Determination of Residues of Phorate and Its Insecticidally Active Metabolites by Cholinesterase Inhibition

Part I. Basic Method
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Part II. Alternative Sample Preparation, and Recovery Data
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A cholinesterase inhibition method has been developed for determination of residues of phorate and its active metabolites in vegetable crops. In addition to the water-maceration sample preparation technique used originally for most crops, alternative methods are applicable to oily crops, potatoes, milk and butterfat, citrus, and certain leafy crops which give unacceptably low recovery figures by the original procedure. Recovery values are listed for approximately two dozen vegetable crops and other food materials analyzed by the basic method and its variants. The effect of varying experimental conditions of residue methods based on cholinesterase inhibition is discussed. The present status of oxidative techniques for enhancing the sensitivity of enzyme methods used for phorate residues is reviewed.

Residues of phorate, *O,O*-diethyl *S*-(ethylthio)methyl phosphorodithioate, and its insecticidally active metabolites have been determined in over 20 vegetable crops and other food products by cholinesterase inhibition. The present paper presents the basic water-maceration sample preparation method used for many of these crops and the cholinesterase technique used in conjunction with this and other sample preparation methods. The water-maceration method has been found suitable for analysis of alfalfa plants, apples, broccoli foliage, broccoli heads, cotton plants, cotton squares, grapes, grapefruit and orange juices (canned, single strength), peaches, pears, canned peas, strawberries, sugar beets (roots), sugar beets (foliage), turnips, and wheat.

Certain foodstuffs such as oily crops, potatoes, milk and butterfat, and citrus offer special problems and cannot be handled by the water-maceration procedure. In others, such as green beans and rutabagas, recoveries of known added amounts of insecticide are unsatisfactorily low (15 to 40%). For all of these, special sample preparation techniques have been developed. Their applicability was tested by analyzing prepared samples containing known added amounts of the insecticidal compounds.

The effects of varying pH values and standing times during the enzyme inhibition step are discussed, together with the nature and proper choice of adequate control runs. In conjunction with the problems of recovery and of sensitivity, the current status of attempts to enhance the activity of phorate itself and of its thiono metabolites by an oxidative step incorporated into the analytical procedure is reviewed.

PART I. THE BASIC METHOD

Water-Maceration Procedure

Cut a 100- to 200-gram representative sample of the material to be analyzed into small pieces, and macerate it thoroughly in a Waring Blendor, or similar homogenizer, preferably with Cenco Pinto hardened steel blade assembly (Central Scientific Co., Catalog No. 17248). Add ice water, if needed, but no more than necessary, since dilution lowers the sensitivity of the method. Record the amount of water added (it is advisable to adopt a standard amount for each crop or type of crop). Pour the macerate onto three or four thicknesses of cheesecloth stretched over the top of a beaker, gather the corners of the cheesecloth, and squeeze gently until sufficient extract has been collected for analysis (the largest aliquot required is 35 ml.). Neutralize the extract to pH 7.5 with sodium hydroxide solution, transfer a suitable aliquot to a 50-ml. volumetric flask, and dilute to 35 to 40 ml. If the order of magnitude of the residue is unknown, take several aliquots of graded size in several 50-ml. flasks to ensure being on-scale in the subsequent cholinesterase inhibition measurement.

In the same manner, prepare the same weight of a control sample, which has not been treated with insecticide, and treat as above.

Cholinesterase Procedure

Apparatus. pH meter, Beckman Model G or equivalent, with small electrodes (Beckman Nos. 39290 and 39270, or equivalent) to fit in microbeakers.

Microbeakers, and watch glasses to fit. The 5-ml. microbeakers supplied with the Model G pH meter can be used; however, more satisfactory containers can be made by cutting down 23-mm. o.d. shell vials (Fisher Catalog No. 3-330) to a height of 25 or 26 mm.

Filter tip for 1-ml. pipet. From a 3 × 2 inch rectangular piece of 150-mesh copper or bronze wire cloth, construct a 3-inch cylinder $^1/_4$ inch in diameter, and close one end by pinching it together.

Reagents. Keep all reagents refrigerated when not in use.

Calibration Standard. *O,O*-Diethyl *S*-(ethylsulfonyl)methyl phosphorothioate (phorate oxygen analog sulfone), obtainable from American Cyanamid Co., P.O. Box 672, Princeton, N. J.

Human Blood Plasma (corpuscles centrifuged out), obtainable as outdated blood from local hospitals or blood bank. Bottled under clean, though not sterile, conditions, such plasma will keep (unopened) for 6 months and more at 35° to 40° F. Opened pints should be used within 1 or 2 months.

Buffer Solution. Dissolve 3.71 grams of sodium barbital ($0.036M$), 44.7 grams of potassium chloride ($1.20M$), and 0.545 gram of potassium dihydrogen phosphate ($0.008M$) in 450 ml. of water, and warm to room temperature. Adtust jo pH 8.1 with $0.5N$ hydrochloric acid. Transfer the solution to a 500-ml. volumetric flask and dilute to volume with water. Since the initial pH and buffer capacity of the solution decrease on standing, prepare this buffer fresh monthly.

Acetylcholine Chloride (AChCl) Substrate Solution. Dissolve 1.2 grams of acetylcholine chloride (Merck or equivalent) in 25 ml. of water. Prepare fresh every 2 weeks.

Procedure. Prepare, by the above water-maceration method or other appropriate procedure (Part II), an aqueous solution of the phorate residue toxicants extracted from the material under test, and transfer a suitable aliquot to a 50-ml. volumetric flask. In the same manner, prepare the same weight and aliquot portion of an untreated control sample. Proceed as given under Calibration Curve, commencing with the addition of blood plasma. Also carry through a reagent blank (plasma blank) consisting of about 40 ml. of distilled water in a 50-ml. volumetric flask.

With samples prepared by the water-maceration procedure, which usually contain a considerable amount of finely divided solids in the inhibition flask, agitate the flasks a few minutes before the end of the 70-minute incubation and inhibition period, and return them to the bath. The wire cloth filter tip will minimize clogging of the 1-ml. pipets used to sample from these 50-ml. flasks.

Calculate the amount of inhibition of the treated sample solutions and determine the amount of phorate oxygen analog sulfone equivalent thereto by reference to the calibration curve, as described under Calculations.

Calibration Curve. On the day the standard solution is to be used (overnight storage is not recommended), accurately weigh about 20 mg. of the calibration standard into a 250-ml. glass-stoppered volumetric flask, dissolve in 95% ethyl alcohol, dilute to volume with alcohol, and mix. Transfer a 2-ml. aliquot of this solution to a 1-liter glass-stoppered volumetric flask, dilute to volume with water, and mix. One milliliter of this solution contains approximately 0.16 μg. of phorate oxygen analog sulfone. Transfer 1-, 2-, 3-, 4-, 5-, 6-, 7-, and 8-ml. aliquots of the solution to 50-ml. volumetric flasks; carry a blank throughout. Dilute each solution to 35 to 40 ml. with water. Starting with the blank, carry the solutions successively, at 2-minute intervals, through each step of the remaining procedure. Add 5.0 ml. of blood plasma, dilute to volume, and mix. Place immediately in a constant temperature bath at 37.5° C. for 70 ± 5 minutes. At the end of the inhibition period, transfer a 1-ml. aliquot from the flask into a microbeaker. Add exactly 1 ml. of barbital-phosphate buffer, mix by swirling, and place the beaker in the constant temperature bath for at least 10 minutes to come to temperature equilibrium. Process the other beakers similarly, in succession, at their 2-minute intervals.

Calibrate the pH meter *vs.* the standard buffer at room temperature. Turn the temperature compensation dial to 33° C., readjust the meter controls, withdraw the beaker from the bath, and immediately measure the pH of the solution. Record this as the initial pH. Add 0.5 ml. of AChCl solution, mix by swirling, and return the beaker to the constant temperature bath, recording the time. The time interval from the beginning of the incubation with the blood plasma to the introduction of the AChCl should be controlled at 90 ± 5 minutes.

Record the length of time required for the plasma blank to decrease to a pH near (but not less than) 6.0, and measure the final pH of each solution after the same time interval as the plasma blank. Determine proper time conveniently by taking two 1-ml. aliquots instead of one (in separate microbeakers) from the plasma blank flask, withdrawing one of them and reading its final pH after 1 hour of the hydrolysis reaction, and extrapolating the observed pH-time curve to pH 6.05, which will give the approximate desirable time interval for the other beakers. The ΔpH per hour figure thus determined should remain constant for the particular batch of plasma.

Calculate the amount of cholinesterase inhibition exhibited by each solution as follows:

$$\% \text{ inhibition} = \frac{[(\Delta pH)_b - (\Delta pH)_a] \times 100}{(\Delta pH)_b} \quad (1)$$

where

$(\Delta pH)_b$ = initial pH − final pH of plasma blank
$(\Delta pH)_a$ = initial pH − final pH of calibration standard solution.

Prepare a calibration curve on three-cycle semilogarithmic paper by plotting per cent inhibition on the linear scale *vs.* concentration of phorate oxygen analog sulfone (μg. per 50 ml.) on the log scale. The curve should be linear between about 25 and 80% inhibition.

Calculations.

% apparent inhibition of control (untreated) sample = (defined later)

$$\frac{(\Delta pH)_b - (\Delta pH)_c}{(\Delta pH)_b} \times 100$$

% inhibition of insecticide-treated sample =

$$\frac{(\Delta pH)_c - (\Delta pH)_s}{(\Delta pH)_c} \times 100$$

Water-Maceration Method

Phorate residue toxicants, calculated as p.p.m. phorate oxygen analog sulfone $= \dfrac{W(G + a)}{G \times b}$

Nonaqueous Extraction Methods (Part II)

Phorate residue toxicants, calculated as p.p.m. phorate oxygen analog sulfone $= \dfrac{W}{G} \times \dfrac{c}{d}$

where

$(\Delta pH)_b$ = initial pH − final pH of plasma blank
$(\Delta pH)_c$ = initial pH − final pH of control (untreated) sample
$(\Delta pH)_s$ = initial pH − final pH of insecticide-treated sample
W = μg. of phorate oxygen analog sulfone/50 ml. (from calibration curve)
G = grams of sample
a = ml. of water added to the sample during maceration
b = ml. aliquot of water extract used
c = ml. of nonaqueous solvent used in extraction
d = ml. of nonaqueous extract used in analysis.

Discussion

Cholinesterase Inhibition Techniques. The principle of using cholinesterase as a reagent and cholinesterase inhibition as a technique for measuring trace residues of organophosphate insecticides in plants was first enunciated by Giang and Hall (*5*), who employed the potentiometric technique developed by Michel (*9*) for measuring cholinesterase activity of blood and blood plasma. Following Giang and Hall's pioneering work, the first method developed and used routinely for a particular insecticide was that of Hensel *et al.* (*7*), who applied it to the determination of demeton residues. The water-maceration procedure described here is a modification of the latter, differing principally in pH control during the inhibition reaction, time control during and after the inhibition reaction, method of calculation, and particularly nature and concept of calibration curves, recovery runs, and controls. Methods of sample preparation developed subsequently by us for other crops (Part II) involve primarily nonaqueous extractants though all methods have been designed to utilize the same cholinesterase inhibition and measuring technique.

Cholinesterase procedures for measuring phosphate insecticide residues are becoming increasingly common in the literature, most of them based more or less closely on Giang and Hall's and Hensel's work. Since no discussion has, to the writer's knowledge, ever appeared on the effects of varying some of the conditions, and since a disregard for such possible effects still turns up from time to time in recommended procedures, some comment on such variations is offered here.

Inhibition Reaction. A reagent blank ("plasma blank") on 5 ml. of blood plasma plus 45 ml. of water buffers naturally at around pH 7.3 or 7.4 during the 70-minute inhibition incubation. Since a sample of plant juice neutralized to an initial pH of 7.5 to 7.6 will generally fall into this normal blood pH range during this inhibition incubation, 7.5 was chosen as the standard neutralization pH.

The inhibition reaction is somewhat sensitive to pH. Inhibitions by phorate and its relatives run at initial pH 7.9 instead of 7.5, for instance, gave per cent inhibition figures significantly higher than those run at initial pH 7.5. These resulted in apparent recoveries of added insecticide of 110 to 115% *vs.* calibration curves constructed from known amounts of insecticide in water (pH ca. 7.4). For this reason, conditions were chosen such that all inhibitions would occur in the normal blood range, pH 7.3 to 7.4.

The increased inhibition observed at higher pH is probably due more to a faster rate of the inhibition reaction than to a shift of the (ordinarily unattained) end point inhibition value. The inhibition reaction is not generally complete at 70 minutes, but has merely reached a flatter portion of the inhibition *vs.* time curve, so that, though the distinction is immaterial for purposes of the analytical method, the actual comparison being made is related as much to inhibition rates as to final values. Thus, the time from addition of blood plasma to addition of acetylcholine chloride is controlled at 90 = 5 minutes in this procedure, since the inhibition reaction is still continuing after the 1-ml. aliquot has been transferred from the 50-ml. inhibition flask to the microbeaker.

Buffer Capacities of Plant Juices. The rate of acetic acid production by the active enzyme remaining after the inhibition step is determined by measuring the rate of pH change of the solution in the presence of Michel's barbital-phosphate buffer (*9*). Since any extraneous buffer capacity in the range pH 6 to 8 introduced into the solution would decrease the observed ΔpH caused by production of acetic acid relative to the ΔpH caused by the same amount of acid produced in the presence of Michel's buffer alone, the presence of control plant juices, which generally have buffer capacity in this range, will shorten the observed ΔpH relative to that of an equivalent plasma blank. The effect can be estimated by curves such as those of Figure 1, which relate the buffer capacities of Michel's buffer (50 ml., pH 8.10), of blood plasma (5 ml., pH 7.23), and of several typical plant extracts (35 ml.) prepared as described in the method. The above amounts are in the ratios in which these materials are mixed in the analysis.

The shortening of ΔpH caused by the control (untreated) plant itself can thus be predicted from a simple acid-base potentiometric titration of an unused portion of the control plant extract of the same size as that used in the cholinesterase procedure. Agreement (to within 10 to 15%) between predicted and observed figures is sufficient indication that the observed shortening of ΔpH by the untreated plant is due to its buffer capacity and not to the presence of any naturally occurring cholinesterase inhibitor. Such an acid-base titration and accompanying calculation are usually made for each new crop under investigation. A plasma blank is usually included in each set of inhibition runs, and the comparison is made each time between the ΔpH of the control plant sample and of the plasma blank. This comparison figure, designated in the Calculations section as "% apparent inhibition of control (untreated) sample," is seldom reported in the final results but is reasonably consistent for a particular plant species. Hence its value serves as a check on the procedure and an indicator for possible contamination or spoilage of the control sample prior to analysis. In the nonaqueous extraction methods of Part II, it is also a check on faulty processing during sample preparation.

Calibration Curves. The I_{50} values for the phorate series compounds, as measured by this method, are given in Table I. Corresponding values, by slightly different cholinesterase procedures, have been reported by Metcalf, Fukuto, and March (*8*) and by Bowman and Casida (*3*). Agreement among the three sets of data is, in general, reasonably good.

The calibration curve for the phorate residue method and for the oxygen analog sulfone is given in Figure 2. Since it is independent of the food material being analyzed and of the sample preparation technique chosen, it is common to all the methods listed in this paper. It reflects none of the losses that may occur in the various steps of the sample preparation procedures.

Recoveries from Plant Tissue. Recovery figures, in the concentration ranges to be encountered in practice, were run on each new crop analyzed by the method. Known amounts of the desired insecticidal compound, dissolved in a small amount of aqueous alcohol, were added to a control sample of the crop either immediately before or during the Waring Blendor maceration. The resulting mixture was thoroughly processed in the blender and carried through the entire procedure. The per cent inhibition found, relative to an unspiked control, was expressed as micrograms of phorate oxygen analog sulfone found, using the curve for known amounts of the sulfone in water (Figure 2); and the micrograms found were compared with the micrograms added. The recovery figures for the various procedures are included in Table III.

The phorate oxygen analog sulfone (compound VI, Table I) and the P=S sulfone (compound III) were used for recovery runs. The latter was included as typical of the less polar compounds in the series; however, the metabolism has ordinarily proceeded beyond this metabolite in plant samples. In the case of

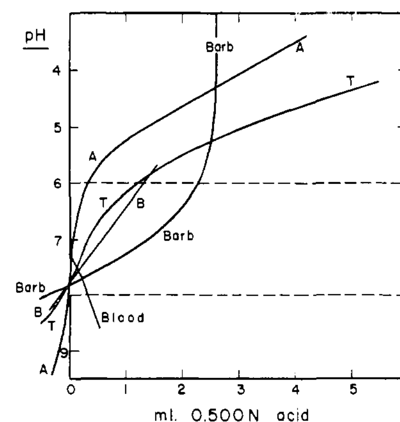

Figure 1. Buffer capacity curves for materials present in analyses

The neutralization curves for the vegetable crops have been arbitrarily reversed, and all curves except that for blood have been displaced along the horizontal axis so that ml. titrant = 0.00 at pH 7.8, the approximate initial pH for hydrolysis runs, in order that the relative buffer capacities between pH 8 and pH 6 will be more readily apparent.

A. Filtered extract from McIntosh apples. Initial pH, 3.4

B. Filtered extract from snap beans. Initial pH, 5.7

T. Initial extract from ripe tomatoes. Initial pH, 4.2

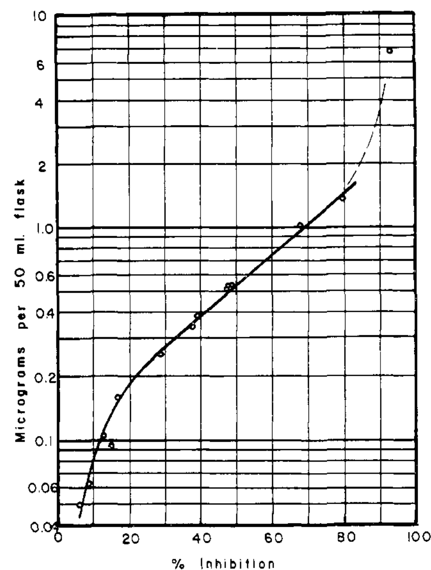

Figure 2. Plasma cholinesterase inhibition *vs.* concentration of O,O-diethyl S-(ethylsulfonyl)methyl phosphorothioate

compound III, recovery figures were calculated from the inhibition curve of the pure P=S sulfone in water, analogously to the calculation of P=O sulfone recoveries from Figure 2. The percentage recovery of the P=S sulfone was generally the same as for the P=O sulfone.

Plant Metabolism and Cholinesterase Techniques. Cholinesterase residue techniques are subject to an important limitation in the case of endometatoxic insecticides, such as phorate,

Table I. I_{50} Values for Phorate Series Compounds

Human plasma cholinesterase

Compound	I_{50} Value (Molar)
O,O-Diethyl phosphorodithioates	
I. *S*-(Ethylthio)methyl	3.7×10^{-5}
II. *S*-(Ethylsulfinyl)-methyl	7.1×10^{-6}
III. *S*-(Ethylsulfonyl)-methyl	2.2×10^{-6}
O,O-Diethyl phosphorothioates	
IV. *S*-(Ethylthio)methyl[a]	4.5×10^{-7}
V. *S*-(Ethylsulfinyl)-methyl	1.2×10^{-7}
VI. *S*-(Ethylsulfonyl)-methyl	4.1×10^{-8}

[a] Compound apparently not produced in plant metabolism of phorate.

Table II. Sensitivity of Water-Macerate Procedure to Various Compounds of Phorate Series

Compound	I_{50} (Molar)	Inhibitory Activity, vs. Parent Compound	Concn. to Give 10% Inhibition, µg./50-Ml. Flask	Method Sensitivity for Compound,[a] P.P.M.
I	3.7×10^{-5}	(1)	52	4.0
II	7.1×10^{-6}	5	10.0	0.8
III	2.2×10^{-6}	17	3.3	0.3
IV	4.5×10^{-7}	82	0.85	0.07
V	1.2×10^{-7}	308	0.25	0.008
VI	4.1×10^{-8}	910	0.085	<0.005

[a] Figures calculated assuming typical experimental conditions of 60% dilution of the sample with added water in blender and 60% over-all recovery of compound.

since the plant changes such compounds, by normal oxidative or other metabolism processes, into a series of compounds of increasing inhibitory activity toward cholinesterase. In the case of phorate, the increase in activity from the first to the last compound in the series is almost a thousandfold (Table II). It is necessary to know accurately and reliably, by independent means, the state of metabolism of the material to be analyzed, to measure recoveries and to choose a meaningful calibration curve. In addition, the sensitivity of the cholinesterase method is a function of the I_{50} of the inhibitory compound involved. The sensitivity of the water-macerate method toward the first two compounds of the phorate series is relatively poor (Table II).

The plant metabolism of phorate has been studied by Metcalf (*8*) and by Casida (*3*). They show that the oxidative metabolism process proceeds steadily in the plant, but that a period of some weeks is required for the insecticidal material to be converted entirely into the final two most active metabolites, the phosphorothiolate sulfoxide and phosphorothiolate sulfone (compounds V and VI), the sulfone being the more persistent of the two (*8*). This period is 4 to 5 weeks after final treatment in the case of such treatment methods as foliage spray, and a week or two longer for such methods as seed treatment, where a reservoir of phorate requires several weeks to move into the plant (*2, 3, 8*). A cholinesterase method, with calibration curve based on the final sulfone, affords an accurate and highly sensitive determination of the insecticidal compounds existing in samples taken after this date, and it is for such samples, and especially harvest samples, that the method was designed. Used before such date, and before the metabolism has run its course, the cholinesterase method would yield results difficult of interpretation, because of the mixed inhibition coefficients of the several compounds present (Table II).

Oxidation of Phorate Prior to Cholinesterase Measurements. In anticipation that possible future uses of phorate might extend to crops and situations where less than the necessary 4 to 6 weeks might elapse between final treatment and desired time of sampling or harvest, it was recognized shortly after the study of cholinesterase methods was begun in this laboratory that incorporation of a suitable oxidation step in the analytical procedure offered the possibility of unequivocally converting all phorate compounds present to the final sulfone (*2, 4*). Such an oxidative procedure would remove any time restrictions from sampling and permit analysis of crops by the highly sensitive cholinesterase procedure at any stage of their development and as soon after phorate application as desired.

Attempts at incorporating this step into the phorate residue cholinesterase procedure have been carried out intermittently but exhaustively over a period of 4 years (*2*). The problem has also engaged the attention of other workers (*1, 10*). Its present status may best be summarized as follows: In general, it is possible to add phorate to a pure solvent and oxidize it, with good yield and recovery (>65%), to the oxygen analog sulfone. This has been done with bromine and with *N*-bromosuccinimide, both here (*2*) and elsewhere (*1*). This oxidation can also be done with peracetic acid, though not all investigators who have reported this have used conditions capable of performing the P=S to P=O portion of the oxidation. Neither in our own work, nor in the published literature, nor in communications submitted to us on a private basis, however, has any evidence been put forward that this oxidation has been successfully performed in the presence of plant material, or in fractions containing phorate series compounds separated from plant material. [See, however, note at end of paper.] The difficulties involved are well exemplified in the communication of Miskus and Hassan (*10*); comparison of the 83-fold oxidative enhancement factor reported there for phorate with the known factor of 910 (Table II) suggests an over-all recovery of less than 10%, due either to insufficient oxidation or to degradation by the oxidant or to both. An equally common difficulty encountered in working with plant material is the occurrence of high and erratic inhibition blanks after oxidation (*1, 2*). In the case of phorate, then, incorporation of an oxidation step into a practicable cholinesterase plant residue method remains a desirable goal as yet unachieved.

Recommendations. The cholinesterase method, together with its sample preparation variants, is recommended for the determination of phorate residues in harvest samples of crops and, in general, for crop samples where the period between final application of the insecticide and sampling exceeds about 6 weeks. The method is sensitive down to about 0.005 p.p.m. The analysis by cholinesterase technique of samples taken sooner than this after final insecticide treatment is not recommended because of the multiplicity of metabolites then present—although the method is valid, or can be made valid, for such samples if one is willing to accept the considerably lower sensitivity attainable under such conditions (Table II).

Giang and Schechter have recently described (*6*) a colorimetric formaldehyde method for determining phorate and its metabolites in plant materials, and this method possesses the important advantage of being approximately equally sensitive to all compounds of the phorate series. Though its stated sensitivity of 0.10 p.p.m. would make it inapplicable to situations where very high sensitivity is required, such as registering crops on the basis of no residue present at harvest, its advantages over the cholinesterase approach in many cases where extent of metabolism has not been established or is known to be incomplete are obvious. Judicious choice between, or combination of, the two types of method should afford a satisfactory approach to most phorate residue problems.

Table III. Recovery Values of Phorate and CL 12,008 Series Compounds from Vegetable Crops and Other Food Materials

Crop	Method[a]	Water–Plant Ratio	Compound Added	Dosage, P.P.M.	No. of Runs	Recovery, %	Estd. Limit of Detection of Compound, P.P.M.
Alfalfa plants	A	2.66	P=O sulfone	0.12	1	75	0.007
				0.05	2	52, 59	0.007
Apples	A	1	P=O sulfone	0.05	4	93–111	0.004
				0.09	2	103, 106	0.004
Beans (see green beans, green bean plants, Lima beans)							
Birch leaves	A	4	P=O sulfone	0.2	6	22–31	0.010
	B	...	P=O sulfone	0.16	2	59, 61	0.001
				0.33	4	67–74	0.001
Broccoli							
Foliage	A	1.2	P=S sulfone	1.4	1	57	0.3
				2.2	1	50	0.3
Heads	A	0.8	P=S sulfone	1.7	1	54	0.2
				3.3	1	54	0.2
Butterfat	D	...	P=O sulfone	0.44	2	62, 63	0.001
				0.88	1	64	0.001
Cotton							
Whole plants	A	1.75	Compound VIII	2.9	2	66, 68	0.2
			P=O sulfone	0.9	1	99	0.005
Leaves	A	4	Compound IX	0.48	2	70, 74	0.06
				0.97	2	70, 72	0.06
			Compound X	0.14	2	107, 112	0.004
Squares	A	4	Compound X	0.10	2	82, 90	0.004
Cottonseed	C	...	P=O sulfone	0.13	3	50–61	0.001
Cottonseed oil	C	...	P=O sulfone	1.5	4	63–68	0.002
				0.3	2	58, 68	0.002
				0.09	3	62–67	0.002
				0.018	2	57, 58	0.002
				0.007	1	60	0.002
Grapes	A	1	P=S sulfone	1.5	2	61, 66	0.2
Grapefruit							
Whole fruit	A	1	P=S sulfone	2.2	2	26, 26	0.2
			P=O sulfone	0.04	2	28, 29	0.004
Peeled fruit	A	1	P=S sulfone	1.4	1	36	0.2
Peel	A	4	P=S sulfone	6.0	1	9	0.4
Grapefruit juice (canned, single strength)	A	1	P=S sulfoxide	3.9	2	51, 56	0.4
Grean beans (snap beans, string beans)							
Pods	A	0.66	P=S sulfone	2.2	4	27–34	0.2
			P=O sulfone	0.04	3	27–28	0.003
	B	...	P=O sulfone	0.047	2	71, 75	0.001
				0.093	3	81–81	0.001
				0.14	3	70–71	0.001
Whole plants	A	1.33	P=S sulfone	2.2	3	45–51	0.3
		1.66	P=O sulfone	0.05	4	24–25	0.005
	B	...	P=O sulfone	0.10	3	76–77	0.001
Johnson grass	A	2	P=S sulfone	5.2	1	22	0.4
Lima beans (whole pods)	B	...	P=O sulfone	0.10	2	78, 81	0.001
Milk	D	...	P=O sulfone	0.07	4	78–85	0.001
				0.14	2	91, 91	0.001
Onions	A	0.8	P=S sulfone	1.1	1	16	0.3
				1.7	1	15	0.3
Oranges							
Whole fruit	E	3	P=S sulfone	4.4	4	36–59	0.4
				4.1	4	43–46	0.4
			P=O sulfone	0.08	6	38–46	0.008
Peeled fruit	E	2	P=S sulfone	3.3	3	64–65	0.4
Orange juice (canned, single strength	A	No water added	P=S sulfoxide	2.5	2	106, 108	0.3
Peaches	A	0.5	P=S sulfone	2.6	1	93	0.2
Peanuts	F	...	P=O sulfone	0.05	2	59, 64	0.001
Pears	A	0.6	P=S sulfone	1.2	1	125	0.3
Peas, canned	A	No water added	P=O sulfone	0.07	3	91–100	0.002

(*Continued on next page*)

(*Table III. Continued*)

Crop	Method[a]	Water–Plant Ratio	Compound Added	Dosage, P.P.M.	No. of Runs	Recovery, %	Estd. Limit of Detection of Compound, P.P.M.
Potatoes	G	...	P=S sulfone	3.1	4	65–68	0.1
			P=O sulfone	0.10	2	79, 85	0.001
			Compound X	0.57	2	80, 80	0.001
				0.11	2	97, 100	0.001
				0.023	1	82	0.001
				0.011	2	68, 72	0.001
Rutabagas (yellow turnips)	A	2	P=O sulfone	0.1	4	47–48	0.006
	B	...	P=O sulfone	0.093	3	62–63	0.001
Strawberries	A	No water added	P=S sulfone	1.1	1	97	0.2
Sugar beets							
Roots	A	1.2	P=S sulfone	0.8	1	103	0.2
				1.7	1	104	0.2
Foliage	A	0.5	P=S sulfone	0.9	1	94	0.2
				1.4	1	96	0.2
	A	0.2	P=O sulfone	0.1	2	61, 62	0.002
Turnips	A	0.5	P=S sulfone	0.9	1	76	0.2
				1.9	1	58	0.2
Wheat grain	A	4	P=O sulfone	0.092	2	59, 61	0.010
	B	...	P=O sulfone	0.082	3	69–71	0.001
Wheat plants	A	6	P=O sulfone	1.33	1	102	0.014
				0.14	4	84–98	0.014
				0.058	2	67, 70	0.014
				0.029	2	71, 71	0.014

[a] A. Water-maceration method. B. Carbon tetrachloride extraction method. Special sample preparation procedure for C. Cottonseed and cottonseed oil. D. Milk and butterfat. E. Oranges. F. Peanuts and peanut oil. G. Potatoes.

PART II. ALTERNATIVE SAMPLE PREPARATION, AND RECOVERY DATA

In addition to the special apparatus recommended for individual methods, a centrifuge equipped with heads and trunnion cups capable of carrying 90-ml. centrifuge tubes and 250-ml. centrifuge bottles is required for several procedures. A variable autotransformer (Variac, Powerstat, etc.) for use with the homogenizer is also desirable.

Carbon Tetrachloride Extraction Method

This method has been shown to be applicable to Lima beans, snap beans, snap bean plants, birch leaves, wheat grain, and rutabagas (yellow turnips). It is probably suitable for many other crops; however, it should be tested by determination of the recovery of known amounts of phorate compounds from the plant tissue.

Procedure. Weigh a representative sample (25 to 100 grams) and transfer to a Waring Blendor. Macerate with 200 ml. of carbon tetrachloride, and filter the macerate through a Büchner funnel fitted with No. 40 Whatman filter paper into a 1-liter filter flask. Return the solids to the Waring Blendor, add 200 ml. of carbon tetrachloride, and macerate and filter as before. Repeat the maceration and filtration a third time. Place the 1-liter filter flask containing the combined carbon tetrachloride filtrates on a steam bath and evaporate with the aid of an air jet until the volume has been reduced to approximately 50 ml. If necessary, the sample may be stored overnight under refrigeration at this point. If the sample has been processed beyond this step, the analysis should be completed without delay. Remove the flask from the steam bath; add a magnetic stirrer bar and 20 ml. of distilled water. Place the flask on a magnetic stirrer, close the flask with a stopper which has a groove cut along its side to serve as an air bleed, and attach a vacuum line.

Continue the vacuum stripping until the carbon tetrachloride layer is completely evaporated and the water layer separates from the residue of plant material. Pour the water layer through a funnel containing a small plug of cotton into a 50-ml. volumetric flask, using care so that most of the solid residue is retained in the filter flask. Wash the walls of the flask with distilled water sparingly, and add the washings to the 50-ml. flask. Return the solids in the funnel to the filter flask and redissolve with 20 ml. of carbon tetrachloride. Add 15 ml. of distilled water and vacuum strip as before. Repeat the vacuum stripping operation once more, and combine the water extracts in the 50-ml. volumetric flask. The total volume of the combined water extracts and wash water should not exceed 45 ml. [During the vacuum stripping operation, evaporation will reduce the volume of water added to the flask by approximately one half, so that if the specified amount (50 ml.) of water is added the three vacuum strippings will yield approximately 25 ml. of water extract exclusive of wash water necessary to effect transfer.]

Carry the same weight of a control sample (untreated with insecticide) through the entire sample preparation procedure along with the treated samples. Proceed without delay to the inhibition and determination of cholinesterase activity by the standard procedure.

Procedure Variant. If, during the maceration and filtration steps, the amount of water in the plant tissue being analyzed is great enough to cause the separation of an aqueous layer or to form an emulsion which is difficult to filter, follow this alternative procedure:

Centrifuge the macerate in two 250-ml. centrifuge bottles at high speed for 15 minutes. Siphon or drain the carbon tetrachloride layer into a 1-liter filter flask, return the aqueous layer and the solids to the Waring Blendor for the second maceration, and repeat the process twice more.

Method for Milk and Butterfat

Special Apparatus. Freeze-drying apparatus. Centrifuge bottles, 1-liter, heavy-walled.

Procedure. Weigh a sample of approximately 200 grams of milk and transfer to a 1-liter heavy-walled centrifuge bottle. Rotate the stoppered bottle slowly in a nearly horizontal position in a dry ice–acetone mixture until the milk has frozen in a uniform coating on the inside walls. Continue until the coating breaks away from the glass. Freeze-dry the material; this may require 12 to 16 hours. (Store the dry milk solids in the refrigerator if the extraction is not to be made immediately.) Transfer the dry milk solids to an 800-ml. beaker, and slurry with 150 ml. of carbon tetrachloride. Thoroughly break up the lumps with a large spoon or spatula and mix well. Filter through a large Büchner funnel fitted with No. 42 Whatman filter paper into a 1-liter filter flask.

Return the solids to the beaker, slurry with 100 ml. of carbon tetrachloride, mix well, and again filter. Repeat the extraction of the milk solids four more times in a similar manner.

Place the 1-liter filter flask containing the combined carbon tetrachloride filtrates on a steam bath, and evaporate with the aid of an air jet. When the volume has been reduced to about 75 ml., remove the flask from the steam bath, add a stirrer bar, and place the flask on a magnetic stirrer. Attach a vacuum line to the side arm and close the flask with a stopper which has a groove cut in its side to serve as an air bleed. Continue the evaporation until the last traces of solvent are removed from the butterfat. Drain as much as possible of the butterfat into a 125-ml. separatory funnel. Swirl 10 ml. of warm (approximately 50° C.) distilled water in the filter flask to wash the butterfat remaining in the flask. Transfer to the separatory funnel and extract by gently swirling and shaking the separatory funnel. Drain the water layer into a 50-ml. volumetric flask. Repeat the extraction three more times in a similar manner. Each time first swirl the 10-ml. portion of wash water in the filter flask to wash the remaining butterfat, then use the same portion of wash water to extract the butterfat in the separatory funnel.

Carry the same weight of a control sample (untreated with insecticide) through the entire sample preparation procedure along with the treated samples. Proceed without delay to the inhibition and determination of cholinesterase activity by the standard procedure.

Procedure Variant. If the original sample is butterfat rather than milk, start at the point in the procedure where the butterfat is transferred to the 125-ml. separatory funnel. Transfer 50 grams of sample, which has been warmed slightly until melted, to a separatory funnel and extract with four 10-ml. portions of warm water.

Chloroform Method for Potatoes

Procedure. From several washed, whole, unpeeled potatoes, cut sufficient pieces to constitute a representative 100-gram sample. Transfer the weighed sample to a Waring Blendor and macerate with 100 ml. of distilled water. Add 100 ml. of chloroform (reagent grade, washed twice with an equal volume of water before use) and blend at high speed for a few minutes. Transfer the entire macerate in approximately equal portions to two centrifuge bottles; centrifuge for 15 to 20 minutes at high speed. Siphon off and discard the aqueous layer lying above the intermediate pulp layer. In order to improve separation of the chloroform from the pulp, add 50 ml. of distilled water to each of the centrifuge bottles, mix the entire contents thoroughly with a stirring rod, and again centrifuge for 15 to 20 minutes at high speed. Siphon off and discard the aqueous layer; then filter the chloroform layer through a small pledget of cotton into a 100-ml. graduate or other suitable container. Repeat the centrifugation with additional water until at least 50 ml. of chloroform has been recovered.

Transfer the combined chloroform solutions to a separatory funnel with Teflon or other greaseless stopcock and, swirling gently to avoid forming an emulsion, wash with two 75-ml. portions of distilled water. Filter the washed chloroform extract through dry cotton. (The procedure may be interrupted at this point, if desired, and the chloroform extract stored overnight under refrigeration. After the insecticide residue has been processed into water, however, the determination should be completed without delay.)

Transfer a suitable aliquot of the washed extract to the evaporation flask. Add 15 ml. of distilled water and evaporate the chloroform with the aid of vacuum and a slow stream of air entering through the air bleed, while agitating constantly with the magnetic stirrer. When the chloroform has been completely removed, filter the aqueous solution through a small pledget of cotton into a 50-ml. volumetric flask. Rinse the evaporation flask with 5 ml. of distilled water, and add the washings to the volumetric flask. Pour 10 ml. of chloroform through the same cotton filter into the evaporation flask. Swirl to dissolve any solid residue in the flask, add 10 ml. of distilled water, and again remove the chloroform completely with vacuum as before. Filter the water solution through a small pledget of cotton into the volumetric flask containing the first water filtrate. Wash sparingly to effect the transfer. The total volume of the combined filtrates and washings should not exceed 40 ml.

Carry the same weight of a control sample (untreated with insecticide) through the entire sample preparation procedure along with the treated samples. Add one drop of 0.1N sodium hydroxide to each extract and proceed without delay to the inhibition and determination of cholinesterase activity by the standard procedure.

CAUTION. Complete removal of the chloroform following the evaporative transfer into water is essential, since chloroform is a strong inhibitor of cholinesterase. After nearly all of the chloroform has been removed in each evaporation, the final traces of solvent are usually evidenced by vapor bubbles which adhere to the particles of waxy residue. When this stage has been reached, 10 to 15 minutes' additional evaporation will usually prove sufficient to eliminate the bubbles; at this point solvent removal may be considered complete.

Alcohol Extraction Method for Cottonseed and Cottonseed Oil

Special Apparatus. Soxhlet extractors and thimbles. Arthur H. Thomas Co. Catalog No. 4987-A extractor, size extra large, and single thickness thimbles No. 4964, 60 × 180 mm., are suitable.

Water bath, 50° to 65° C.

Manifold for directing filtered compressed air downward into 50-ml. beakers in the water bath.

Procedure. Weigh about a 200-gram sample of ginned cottonseed and break the seed up thoroughly in a Waring Blendor. This is best done dry (no solvent), with the blender on a moderately fast speed setting, and by taking about 20-gram portions of the cottonseed at a time. (The seed should end up with the hulls thoroughly broken open, so that the ether will be able to penetrate readily to the meats, but it is not necessary that the meats be completely pulverized. Two hundred grams of seed can be broken up in about half an hour in this manner.) Collect the seed in a large dish or similar container and pack it into a large extraction thimble. Place some glass wool on the top of the thimble to prevent splashing.

Extract the seed with ethyl ether in a Soxhlet extractor on the steam bath for 5 or 6 hours or until the effluent from the extraction chamber is clear. Filter the ether extract through a sintered-glass filter crucible of medium porosity into a 1-liter filter flask. Add one or two glass beads and evaporate off the bulk of the ether on the steam bath with the aid of an air jet. When most of the ether has evaporated (approximately 20 to 30 minutes) as indicated by the slowing of ebullition, remove the flask promptly from the steam bath, since exposure to full steam bath temperature after the ether has boiled off will cause slow degradation of the phorate compounds. Add a stirrer bar and place the flask on a magnetic stirrer. Attach a vacuum line to the side arm and close the flask with a rubber stopper which has a groove cut along its side to serve as an air bleed. Adjust the stopper so that a slow stream of air sweeps through the flask as the oil is stirred magnetically and vacuum stripped. (Forty-five minutes of vacuum stripping is usually sufficient to remove the last traces of ether from the oil.)

Transfer all of the oil to a 125-ml. separatory funnel. (If the submitted sample is cottonseed oil rather than seed, take a 40-ml. portion and start the procedure at this point.) Extract with 20 ml. of 95% ethyl alcohol by shaking the materials together intermittently for 5 minutes. Pour the mixture through the top opening of the separatory funnel into a 90-ml. centrifuge tube, and centrifuge it at high speed for 10 minutes. Place the centrifuge tube in the dry ice bath until the oil (lower) layer has solidified. Pour off the alcohol (upper) layer into a 50-ml. beaker, being careful not to allow any of the oil layer to run into the beaker. Place the beaker containing the alcohol layer in a water bath at 50° to 65° C. and commence evaporation of the alcohol, with the aid of a jet of filtered air blowing on its surface.

Warm the lower (cottonseed oil) layer until it liquefies, return it to the original separatory funnel, add 15 ml. of fresh 95% alcohol, and extract, centrifuge, freeze, and decant as before, combining the decantate with the first

decantate in the 50-ml. beaker. Resume evaporation of the alcohol layers. Thaw the lower layer again and return it to the separatory funnel, add another 15-ml. portion of 95% alcohol, and extract, centrifuge, freeze, and decant once more.

Take the three combined alcohol layers down on the water bath with the aid of the air jet until it is apparent to the eye that essentially all of the alcohol has been driven off. Continue the evaporation process about 10 or 15 minutes beyond this point, rotating and tipping the beaker so that the impinging air jet stirs up the film on the bottom of the beaker. It is important that all of the alcohol be removed at this point before proceeding to the final cholinesterase inhibition measurement. Remove the beaker from the water bath and allow it to cool to room temperature. (The procedure may be interrupted here, if desired, and the beakers stored in the refrigerator overnight. Allow them to warm to room temperature before proceeding to the next step of the procedure.)

Extract the residue with about 10 ml. of water, adding this in a hard, fine stream from a wash bottle in order to break up the residue, and dispersing and mincing the residue still further by vigorous slicing motions with a stirring rod. Making use of a filter flask, prepare a Gooch crucible with an asbestos mat slightly thicker than that usually used. Filter the water extract through this, being careful to retain as much of the oily residue as possible in the beaker for subsequent washing. This can best be done on most samples by laying a microscope slide flat against the top of the beaker on the side away from the pouring spout, tipping the beaker to that side to a point beyond the horizontal, and carefully sliding the microscope slide upward so that the water may run into the Gooch crucible. In this manner, with care, most of the water layer may be transferred cleanly to the Gooch crucible and most of the oily material retained by the cover glass.

Wash the residue in the beaker with two further 8- to 10-ml. portions of water from the wash bottle, again using slicing motions of the stirring rod to bring the oil residue and the water phase into intimate contact. Filter these washings also through the Gooch crucible, collecting them in the same vessel with the original filtrate. Transfer the combined filtrate to a 50-ml. volumetric flask; total volume of the filtrate should not exceed 40 ml.

Carry the same weight of a control sample (untreated with insecticide) through the entire sample preparation procedure along with the treated samples. Add one drop of $0.1N$ sodium hydroxide to each extract and proceed without delay to the inhibition and determination of cholinesterase activity by the standard procedure.

Alcohol Extraction Method for Peanuts and Peanut Oil

This method is a variant of the alcohol extraction method for cottonseed and cottonseed oil. The lesser bulk and absorptive capacity of peanuts, relative to that of cottonseed and its linters, permit leaving out the Soxhlet extraction step and introducing the alcohol extractant at the maceration stage. The somewhat different properties of peanut oil relative to cottonseed oil (especially lower phospholipide content) also permit a different technique for processing the insecticidal compounds into water solution for determination by the usual cholinesterase inhibition method. This procedure is applicable to shelled peanuts, unshelled peanuts, and peanut oil.

Procedure. Weigh approximately 200 grams of peanuts. (Unshelled peanuts should be thoroughly washed with water to remove soil clinging to the surface, and dried.) Macerate the sample in the Waring Blendor with 200 ml. of anhydrous 2B ethyl alcohol, adding the sample a little at a time to avoid overloading the blender. After the sample has been added, continue the maceration for several minutes, then filter the macerate through the Büchner funnel into a 1-liter filter flask. Return the filter cake to the Waring Blendor, add 150 ml. of 2B alcohol, and again blend for serveral minutes. Filter the macerate through the Büchner funnel into the filter flask. Repeat the maceration and filtration once more with 100 ml. of 2B alcohol.

Evaporate the bulk of the alcohol filtrate on the steam bath, with the aid of an air jet. Place the flask on a magnetic stirrer, attach a vacuum line to the flask, and strip off the last traces of alcohol by evacuating the flask while stirring for 45 to 60 minutes. When all the alcohol has been removed, drain the oil from the flask into a 90-ml. centrifuge tube, leaving the solid residue in the flask. Add 10 ml. of distilled water to the flask and swirl gently to leach the solid residue. Transfer the water to the 90-ml. centrifuge tube containing the oil, and mix thoroughly by stirring vigorously with a glass rod. Separate the water and oil layers by centrifuging the mixture at high speed for 10 minutes. Recover the water layer and filter it into a 50-ml. volumetric flask through a funnel fitted with a small cotton plug. Repeat the leaching and extraction steps as above twice more with two additional 10-ml. portions of distilled water, filtering the water extracts through the cotton plug into the 50-ml. volumetric flask. The total volume of the water extracts plus any water used to effect transfer should not exceed 40 ml.

Carry the same weight of a control sample (untreated with insecticide) through the entire sample preparation procedure along with the treated samples. Proceed without delay to the inhibition and determination of cholinesterase activity by the standard procedure.

Procedure Variant. If the original sample is peanut oil rather than whole peanuts, take 30 to 50 grams of the oil and commence the procedure at the point where the oil is transferred to the 90-ml. centrifuge tube for extraction with the three 10-ml. portions of water.

Water Maceration Method for Whole Oranges

Procedure. From several whole oranges, cut enough segments consisting of both pulp and peel to obtain a representative 100-gram sample. Transfer the weighed sample to a Waring Blendor, add 300 ml. of distilled water, and macerate at high speed. Pour the macerate through three or four thicknesses of cheesecloth stretched over the top of a 600-ml. beaker, and allow to filter until sufficient filtrate has been obtained for analysis. Do not attempt to hasten filtration by applying pressure to the cheesecloth, since introduction of pulpy matter into the juice may result in gel formation during subsequent steps in the procedure.

Transfer a measured portion of the filtrate (a maximum of 30 ml.) to a small beaker, place in a water bath, warm it to 50° C., and maintain it at this temperature for several minutes. Remove from the bath, allow to cool to room temperature, then neutralize with $0.5N$ NaOH to pH 7.5, using a pH meter. (The neutralization should not be considered complete until the solution remains at pH 7.5 for approximately 1 minute with vigorous stirring.) Transfer the neutralized solution to a 50-ml. volumetric flask.

Carry the same weight of a control sample (untreated with insecticide) through the entire sample preparation procedure along with the treated samples. Proceed without delay to the inhibition and determination of cholinesterase activity by the standard procedure. The transfer of the 1-ml. aliquot from the volumetric flask to the inhibition beaker may be facilitated by the use of a pipet equipped with the wire cloth "filter tip" described previously.

Discussion

Some of the recovery figures listed in Table III were obtained on compounds related to American Cyanamid CL 12,008, the isopropyl analog of phorate. This compound has a series of metabolites completely analogous to those of phorate (*2*) and, since recovery figures run the same as those for the corresponding compounds of the phorate series, these values also are listed in Table III. The CL 12,008 compounds which appear in Table III are compounds VII to X of Table IV.

The "estimated limit of detection of compound, p.p.m." in Table III is calculated from the amount of that particular compound required to give 10% inhibition of cholinesterase in the procedure used for that recovery run—10% inhibition being taken as the lowest amount reliably distinguishable from an untreated blank run simultaneously. This column was constructed on the

Table IV. Phorate and CL 12,008 Series Compounds

O,O-Diethyl Phosphorodithioates
- I. *S*-(Ethylthio)methyl (phorate)
- II. *S*-(Ethylsulfinyl)methyl ("P=S sulfoxide")
- III. *S*-(Ethylsulfonyl)methyl ("P=S sulfone")

O,O-Diethyl Phosphorothioates
- IV. *S*-(Ethylthio)methyl
- V. *S*-(Ethylsulfinyl)methyl
- VI. *S*-(Ethylsulfonyl)methyl ("P=O sulfone")

O,O-Diethyl Phosphorodithioates
- VII. *S*-(Isopropylthio)methyl (CL 12,008)
- VIII. *S*-(Isopropylsulfonyl)methyl

O,O-Diethyl Phosphorothioates
- IX. *S*-(Isopropylthio)methyl
- X. *S*-(Isopropylsulfonyl)methyl

assumption of 100% recovery of the compound from the crop. The figures given would consequently require modification for the recovery figure actually found for each case.

Table III includes all crops and food materials examined to date, including some for which the recovery figures are unacceptably low (<50 to 60%). Some of these low values represent early data, and results could now probably be improved in many cases by better choice of method. Several crops (green beans, rutabagas, etc.) which gave unacceptably low recoveries by the water-maceration technique gave acceptable figures when analyzed by the subsequently developed carbon tetrachloride extraction procedure. Unless a recovery figure of better than 50% can be demonstrated for a particular crop by a particular method, analysis of such crop by that method would seem difficult to justify.

The following comments apply to the various sample preparation procedures described herein.

Carbon Tetrachloride Extraction Method. This, the most versatile of the sample preparation methods, noticeably improved the recovery figures on a number of crops—e.g., green beans, rutabagas—for which the water-maceration technique gave unacceptably low values (Table III). It is probably capable of handling any crops now analyzed by the latter method. Since, however, the water-maceration method requires about half the working time of the carbon tetrachloride procedure, it is probably the routine method of choice for all crops for which recovery figures can be demonstrated to be acceptably high.

Method for Milk and Butterfat. Extraction of the insecticide residues from whole milk by a water-immiscible solvent was not practical because of the formation of very stable emulsions. This difficulty was avoided by removing all the water from the milk by freeze drying. Carbon tetrachloride proved more effective than ethyl ether, chloroform, or alcohol for extracting out the butterfat plus insecticide residues. The insecticide residue could be extracted from the butterfat with warm water if the butterfat was also warmed slightly.

Chloroform Method for Potatoes. Potatoes have been found to contain an unidentified water-soluble substance which interferes in the determination of phorate insecticide residues by the cholinesterase inhibition method. Since this interfering material is insoluble in chloroform, it is eliminated by a preliminary extraction of the residue into chloroform.

Alcohol Extraction Method for Cottonseed and Cottonseed Oil. Snell and Snell (*11*) mention that triglyceride fats and oils, though readily miscible with most organic solvents and with each other, are but very slightly soluble in 95% ethyl alcohol. Extraction of the insecticide residues from the cottonseed oil with pure 95% alcohol proved feasible, and should work well with other triglyceride oils as well.

In this cottonseed oil procedure, the efficiency of extraction in the water leaching step on the extracted residue was initially a source of concern. Repeated recovery runs (about 20) have shown, however, that amounts of the P=O sulfone ranging all the way down to 0.25 μg. can be recovered and determined consistently and reliably. Experiments in which the P=O sulfone was added at various points in the procedure have shown that the 65% average recovery figure of the method is the result of about 15% of the compound remaining in the oil phase after the three alcohol extractions, plus a further 20% loss in the combined steps of alcohol evaporation, water leaching, and filtration.

Water Maceration Method for Whole Oranges. This method is a variant of the standard water-maceration technique. Greater dilution, careful filtration, and preliminary warming to 50° C. are used to destroy the tendency of the juice to form a stable gel with the blood plasma. Recovery figures by this method are somewhat below acceptable levels. It is probable that considerably better recovery figures could be obtained on this crop by use of the subsequently developed carbon tetrachloride extraction procedure.

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Note Added in Press

To the comments expressed in the above paper on oxidative cholinesterase techniques for phorate, which adequately describe the situation as of September 1960, it should be added that several workers have come forward independently in 1961 with oxidative procedures in advanced stages of development. Although these have not yet been evaluated in our laboratory, advance reports would suggest that some of these procedures may be more successful than earlier attempts at meeting the necessary criteria of low and reproducible blanks, adequate and reproducible oxidative enhancement of activity of the less active inhibitors, and satisfactory recoveries as measured versus a suitable reference curve. We await with interest further reports on these methods.