MECHANISM OF INSECTICIDE ACTION

Preparation, Purification, Isomerization, and Biological Properties of Octamethyl-pyrophosphoramide N-Oxide

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Octamethylpyrophosphoramide (schradan) is activated through enzymatic or chemical oxidation processes. Oxidation with neutral permanganate yields an initial oxidation product almost a million times more effective as an inhibitor of cholinesterase or chymotrypsin than the original phosphoramide; further oxidation results in a complex demethylation reaction. The first product, octamethylpyrophosphoramide N-oxide, appears to be the most efficient inhibitor of cholinesterase, whereas inhibitory activity towards chymotrypsin is more directly related to the hydrolytic lability of other phosphoramides subsequently formed. This specificity towards cholinesterase is apparently conferred by the electron-attracting N-oxide group. This oxidation product phosphorylates chymotrypsin in a mole for mole reaction in which the enzyme combines with either phosphorus moiety of the oxidized pyrophosphoramide. Chromatographic purification of oxidation products has yielded an unstable, active anticholinesterase and a more stable oxidation product.

OCTAMETHYLPYROPHOSPHORAMIDE (schradan) has recently been shown (6–8, 11) to be susceptible to biological oxidation to form the corresponding monophosphoramide oxide. This enzymatic oxidation increases the chymotrypsin inhibitory activity of schradan almost a millionfold (7, 8, 10, 11).

Chlorination of schradan (36) yields effective anticholinesterase products, but these chloro derivatives differ from the biological metabolite. Hartley (21) reported that the main initial product of the permanganate reaction probably has an amide oxide structure, but claimed that the inhibitor produced in major quantity by permanganate oxidation is not the same as the biologically oxidized product whose distribution coefficient is like that of —N(CH₃)H or —N(CH₃)-CH₂OH. In their study of the decomposition of schradan in the living plant, Heath, Lane, and Llewellyn (23) could not find any decomposition product consistent with the —N(CH₃)CH₂-OH structure and ruled out the N-oxide structure because "the introduction of a C—N(O)—P group involved a far more drastic withdrawal of electrons from the neighboring phosphorus atom, and hence might well produce immediate aqueous hydrolysis of the P—O—P bond." Casida, Allen, and Stahmann (6, 8) obtained evidence that the products from liver and permanganate oxidations were effective inhibitors of chymotrypsin at

the same molar concentrations and proposed the name phosphoramide oxide for the product from enzymatic and chemical oxidation of schradan. Indeed, Hartley (22) reported agreement with this N-oxide structure and further indicated that the more stable derivative, because of a consecutive or parallel reaction, might be fitted by either the —NCH₂OH or —NH compounds. Recently, O'Brien and Spencer (32) found that permanganate oxidation of schradan at pH 7.4 yielded a very active anticholinesterase identical to the active insect metabolic product, along with a more stable less active product. An amide oxide or a methylol structure was suggested for the more active anticholinesterase, but no structure was proposed for the more stable derivative.

In this communication further data are presented in support of the N-oxide structure, the identity of the chemical and biological oxidation product of schradan, and the relationship and properties of the unstable to the more stable oxidation products (37) obtained from schradan. Further investigations into the nature of activation of schradan by chemical oxidation revealed that a host of other oxidizing agents such as sodium hypochlorite, bromine water, and potassium dichromate are equally effective in this chemical conversion. Of these several oxidizing agents, sodium hypochlorite was found to be the most effective. The oxidation product, which was

purified chromatographically using a silica gel column, was demonstrated to be identical to the biologically oxidized schradan. Peracetic acid oxidation, however, produced a product that was found to be distinctly different with respect to biological activity and solubility when compared with the hypochlorite-oxidized schradan.

The loss of a hundred- to a thousand-fold in antiesterase activity of both the liver metabolite and the hypochlorite-oxidized schradan on storage at room temperature was found to be associated with the transformation of the initial oxidation product to a compound less soluble in water than the schradan N-oxide or even schradan itself. This less active compound was found to be identical to the product formed by peracetic acid oxidation of schradan. A similar transformation of the product from hypochlorite or biological oxidation of schradan could be effected by heat, weak acid, and weak alkali.

Hartley (22) has observed the presence of a compound in schradan-treated clover plants, and O'Brien and Spencer (32) recently reported on a compound from permanganate oxidation of schradan which could well be identical to the peracetic acid oxidation product or the product that rapidly formed from the schradan N-oxide in weak acid or base.

As a metabolic activation of schradan is required before it is toxic to insects

(10, 17, 31) and as the product formed in this manner is identical to chemically oxidized derivatives, it appeared that the insecticidal activity of schradan and other dimethylphosphoramides might be increased by oxidation, which might prove a more certain activation mechanism than slow conversion by the insect. Accordingly, oxidized dimethylphosphoramides were tested as contact and systemic insecticides.

Synthesis and Biological Properties of Octamethylpyrophosphoramide N-Oxide

Experimental Procedure and Results

Schradan [melting point 8–10° C., uncorrected; 14° to 20° C. (35)] was purified from a commercial preparation as previously reported (7). Assays of chymotrypsin and cholinesterase were conducted manometrically (7, 9). Formaldehyde was determined colorimetrically after reaction with chromotropic acid (10). Hydrochloric acid hydrolyzates were used for determination of dimethylamine (20) and monomethylamine (3, 14, 33). Infrared absorption studies were made with a Baird I-R spectrophotometer using a sodium chloride prism and anhydrous chloroform as the solvent.

Oxidation of Schradan. Neutral permanganate was the first oxidizing agent studied. Product hydrolysis was reduced by buffering the aqueous reaction mixture at pH 7.0 with phosphate buffer, or by precipitating the manganate ion as rapidly as it was formed by using an excess of barium ions. A typical oxidation method was as follows:

To a solution of potassium permanganate (analytical reagent grade) and barium chloride, at 0.05 and 0.075M, respectively, at 37° C., schradan was added until the solution was 0.05M, mixed, and allowed to react until the permanganate was decolorized (about 3 hours if no manganese dioxide is added initially for catalysis). The reaction mixture was then extracted with three successive equal volumes of chloroform, the chloroform extract dried with anhydrous sodium sulfate, and the solvent removed under reduced pressure. Only phosphorus compounds with their pyrophosphate structure intact would be extracted by the chloroform.

Phosphorylation of Chymotrypsin by Oxidized Dimethylphosphoramides. The dimethylphosphoramides were oxidized with neutral permanganate, separated by chloroform extraction, and made to react in aqueous solution with crystalline chymotrypsin (Worthington Biochemical). The addition of 0.017 mole (4.8 grams) of schradan which had been oxidized, to 3.2 × 10⁻⁵ mole (860 mg.) of chymotrypsin in 250 ml. of 0.2M phosphate buffer

at pH 7.0, yielded 98% esterase inhibition (7) after 24 hours' incubation at 5° C. Three successive ammonium sulfate precipitations of the chymotrypsin which was made to react with a non-oxidized sample of schradan, followed by exhaustive dialysis against distilled water, left an active enzyme which yielded no phosphorus, formaldehyde, or methylamines on hydrolysis. The purified enzyme which was inhibited by the oxidized schradan was then analyzed for residues of the organophosphate which were introduced during the inhibition.

When concentrated hydrochloric acid was added to 2.4N to the inhibited chymotrypsin and the product was rapidly distilled almost to dryness at 100° C., about 0.5 mole (actual, 0.51) of formaldehyde (10) was liberated per mole of enzyme [enzyme concentration based on Kjeldahl (24), 16.0% nitrogen (30), and molecular weight of 27,000 (30)]. To the residue was then added sufficient sodium hydroxide to allow the free amines to be steam-distilled into a 0.1N hydrochloric acid solution (20). The amine hydrochlorides in the distillate were dried down in a vacuum desiccator onto clean quartz sand. The dimethylamine hydrochloride was separated by successive washes of the sand with hot chloroform (3), and analyzed by formation of the cupric dimethyldithiocarbamate (20). Monomethylamine hydrochloride was then removed from the white sand with hot water (3) and analyzed by oxidation to formaldehyde with ninhydrin (14), followed by colorimetric determination of the formaldehyde (10). The dimethylamine residues introduced into the enzyme approached 1.5 moles (actual, 1.48) and the monomethylamine 0.50 (actual, 0.60) mole per mole of chymotrypsin. Phosphorus determination following digestion with perchloric acid (2) yielded 0.95 mole per mole of the enzyme.

The oxidation product of schradan which had reacted with chymotrypsin released approximately 0.5 mole of formaldehyde per phosphate residue. This ratio indicates that the amine oxide structure (6) was the effective phosphorylating agent which combines with the enzyme at either phosphorus moiety from the pyrophosphate. This phosphoramide oxide moiety appeared to be fairly stable when attached to chymotypsin, as even after 72 hours' incubation at 5° C. in distilled water the formaldehyde liberated from the protein on hydrolysis was about the same as the monomethylamine content. This observation indicated that the N-oxide group had rearranged to the more stable isomer, the N-methoxide, on the phosphorylated protein.

Further studies with bis-(dimethylamino) p-nitrophenyl phosphate and tetramethyl phosphorodiamidic fluoride [bis-(dimethylamino) fluorophosphine

oxide] showed a similar increase in phosphorylation ability after permanganate oxidation as measured by enzymatic inhibition and determination of phosphorus in the inhibited chymotrypsin. A sample of liver metabolite (7) also inhibited this enzyme by introduction of phosphorus into the chymotrypsin molecule.

Identity of Liver Metabolite and Active Permanganate Oxidation Product. Experiments were conducted to determine if the oxidation product which phosphorylates chymotrypsin and that which liberates formaldehyde on treatment with acid were the same as the liver metabolite. The partition coefficients between chloroform, methylene chloride, or carbon tetrachloride and water of the formaldehyde-yielding oxidation products were the same for the two materials, as well as the molar concentration required for 50% inhibition of cholinesterase and chymotrypsin (concentration of oxidation product based on formaldehyde analysis) (11). These properties thus indicate that the initial active inhibitory product from permanganate oxidation is the same as the active biological derivative of schradan—i.e., the monophosphoramide N-oxide of octamethylpyrophosphoramide. However, on standing in aqueous solution the anticholinesterase activity progressively decreased and more formaldehyde-yielding material was extractable with chloroform. This material was not free formaldehyde, and its origin is described below.

Effect of Varying Degrees of Oxidation. The varying yield of active enzyme inhibitor and the low yield of formaldehyde-liberating material suggested that rather complicated oxidation processes were involved. This was confirmed by the finding that 1 mole of schradan reacted with 36 equivalents of permanganate under neutral conditions in the presence of excess permanganate and barium chloride.

Figure 1 shows the effect of the degree of oxidation on the enzyme-inhibitory activity, amine content, and stability of the organophosphates which were extracted into chloroform from the reaction mixtures. Table I gives certain properties of these products. With increasing oxidation there is a rapid initial increase in the cholinesterase inhibitory activity, followed by a decrease on further oxidation (Figure 1, A), whereas the change in inhibitory effect towards chymotrypsin is more gradual and continues to increase throughout the oxidation. A comparison of the formaldehyde analysis and the anticholinesterase activity suggests that the most effective cholinesterase inhibitor is the initial oxidation product formed, which liberates formaldehyde on treatment with acid, and that possibly other formaldehyde-yielding antiesterase materials ap-

pear at later stages in the oxidation. The inhibitory activity towards chymotrypsin (Figure 1, A) generally paralleled the relative hydrolysis rate at pH 8 (Figure 1, B). A similar observation regarding stability and effectiveness as chymotrypsin inhibitors has been made with a series of dimethylphosphoramides (8). Amine determination (Figure 1, C) showed that the decrease in stability was associated with a conversion of dimethylamino to monomethylamino groups. The phosphorus content of the
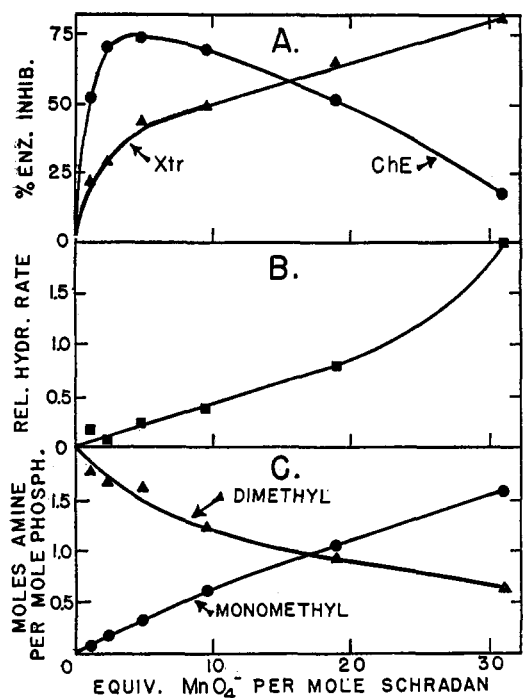

Figure 1. Effect of degree of oxidation on properties of schradan

A. Enzyme inhibitory properties. Cholinesterase (ChE) curve, inhibition of rat brain cholinesterase from 1-hour incubation at 37° C. with 2.5 × 10⁻⁵M pyrophosphate. Chymotrypsin (Xtr) curve, inhibition of purified enzyme after 16-hour incubation with 5 × 10⁻⁸M pyrophosphate. Assays made on chloroform extracts of reaction mixtures indicated

B. Stability. Relative hydrolysis rate is based on per cent hydrolysis in 14 hours at 37° C. and pH 8.0, 2.0 representing 41.5% hydrolysis. Assays made on chloroform extracts of reaction mixtures indicated

C. Amine content. Analyses made on hydrochloric acid hydrolyzates of chloroform extract of reaction mixtures indicated

chloroform-soluble reaction products increased from 21.7 to 26.3% in the course of successive oxidation, which is consistent with this oxidative degradation. The yield and the partition coefficients declined, as shown in Table I, in a manner somewhat similar to the conversion of dimethylamino to monomethylamino groups. As the pH of the essentially unbuffered solution dropped only 0.7 pH units, little hydrolysis occurred during this oxidation.

Infrared spectrophotometry was utilized to follow the oxidation process.

Several of these spectra are shown in Figure 2. As the oxidation progressed, the absorption bands at 2.97, 3.35, 4.10, 6.70, 6.83, and 10.80 microns decreased, that at 10.05 increased, and new bands were introduced at 5.90, 7.02, 7.80, and 9.30 to 9.50 microns. These spectra generally support the hypothesis of an initial oxidation on the amide nitrogen followed by an oxidative demethylation (34).

Insecticidal Activity. The products indicated in Table I were compared with tetraethyl pyrophosphate (TEPP) for contact insecticidal activity against the fruit fly (Drosophila melanogaster Meig.) by determining the mortality after 40 hours' exposure to 10 micromoles of the chemical impregnated in an 8-cm. filter paper in a petri dish. The tetraethyl pyrophosphate produced 100% mortality, while the phosphoramides did not differ significantly and gave about 30% kill. Preparations of schradan, bis-(dimethylamino) p-nitrophenyl phosphate, and bis-(dimethylamino) fluorophosphine oxide (tetramethyl phosphorodiamidic fluoride) were oxidized with peracetic acid (1.1 moles of peracetic acid to 1.0 mole of phosphoramide) by the general method of oxidation outlined below and also with permanganate, and were compared in the field with the nonoxidized products on potato insects. The kill of aphids, leaf hoppers, and flea beetles was not markedly affected by the oxidation. Where
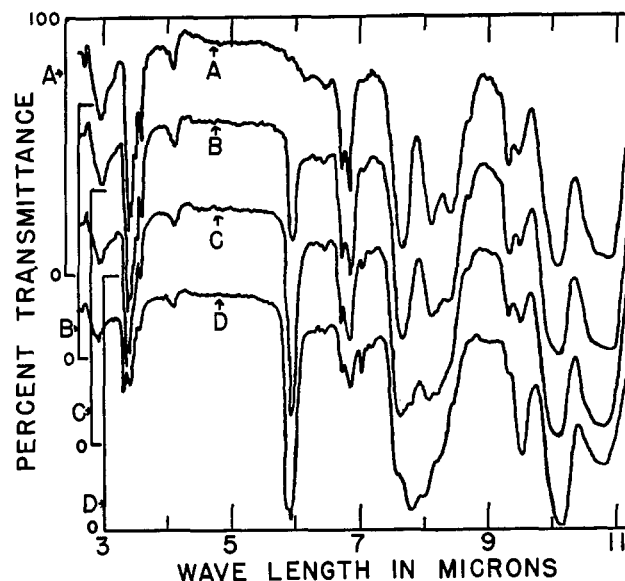

Figure 2. Effect of degree of oxidation on infrared spectra

Determinations at 0.25M pyrophosphate in chloroform in NaCl prism with Baird spectrophotometer. Curves made on chloroform extract of reaction mixtures with: A, 0; B, 2.3; C, 9.6; D, 31.1 equivalents of KMnO₄ per mole of schradan

tested for systemic insecticidal properties (small excised cabbage leaves were exposed 4 days to 10-ml. solutions containing 10 micromoles of insecticide and were bioassayed with the cabbage aphid, Brevicoryne brassicae L.), there was no activity with tetraethyl pyrophosphate and little with the least stable of the oxidized materials, but the other phosphoramides produced 60 to 90% mortality and did not differ significantly among themselves.

Isomerization of Octamethylpyrophosphoramide N-Oxide to Heptamethylpyrophosphoramide N-Methoxide

Experimental and Results

Selection of Oxidizing Agents. In the general procedure employed for screening the various oxidizing agents for their ability to convert

Table I. Effect of Degree of Oxidation on Schradan Oxidation Mixture

Equiv. MnO₄⁻/ Mole Schradan[a]	Yield[b], %	Part. Ratio[c], CHCl₃/H₂O	Molar Ratio HCHO/P[d]	Final pH[e]
0.0	100	7.1	0.000	6.9
1.1	87	7.2	0.017	6.8
2.3	75	6.8	0.027	6.8
4.8	67	7.1	0.043	6.8
9.6	56	6.0	0.056	6.8
19.0	36	4.9	0.036	6.7
31.1	14	2.8	0.027	6.2

[a] 1.98 millimoles pure schradan reacted with appropriate amount of 0.15M MnO₄⁻ with excess BaCl₂ for 3 hours at 37° C. and then extracted with equal volume of chloroform. Unreacted MnO₄⁻ then titrated.
[b] Total phosphorus analysis of chloroform extract.
[c] Based on total phosphorus in two solvent phases.
[d] HCHO liberated by 8N H₂SO₄ at 100° C. for 30 minutes.
[e] pH of oxidation mixture just prior to extraction.

schradan to an active anticholinesterase, 0.03 micromole of schradan was allowed to react with an oxidizing equivalent of various oxidizing agents at room temperature for a specified number of hours in 0.5M phosphate buffer pH 7.5. At intervals, 1-ml. aliquots of the reaction mixture were extracted once into chloroform and suitable aliquots of this chloroform extract corresponding to 1 micromole of the original schradan were assayed manometrically (7, 9) for cholinesterase inhibition (Table II). Of the various oxidizing agents tested, potassium permanganate, potassium dichromate, bromine water, and sodium hypochlorite were effective. Hydrogen peroxide gave good but not reproducible results on several trials. Of these agents, sodium hypochlorite prepared according to the method of Fieser (18) gave the highest yield of the anticholinesterase agent from schradan.
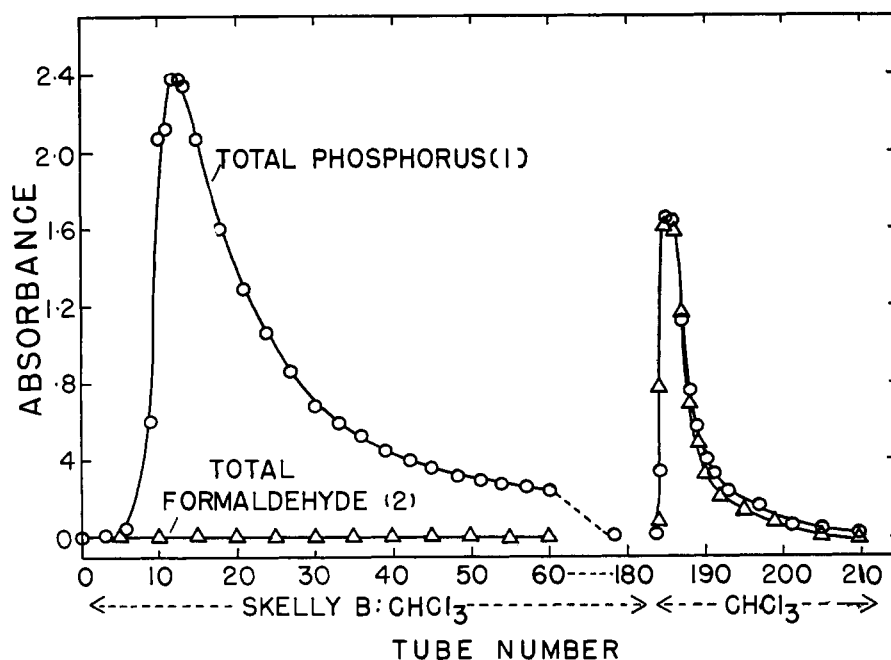

Figure 3. Separation of hypochlorite-oxidized schradan on silica gel column

0.6 gram of oxidized schradan separated on 10 grams of silica gel containing 7 ml. of 0.5M phosphate buffer at pH 8 as inside phase. 5-ml. fractions collected. Separation followed by total phosphorus determination (1) and antiesterase active fraction followed by formaldehyde determination (2)

Table II. Selection of Oxidizing Agents

Oxidizing Agent	Reaction[a] Time, Hours	Cholinesterase Inhibition, %	Yield, %
K₃Fe(CN)₆	24	0	0
K₂Cr₂O₇	24	55	0.1
KMnO₄	3	86	0.3
CH₃CO₃H	24	0	0
Br₂ water	1	22.7	0.016
NaClO	1	60.6[b]	1.0
Bleaching powder	20	80	0.3

[a] Time at which maximum cholinesterase inhibition obtained.

[b] Contained 0.1 μmole of original schradan in Warburg flask.

Typical Oxidation of Schradan with Hypochlorite. Five portions, each containing 7 ml. of 5N sodium hypochlorite (35 meq.) and 25 ml. of phosphate buffer (1M, pH 7.0), were added to 5 grams (17.5 millimoles) of schradan at 18-minute intervals over a period of 90 minutes and at a temperature of 55° C. The reaction mixture was cooled quickly and immediately extracted five times with equal portions of chloroform. The chloroform extract was dried over anhydrous magnesium sulfate.

An analysis of the resulting oxidation product from several typical oxidations showed that between 35 and 40% (based on formaldehyde analysis) of the original schradan had been oxidized. Of this amount of oxidized schradan between 4 and 12% was extractable into the chloroform. This fraction displayed a strong anticholinesterase activity and represented the oxidation products whose pyrophosphate bond remained intact. However, above 30% of the formaldehyde-yielding oxidized product remained in the aqueous phase even

after 12 hours' continuous liquid to liquid extraction in a Kutscher-Steudel extractor. This fraction was probably the oxidized schradan hydrolyzed at the pyrophosphate linkage or free formaldehyde, for it did not inhibit cholinesterase and could not be extracted into chloroform from the reaction mixture buffered at pH 7.5.

Typical Oxidation of Schradan with Peracetic Acid under Anhydrous Conditions. Schradan (2.5 grams, 8.75 millimoles) was oxidized with 43 meq. of peracetic acid in 25 ml. of anhydrous chloroform at room temperature for 3 hours. The entire reaction mixture was shaken with 60 ml. of saturated solution of sodium bicarbonate to remove the excess peracetic and acetic acids and extracted thrice with 50-ml. portions of fresh chloroform. In a typical example, 697 micromoles of formaldehyde-yielding chloroform-soluble and 300 micromoles of formaldehyde-yielding chloroform-insoluble material were obtained.

Chromatographic Separation of Hypochlorite-Oxidized Schradan. The column was prepared by uniformly mixing 40 grams of silicic acid (Mallinckrodt chromatographic grade) with 28 ml. of 0.5M phosphate buffer at pH 7.5. The mixture was then mixed with the outside phase (75 parts of Skellysolve B and 25 parts of chloroform), poured into the column and packed under a positive pressure of 1 to 2 pounds.

In a typical separation procedure, 2.5 grams of the oxidized schradan was layered on top of the column, washed into the silica gel with a small volume of chloroform, and eluted with Skellysolve

B-chloroform (75 to 25 by volume). Then 10-ml. fractions were collected by an automatic fraction collector at a flow rate of 3 to 4 minutes per fraction. Generally, about 3500 ml. of the solvent was required to elute out all the non-formaldehyde-yielding material, which consisted chiefly of schradan. Then chloroform was introduced to elute out the formaldehyde-yielding antiesterase compound. The partition ratio of schradan in Skellysolve B (75)–chloroform (25) to water was found to be 0.146, while that of the purified oxidized schradan was 0.062.

The progress of separation was followed by determining total phosphorus and formaldehyde in the fractions. In Figure 3 are presented the results of a typical separation of the oxidized schradan from schradan. It is evident that Skellysolve B–chloroform (75 to 25) removes all the inactive phosphate antiesterase material without eluting out the active antiesterase, as is shown by the negative formaldehyde color. When the proportion of chloroform was increased to 30%, the formaldehyde-positive material was eluted out along with the schradan. As the ratio of the absorbances of the formaldehyde and the total phosphorus remained constant, this component represents the purified oxidized product.

Chromatographic Separation of Peracetic Acid–Oxidized Schradan. The column was prepared and the separation conducted in a manner similar to that for the separation of the hypochlorite-oxidized schradan. The degree of separation was likewise followed by deter-

mination of total phosphorus and formaldehyde.

The results in Figure 4 show the appearance of a formaldehyde-positive peak which was eluted out with the Skellysolve B–chloroform mixture. The formaldehyde-positive material appeared to have solubility characteristics almost identical with those of schradan and could not be effectively separated from schradan by a single passage through a column. The larger amount of total phosphorus eluted with chloroform compared with the results of Figure 3 is due to the overloading of the column. The production of a formaldehyde-yielding, phosphorus-containing, anticholinesterase compound which was less water-soluble than schradan was unexpected, as the hypochlorite-oxidized schradan proved to be more water-soluble than schradan. Consequently, these results were investigated further and are discussed later.

The formaldehyde-positive material was pooled, the solvent concentrated, and the residual sirup rechromatographed on silica gel. Figure 5 shows that this material upon rechromatography may be separated from schradan, as is evident from the curve for total phosphorus.

Upon longer oxidation with peracetic acid another formaldehyde-positive, phosphorus-containing peak was observed at a position corresponding to the hypochlorite-oxidized schradan. This

Figure 5. Rechromatography of antiesterase fraction from peracetic acid–oxidized schradan

Tubes 20 to 45 represented by Figure 4 combined, solvent removed by vacuum distillation, and residual oil transferred to column containing 40 grams of silica gel (containing 28 ml. of 0.5M, phosphate buffer pH 8, as inside phase). 10-ml. fractions collected. Separation followed by total phosphorus determination (1) and antiesterase active fraction followed by formaldehyde determination (2)
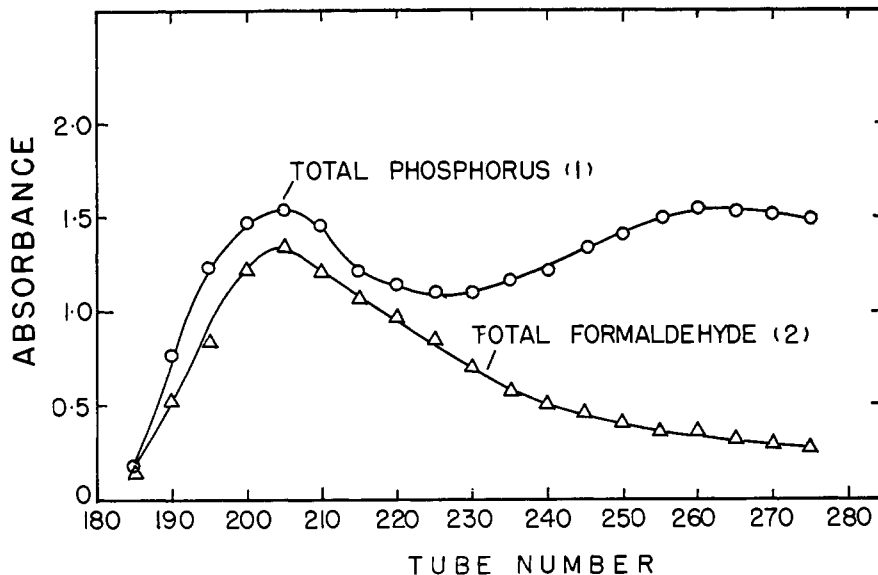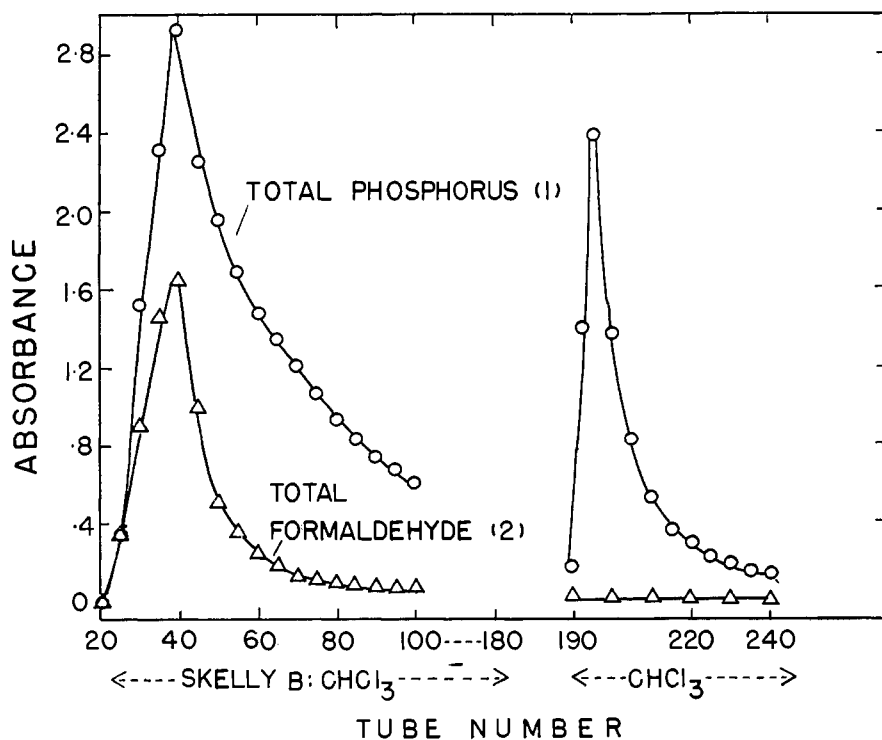
compound could be one in which one of the nitrogens is oxidized as with the peracetic acid–oxidized schradan but in which an additional oxidation occurred as with the hypochlorite-oxidized schradan. The presence of this N-oxide

structure in the peracetic acid–oxidized schradan may alter the solubility characteristics sufficiently to show this type of behavior in a silica gel column.

Countercurrent Solvent Fractionation of Peracetic Acid-Oxidized Schradan. The peracetic acid–oxidized schradan was also separated by solvent fractionation utilizing a 98-tube countercurrent distribution apparatus (*13*). Two grams of the chloroform-soluble oxidized product was distributed in 10-ml. volumes of chloroform and water over a period of 3 hours. Total phosphorus and formaldehyde were determined in both upper and lower phases. From Figure 6 it is apparent that in the chloroform phase both the phosphorus and the formaldehyde are present in only the initial tubes (tubes 4 to 25, inclusive). The formaldehyde peak which has a partition coefficient of 8.9 between chloroform and water appears a few tubes before the large phosphorus peak, indicating that the formaldehyde-yielding oxidation product is more chloroform-soluble than schradan, which has a partition coefficient of 7.0 between chloroform and water. In the aqueous phase the phosphorus and formaldehyde appear in the same relative positions as in the chloroform phase, but the absolute amounts of the two components are governed by their relative partition coefficients between chloroform and water. Another formaldehyde-positive peak appears in the aqueous phase at tube 50. This material, which is almost totally lacking in phosphorus, has not been studied further, but may be para-

Figure 4. Separation of peracetic acid–oxidized schradan on silica gel

2.5 grams of oxidized schradan separated on 40 grams of silica gel containing 28 ml. of 0.5M phosphate buffer at pH 8 as inside phase. 10-ml. fractions collected. Separation followed by total phosphorus determination (1) and antiesterase active fraction followed by formaldehyde determination (2)

formaldehyde. The third formaldehyde positive peak appearing in tube 90 in the aqueous phase appears to check with free formaldehyde, whose distribution coefficient between chloroform and water is 0.15. The presence of this free formaldehyde peak might be due to the free formaldehyde which passed into the chloroform phase during the extraction of the oxidized product from sodium bicarbonate solution or to the slow decomposition of the oxidized product prior to the countercurrent separation. Only the formaldehyde-yielding peak from the chloroform phase was checked for anticholinesterase activity, as this material was the only one containing both formaldehyde and phosphorus. The 50% cholinesterase inhibition concentration value was within the range (5 to 6 $\times$ 10^{-4}M) of that found for the first peak in the peracetic acid–oxidized schradan separated by silica gel column.

Anticholinesterase Activity of Hypochlorite-Oxidized Schradan. The product from oxidation with hypochlorite separated with silica gel was tested for its inhibition of acetylcholinesterase. Both the purified enzyme (obtained from Winthrop-Stearns, Inc., New York, N. Y., lot N331UN) and the enzyme from rat brain homogenates were used in this measurement. The results in Table III show that the rat brain homogenate enzyme is inhibited 50% at a concentration level of 7.5 $\times$ 10^{-6}M of the oxidized schradan, while the purified enzyme shows the same amount of inhibition at a concentration level of 8 $\times$ 10^{-6}M of the oxidized schradan. Thus it is evident that oxidation increases the cholinesterase inhibitory ability of schradan about a millionfold.

Stability of Hypochlorite-Oxidized Schradan. Oxidized schradan was found to be stable for about 3 months when stored in the refrigerator in anhydrous chloroform. However, in aqueous solution the oxidation product loses its antiesterase activity rapidly. Its stability was tested therefore in an aqueous solution at pH 7.8 and at room temperature.

Ten milliliters of a chloroform solution containing 3 micromoles of the oxidized schradan (based on formaldehyde assay) were freed of solvent and dissolved in 20 ml. of sodium bicarbonate buffer (0.025M, pH 7.8). At various intervals of time, aliquots of the aqueous solution were tested for their anticholinesterase activity. The oxidation product analyzed in this manner showed a half life of 18 minutes (Figure 7).

Anticholinesterase Activity of Peracetic Acid–Oxidized Schradan. The formaldehyde-positive peak obtained by the separation in a silica gel column of the peracetic acid–oxidized schradan showed a 50% inhibition of purified cholinesterase at a concentration of 6 $\times$ 10^{-4}M. Thus this material is 100 times less effective in inhibiting cholinesterase than the product from hypochlorite oxidation. However, when its stability in anhydrous chloroform was tested, it was found that even after 1 year at room temperature the anti-enzyme activity was retained completely.

A comparison of the antiesterase activity of the oxidized schradan from both oxidation procedures indicated that the one with the lower activity (peracetic oxidation) might be a transformation product of the compound with higher activity (hypochlorite oxidation). When the hypochlorite-oxidized product was allowed to stand at room temperature in anhydrous chloroform, within a month it lost the high original antiesterase activity but still partitioned into chloroform from water, suggesting that the pyrophosphate linkage had remained intact. If the loss in the high activity was due to the cleavage of the pyrophosphate linkage, a large change in solubility would be expected, as then the polar phosphate grouping becomes exposed.

It was decided to investigate such a possible chemical relationship by the determination of the change in partition coefficients between chloroform and water of the hypochlorite-oxidized schradan as a result of various treatments.

Partition Coefficients of Oxidized Schradan Derivatives. In Table III are listed the partition coefficients in chloroform to water of the various oxidation products. Prior to the determination of the partition coefficients, the chloroform solutions of the compounds

Figure 6. Separation of peracetic acid–oxidized schradan by countercurrent distribution

2.5 grams (8.75 mmoles) of schradan oxidized with 43 meq. of peracetic acid in 25 ml. of anhydrous chloroform at room temperature for 3 hours. Entire reaction mixture extracted with 60 ml. of saturated solution of sodium bicarbonate, extracted thrice with 50-ml. portions of fresh chloroform. Solvent removed and 2.0 grams of oxidized schradan separated by 98-tube all-glass countercurrent distribution apparatus described by Craig, using chloroform–water solvent system. 10 ml. of each solvent used per tube

A. Total phosphorus (1) was determined in chloroform layer to follow separation of unoxidized schradan, and total formaldehyde (2) to follow separation of anticholinergic product.

B. Total phosphorus (1) was determined in aqueous layer to follow separation of unoxidized schradan, and total formaldehyde (2) to follow separation of formaldehyde-yielding hydrolytic products.
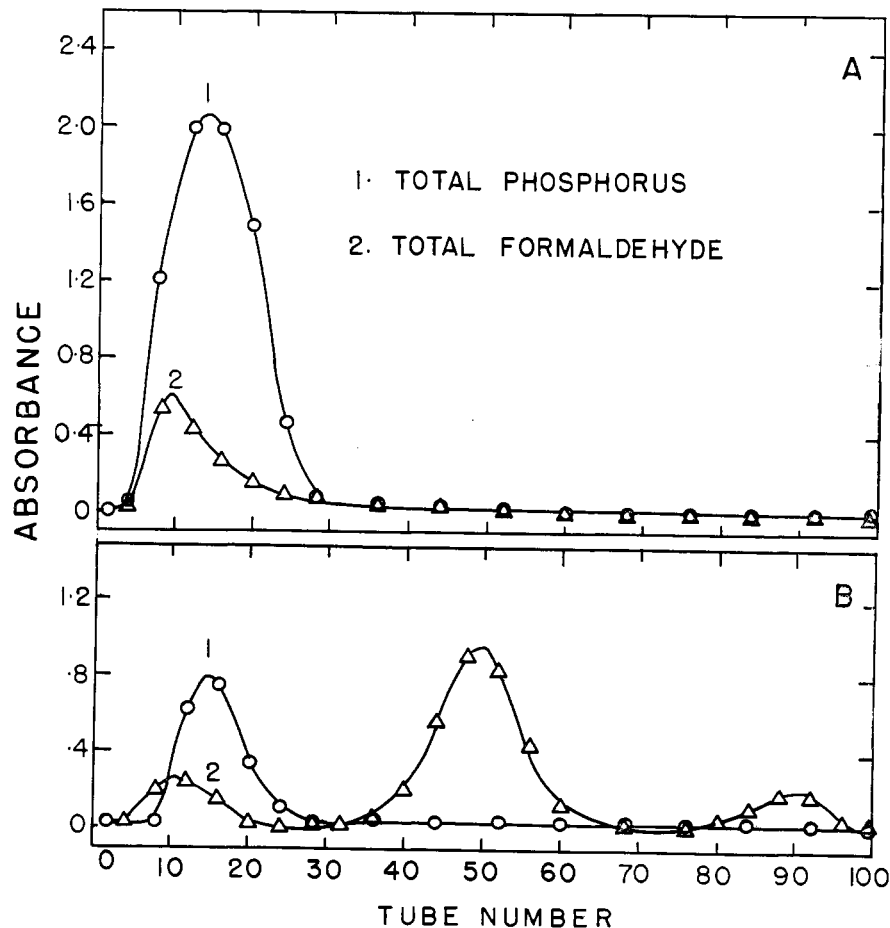
to be partitioned were rapidly extracted once with an equal volume of a saturated solution of sodium bicarbonate to remove any acidic formaldehyde-yielding components (by hydrolysis at the pyrophosphate linkage) and free formaldehyde that may be liberated by the decomposition of the oxidized product. The partition ratio was then determined by equilibrating the chloroform phase with an equal volume of fresh distilled water.
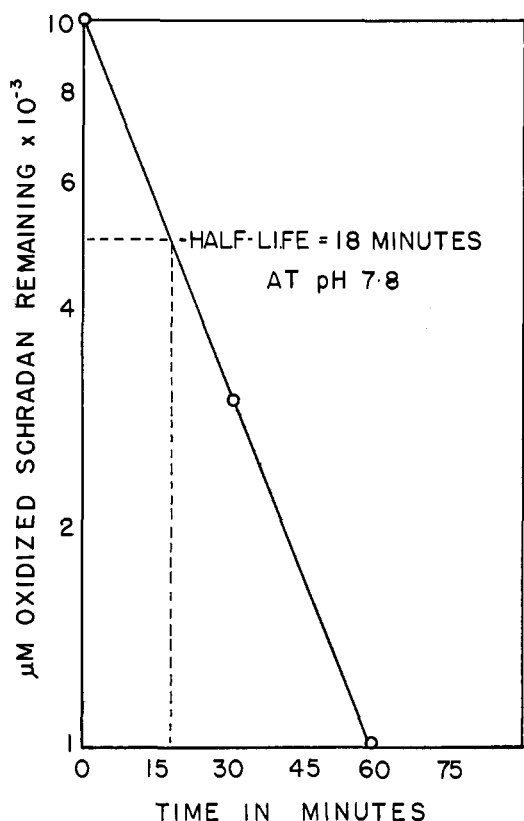

Figure 7. Half life of sodium hypochlorite–oxidized schradan at pH 7.8

3 μmoles of oxidized schradan dissolved in 20 ml. of sodium bicarbonate buffer (0.025M, pH 7.8) and at various intervals of time aliquots of aqueous solution tested manometrically in Warburg for inhibition against 0.1 mg. of purified cholinesterase. Log of micromoles of oxidized schradan remaining plotted against time in minutes

The partition coefficients of the hypochlorite- and permanganate-oxidized schradan and the liver metabolite are the same; this indicates that these three are the same product. This product which can be produced by the three oxidative procedures is more polar than schradan and its molar concentration for 50% inhibition of cholinesterase is also within the same range. The slightly lower inhibitory activity of the hypochlorite-oxidized schradan may be due to differences in the rat brain homogenates used for assay or partial isomerization of the product.

The partition ratio of the peracetic acid–oxidized schradan is distinctly different from the hypochlorite- and permanganate-oxidized schradan. The ratio indicates a far less polar product, even less than schradan. The anticholinesterase activity is likewise 100 to a 1000 times less.

Isomerization of Hypochlorite-Oxidized to Peracetic Acid–Oxidized Schradan. During this investigation it was observed that when schradan oxidized by rat liver homogenate was separated by silica gel, the formaldehyde positive peak was not eluted until chloroform was used as the eluent. In other words, this liver metabolite displayed the same solubility characteristics as the hypochlorite-oxidized schradan. However, upon rechromatography, the solubility characteristic of this biologically active fraction changed to that of a less polar compound, and was eluted by Skellysolve B–chloroform (75 to 25 by volume) in approximately the same position as the peracetic acid–oxidized schradan. This shift was not due to free formaldehyde formed by decomposition, as its partition ratio in chloroform to water is 0.15.

Table III shows that the partition coefficient of the hypochlorite-oxidized schradan, when treated in several ways, shifts to that of the peracetic acid–oxidized compound, which is far more soluble in chloroform. This change in partition coefficient is not due to either free formaldehyde or to some form-

aldehyde-liberating hydrolytic product of the oxidized schradan, as these have been removed by a previous extraction with bicarbonate.

Thus weak alkali (pH 7.8) caused a shift in partition coefficient from 1.41 to 8.4, showing that the hypochlorite-oxidized schradan was converted to the peracetic acid–oxidized schradan.

Treatment of the hypochlorite-oxidized schradan in anhydrous chloroform under reflux conditions for 1.5 hours causes a shift in partition coefficient to 7.1. A preliminary extraction of the chloroform solution with bicarbonate had removed approximately 50% of the original oxidized schradan as measured by formaldehyde determination. This 50% probably represents destruction of the oxidized schradan to free formaldehyde and other formaldehyde-liberating hydrolytic products. The remaining 50%, which has a partition ratio of 7.1, appeared to be similar to the peracetic acid–oxidized schradan.

Treatment of the hypochlorite-oxidized schradan in chloroform containing acetic acid was selected because oxidation with peracetic acid in chloroform led to the formation of the oxidized schradan with the lower anticholinesterase activity. From Table III it is evident that even 10-minute treatment at room temperature caused a complete shift in the partition coefficient of the hypochlorite-oxidized schradan to that of the peracetic acid–oxidized schradan. When the anticholinesterase activity of

Table III. Partition Coefficients and Molar Concentrations for 50% Enzyme Inhibition of Oxidized Schradan and Its Transformation Product

	$CHCl_3/H_2O^a$	Molar Concn., 50% Enzyme Inhibition
Schradan	7.0	1.5×10^{-1}
Oxidized schradan (NaClO)	1.41	7.5×10^{-8}
Liver metabolite of schradan[b]	1.42	3.6×10^{-7}
Oxidized schradan		
KMnO₄[b]	1.27	4.0×10^{-7}
Peracetic acid	8.0	6.0×10^{-4}
Free formaldehyde	0.15	...
Oxidized schradan (NaClO)		
Treated with acid 10 minutes[c]	9.13	...
Treated with acid 30 minutes[d]	10.1	...
Heated for 1.5 hours[e]	7.1	...
At pH 7.8 for 3 hours[f]	8.4	...

[a] 3.0 μmoles of compounds dissolved in 10 ml. of chloroform, extracted once with equal volume of saturated solution of sodium bicarbonate. Chloroform phase was then equilibrated with equal volume of distilled water and formaldehyde determined in both upper and lower phases to determine partition coefficients.
[b] Partition ratios and molar concentration for 50% inhibition of cholinesterase represent values reported by Casida (5).
[c,d] 3.0 μmoles of sodium hypochlorite–oxidized schradan were dissolved in 10 ml. of chloroform containing 1 ml. of glacial acetic acid and allowed to stand at room temperature for 10[c] and 30 minutes[d]. Then chloroform solution was extracted once with equal volume of saturated solution of sodium bicarbonate. Chloroform phase was equilibrated with equal volume of distilled water.
[e] 3.0 μmoles of hypochlorite-oxidized schradan were dissolved in 10 ml. of chloroform and refluxed for 1.5 hours. Chloroform solution was extracted once with equal volume of sodium bicarbonate solution, separated, and organic phase again equilibrated with equal volume of distilled water.
[f] 3.0 μmoles of hypochlorite-oxidized schradan were dissolved in 10 ml. of 0.025M bicarbonate buffer and allowed to stand at room temperature for 3 hours. Aqueous solution was extracted into 10 ml. of chloroform, and organic phase equilibrated with equal volume of distilled water.
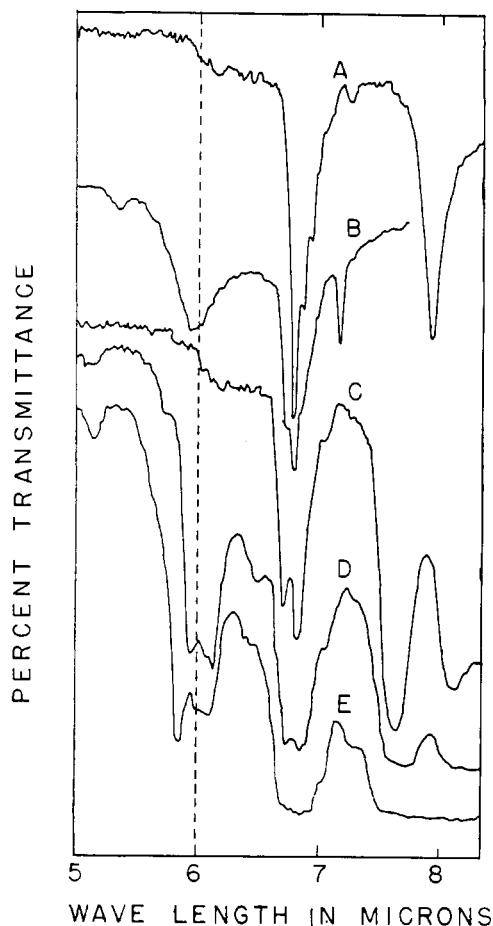
Figure 8. Infrared absorption spectra of trimethylamine in chloroform, A

Trimethylamine oxide dihydrate solid film (B), schradan liquid film (C), column-separated hypochlorite-oxidized schradan liquid film (D), and column-separated peracetic acid–oxidized schradan liquid film (E). Determinations made in NaCl prism with Baird spectrophotometer

this isomerized product was tested, it was found that the range of molar concentration for 50% inhibition of the enzyme was in the neighborhood of that for the peracetic acid–oxidized schradan—namely, 5 to 6 $\times$ $10^{-4}M$.

Ultraviolet Absorption. It has been reported that both the insect- and permanganate-oxidized schradan showed a characteristic absorption in the ultraviolet region with a maximum at 274 mμ (32). However, when the chromatographically purified hypochlorite-oxidized schradan dissolved in spectrographic grade chloroform was tested with the Cary recording spectrophotometer, the compound did not show any significant absorption between 260 and 300 mμ. Chromatographically purified peracetic acid–oxidized schradan likewise did not show any significant absorption in the same region of the ultraviolet spectra. However, the solvent used for the elution of the oxidized schradan indicated the presence of benzene. Efforts directed toward find-

ing out the interfering material in reagent grade chloroform, which was used for the initial extraction of the oxidized schradan, contained traces of benzene in addition to a substance showing absorption between 265 and 295 mμ with a maximum at 274 mμ. This absorption displayed by the reagent grade chloroform corresponds closely to that reported by O'Brien and Spencer (32) for insect and permanganate-oxidized schradan, which shows absorption between 265 and 295 mμ with a maximum at 274 mμ.

Infrared Absorption Spectra. In Figure 8 are shown the infrared absorption curves of trimethylamine and trimethylamine N-oxide dihydrate. Figure 8, B, shows a new absorption peak at 5.97 microns, which represents the absorption due to the N → O bond of $(C_2H_5)_3N$ → O (34). Trimethylamine N-oxide was selected because the N-oxide of this compound is structurally similar to the N-oxide grouping of octamethyl-pyrophosphoramide N-oxide.

It can be seen from Figure 8, C, that schradan displays no absorption in the region of 6 microns, whereas both the hypochlorite-oxidized schradan (Figure 8, D) and the peracetic acid–oxidized schradan (Figure 8, C) show strong absorption in this region, corresponding to the —N → O bond of trimethylamine N-oxide. Hypochlorite-oxidized schradan has absorptions at 5.95 and 6.13 microns, whereas peracetic acid–oxidized schradan shows absorptions at 5.85 and 6.13 microns. Thus there is a definite shift in the absorption peaks of peracetic acid–oxidized schradan. The two oxidized schradans, therefore, do not appear to be identical but rather represent a structural change. It was noted earlier that there was a change in biological activity and solubility characteristics when the hypochlorite-oxidized schradan was treated with acid, alkali, and heat, and that this change produced a compound identical to the peracetic acid–oxidized schradan.

Identity of Liver Metabolite and Chemically Oxidized Schradan. It has been reported (5, 8) that the liver-oxidized schradan is identical with the initial permanganate-oxidation product of schradan. The results compiled in Table III indicate that the hypochlorite-oxidized schradan is also similar to the liver metabolite, for the partition coefficients between chloroform and water and the molar concentration for 50% inhibition of acetylcholinesterase are identical. An infrared absorption on the liver metabolite of 15% purity based on ratio of formaldehyde to phosphorus displayed a new absorption peak at 5.90 microns. The infrared absorption curves of chemically oxidized materials (Figure 8, A, B, C, D, E) likewise show absorption in the same region. The fact that both the liver-oxidized and hypochlorite-oxidized

schradan transform hemselves to the same product—namely, the peracetic acid–oxidized schradan—also indicates that the liver- and chemically oxidized schradan are one and the same compound.

Discussions and Conclusions

Chemical and Biological Oxidation of Schradan to Schradan N-Oxide. The initial step in the chemical oxidation of the dimethylphosphoramides appears to be an attack on one of the amide nitrogen atoms to produce a new type of functional group for which the new name, phosphoramide N-oxide (8) is proposed.

Evidence that the pyrophosphate linkage remains intact in this oxidation product is shown by the constant ratio of formaldehyde to phosphorus of the column-separated oxidized schradan, the chloroform distribution, infrared curves, cholinesterase inhibition, and neutrality of the oxidation product. The apparent weakly basic properties reported for the schradan N-oxide (8) were based upon an experimentally observed change in the distribution properties between chloroform and water from neutral and acidic solutions. [It is now realized that this change might also result from the isomerization of the N-oxide, a reaction that was unknown when the distribution experiments were carried out.] Only one nitrogen is oxidized as analysis of the antiesterase from oxidized schradan shows 3 moles of dimethylamine to every mole of monomethylamine.

Oxidation of more than one dimethyl amide to the N-oxide probably produces a compound which is so unstable that it is hydrolyzed instantaneously.

A study of the specificity of the permanganate oxidation products for cholinesterase inhibition indicated that the oxidation of only one nitrogen yields the most effective anticholinesterase, whereas further oxidation yields other derivatives which liberate formaldehyde on acid hydrolysis but are of lower anticholinesterase activity. The higher oxidation products liberate free formaldehyde from the neutral reaction mixture on heating, but most of this formaldehyde may be further oxidized in the oxidation mixture at room temperature to carbon dioxide and water. This is indicated by the strong odor of formaldehyde liberated from permanganate oxidation mixtures at 98° C. However, no formaldehyde odors were apparent at room temperature and less than the 36 equivalents of permanganate consumed at room temperature were taken up at the higher temperature. The extent of the decrease in dimethylamine groups on oxidation directly corresponds to the formation of monomethylamino groups in the chloroform-soluble products. The pyrophosphate structure remains intact during oxidation to the stage at which
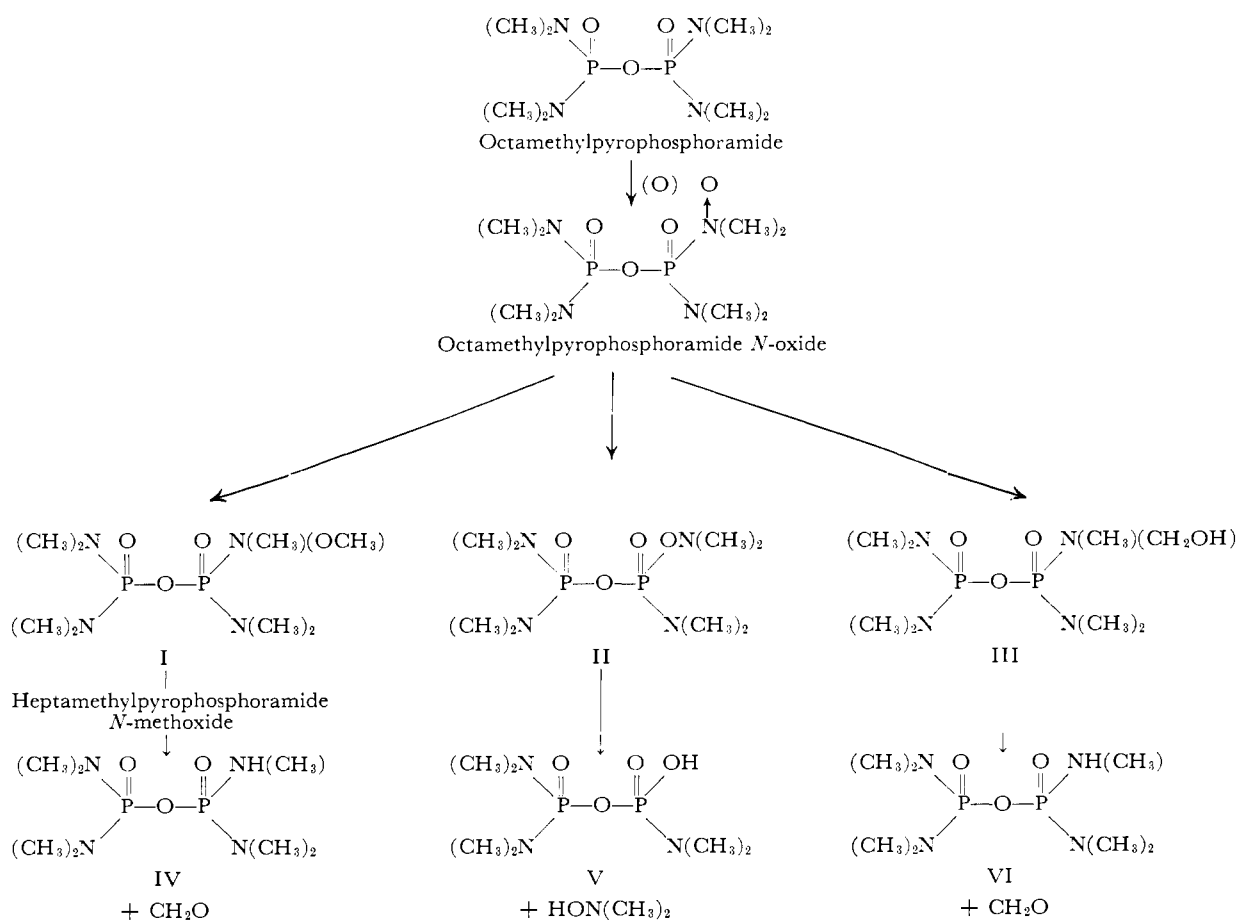
hydrolysis yields three monomethyl-amino and one dimethylamino groups (over-all average of products in reaction mixture) as shown by the infrared curves, chloroform distribution, chymotrypsin inhibition, and neutrality of the oxidation product.

Rearrangement of Octamethylpyrophosphoramide N-Oxide. Oxidation with peracetic acid revealed an unexpected difference in the distribution coefficient and anticholinesterase activity as compared to the hypochlorite-oxidized schradan whose identity with the liver metabolite has been established. It is proposed that the peracetic acid–oxidized schradan represents a rearranged product of the octamethylpyrophosphoramide N-oxide. Weak alkali, weak acid, and heat all cause this transformation. The liver metabolite also appears to be subject to this type of isomerization on the basis of the solubility characteristics during separation on a silica gel column. The chemical oxidation of schradan to octamethylpyrophosphoramide N-oxide and the subsequent rearrangement of this N-oxide may be schematically represented as:

a methyl group has migrated to the oxygen is analogous to the known rearrangements of amine oxides. It is known that benzyldiethylamine oxide rearranges to O-benzyl-N,N-diethylhydroxylamine (12), and that N-methyl-and N-ethyltetrahydroquinoline N-oxides produce tetrahydroquinoline and formaldehyde and acetaldehyde, respectively (15), through the intermediary formation of the substituted hydroxylamines. The liberation of formaldehyde from octamethylpyrophosphoramide N-oxide and its rearranged product, compound I, upon treatment with strong sulfuric acid also finds analogy in the decomposition of the tertiary amine oxides to produce aldehydes. For example, it has been reported that trimethylamine oxide produces dimethylamine and formaldehyde when heated with 33% sulfuric acid at 110° C., and that triethylamine oxide sulfate decomposes to diethylamine and acetaldehyde condensation products (16). N-Methyl- and N-ethyltetrahydroquinoline N-oxides likewise liberate formaldehyde and acetaldehyde, respectively, upon decomposition (15). The fact that form-

aldehyde and the infrared absorption spectrum indicates the formation of a —NH group. The product showing absorption in this region was soluble in chloroform and contained phosphorus. A shift in the infrared absorption of the N-oxide when it is rearranged to a more chloroform-soluble material indicates that the rearranged product, which liberates formaldehyde and in which the pyrophosphate linkage is intact, is not the same as the N-oxide.

The partition coefficient between chloroform and water of the N-oxide was found to be 1.41, while that of the rearranged product was 9, a value indicating that it is less polar than the N-oxide. The compound represented by structure I would be in agreement with this change in solubility. A consideration of the antiesterase property shows that the N-oxide is approximately 100- to 1000-fold more active than the rearranged product. It is known that if the pyrophosphate linkage is broken, this antiesterase agent cannot phosphorylate cholinesterase and consequently will show no inhibitory activity. This phenomenon also holds true

Octamethylpyrophosphoramide

Octamethylpyrophosphoramide N-oxide

I
Heptamethylpyrophosphoramide N-methoxide

IV
+ CH₂O

II

V
+ HON(CH₃)₂

III

VI
+ CH₂O

Compounds with structures I, II, and III were considered as possible structures of the transformed product of the N-oxide.

Structure I. The rearrangement of the N-oxide to compound I in which

aldehyde is liberated from compound I finds analogy in the liberation of formaldehyde from O,N,N-trimethylhydroxylamine (26). During the permanganate oxidation of schradan (5), there is a rapid liberation of free form-

for the inhibition of the enzyme chymotrypsin. It is reasonable to expect, then, that a compound of structure I would have a more stable phosphoric anhydride linkage than the N-oxide, a fact substantiated by the inhibition data

which was obtained with chymotrypsin.

STRUCTURE II. The rearranged product represented by structure II, in which the oxygen has migrated to the phosphorus, must also be considered. A decrease in the water solubility would be expected to follow such a rearrangement. This compound upon degradation may be expected to yield N,N-dimethylhydroxylamine, which does not liberate formaldehyde upon decomposition. The infrared absorption at 5.9 microns on the permanganate-oxidized schradan indicated the presence of an —NH bond as shown in structure IV rather than one of structure V. A compound of structure II, therefore, does not appear to be in accord with all the facts and consequently must be eliminated.

STRUCTURE III. The third alternative structure as represented by structure III, in which the oxygen has migrated to a carbon atom, satisfies our results only in so far as the liberation of formaldehyde upon treatment with strong acid is concerned. Infrared curves on the rearranged product failed to show any absorption due to the —OH group. The structures closest to III in the carboxylic acid series are the N-methylols such as methylol benzamide, which are not very stable and liberate formaldehyde quantitatively upon very mild treatment with acids (4). However, the presence of an alcohol group in this structure suggests a compound more water-soluble than schradan. The partition coefficient of 9 found for the rearranged product and the more drastic conditions required for formaldehyde liberation appear to rule out this possibility.

Biological Significance of Schradan Oxidation and Rearrangement. The activation of schradan occurring in vivo appears to be oxidative by the formation of an N-oxide (6–8, 11). This enhanced anticholinesterase activity of schradan N-oxide may be explained by the labilization of the pyrophosphate bond to facilitate phosphorylation of the enzyme.

The rearrangement of schradan N-oxide may also have significance in the biological activity of this insecticide. In treated plants a very unstable as well as a more stable schradan derivative has been observed (22). The authors have found that on prolonged storage under anhydrous conditions at room temperature, exposure to acid or alkali, or passage through a silica gel column the rat liver metabolite is greatly decreased in antiesterase activity and becomes less water-soluble than schradan. The less stable antiesterase from cockroach or rat liver metabolism is identical to the less stable product from permanganate oxidation (5, 32, 37), which is a transitory material forming a less active, more stable oxidation product. All of these observations indicate that schradan in a

biological system undergoes an oxidation to the active, labile N-oxide, some of which then rearranges to the substituted hydroxylamine structure. Both oxidation products might contribute to the toxicity, but the rapid rate of schradan oxidation by cockroach nervous tissue (37) to yield the N-oxide and the high activity of this material in blocking cockroach cholinesterase indicate that at least in this one case the N-oxide formed in the nerve probably is the effective poisoning agent. As the more stable substituted hydroxylamine (37) acts as a weaker anticholinesterase than schradan (22), it might also be activated by a further oxidation, as apparently can occur chemically (37). However, the substituted hydroxylamine could also act directly as an anticholinesterase agent.

The N-oxide might also rearrange after reaction with the esterases. If this occurred, the inactivation might become essentially irreversible, for the phosphorus-enzyme bond would thereby be stabilized and hence the tendency for esterase reactivation through cleavage of the phosphorus-enzyme bond would be less. The isomerization of the N-oxide after attachment to the enzyme might help to explain this stability observed here of the formaldehyde-yielding group of the phosphoramide when attached to chymotrypsin and the prolonged in vivo anticholinesterase effect of schradan (19). The selectivity of schradan for certain insects does not appear to be due to their ability to form the N-oxide (10). Rather, it might be based on a difference in the rate at which isomerization occurs in vivo. A difference in their internal pH might effect rapid isomerization of the N-oxide in the resistant forms before reaction with the insect cholinesterase but not in the susceptible forms.

Stability Relationships. The stability relationships are interesting. Organophosphate esters inhibit cholinesterase and chymotrypsin by phosphorylation (25, 29). There is some correlation of the alkaline hydrolysis rate of the organophosphate and the ability to inhibit both enzymes (7, 8). It has been frequently demonstrated that the specificity of organophosphate inhibitors for different esteratic enzymes varies to a large extent. This specificity was particularly evident with the phosphoramide oxidation products in the present study, as the monophosphoramide oxide derivative appeared to be selective for cholinesterase, while the inhibition of chymotrypsin was more directly related to the reactivity of the anhydride bond in the products formed on further oxidation. The spatial similarity of the phosphoramide oxide to the normal cholinesterase substrate, acetylcholine, may contribute to this specificity. In no case did the chemical

oxidation of schradan appear greatly to enhance its insecticidal activity through either contact or systemic application. It is possible that the N-oxide might not penetrate readily into the insect nerve sheath (27, 31, 36), or that it rapidly isomerizes to a less active and less polar derivative. The authors have found, as did Hartley (22), that the more stable oxidation product is somewhat weaker than schradan in insecticidal activity. It therefore appears that the stability of the oxidized derivatives are such that only those formed within the susceptible organism can be effective as antiesterase agents. A somewhat analogous situation occurs when heat isomerization of thionophosphates increases their anticholinesterase activity but reduces their toxicity to insects and mice (28).

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GRANARY INSECTICIDES

Protection of Stored Grain with Sprays of Pyrethrins–Piperonyl Butoxide Emulsion

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Water-miscible emulsions of piperonyl butoxide and pyrethrins, completely free from mineral oils or objectionable ingredients, can be applied directly to grain to control insects in stored grain. Using a standard concentration of 2% piperonyl butoxide and 0.2% pyrethrins in oil-free emulsion sprays, studies showed satisfactory results from 2 to 12 gallons of emulsion per 1000 bushels of grain. This development opens an immense field of usefulness of a simple spray method, which should meet with complete approval of government agencies concerned with foods.

THE PROTECTION OF STORED GRAIN from insect damage is a serious problem, which is even more serious in times of overproduction when it is necessary to store surpluses for long periods. In order to reduce to a minimum the deterioration and losses that result from insects, good methods of handling and storage have aided in holding down insect populations, but alone these are not sufficient to handle the problem. Because grain in storage is always vulnerable to attack by insects (7) and the destruction of an insect population already established in grain does not undo the damage done, the primary need is for a material and method that will prevent insects from becoming established in stored grain.

For a number of years, protectant powders (2, 4, 6) have been available, which, when mixed intimately with grain, effectively prevent infestations of insects and offer prolonged protection from reinfestations. The active ingredients in these protectants are pyrethrum, long known for its rapid action against insects and its unusual safety,

and piperonyl butoxide, a pyrethrum synergist, also of extreme safety. This combination has been most widely used in liquid form as sprays and aerosols, particularly where freedom from toxic hazards (3) is an important consideration. The use of the same ingredients applied as liquids to grain would, in some circumstances, have advantages that are not obtained from powders.

In many fields, insecticides are being used in rather highly concentrated form, but at very low rates of application. Such a method of treatment carries a strong appeal for the treatment of stored grains. Tests were undertaken in the laboratory to determine the effectiveness of different sprays applied to grain, the importance of the dosage-concentration relationship, and the effectiveness of a new treatment with oil-free emulsion spray.

Materials and Methods

In comparing oil sprays and water emulsions, soybean oil was used as representative of the more readily available

vegetable oils. Ultrasene was used as representative of deodorized petroleum-base oils. These oils were used as diluents for a commercially available oil-base concentrate containing 50 grams of piperonyl butoxide and 5 grams of pyrethrins per 100 ml. of concentrate. The water emulsion was prepared by diluting with water an oil-base emulsifiable concentrate containing 10 grams of piperonyl butoxide and 1 gram of pyrethrins per 100 ml. of concentrate. The protectant powder used for comparative purposes was a commercially available product.

The oil-free emulsions were prepared by adding 20% of an emulsifier, polyoxyethylene (8) stearate (available as MYRJ-45 and also under different trade names from various sources), to 80% of an oil-free concentrate and then diluting with water to give the desired concentration. The oil-free concentrate consists of 75% of piperonyl butoxide, 7.5% of pyrethrins, and 17.5% of inert material, mostly other pyrethrum extractives. The resulting oil-free emulsifiable concentrate (T-647) contained