SCHRADAN

Enhancement of Anticholinesterase Activity in Octamethylpyrophosphoramide by Chlorine

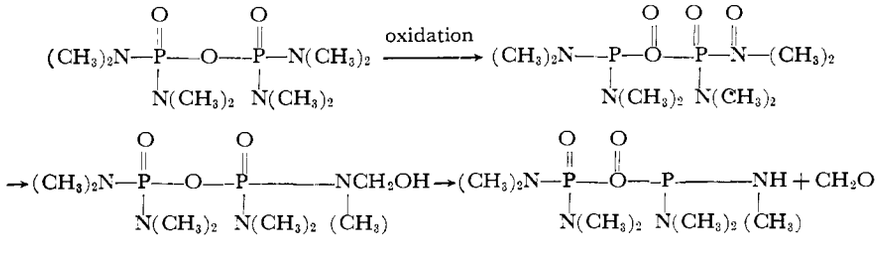
E. Y. SPENCER, Science Service Laboratory, Department of Agriculture, London, Ont., Canada
R. D. O'BRIEN, Department of Chemistry, University of Western Ontario, London, Ont., Canada

Schradan has a low in vitro anticholinesterase activity while in mammals and certain insects its toxicity is high and appears to be due to cholinesterase inhibition. There has been speculation as to a conversion to a more active compound. Chlorination of schradan was undertaken in order to determine whether the introduction of electrophilic groups would activate the molecule and increase its anticholinesterase activity. Studies on the hydrolysis of chlorinated schradan indicated that the chlorination was heterogeneous. Di- and higher chlorinated derivatives were so reactive toward water that they showed little anticholinesterase activity while the monochlorinated derivative had an activity almost 100,000 times as great as that of schradan itself. Formaldehyde was isolated as a hydrolysis product of chlorinated schradan, thus paralleling the decomposition of the naturally produced active material. The relative reactivities of the organophosphate insecticides are of practical importance. This work has indicated that the rate of hydrolysis of an organophosphorus insecticide (schradan) and its anticholinesterase activity can be increased simultaneously (in this case by chlorination). However, beyond a certain degree of activation (chlorination) a point is reached at which the hydrolyzability is so great that the anticholinesterase activity decreases.

SCHRADAN (OCTAMETHYLPYROPHOSPHORAMIDE; OMPA) is an insecticide which has two properties of considerable practical importance; it is systemic, so that when applied to one part of a plant it is translocated and renders other parts toxic; and it is selective, killing only plant-sucking insects, such as aphids. It is also of interest from a more fundamental point of view, in that although it is in itself a very weak anticholinesterase; its toxicity to mammals and some insects is of the same order as organophosphorus compounds which are strong anticholinesterases.

A conversion of schradan in the liver and some plants, to a strong anticholinesterase was first demonstrated by DuBois *et al.* (*9*), while Duspiva (*10*) has shown a conversion to be made in vitro by the tissues of certain insects susceptible to schradan poisoning.

The anticholinesterase activity of organophosphorus compounds appears to depend to a large extent on the hydrolyzability of the compound (*6*, *17*, *20*). This has been shown to be the case by Aldridge and Davison (*2*) in a series of substituted diethylphosphoric acids. Unaltered schradan is very stable to hydrolysis (half life of 10 years at pH 7) (*19*). It thus appears that its enhanced in vivo activity may be due to weakening of the anhydride link caused by the introduction of electrophilic group(s). Hartley (*15*) suggested the following mechanism:

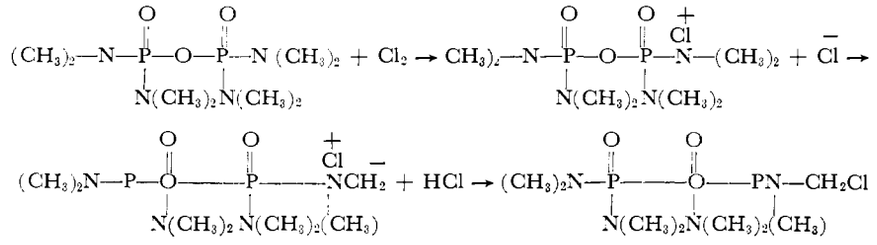


He succeeded in producing a strong anticholinesterase by oxidation of schradan with potassium permanganate. Presumably the active compound contains the amide oxide, and the electrophilic nature of this new substituent renders the anhydride bond less stable and, therefore, the compound is more hydrolyzable and a better phosphorylating agent. Casida *et al.* (*7*) recently confirmed this conversion and isolated formaldehyde from the hydrolyzed end product.

In the present investigation, schradan

was treated under anhydrous conditions with chlorine in order to study further the effect of introducing electrophilic groups. The effect on schradan of increased chlorine substitution as measured by anticholinesterase activity and hydrolyzability has been studied.

The proposed mechanism for chlorination of schradan is based on the assumption that it is similar to the mechanism proposed by Böhme and Krause (*4*) for tertiary amines.



This chlorinated end product is assumed to be the active anticholinesterase. On refluxing with acid the products should be as follows:

$$\xrightarrow{5H_2O} 3(CH_3)_2NH + H_3PO_4 + (CH_3)NH_2 + CH_2O + HCl$$

If it is possible to introduce up to four chlorine atoms per molecule of schradan, then for each atom of chlorine introduced, one mole of formaldehyde should be formed, one monomethylamine should appear, and one dimethylamine disappear from each molecule.

The ease and extent of chlorination were investigated. This was important if data leading to an explanation concerning the reactivity of the chlorinated product were to be obtained. Presumably increased chlorination should activate the anhydride to such an extent as to render it very unstable in water. Thus, the anticholinesterase activity would pass through a maximum and finally fall off with chlorination beyond the monochlorinated derivative (*6*, *17*, *20*). The extent and type of chlorination were studied by determining the rate of hydrolysis at different temperatures.

The effect of chlorination of schradan on its anticholinesterase activity was examined by measuring the extent of inhibition of serum cholinesterase. Analyses were also made for total chlorine, phosphorus, formaldehyde, dimethylamine, total amine, and monomethylamine (by difference between the last two).

In order to elucidate further the mechanism of chlorine activation of schradan, the ethylamido homolog of schradan (octaethylpyrophosphoramide) was synthesized. If the mechanism of decomposition of the chlorinated product is similar to that postulated for chlorinated schradan then acetaldehyde should be found.

The possible chlorine activation of ethyl bis(dimethylamido)phosphate as an anticholinesterase was also investigated. The effect of bromine addition to schradan was also examined to determine the effect of introducing a group less electrophilic than chlorine.

Materials and Methods

Schradan Synthesis In the synthesis of schradan, both dimethylamidophosphoryl dichloride and bis(dimethylamido)phosphoryl chloride were synthesized by the method of Gardiner and Kilby (*12*).

Later, Holmstedt's (*16*) slight variation was found more convenient. In the final step in the synthesis, ethyl bis(dimethylamido)phosphate was reacted with bis(dimethylamido)phosphoryl chloride in the absence of a solvent for 5 minutes at 200° C. essentially according to the method of U. S. patent 2,610,139 (*11*).

Analysis revealed phosphorus found 21.3%, theoretical 21.6%; dimethylamine found 63.4%, theoretical 63.0%; b.p. 112° to 116° C. at 0.01 mm.; n_D^{22} 1.4625.

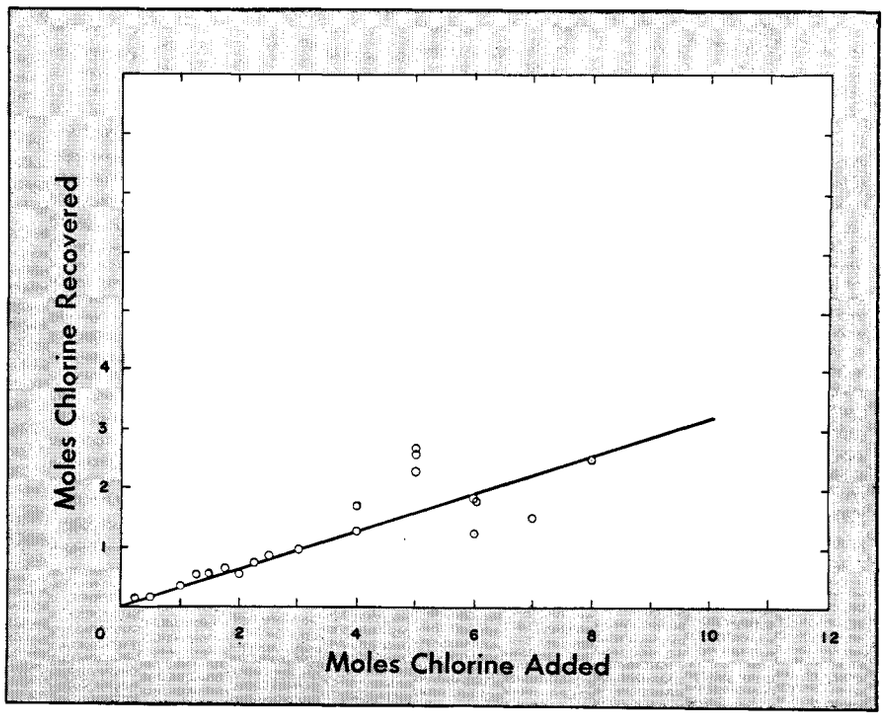
Octaethylpyrophosphoramide was synthesized essentially according to the method of Gardiner and Kilby for schradan (*12*).

Schradan Chlorination The chlorination of schradan was carried out as follows: The quantity of chlorine required was obtained by taking the calculated volume of a standardized solution of chlorine in carbon tetrachloride and making it up to 10 ml. with carbon tetrachloride. This was then added to the schradan in a dry flask, stoppered, and let stand overnight. An immiscible layer or crystals formed. Dry nitrogen was bubbled through the mixture to drive off any unreacted chlorine. The remainder of the solvent was removed in vacuo, and the residue was taken up in buffer. Acetate buffer pH 5.6 had to be used because more convenient buffers interfered in the cholinesterase assays. The solution was adjusted immediately to pH 7 with sodium hydroxide, using a glass electrode, and made up to volume. It was assayed for anticholinesterase activity against human serum as a source of cholinesterase, using a standard Warburg technique.

Serum Assay The serum was prepared by centrifuging whole blood and diluting the resultant serum to a convenient cholinesterase activity (b_{30} = 260 microliters of carbon dioxide). Acetylcholine bromide, 0.2 ml. of 5%, was the substrate. One milliliter of serum was used with one milliliter of bicarbonate–Ringer's solution (*3*). The assay was carried out at 25° C. under a 95% nitrogen–5% carbon dioxide atmosphere. The chlorinated schradan in buffer was serially diluted with more buffer, and added to the enzyme 30 minutes after the initial buffer addition. Ninety minutes incubation was allowed before tipping in substrate. Readings were taken and activities calculated by the procedure of Aldridge, Berry, and Davies (*1*). The pI_{50} (negative log of molar concentration to produce 50% inhibition) was read from the graph made of *pI vs.* per cent enzyme inhibition. Four concentrations, in duplicate, were used for each assay.

Hydrolysate Analysis Dimethylamine in the hydrolysate was determined colorimetrically by the method of Hall *et al.* (*14*). Total amines were estimated by distillation into standard acid, and monomethylamine was calculated by difference. Formaldehyde was determined before hydrolysis of the sample by the method of Daughaday *et al.* (*8*), using chromotropic acid with fresh reagent being made up each day. No distillation of the sample was necessary.

Figure 1. Effect of chlorine concentration on chlorination of schradan



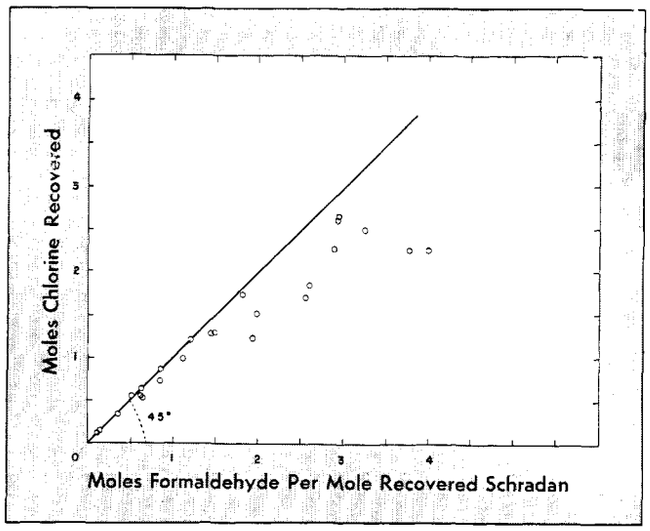


Figure 2. Comparison of chlorine recovered with formaldehyde produced

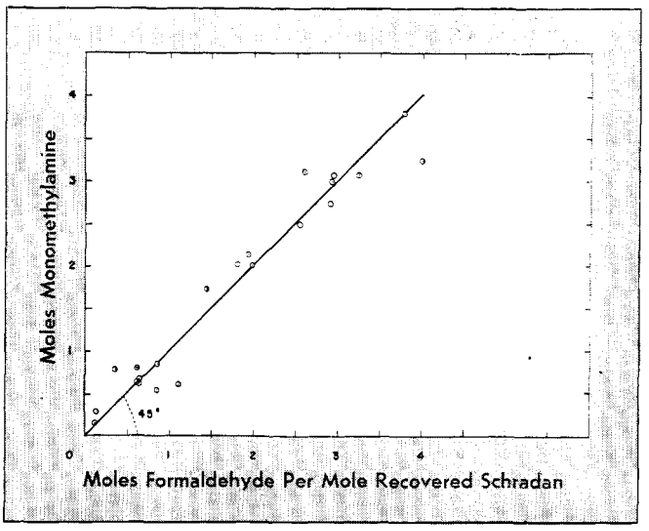


Figure 3. Comparison of monomethylamine and formaldehyde recovered per mole of schradan

Solutions were allowed to remain in the boiling water bath for 40 minutes (rather than the prescribed 30 minutes); resulting color could be determined before allowing the solutions to return to room temperature.

Acetaldehyde was determined according to the method of Veksler using Schiff's reagent (*21*). Phosphorus was determined colorimetrically by the method of Nakamura using a Beckman model B spectrophotometer with readings at 680 mμ (*18*). Total chloride was assayed by the Volhard method after warming the aliquot sample in dilute alkali. The final ratios of amines and formaldehyde to schradan were calculated from phosphorus values expressed in terms of schradan.

Hydrolysis of Chlorinated Schradan

The rates of hydrolysis of the chlorinated products at various temperatures were determined on the chlorinated residue after removing the solvent and dissolving the residue in about 400 ml. of water previously cooled to the temperature at which the hydrolysis rate was to be determined. The solution was stirred and maintained under an atmosphere of nitrogen in a thermostatic bath. At timed intervals, depending on the rate of hydrolysis, standard alkali was added to the color change of phenolphthalein, and thus the pH was maintained within narrow limits. From these values the hydrolysis constants of the mono-, di-, and trichloro-substituted schradan were calculated as well as the activation energy as outlined by Brown and Fletcher (*5*) for a two component system and by Hall and Jacobson (*13*) for tetraethyl pyrophosphate.

The technique of measuring the rate of hydrolysis of the anhydrides by titration in situ is a departure from such normal procedures as that of Hall and Jacobson (*13*), where aliquots were removed. However, since the time element and the effect of a large change in pH were so important in the determination of the rate of hydrolysis [c.f., importance of pH in rate of schradan hydrolysis (*19*)], it was felt that the slight error of dilution introduced was a minor consideration. By our technique the time element error was reduced to a minimum, and the pH was maintained at a relatively constant level.

Results and Discussion

The extent of reaction of chlorine with schradan in an anhydrous solution was studied by increasing the amount of chlorine added in solution and determining the chloride recovered after removal of the solvent. From the hypothetical mechanism of the reaction, for every mole of chlorine added, one-half mole would be lost as hydrogen chloride. Using same total volume of solution, but varying concentration, Figure 1 shows that efficiency of chlorination decreases as the chlorine concentration is increased.

Since chlorine reacted readily with schradan in an anhydrous medium at room temperature, it was of interest to see whether formaldehyde was ultimately produced on hydrolysis of the chlorinated product. This would offer a comparison with Hartley's (*15*) postulation and Casida's *et al.* (*7*) findings with the oxidized schradan as cited above. If the mechanism of production is similar, then for every atom of chlorine introduced one mole of formaldehyde should be produced and one mole of monomethylamine should be released on acid hydrolysis of the final product. This was confirmed, and the direct correlation between chlorine introduced and formaldehyde determined is indicated in Figure 2. A similar correlation is shown between monomethylamine and formaldehyde in Figure 3.

As a final test of the effect of chlorine substitution on the anticholinesterase activity of schradan, the pI_{50} values were determined for different levels of chlorination (which corresponds to formaldehyde recovered). These are shown in Figure 4. The activity increased 10,000 times from a pI_{50} of 1.8 for schradan to a maximum of 5.8 and then fell off on further chlorination. This can best be explained by the greater reactivity of the more highly chlorinated products which hydrolyze before reaching the enzyme. Since maximum anticholinesterase activity is expected from the monochlorinated schradan, one would expect to find the peak of the pI_{50} *vs.* monomethylamine curve at 1.0 mole of monomethylamine. Examination of Figure 4 indicates, however, that the greatest activity is shown when approximately 0.6 mole of monomethylamine is recovered (which is equivalent to the recovery of the same amount of chloride). This could be explained, as is discussed later, on the assumption that chlorination is heterogeneous. That is, complete monochlorination does not occur before the production of di- and possibly trichlorinated derivatives.

In order to clarify this point a kinetic study of the rate of hydrolysis of the chlorinated schradan was undertaken.

Figure 4. Comparison of anticholinesterase activity with formaldehyde recovered per mole of schradan

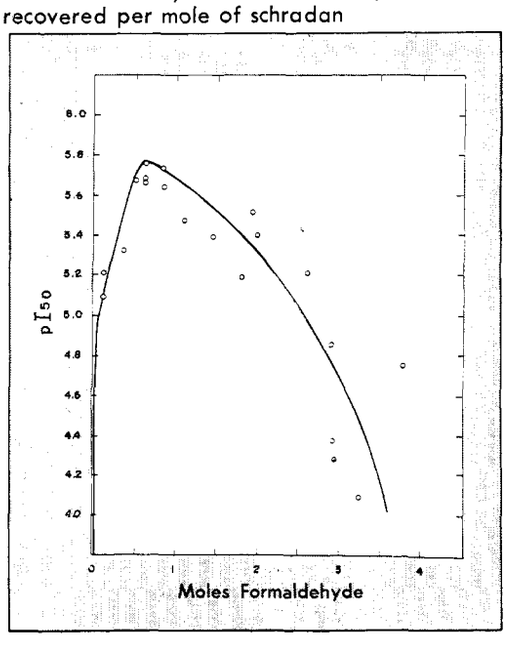


Table I. Kinetic Constants for Chlorinated Schradan Derivatives

Temp., °C.	Derivative	Rate Constant k (Min.$^{-1}$)	Half-Life, Min.	E (Kg. Cal./Mole)
5	Monochloro	2.27×10^{-3}	310	...
15	Monochloro	5.88×10^{-3}	118	15.8
26	Monochloro	17.3×10^{-3}	40	...
5	Dichloro	2.36×10^{-2}	30	...
15	Dichloro	4.81×10^{-2}	15	11.3
26	Dichloro	17.4×10^{-2}	4	...
5	Trichloro	1.79×10^{-1}	4	...
15	Trichloro	2.68×10^{-1}	2.6	6.4

This could be readily followed by titrating at frequent intervals the monobasic substituted phosphoric acid produced on hydrolysis. The logarithm of the per cent of anhydride not hydrolyzed was then plotted against time at 5°, 15°, and 26° C. The results obtained at 5° are shown in Figure 5, curve 1. Examination of the curve would indicate at least two compounds are hydrolyzing in the early stages with one component later. The contribution of the more stable component was removed by projecting the straight line part of the curve to the ordinate and subtracting its contributions from the titration values. The logarithm of the per cent of the more reactive component was then plotted against time, and curve 2 was obtained. Since this was not a straight line, this would indicate a third and yet more reactive component. The same projecting, recalculating, and plotting repeated resulted in line 3. Since this line has a slight curve there is possibly a very small amount of the tetrachlorinated material also present. It therefore appears that chlorination of schradan with two mole equivalents of chlorine results in the production of mono-, di-, tri-, and even some tetrachlorinated schradan, as well as leaving some unreacted material. The exact proportions are calculated below.

The rate constant, k, was calculated where possible for the three chlorinated derivatives at 5°, 15°, and 26° C. by multiplying the slope of the lines in Figure 5 by -2.303 with the results shown in Table I.

The hydrolysis of the trichloro derivative was too rapid at 26° C. under the conditions of the experiment to allow calculation of the rate constant at this temperature. Since the hydrolysis appeared to be first order as indicated by the results illustrated in Figure 5, the half lives or the times for 50% of the derivatives to hydrolyze could be calculated from the first order reaction equation. They could also be obtained by inspection from Figure 5 and from the curves obtained at the other two temperatures. The calculated values are included in Table I.

Substituting the values of two rate constants in the Arrhenius equation integrated between two values of k, the energies of activation for the three chlorinated derivatives are as follows: monochloro, 15.8; dichloro, 11.3; and trichloro, 6.4 kg. cal. per mole. The energy of activation for the monochlorinated derivative can be read from Figure 6.

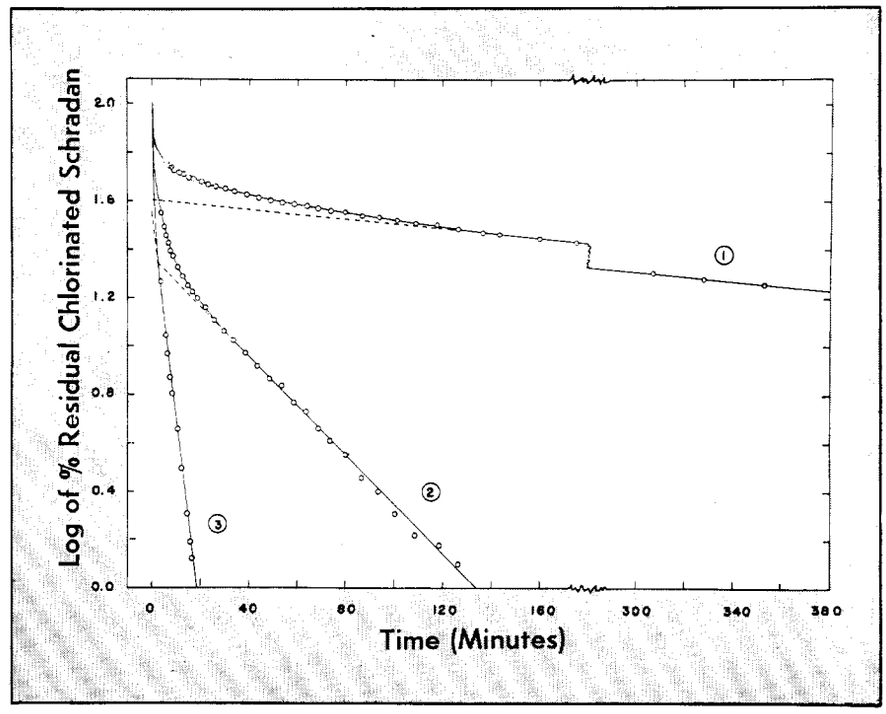
Since the half lives of the dichloro and trichloro derivatives are short as shown in Table I, these components of chlorinated schradan would have been hydrolyzed before reaching the enzyme in the anticholinesterase assay at room temperature. On the other hand, the unreacted schradan with a half life of 10 years has such a low pI_{50} (1.8) that it would also be ineffective. Thus the effective anticholinesterase component would be largely the monochloro derivative. However, the monomethylamine values from the acid hydrolysis of the final mixture would include contributions from all the chlorinated derivatives. This, therefore, is an explanation for the maximum anticholinesterase activity of chlorinated schradan at a monomethylamine value of 0.6 mole per mole of schradan as read from Figure 4. In the example cited in Figure 5 where two moles of chlorine were used per mole of schradan, the relative mole fractions of chlorinated and unreacted material in the final product (calculated from the alkali consumed to neutralize the acid liberated on hydrolysis) were 0.21, monochloro; 0.076, dichloro; 0.079, trichloro; and 0.64, unreacted schradan. This would yield a total mole fraction of monomethylamine of 0.60 mole which is the value for the optimum pI_{50} value. Higher chlorination would result in less unreacted schradan but more di-, tri-, and ultimately tetrachlorinated schradan, all of which would contribute to the monomethylamine value but would be lost for anticholinesterase activity.

As chlorination of schradan was increased beyond the addition of two mole equivalents the resulting product was less water soluble, and transfer became difficult. This insoluble material was soluble in acetone, but after a few minutes a voluminous precipitate appeared. This factor as well as the effect of concentration and room temperature variation would affect the ratio of chlorinated schradan derivatives and therefore render exact reproducibility of results difficult. This is shown by some scattering of values in Figures 1, 2, and 4.

Brief experiments with bromine addition to schradan indicated that the anticholinesterase activity of the brominated product was enhanced to a pI_{50} of 4.9 from that of 1.8 for schradan. This enhancement is less than with chlorine and is to be expected from the less electrophilic nature of bromine.

The anticholinesterase activity of ethyl bis(dimethylamido)phosphate was enhanced on chlorination from a pI_{50} of 1.3

Figure 5. Hydrolysis rate curves for mono-, di-, and trichlorinated schradan
1, mono-; 2, di-; 3, tri-.



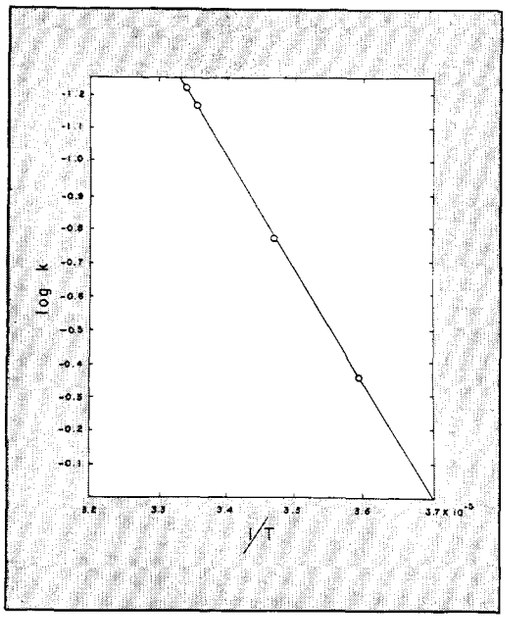


Figure 6. Activation energy curve for monochlorinated schradan

to 4.7. This would indicate an increase in phosphorylating ability of even a phosphate ester on introduction of an electrophilic group. A similar enhancement was observed on the chlorination of octaethylpyrophosphoramide, and acetaldehyde was identified after adding the product to water. This substantiates the proposed mechanism for chlorination of schradan when formaldehyde was found.

The optimum pI_{50} value of 5.8 as shown in Figure 4 is below the actual value for the monochlorinated derivative, since the di- and trichlorinated schradan have been lost by hydrolysis, and the product is diluted with unchanged schradan. If one corrects for the amount of monochlorinated derivative hydrolyzed before assaying (at the end of 30 minutes) together with the value for the actual amount of monochlorinated derivative in the mixture (21%), then the pI_{50} value is increased to 6.7, or almost a 100,000-fold increase in anticholinesterase activity.

Summary

Chlorination of schradan in an anhydrous medium proceeds with the release of hydrogen chloride in a heterogeneous manner and not stepwise. The amount of chlorine added determines the ratio of schradan and mono-, di-, tri-, and tetrachlorinated products. Formaldehyde is produced in amounts directly proportional to the amount of chlorine introduced.

Increasing substitution renders the compound increasingly reactive to water so that schradan with a half life of ten years is reduced in the dichloro derivative to a half life of 4 minutes at room temperature. The increase in activity is indicated by a net increase in the anticholinesterase activity of almost 100,000 times. However, an optimum is reached, due to the instability of the more highly chlorinated material which is hydrolyzed before being able to react with and inactivate the enzyme.

Conclusions

Evidence is offered to support the concept of activation of schradan. The introduction of an electrophilic group, in this case chlorine, renders the anhydride less stable and therefore a better anticholinesterase in vitro. Increased chlorination renders the compound so unstable that its anticholinesterase activity is greatly reduced due to rapid hydrolysis.

The chlorination of schradan in an anhydrous medium has been shown by a kinetic study of rates of hydrolysis to be heterogeneous and not stepwise.

Acknowledgment

The authors are indebted for technical assistance and suggestions to Mrs. S. M. Eichenberg, and for many of the analyses to J. R. W. Miles and Miss M. B. Carruthers. To D. M. Miller we acknowledge helpful discussions concerning the kinetics. The use of a low temperature bath was provided through the courtesy of R. H. Pearce of the University of Western Ontario Medical School. The second author was the recipient of a Canadian Industries, Ltd., fellowship.

Literature Cited

(1) Aldridge, W. N., Berry, W. K., and Davies, D. R., *Nature*, **164,** 925 1949.
(2) Aldridge, W. N., and Davison, A. N., *Biochem. J.*, **51,** 62–70 (1952).
(3) Augustinsson, K. B., *Acta Physiol. Scand.*, **15,** Suppl. 52, 1948.
(4) Böhme, H., and Krause, W., *Chem. Ber.*, **84,** 170–181 (1951).
(5) Brown, H. C., and Fletcher, R. S., *J. Am. Chem. Soc.*, **71,** 1845 (1949).
(6) Burgen, A. S. V., *Brit. J. Pharmacol.*, **4,** 219 (1949).
(7) Casida, J. E., Allen, T. C., and Stahmann, M. A., *J. Am. Chem. Soc.*, **74,** 5548 (1952).
(8) Daughaday, W. H., Jaffe, H., and Williams, R. H., *J. Clin. Endocrinol.*, **8,** 166 (1948).
(9) DuBois, K. P., Doull, J., and Coon, J. M., *J. Pharmacol. Exptl. Therap.*, **99,** 376 (1950).
(10) Duspiva, F., *Pflanzenschutz Tag.*, **70,** 91 (1951).
(11) Dye, W. T. (to Monsanto Chemical Co.), U. S. Patent 2,610,139 (Sept. 9, 1952.)
(12) Gardiner, J. E., and Kilby, B. A., *J. Chem. Soc.*, **1950,** 1769.
(13) Hall, S. A., and Jacobson, M., *Ind. Eng. Chem.*, **40,** 694 (1948).
(14) Hall, S. A., Stahlmann, J. W., and Schechter, M. S., *Anal. Chem.*, **23,** 1866 (1951).
(15) Hartley, G. S., paper presented at the XII International Congress of Pure and Applied Chemistry, New York, September 1951.
(16) Holmstedt, B., *Acta Physiol. Scand.*, **25,** Suppl. 90 (1951).
(17) Metcalf, R. L., and March, R. B., *J. Econ. Entomol.*, **46,** 288 (1953).
(18) Nakamura, G. R., *Anal. Chem.*, **24,** 1372 (1952).
(19) Ripper, W. E., Greenslade, R. M., and Hartley, G. S., *J. Econ. Entomol.*, **44,** 448 (1951).
(20) Topley, B., *Chemistry & Industry*, **1950,** S 859.
(21) Veksler, R. I., *Zhur. Anal. Khim.*, **5,** 32 (1950).

Received for review June 29, 1953. Accepted August 7, 1953. Presented in part before the Division of Agricultural and Food Chemistry at the 123rd Meeting of the AMERICAN CHEMICAL SOCIETY, *Los Angeles, Calif. Contribution Number 19 of the Science Service Laboratory, London, Ontario, Canada.*

EMULSION TESTING

A Basis for Tests for Emulsifiable Concentrates of Agricultural Chemicals

R. W. BEHRENS and W. C. GRIFFIN
Central Research Laboratory, Atlas Powder Co., Wilmington 99, Del.

ESTABLISHMENT OF RECOGNIZED methods of evaluating emulsifiers by test emulsions is of critical interest to several industries, but data that would provide a basis for devising suitable test procedures are not available. This is due in part to the complexity of the problem, confusion resulting from the many tests that have been devised for divergent applications, and lack of reproducibility of results even with a given test. The differences observed are often due to a lack of