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Received for review June 29, 1959. Accepted October 2, 1959. Division of Agricultural and Food Chemistry, 133rd Meeting, ACS, San Francisco, Calif., April 1958.

INSECTICIDE-MITICIDE RESIDUES

Determination of Trithion Crop Residues by Cholinesterase Inhibition Measurement

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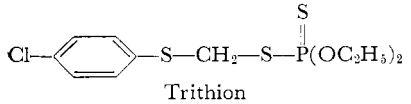
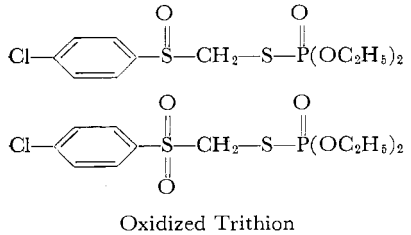
A two-phase hydrogen peroxide–acetic acid–benzene system has been developed for oxidizing Trithion to strong cholinesterase inhibitors with subsequent determination by measuring its inhibition of cholinesterase in human blood plasma. The method is sensitive to 0.01 γ of Trithion and will detect 0.005 p.p.m. residue in crops having low background values. Derived anticholinesterase metabolites which may occur as a spray residue are also determined. Interference levels of some other cholinesterase-inhibiting pesticides are also discussed.

TRITHION (formerly Stauffer R-1303), or *S*-(*p*-chlorophenylthio)methyl *O*,*O*-diethyl phosphorodithioate, is an insecticide-miticide exhibiting moderately long residual activity.



Trithion

It is a poor cholinesterase inhibitor, but is converted into strong inhibitors by oxidation in a two-phase hydrogen peroxide–acetic acid–benzene system. The products of oxidation have been determined by column chromatography and ultraviolet spectroscopy to be a mixture of *S*-(*p*-chlorophenylsulfinyl)methyl *O*,*O*-diethyl phosphorothioate and *S*-(*p*-chlorophenylsulfonyl)methyl *O*,*O*-diethyl phosphorothioate which result from oxidation at two sites on the Trithion molecule.



Oxidized Trithion

Trithion, the two above oxidation products, and the three other possible intermediate oxidation products effect a similar cholinesterase inhibition when the pure materials are subjected to the oxidation procedure. The detection of the oxidation products which may be present in aged spray residues is the chief advantage of the cholinesterase method over the previously employed colorimetric method (*9*) which is specific for the parent compound. In the latter method the chromatographically isolated Trithion is hydrolyzed to *p*-chlorothiophenol and reacted with 2,6-dibromo-*N*-chloro-*p*-quinoneimine at pH 6.6 to form an orange color ($\lambda_{max.}$ 480 mμ). A total chloride procedure employed by Gunther and Blinn (*5*) has been used to determine Trithion residues in oranges and lemons (*6*), but residue values tend to be high because of the detection of hydrolyzed as well as oxidized forms of Trithion. The high sensitivity of the cholinesterase method greatly facilitates sample extraction by permitting the processing of smaller samples.

Earlier attempts to oxidize Trithion crop extracts with dilute bromine water by the method of Fallscheer and Cook (*2*) were not successful. Trithion could be satisfactorily oxidized with extremely small concentrations of bromine, but not in the presence of bromine-reacting crop extractives. Use of higher concentrations of bromine, such as those successfully employed with parathion, etc., resulted in the destructive oxidation of Trithion. The use of *n*-bromosuccinimide as described by Cock (*1*) and of bromine in carbon tetrachloride did not result in the desired conversion of Trithion to strong inhibitors.

A less destructive oxidizing agent was sought which could be used in excess to minimize interference from crop extractives. Also required was the incorporation of an organic solvent which would dissolve the extracted fats and waxes and eliminate the anticipated mechanical problems. The organic solvent would preferably be one that could be employed for crop extraction. These requirements were met by the development of the oxidation system employing hydrogen peroxide, acetic acid, and benzene which has been proved reliable during 3 years of application.

The cholinesterase procedure employed is an adaptation of the method of Giang and Hall (*3*) as modified by Hensel *et al.* (*7*).

Analytical Procedures

Apparatus. Beckman Model G pH meter or equivalent which has a precision of ±0.01 pH unit.

Glass-stoppered test tubes. 160 × 16 mm. in outside diameter with ⅌ 14 flat-head stoppers. These tubes must be made to order.

Constant temperature bath maintained at 37.5° ± 0.5° C. equipped with racks for supporting 5- and 20-ml. beakers.

Constant temperature bath maintained at 75° ± 1° C. equipped with a rack for holding glass-stoppered test tubes in a vertical position and immersed to a depth of 4 to 5 cm.

Solvent evaporator. A manifold of air jets suspended with rubber couplings directly over a hot plate maintained at about 40° to 60° C. Means are provided for adjusting the height of the jets above the hot plate. The air line should contain a trap and filter.

Waring Blendor or suitable substitute for macerating samples.

Rotary tumbling device similar to that described by Gunther and Blinn (*4*), geared to revolve at 29 r.p.m. Compartments are made to hold 1-gallon wide-mouthed glass jars, and also combinations of half-gallon, quart, or pint jars with the aid of cardboard separators.

Reagents. Pooled human blood plasma (as source of cholinesterase). Purchase plasma at local blood banks and keep it constantly refrigerated or in an ice bath. Test individual batches and mix them so that a 1-ml. aliquot will effect an acidity between 1.8 and 2.0 pH units. Plasma can be stored in sterile screw-cap bottles for several months under refrigeration without detectable change in activity. Frozen plasma can be stored for more than a year.

Buffer Solution. Add 2.67 grams of barbital, 0.545 gram of potassium dihydrogen phosphate, 44.7 grams of potassium chloride, and 12 ml. of 1.0*N* sodium hydroxide to a 500-ml. glass-stoppered, volumetric flask and dissolve in about 480 ml. of distilled water. Shake vigorously if necessary to dissolve the barbital, and remove any flocculent material by filtration. Adjust the pH to 7.9 to 8.0 with about 1 ml. of 1*N* hydrochloric acid and dilute to the 500-ml. mark. Add a drop of toluene as preservative and store in the refrigerator when not in use.

Acetylcholine Solution. Transfer 12.0 grams of acetylcholine chloride (Matheson, Coleman, and Bell) into a 100-ml. volumetric flask, dissolve in distilled water, and bring to mark. Add a drop of toluene as preservative and store in the refrigerator when not in use.

Acetic Acid–Peroxide Solution. Mix 1 volume of 30% hydrogen peroxide with 5 volumes of glacial acetic acid. Prepare this mixture just before use and discard the excess.

Trithion Stock Solution. Prepare a solution containing 1.00 γ of Trithion per ml. of benzene and store in the absence of direct light in a tightly stoppered container

Nujol Solution. Weigh 1.0 gram of Nujol or other refined mineral oil into a 60-ml. dropper bottle and dissolve in 50 ml. of benzene.

Boiling Chips. Hengar granules or equivalent—preferably crushed and screened to about 10- to 15-mesh.

Sample Extraction. Weigh 100 grams of sample, selected so as to be representative, into a 1-quart Waring Blendor cup. Add 200 ml. of benzene, blend intimately with pulp for 5 minutes, and transfer to a 1-pint, wide-mouthed jar. Tumble for 1 hour in a mechanical tumbler and filter a portion of the benzene extract into a suitable container.

Trithion Oxidation. Transfer 5-ml. portions of the benzene extract of the sample to glass-stoppered test tubes. Add 3 ml. of acetic acid–peroxide solution, and a boiling chip to each tube. Stopper the test tubes, shake briefly, loosen the stoppers and place in a 75° C. bath for 20 ± 0.5 minutes. At the end of the heating period, remove the test tubes and cool them in an ice bath. Add 5 ml. of distilled water to the tubes, stopper, and shake well. When the benzene layer (top) has cleared, transfer measured aliquots into 20-ml. beakers with the aid of a serological pipet. (Aliquots should contain between 0.02 and 0.15 γ of Trithion. Use a Propipette to assist in taking 4-ml. aliquots.) Add 4 drops (about 0.1 ml.) of Nujol solution to each 20-ml. beaker and mix with the sample aliquot. (The Nujol keeps waxy extractives soft and facilitates the subsequent water extraction.) Remove the benzene with solvent evaporator. Allow a few minutes after the beakers appear dry to remove traces of acetic acid which may remain (can be detected by odor). Add 9.0 ml. of water to each beaker and heat for 10 minutes on a hot plate set at 110° to 130° C. Cover the beakers tightly with a piece of aluminum foil during the heating period to minimize evaporation (heating to near boiling temperature is necessary to extract the oxidized Trithion from sample waxes and oils). After the heating period, remove the foil and cool the beakers in a water bath. Samples may be capped and stored overnight at this point, if necessary.

Cholinesterase Inhibition. Determinations are conveniently run in sets of 24, typically including two blanks and several standards or fortified control samples.

Add 1.0 ml. of plasma to the 20-ml. beakers containing the oxidized Trithion samples. Swirl to effect homogeneous solutions and place in a 37.5° C. bath for 70 minutes. (Beakers need not be individually covered, as a loose cover over the water bath minimizes evaporation and prevents contamination.) After the heating period, remove the samples from the bath and transfer a 1.0-ml. aliquot from the 20-ml. beakers to 5-ml. beakers to which has been added 1.0 ml. of buffer solution. Take the pH of the beaker solutions and record them as the initial pH. Carefully add exactly 5 drops (0.2 ml.) of the acetylcholine solution to the 5-ml. beakers. Use the same dropper for each sample. Place the beakers in a 37.5° C. bath for 120 minutes. At the end of this period, remove the beakers, allow them to cool for 5 minutes, and take the pH of the solutions without unnecessary delay and record them as the final pH. Calculate the per cent inhibition as follows:

$$\% \text{ cholinesterase inhibition} = 100\left[1 - \left(\frac{\text{sample } \Delta\text{pH}}{\text{blank } \Delta\text{pH}}\right)\right]$$

The ΔpH is the difference between the initial and final pH, and the blank is a sample of distilled water (containing no Trithion), which is processed in a manner identical to the standards and samples. The ΔpH of the blank should range from 1.8 to 2.0 pH units. A higher ΔpH may decrease the sensitivity of the method, so it should be lowered by diluting the plasma. The ΔpH can also be adjusted by changing the acetylcholine chloride concentration.

If two blanks are run as the first and the last sample of a 24-sample run, the last blank will average about 0.15 pH unit lower than the first because of the continuing, but slower, hydrolysis of acetylcholine at the lower temperature. For maximum accuracy this effect is compensated for in the calculations.

Standard Curve Preparation. Oxidize a 5-ml. portion of Trithion stock solution containing 1 γ per ml. of benzene as described under Trithion oxidation. Determine the cholinesterase inhibition of an appropriate number of aliquots of the oxidized Trithion solution and plot per cent inhibition *vs.* amount of Trithion in the aliquot. The curve approximates a straight line between 75 and 20% inhibition when the per cent inhibition is plotted on the linear scale of semilog graph paper. The slope of the curve increases below 20% inhibition, but is still considered useful. Typical values are listed in Table I.

Sample Analysis. Dilute the sample extract with benzene if the Trithion concentration exceeds 2 γ per ml. and proceed as in the preparation of the standard curve. Refer the resulting per cent inhibition values to the standard curve to determine the Trithion content of each sample aliquot.

Special Oil-Removal Procedure. The presence of excessive quantities of oil and/or wax in the extract tends to decrease the extractability of the oxidized Trithion with hot water. Crops such as almonds, walnuts, and cottonseed must therefore be subjected to an oil-removal step. The acetonitrile extraction of Jones and Riddick (*8*) is employed to separate the oils and waxes from the Trithion and derived anticholinesterase products in the following procedure:

Table I. Typical Cholinesterase Inhibition Values

Trithion, γ	*Inhibition*, %
0.000	0
0.010	4
0.015	7
0.020	10
0.030	18
0.050	33
0.080	47
0.120	58
0.190	71

Table II. Cholinesterase Inhibition of Some Other Pesticides

Pesticide	γ Required to Effect 50% Inhibition
Delnav	1.0
Demeton	4.3
Diazinon	0.08
Di-syston	4.2
EPN	0.2
Ethion	0.09
Guthion	4.4
Malathion	4.3
Methyl parathion	3.1
Methyl trithion	15.5
OMPA	50.0
Parathion	0.03
Phosdrin	1.5
Sevin	77.0
TEPP	0.06
Thimet	5.5
Trithion	0.09

Table III. Recovery of Trithion Added to Crop Extracts

Crop	Added, P.P.M.	Recovered, %
Almond hulls	5.0	102
Apples	1.0	90
Beans, fresh	0.10	81
Beans, dried	0.05	95
Beets, red	0.05	110
Beets, sugar	0.05	116
Eggplant	0.06	109
Grapes	1.0	95
Hops, dried	10.0	30
Lemon peel	10.0	90
Lemon juice	0.10	98
Olives	2.0	95
Orange peel	8.0	100
Orange juice	0.10	92
Peaches	1.0	87
Pears	0.10	99
Plums	0.50	96
Sorghum	1.0	89
Almonds[a]	0.10	66
Cottonseed[a]	0.10	84
Walnuts[a]	0.05	70

[a] These crops were subjected to the special oil-removal procedure and are calculated on the basis of 80% theoretical recovery from the acetonitrile partition.

Transfer 10 ml. of the sample to a 100-ml. beaker and remove the benzene on the solvent evaporator. Dissolve the residual material in 10 ml. of iso-octane and transfer to a centrifuge tube with the aid of 10 ml. of acetonitrile. Stopper the tube and shake well. Stable emulsions form in most cases and the sample must be centrifuged. Addition of mineral acids to prevent emulsions can cause interferences. Transfer 5.0 ml. of the acetonitrile phase (lower) to a glass-stoppered test tube (the Propipette pipet filler will aid in this operation). Immerse the test tube in a beaker of warm water at 40° to 50° C. and remove the acetonitrile under a gentle stream of air. Dissolve the residual material with 5 ml. of benzene and oxidize as previously described.

The partition coefficient for Trithion is 80% into the acetonitrile, and is greater than 87% for the five oxygen analogs.

Other Pesticides. Table II lists the cholinesterase-inhibiting pesticides which have been subjected to the oxidation procedure. Many of these could seriously interfere with the determination of Trithion residues unless selectively removed by reduction, hydrolysis, etc. These possibilities are being investigated.

Discussion

Oxidation Conditions. When the proportions of benzene, acetic acid, and peroxide are adjusted to effect a miscible system, the conversion to good cholinesterase inhibitors is greatly reduced. In the oxidation procedure a small interfacial area between the organic and inorganic phases is essential, as exemplified by the poor results obtained in an Erlenmeyer flask as compared to a test tube. Optimum time and temperature for the two-phase system have been determined. Excessive or insufficient oxidation will occur at the rate of 2 to 3% per minute of deviation from the 20-minute oxidation time. Change in temperature is less critical, unless it reaches about 78–79° C., where the solutions begin to boil with subsequent increased oxidation rate. This is to be avoided, because of the inability to maintain uniform boiling times. Originally, the oxidation system employed an 80° C. bath without addition of boiling chips. The solutions did not boil and were shaken by hand at intervals. Shaking is now unnecessary, because of uniform evolution of small bubbles from the boiling chips which result in sufficient mixing as they rise through the benzene phase.

Extraction Efficiency. Trithion and its five oxygen analogs will all partition completely into benzene from water or aqueous fruit pulp, and very good recovery has been effected when these compounds were added to pulverized samples. Sample types tested include aqueous and oily fruits, green and dried beans, grain crops, and nuts.

Extensive tests on pippin apples showed the absence of any potential cholinesterase inhibitor which was not extracted by benzene. These tests were based upon water extractions of the samples which were checked for cholinesterase activity before and after bromine oxidation.

Analytical Recovery. Table III lists some typical values for the recovery of Trithion which had been added to a variety of crop extracts in the indicated amounts. Many other crops have resulted in similar recoveries. Interferences are generally small and mostly of a mechanical nature. The recovery percentage is usually lower for crops having a high oil or wax content, although the acetonitrile partition reduces this interference markedly. Dried hops gave a very low recovery, although extractives in the final aliquot were low.

Natural inhibitors are suspected to be present in hops and celery.

Stability of Trithion Solutions. Trithion and its five oxygen analogs have exhibited no decomposition, when stored for 7 weeks in the absence of direct light as dilute solutions in benzene. A dilute solution of Trithion in iso-octane was stored under similar conditions for 19 weeks without detectable decomposition as determined by ultraviolet spectroscopy and anticholinesterase activity. Dilute solutions of Trithion in iso-octane and methanol are not stable when subjected to strong ultraviolet radiation.

The half life of Trithion in distilled water at laboratory temperatures is 102 days (solubility is 0.33 p.p.m.), whereas the half life for some of the more water-soluble oxidation products is about 30 days, as determined by the cholinesterase method.

Tests on sample extracts stored at laboratory temperatures in clear glass containers have exhibited some changes after 1 to 2 months. The magnitude of the change appears to be dependent upon the type of crop. It is thus recommended that samples be analyzed soon after extraction. If this is not possible, the extracts should be refrigerated, along with controls fortified with known quantities of Trithion, so that storage stability of the experimental samples can be verified.

Accuracy, Precision, and Sensitivity. The maximum precision of any single cholinesterase inhibition determination is estimated to be ±0.005 γ Trithion in the 30 to 60% inhibition range. Precision is determined by the limitations for making rapid and accurate volumetric measurements of some of the reagents, and by the precision of the pH meter. Increased precision is attained by averaging the results from multiple determinations.

Accuracy of the analysis is dependent upon reliability of the reference curve. Greatest accuracy is attained when a working curve prepared from fortified control samples is determined simultaneously with actual samples.

Quantities of Trithion down to 0.01 γ can be satisfactorily estimated in samples with low background and low interference levels. This represents 0.005 p.p.m. for a 2-gram aliquot (in 4 ml. of benzene) which is the practical limit of the procedure.

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Received for review April 8, 1959. Accepted October 29, 1959.

INSECTICIDES AND FLAVOR

Flavor of Cantaloups as Affected by Treatment with Lindane

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The flavor of cantaloups treated with lindane as a dust, wettable powder, or emulsion concentrate at the rate of 0.35 pound active ingredient per acre in each of four applications was compared to the flavor of untreated cantaloups. Results of these palatability evaluations indicate that lindane applied as a dust had no detrimental effect on flavor. The effect of lindane wettable powder on flavor of melons was less definite, but its use may contribute to off-flavor. Treatment with lindane emulsion concentrate resulted in an adverse effect on flavor of cantaloups.

THE USE OF AGRICULTURAL CHEMICALS has greatly reduced insect damage to food crops and thus has increased yield and quality of these crops, although in certain foods off-flavors have been produced.

A possible residual in the soil by lindane treatments may affect the flavor of subsequent crops grown in the same soil. Present U. S. Department of Agriculture recommendations (*4*) for the control of the pickleworm include the use of lindane applied as dust or wettable powder with the restriction that it not be applied within 1 day before a harvest and not be applied in fields to be planted later to potatoes or other root crops, as it may adversely affect their flavor.

Reported here are the results of a study conducted by the Human Nutrition Research Division in cooperation with the Entomology Research Division to determine whether cantaloups treated with lindane in the form of dust, wettable powder, or emulsion concentrate developed off-flavors or off-odors.

Production and Harvesting

Cantaloups of the Hearts of Gold variety were grown at the Plant Industry Station, Beltsville, Md., by the Crops Research Division, and were treated and harvested by the Entomology Research Division. The melons were grown in three field blocks, each of which contained one plot for the untreated cantaloups and one plot for each of the three treatments. Two rows of cantaloups were grown in each plot.

The insecticide treatment was lindane applied to the vines and melons as dust, wettable powder, or emulsion concentrate at the rate of 0.35 pound of active ingredient per acre. Cantaloups received the first application of lindane treatment the week of July 25, 1957, and treatment was continued at weekly intervals for 4 weeks. The last application of lindane was made the day preceding harvest to allow the greatest possible influence on flavor. This is in accordance with commercial practice in growing the relatively new fall crop. All samples, including the control, were sprayed with a fungicide made with 3 pounds of tribasic copper sulfate and 2 pounds of Dithane to 100 gallons of water per acre.

The cantaloups were harvested from August 26 to September 4 at the "full-slip" stage of maturity in order to obtain melons of uniform ripeness. However, upon cutting the cantaloups, some variation was found in degree of ripeness regardless of the care exercised in selection. At the time of harvest the top of each melon was marked with an X to distinguish it from the bottom, which was unmarked. The top of the cantaloup was considered that portion which did not touch the ground during growth. This procedure made it possible to separate the two portions for palatability evaluation and to ascertain whether both portions were similarly affected by the lindane treatment.

Two melons from each plot (one from each row) of a single field block were harvested and delivered daily to the Human Nutrition Research Division laboratory. The cantaloups, which were of good quality when received, were held overnight at 40° F. They were judged the day following harvest to avoid long holding, as flavor loss in full-slip, yellow melons is rapid in storage (*3*).

Procedures for Palatability Evaluations

The two melons from each of the untreated plots and each of the treated plots were cut in half; top halves were prepared as one sample and bottom halves as another. The rind was removed, any damage from cracks or bruises was carefully trimmed away, and the cantaloups were then cut into 1-inch cubes. To provide a representative sample of cut pieces and juice, the cantaloup was prepared in an electric blender. To avoid development of oxidized flavor, only one eighth of the cubes at one time were chopped for 15 to 20 seconds on low speed. All eight aliquots of a sample were transferred to a glass bowl and any pieces uncut by the blender were cut by hand with a stainless steel paring knife. The bowl was covered with aluminum foil and the melon was chilled in a refrigerator until served—no longer than 20 minutes.

Individual portions of the melon samples, approximately two tablespoons, were served in coded, white procelain dishes to the panel members.

Palatability testing was done at individual tables in a room especially equipped for panel testing and separate from that in which samples were prepared. Five members of the staff, in no way connected with preparation of the samples, evaluated the cantaloups for flavor and odor, and were asked to identify any detectable off-flavors and/or off-odors. Separate 5-point scoring scales were used for flavor and odor: for flavor, 5 represented natural flavor or no off-flavor; 4, slight off-flavor; 3, moderate off-flavor; 2, strong off-flavor; 1, very strong off-flavor. A similar scoring scale was set up for odor.