154*M*

[1] Present address, Institut für Pflanzenpathologie, Göttingen, Germany.
[2] Present address, Pesticide Research Institute, London, Canada.