2-year rats received diets containing 100, 300, or 1000 p.p.m. Comparison of tissue levels as parts per million of 2,2-dichloropropionic acid with dietary levels expressed as parts per million of dalapon sodium shows the results for the two species to be of the same order of magnitude. Quantitatively, the amounts occurring in the meat or other tissues were quite low in relation to the levels administered.

Practical Considerations for Handling and Use

The results of the studies reported here indicate that dalapon sodium is low in acute and subacute oral toxicity. The likelihood of human subjects, livestock, or wildlife ingesting sufficient amounts of the material to cause serious toxic effects is therefore extremely remote. However, salt-hungry stock have been known to ingest lethal amounts of sodium chloride, so it is recommended that dalapon sodium or strong solutions of it be kept in closed containers where it is unavailable to stock or wildlife.

Undiluted dalapon sodium may cause skin irritation, if it is allowed to remain on the skin for a prolonged period. Exposures of short durations are not likely to be injurious. Contact with dust, particularly when sweating, may cause a mild burning sensation. Prolonged contact with dilute aqueous solutions is not likely to cause any appreciable effect. Systemic effects due to absorption through the skin are not likely to occur. Because the ability of the material to cause irritation is markedly reduced by dilution with water, the prompt flooding of exposed areas with water will practically eliminate the possibility of irritation occurring as a result of skin contact.

The material in the solid form or in concentrated solutions is capable of causing appreciable pain, and irritation of the eyes, but is not likely to cause serious damage. Nevertheless, eye protection, such as safety glasses, should be worn when handling the salt or concentrated solutions. Solutions of less than 10% concentration are not likely to cause more than transient pain and inflammation. Prompt washing of contaminated eyes will markedly reduce the severity of any effect caused by the solid or strong solutions and practically eliminate the effect from dilute solutions.

Experience in the manufacturing, handling, and application of dalapon sodium has indicated that it presents no unusual problems. No incidences of irritation or other adverse effects have come to the attention of the authors thus far in the use of this product.

The results of the 2-year, life-span dietary feeding studies to rats, the reproduction and lactation studies in rats, and the 1-year repeated oral administration to dogs also show a low order of toxicity for this herbicidal material. Thus, these data provide a basis for judging the safety of the small amounts of dalapon sodium residues likely to occur when this herbicide is used as directed on edible crops, or on soil in which food crops are grown.

Acknowledgment

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INSECTICIDE RESIDUES

Colorimetric Determination of Residues of Phorate and Its Insecticidally Active Metabolites

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A colorimetric method, based on the chromatropic acid procedure for formaldehyde, has been developed for the estimation of Thimet (phorate) and its insecticidally active metabolites. The limit of detection of the method as indicated by results from analysis of 100-gram samples of treated plant leaves is about 0.10 p.p.m. and with larger samples it is even less. Although the method was developed for the determination of residues of phorate and related materials, it can also be applied with some minor modifications to the analysis of residues of any organic phosphorus insecticide that has a methylene linkage present in its molecule which will produce formaldehyde on hydrolysis.

PHORATE [the recently assigned generic name for *O,O*-diethyl *S*-(ethylthio)methyl phosphorodithioate, the major constituent of the commercial product, Thimet] has recently attracted wide interest as a systemic insecticide (*2, 7, 9*), although it also has considerable contact activity. Like many of the other organophosphorus insecticides, phorate is toxic to warm-blooded animals, being comparable to parathion in this respect (*2*).

In studies of the fate of phorate in plants, Bowman and Casida (*3*) and Metcalf, Fukuto, and March (*8*) found five oxidation products which are insecticidally active and which have high mammalian toxicity. These are the phorate oxygen analog, phorate sulfoxide, the phorate oxygen analog of the sulfoxide, phorate sulfone, and the phorate oxygen analog of sulfone.

Hydrolysis or any further oxidation results only in decomposition products that have little toxicity to insects or mammals. These authors found from in vitro enzymatic studies, that the sul-

Table I. Recoveries of Phorate and Its Oxidation Products Added to 100 Grams of Cabbage without Perbenzoic Acid Oxidation

Chemical	Added, γ	Recovered γ	Recovered %
Phorate	87	47	54
	44	22	50
Phorate sulfoxide	102	86	84
	51	42	82
Phorate sulfone	78	73	94
	39	37	94
Oxygen analog	85	55	65
	42	25	60
Oxygen analog of sulfone	91	83	91
	46	43	93
Mixture of compounds	78	60	77
	39	32	82

fones are ten times more potent as cholinesterase inhibitors than the respective sulfoxides, and that pure phorate itself is a weak inhibitor.

Currently, the only available analytical method for phorate residues is a cholinesterase inhibition method (*2*), which is also used for the determination of residues of many of the other organophosphorus insecticides. Oxidation products of phorate are found in plants in varying amounts depending on time, temperature, light, soil conditions, etc. As each oxidation product possesses a different degree of anticholinesterase activity, the results obtained by this method are difficult to interpret, unless the phorate residue is oxidized (*8*). It is important that any method for the analysis of phorate residues include not only phorate itself, but also all its metabolic products that have high anticholinesterase activity.

The method described herein is based on the observation that, under appropriate conditions, certain types of organophosphorus compounds will yield formaldehyde (*6*), which can be determined by a colorimetric chromotropic acid procedure. Phorate, its oxygen analog, and their sulfoxides produce a red-violet color with this reagent due to the production of formaldehyde. These compounds produce the color whether they have been hydrolyzed by alkali or not, whereas the respective sulfones must be hydrolyzed by alkali before they will produce a color. However, all these compounds can be easily oxidized to their respective sulfones by perbenzoic acid. In experiments on the recovery of each of the compounds without including an oxidation step, the sulfones gave the highest recoveries (over 90%), the sulfoxides about 80 to 85%, and phorate and its oxygen analog about 50 to 60% (Table I). Thus, it is advantageous to include an oxidation step, with perbenzoic acid to convert the compounds to their sulfones, in order to obtain maximum recoveries.

In the method described, the plant material is extracted with chloroform, the extract is oxidized with perbenzoic acid, and then chromatographed through a Nuchar–Hyflo Super-cel column. The eluate is washed with sodium metabisulfite and saturated sodium chloride solutions, and the solvent is evaporated completely. The residue is taken up in water and chilled, and the waxes are removed by filtering through a layer of Attaclay–Hyflo Super-cel mixture. The filtrate is extracted with chloroform, the solvent is again evaporated, and the sodium hydroxide solution is added. The color is developed by heating with chromotropic acid reagent and is read in a spectrophotometer at 570 mμ.

Apparatus

Borosilicate glass test tubes, 20 × 150 mm., with ground-glass stoppers. Scratch a mark on each tube at 6-ml. volume.

Gooch crucible with a perforated bottom, top about 29 mm. and bottom about 18 mm. in diameter.

Reagents

Chloroform, technical grade. Wash the solvent four times with an equal volume of water, dry over anhydrous calcium chloride, decant, and distill.

Perbenzoic acid in benzene. Prepare and analyze as outlined by Adams (*1*) and Braun (*4*). For use in this method, dilute 5 ml. of the prepared benzene solution to 50 ml. with chloroform.

Adsorbent mixture. Boil some Nuchar in a large flask with concentrated hydrochloric acid for 20 minutes, wash by decantation with distilled water, filter, and wash until neutral to litmus. Then wash with two 200-ml. portions of 95% ethyl alcohol, and dry in the hood and finally in a 120° C. oven overnight. Mix 3 parts of this activated Nuchar with 1 part of Hyflo Super-cel.

Sodium metabisulfite ($Na_2S_2O_5$), 0.5% solution, freshly prepared.

Saturated sodium chloride (C.P. grade) solution in distilled water.

Filter mixture. Mix 3 parts of Attaclay with 1 part of Hyflo Super-cel.

Chromotropic acid reagent. With the aid of heat, dissolve 16 grams of chromotropic acid (4,5-dihydroxy-2,7-naphthalenedisulfonic acid sodium salt, practical) in concentrated sulfuric acid (C.P. grade), cool to room temperature, dilute to 1 liter with sulfuric acid, and store in a brown bottle. Prepare fresh reagent every 2 weeks. The reagent is conveniently dispensed from an automatic buret.

Mixed solvent. Using redistilled solvents, add 5 parts of chloroform, 5 parts of carbon tetrachloride, and 7 parts of diethyl ether. Mix the solvents just before use.

Procedure

Preparation of Standard Curve. Transfer an accurately weighed sample of 50 mg. of phorate to a 500-ml. volumetric flask and make up to the mark with chloroform. After mixing, pipet 20 ml. of the solution into a 100-ml. volumetric flask, make up to the mark with chloroform, and mix well. Pipet 0-, 1-, 2-, 3-, 4-, and 5-ml. aliquots of this solution into borosilicate glass test tubes. Add to each tube 1 ml. of the diluted perbenzoic acid solution. Stopper, shake well, and place the tube in a 50° C. water bath for 15 minutes. Then immediately transfer the contents to a 60-ml. separatory funnel. Rinse the tube with two 5-ml. portions of chloroform and add the rinses to the material in the separatory funnel. Extract the chloroform solution with 10 ml. of freshly prepared 0.5% sodium metabisulfite and then twice with 10-ml. portions of saturated sodium chloride solution. Place a small plug of cotton and some anhydrous sodium sulfate (about 3 to 5 grams) in a Gooch crucible holder, and filter the washed chloroform solution into a clean borosilicate glass test tube. Evaporate the solvent carefully in a 60 to 70° C. water bath in the hood. Heat no longer than necessary, to avoid loss of the sulfones.

To develop the color, pipet 3 ml. of 0.5*N* sodium hydroxide solution into each tube, rinse the sides of the tube well, and heat in a hot-water bath for 1 minute. Cool, add 3 ml. of the chromotropic acid reagent, and mix by stoppering the tube tightly and shaking well. Loosen the stopper carefully, place the tube in a boiling-water bath for 45 minutes, and shake occasionally. Remove the tube from the bath and cool at once in a beaker of cold water. Make up to the 6-ml. mark with water and mix the contents well. Fill cuvettes with the solution, cover, and measure in the photometer (Beckman Model B or equivalent) at 570 mμ against a blank solution carried through the same procedure. Prepare the standard curve by plotting the absorbance readings against micrograms of insecticide. The curve follows Beer's law up to about 100 γ, having a slope of 11.6 γ of phorate per 0.100 unit of absorbance on the Beckman Model B spectrophotometer.

Analysis of Plant Samples. Cut a weighed plant sample into small pieces and macerate with a measured volume of chloroform (about 2 ml. per gram of leafy vegetable) in the blender for about 3 minutes. Strain the extract through a piece of cheesecloth, and wash the chloroform with about half its volume of saturated sodium chloride solution. If the chloroform and aqueous layers do not separate readily, centrifugation may be

used; otherwise, filter the chloroform layer through a Gooch crucible holder containing cotton and anhydrous sodium sulfate. Measure the volume collected, and evaporate to about 100 ml. in an Erlenmeyer flask.

For the oxidation add 5 ml. of the diluted perbenzoic acid to the extract, stopper tightly, mix thoroughly, and let stand for 10 minutes. The oxidation should be carried out just before percolation of the extract through the chromatographic column.

For adsorption chromatography use 10 grams of the Nuchar–Hyflo Super-cel mixture for each 100 grams of plant sample. Slurry the mixture with chloroform, pour into the chromatographic tube (about 20 × 400 mm.), and pack the column with a little air pressure. Add approximately 2 inches of anhydrous sodium sulfate at the top of the column followed by more chloroform (50 ml. for each 10 grams of Nuchar mixture).

When the chloroform level reaches the top of the sodium sulfate, quantitatively transfer the oxidized chloroform extract to the column, and apply a little pressure if necessary, so that the speed of percolation will be not more than 60 drops per minute. When the level of the chloroform extract reaches the top of the column, carefully add more chloroform (100 ml. for each 10 grams of Nuchar mixture). Transfer the eluate into a 500-ml. separatory funnel, wash first with 100 ml. of 0.5% freshly prepared sodium metabisulfite solution and then with three 150-ml. portions of saturated sodium chloride solution, and filter through anhydrous sodium sulfate in a Gooch crucible holder.

For distillation of the solvent, place the flask on the steam bath and attach a three-bulb Snyder column. Evaporate the last traces of solvent at room temperature by inserting a glass tube connected to a vacuum line into the neck of the flask while it is being rotated. Heat the waxy residue with 20 ml. of water by swirling on the steam bath until the wax is melted. Chill thoroughly in an ice bath, and then filter with vacuum through a 0.5- to 1-cm. layer of Attaclay mixture on a filter-paper disk in a Gooch crucible. Rinse the flask with two 10-ml. portions of water and filter each through the same filter. At no time during the filtration should the Attaclay filter cake be allowed to dry or crack.

Extract the combined filtrate with 50 ml. of chloroform by vigorously shaking for at least 1 minute. When the two layers have completely separated, filter the chloroform layer through anhydrous sodium sulfate. Extract the aqueous layer again with another 25 ml. of chloroform and filter through the same sodium sulfate. Distill the chloroform extract on the steam bath through a three-bulb Snyder column until about 5 to 10 ml. are left. Quantitatively transfer this extract to a borosilicate glass test tube with two successive 5-ml. chloroform rinsings. Evaporate the solution, almost to dryness, in a water bath at 60° to 70° C. Remove the last traces of solvent with slight vacuum at room temperature.

Add 3 ml. of 0.5*N* sodium hydroxide solution to the material in the tube and finish the determination as described under Preparation of the Standard Curve. Occasionally, the colored solution shows some turbidity, especially when the sample is large and the Nuchar mixture does not remove impurities completely or the Attaclay mixture packed in the Gooch crucible is not thick enough or cracks during filtration. This turbidity can be remedied by cooling the solution, extracting with 5 ml. of the mixed solvent, and centrifuging.

A control sample of untreated plant should be analyzed along with the treated samples. The residues of phorate and all its active metabolites are expressed in terms of phorate equivalents.

Recovery Tests. The percentage recovery of phorate and of each of its oxidation products added to cabbage was determined in the following manner: Known amounts were added to a series of 100-gram samples of cut-up cabbage, extracted with 200 ml. of chloroform in a blender, and analyzed, without the perbenzoic acid oxidation step. The results are shown in Table I.

Additional experiments on the application of the method to each of the metabolites, with the inclusion of the oxidation step, showed that in each case over 90% recovery was obtained. Table II shows results of recoveries of phorate added to a variety of plants when the oxidation step is included.

Table II. Recovery of Phorate Added to 100 Grams of Plant Material with Perbenzoic Acid Oxidation

Plant	Added, γ	Recovered γ	Recovered %
String beans	19	17	89
	38	37	97
	56	54	96
Red cabbage	19	18	95
	38	35	92
	56	53	95
Spinach	19	18	95
	38	36	95
	56	52	93
Kale	11	10	91
	23	22	96
	45	43	95
	91	82	90
Tomatoes	11	10	91
	23	20	87
	45	42	93
Potatoes	23	21	91
	45	40	89
	90	83	92
Beet leaves and roots	19	16	84
	38	33	87

Discussion

For chromatographic cleanup, the charcoal-Celite column of Fukuto *et al.* (*5*) for demeton was adopted. In order to minimize the volume of solvent to be evaporated, only chloroform was used instead of the solvent mixtures used by them.

For their preparation of sulfone and sulfoxide derivatives, Metcalf, Fukuto and March (*8*) employed perbenzoic acid in chloroform overnight in a refrigerator. In the method described herein, however, 10 to 15 minutes with slight heating was found to be sufficient for oxidation of phorate and related compounds for the preparation of the standard curves. For residue determinations, heating is not necessary, because of the long time taken for the chromatography step.

The amount of perbenzoic acid needed in the phorate oxidation procedure is not critical. For the preparation of the standard curve, the use of 0.5 to 1.0 ml. of the chloroform-benzene solution of perbenzoic acid is sufficient. For small samples (about 50 grams), 2 ml. of the diluted perbenzoic acid solution may be used, but for larger samples (100 to 200 grams), 5 ml. are preferred.

In the cleanup procedure, the chromatography with Nuchar–Hyflo Super-cel as the adsorbent would remove most of the plant pigments and interferences other than plant waxes. Washing with sodium metabisulfite is necessary to remove the yellow or brown color produced by the perbenzoic acid with control samples of plant crops. After distillation of the chloroform, plant waxes are removed by dissolving the residue in water, and filtering through the Attaclay–Hyflo Super-cel mixture.

Although practically no interference has thus far been encountered from runs on plant materials not treated with phorate, it is always important to run such control analyses. Occasionally, the results of an analysis can be too high if a suitable control sample is not run or if the cleanup procedure is not carefully followed. Absorbance readings on control samples of various vegetables measured against a reagent blank in the reference beam of the spectrophotometer were in the range of 0.02 to 0.08 for the vegetables shown in Table II.

Many insecticides—such as DDT, BHC, chlordan, aldrin, dieldrin, malathion, parathion, demeton, schradan, and Phosdrin—do not interfere with this colorimetric analysis. However, some of them—such as Guthion, Trithion, and ethion—do liberate formaldehyde in this procedure and therefore should be considered interferences. A procedure based on the liberation of formaldehyde has been developed for Guthion (*6*), and further work is in progress on Trithion and ethion.

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INSECTICIDE-MITICIDE RESIDUES

Determination of Trithion Crop Residues by Cholinesterase Inhibition Measurement

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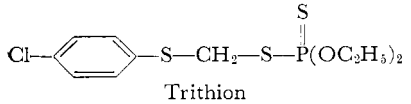
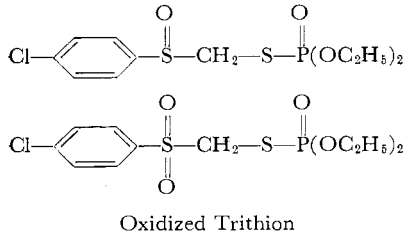
A two-phase hydrogen peroxide–acetic acid–benzene system has been developed for oxidizing Trithion to strong cholinesterase inhibitors with subsequent determination by measuring its inhibition of cholinesterase in human blood plasma. The method is sensitive to 0.01 γ of Trithion and will detect 0.005 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.