Table VI. Diffusion Current Quotients as Affected by Interfering Ions

Concn. of Zinc, mM	Diffusion Current Quotient ($i_{d/c}$), Solution with Interfering Ion	
	Not added	Added
0.00615	11.38	11.06
0.03375	12.00	12.32
0.04245	12.93	12.63
0.19348	11.16	11.01
Average	11.87	11.76

t found, 1.57; t required at 0.01, 5 84.

Concentration of Added Ions

Ion, mM		Equivalent P.P.M. in Leaf
Mn^{++}	1.457	5000
Ni^{++}	0.00136	5
Co^{++}	0.00136	5
Cr^{+++}	0.00154	5
Fe^{+++}	0.0573	200
Al^{+++}	2.965	5000
Cd^{++}	0.000712	5

tions (Table VI) were calculated on the basis of the sample weight used in the zinc determination. Over a wide range of zinc concentrations the presence of these added ions had no effect. The t value was 1.57 when 5.84 was needed for significance at the 1% level.

The effect of the individual ions was tested at about 10 times the maximum concentration likely to occur in leaf solution. Even at this high concentration, only cobalt and aluminum had an effect. Cobalt tended to increase the wave height, while aluminum tended to decrease it.

In a subsequent test, it was found that cobalt did not interfere if its concentration in the plant tissue was below 20 p.p.m., and aluminum, if its concentration was below 50,000 p.p.m. Plant material very rarely contains concentrations as large as these (*2*).

Although manganese did not interfere with the zinc wave, it gave a well-defined wave at −1.69 volts *vs.* S. C. E. Preliminary tests indicated that manganese and zinc could be determined in the same solution.

Acknowledgment

The authors wish to thank Barbara K. Williams for her assistance in both the colorimetric and polarographic determinations.

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MODE OF ACTION OF PESTICIDES

Mechanism of Reaction of Di-*n*-propyl-2,2-dichlorovinyl Phosphate (DDP) with Esterases

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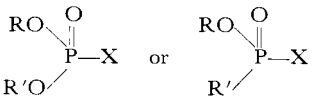
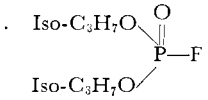
Di-*n*-propyl-2,2-dichlorovinyl phosphate (DDP) is an active antiesterase; its rate of reaction with various esterases is approximately that of diisopropyl phosphorofluoridate (DFP), tetraethyl pyrophosphate (TEPP), and isopropylmethyl phosphonofluoridate (sarin). In contrast with the latter antiesterases, DDP is very stable toward hydrolysis and does not appear to react with catechol and a hydroxamic acid. The reaction of DDP with chymotrypsin is stoichiometric and is accompanied by introduction of phosphorus into the protein. During the reaction chlorine is released in organic alkali-labile form which has been identified as dichloroacetaldehyde. The mechanism of these reactions is discussed with reference to the reactions of other irreversible antiesterases with susceptible enzymes.

ESTERASE INHIBITORS OF THE ORGANOPHOSPHORUS TYPE may be generally divided into two classes.

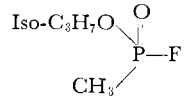
Class I with the general structure



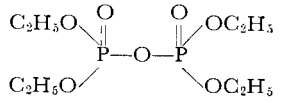
contains a linkage (P—X) which is relatively readily broken by spontaneous hydrolysis, enzymatically catalyzed hydrolysis (*24, 28*) as well as during reactions with various esterases. Representative members of this class include phosphofluoridates, alkyl pyrophosphates, and alkyl-*p*-nitrophenyl phosphates. Chemi-

1. 

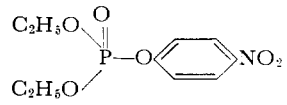
Diisopropyl phosphorofluoridate, DFP

2. 

Isopropyl methyl phosphonofluoridate, sarin

3. 

Tetraethyl pyrophosphate, TEPP

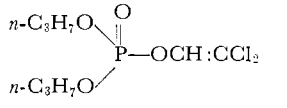
4. 

Diethyl *p*-nitrophenyl phosphate, paraoxon

cally speaking, all compounds belonging to this class may be regarded as acid anhydrides; this applies to paraoxon also since the nitro group confers sufficiently acidic properties upon the phenolic hydroxyl group. From the biochemical point of view, they all react with esterases stoichiometrically (*5*) and irreversibly [in the sense as used in the Michaelis-Menten theory (*23*)] by an alkylphosphorylation mechanism which has recently been reviewed (*16*); within any particular series (X = constant) the rate of reaction with esterases bears a constant relationship to the rate of hydrolysis (*2*).

Class II comprises compounds usually classed as "systemic insecticides"—i.e., compounds stable to hydrolysis at neutral pH which have no in vitro activity prior to a metabolic transformation. Representatives of this class are parathion [transformation to paraoxon (*25*)], octamethyl pyrophosphoramide [oxidation to amine oxide (*7*)] and tri(*o*-cresyl)-phosphate [activation product unknown (*1*)].

Several years ago an anticholinesterase was pointed out as a compound whose chemical structure did not allow for an *a priori* classification as either an acid anhydride—that is, a member of the first class of compounds described above—or as a trialkyl phosphate, a group included in the second class. On preliminary biochemical investigation it likewise appeared to fit into neither of the above classes entirely, being remarkably stable to hydrolysis, and a potent inhibitor of esterases in vitro. This compound is di-*n*-propyl-2,2-dichlorovinyl phosphate (DDP). Its chemical and physical

5. 

Di-*n*-propyl 2,2-dichlorovinyl phosphate
DDP

properties as well as those of its homologs and their completely chlorinated derivatives (dialkyltetrachloroethyl phosphates) have recently been reported by Allen and Johnson (*3*) and by Barthel and coworkers (*6*); several articles have appeared dealing with their anticholinesterase activity, their mammalian and insect toxicity, and their clinical application (*20*, *26*, *27*, *30*).

This paper compares the reactivity of DDP with that of phosphofluoridates toward spontaneous hydrolysis, in their reactions with accelerators of hydrolysis, and in their interactions with esterases leading to enzymatic inactivation.

Materials

Chymotrypsin (ChTr) was a salt-free preparation, obtained from the Worthington Biochemical Sales Co., Freehold, N. J. Its average molecular weight was taken as 25,000 (*5*). Eel cholinesterase was a purified preparation containing 10 units per mg. of dry weight [1 unit = 1 gram of acetylcholine hydrolyzed per hour under conditions as specified by Rothenberg and Nachmansohn (*31*)]. The source of red cell cholinesterase was either a suspension of normal human red cells in saline, or washed and resuspended human red cell stromata. Plasma cholinesterase was a preparation partially purified by the method of Strelitz (*32*). Other chemicals were obtained commercially or synthesized in this laboratory as noted.

Methods

Cholinesterase activity was determined by micropotentiometric titration as described previously (*23*). Chymotrypsin esterase activity was determined by the same technique, using N-acetyltyrosine ethyl ester (final concentration, 0.004 M) as substrate. Proteolytic activity of chymotrypsin was determined, using casein as substrate, according to Kunitz (*21*) with minor modifications. The reaction mixture was deproteinized with trichloroacetic acid, and the absorption of the filtrate at 280 mμ served as a measure of activity. Chloride at the micromole level was determined by a modification of the turbidimetric silver nitrate method of Lamb, Carleton, and Maldrum (*22*). Protein-free aliquots of reaction mixtures containing up to 2.5 μeq. of chloride were diluted to 1.5 ml. with water. To this solution, 1.5 ml. of 95% ethyl alcohol was added with mixing. At intervals of one minute, 3.0 ml. of 0.1M silver nitrate in 0.1N nitric acid was added to each tube with mixing, and the tubes were transferred to a dark box. After standing for 20 minutes, the suspension was read in the Klett-Summerson colorimeter (filter No. 42) previously set to zero with a mixture of 3 volumes of water to 1 volume of ethyl alcohol. A straight line of readings *vs.* chloride concentration was found between 0 and 2.5 μeq. chloride per sample, with a sensitivity of approximately 75 Klett units per μeq.

Alkali-labile chlorine was determined by treating the sample to be analyzed, contained in a volume of 0.5 ml., with 0.5 ml. of 0.2N sodium hydroxide, heating in a boiling water bath for 5 minutes (capped tube), cooling, adding 0.5 ml. of 0.2N nitric acid, and proceeding as in the determination of chloride. In controls the heating period was omitted and the nitric acid was added immediately following the addition of sodium hydroxide.

Arnow's test (*4*) for catechol and its derivatives was adapted for use with the photocolorimeter (filter No. 52); the intensity of the red color is proportional to the concentration of *o*-diphenol up to about 0.6 μmole per ml. of test solution, with a sensitivity of about 50 Klett units per 0.1 μmole. "Alkylating power" of solutions containing DDP was determined by use of the reagent γ-(4-nitrobenzyl) pyridine which appears to be specific for alkylating agents (*10*). The method used was essentially as described by Epstein, Rosenthal, and Ess, with minor modifications. The colored reaction product was extracted into 6 ml. of xylene and read in the photocolorimeter (filter No. 54). The useful range is 0 to 1.5 μmoles of DDP, with a sensitivity of about 120 Klett units per μmole. Protein-free filtrates of reaction mixtures were prepared with the use of barium hydroxide and zinc sulfate according to Nelson (*29*).

Results

Stability to Nonenzymatic Hydrolysis After the preliminary finding that a $10^{-3}M$ solution of DDP in water had lost only 10% of its anticholinesterase activity after standing at room temperature for a full year, the activity of DDP with compounds previously known to react with antiesterases of Class I was studied.

Two examples are presented of stability under conditions where phosphonofluoridates are labile.

Catechol. It has previously been shown that polyhydroxyphenols react in a quantitative fashion with DFP (*18*) and even more rapidly with sarin; the reaction represents a phosphorylation of one (catechol) or two (pyrogallol) phenolic OH groups. The presence of two unsubstituted vicinal OH groups is required for a color formation in the test for catechols (*4*). From the results presented in Table I, it may be seen that under conditions where sarin (as a representative phosphofluoridate) completely reacts with catechol by phosphorylation and consequent disappearance of *o*-diphenolic configuration, no such reaction takes place when an equimolar amount of DDP is substituted for sarin.

One volume of sarin or DDP ($10^{-3}M$) was incubated with 1 volume of catechol

Table I. Reaction of Catechol with Sarin and DDP

Reaction Mixture	Free Catechol, μmole
Catechol	0.20
Sarin	0.00
Catechol + sarin	0.00
DDP	0.00
Catechol + DDP	0.196

($2 \times 10^{-4}M$) in $0.025M$ phosphate, pH 7.4, for 1 hour at room temperature; controls contained water instead of sarin or DDP, or phosphate buffer instead of catechol solution. After incubation, 2-ml. aliquots were treated successively with 1 ml. of $0.5N$ hydrochloric acid, 1 ml. of 10% sodium nitrite in 10% sodium molybdate, 1 ml. of sodium hydroxide, and 5 ml. of water. Color was read in a Klett colorimeter, filter No. 52.

Picolinohydroxamic Acid. It has been shown (*11*, *15*) that hydroxamic acids, in addition to being potent reactivators of alkylphosphorylated enzymes, also have the property of reacting directly with anticholinesterases in a manner which leads to the disappearance of their inhibitory properties.

Picolinohydroxamic acid ($0.1M$) (PHA) was incubated with sarin or DDP ($1.3 \times 10^{-4}M$) for 30 minutes, at pH 7.3 and 25° C. An aliquot of the reaction mixture was added to a preparation of electric eel cholinesterase. This mixture was further incubated for 30 minutes and tested for residual cholinesterase activity. Controls contained no hydroxamic acid.

Table II demonstrates that, as in the case of catechol, a hydroxamic acid capable of reacting with sarin (as shown by absence of inhibition when the reaction mixture between sarin and picolinohydroxamic acid is added to cholinesterase) does not react under the same conditions with DDP; a given amount of DDP produces the identical amount of inhibition whether or not it had previously been incubated with hydroxamic acid.

Table II. Reaction of Picolinohydroxamic Acid with Sarin and DDP

Reaction Mixture	ChE Activity: Div. per min.	ChE Activity: Control, % of
No addition	69.0	100
PHA, no inhibitor	69.0	100
Sarin	0.0	0
PHA and sarin	69.0	100
DDP	29.5	43
PHA and DDP	30.0	44

Reaction with Enzymes

Kinetics. When DDP is incubated with various esterases there is a progressive loss of enzymatic activity. If DDP is in great stoichiometric excess over the enzyme, the reaction follows first-order kinetics of the type $kt = \ln A_o/A_t$ where A_o and A_t represent enzymatic activity at zero time and after incubation of enzyme and inhibitor for t minutes, respectively. This is shown in Figure 1 where proportionality between length of incubation and log A_o/A_t is demonstrated for three enzyme preparations. A plot of first-order constant against concentration of DDP yields a straight line, indicating that the over-all reaction between enzyme and inhibitor is a bimolecular one. The bimolecular velocity constants for the reaction between DDP and various esterases are tabulated in Table III, where they are compared with those for other irreversible anticholinesterases and the same enzymes. The reactivity of DDP with these enzymes is high and is of the same order as the rates of reaction of other inhibitors of this type. DDP is somewhat more reactive toward the serum type of cholinesterase than against the true acetylcholinesterase of red cells and the electric organ, and in this respect it resembles DFP and TEPP.

Table III. Bimolecular Rate Constants of Organophosphorus Inhibitors with Esterases

(pH 7.4, 25° C. Units: liter-mole^{-1} min.$^{-1}$)

	DDP	DFP	TEPP	Sarin
Human red cell ChE	1.3×10^6	8.1×10^4	2.1×10^6	1.5×10^7
Eel ChE	2.8×10^5	1.9×10^4	2.1×10^6	6.3×10^7
Horse serum ChE	4.8×10^7	1.5×10^7	5.0×10^7	4.4×10^6
Chymotrypsin	8.9×10^3	2.0×10^4	1.1×10^3	1.7×10^4

Stoichiometry. When chymotrypsin is incubated to maximal inhibition with increasing amounts of DDP, there is a progressive increase in the degree of inhibition produced (Table IV), which corresponds to the predicted inhibition if one mole of agent is sufficient to inactivate one mole of enzyme. DDP therefore conforms with what has been found previously for DFP (*5*), TEPP (*17*), and other inhibitors of this type (*12*).

Table IV. Stoichiometric Inactivation of Chymotrypsin by DDP

DDP in Reaction Mixture: Total micromole	DDP in Reaction Mixture: Moles per mole ChTr	R_{280}	Residual Activity, %: Found	Residual Activity, %: Expected[a]
0		0.174	(100)	(100)
0.05	0.46	0.085	58	54
0.1	0.93	0.010	7	7
0.2	1.85	0.000	0	0
0.5	4.63	0.000	0	0

[a] On basis of 1:1 stoichiometric reaction.

Chymotrypsin (1 ml. containing 2.7 mg. = 1.08×10^{-7} mole in $0.1M$ phosphate, pH 7.4) was incubated at room temperature with DDP in water for 15 minutes. Volume of incubation mixture was 2.0 ml. At the end of the incubation a 0.2-ml. aliquot was diluted with water to 100 ml. Proteolytic activity was determined on 2.0-ml. aliquots of this dilution. R_{280} = absorbance of protein-free filtrate at 280 mμ, corrected for blank.

Mechanism. The experiments to be described were based on the hypothesis that the mechanism of reaction between DDP and enzymes can be formulated in a fashion analogous to that proved for

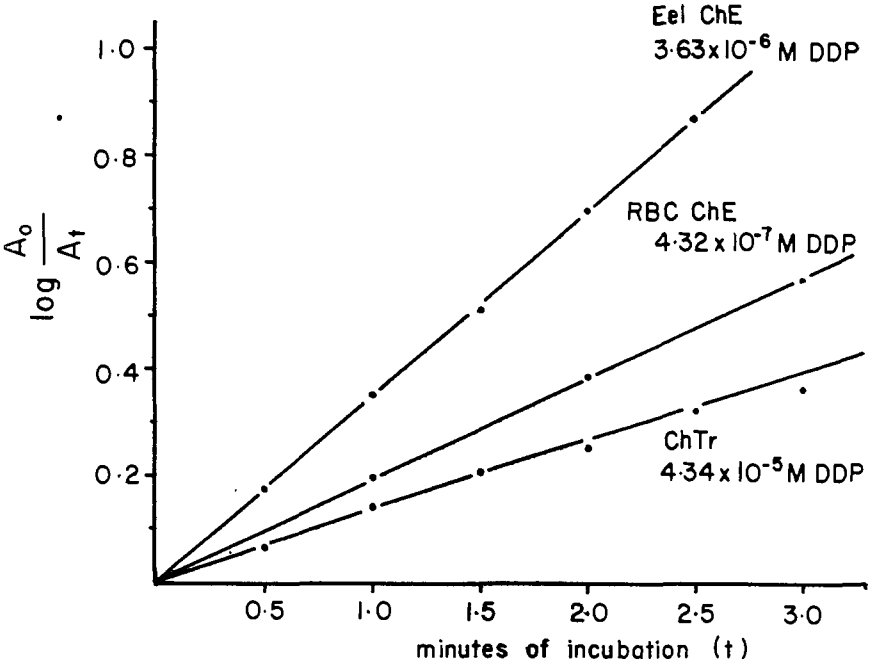


Figure 1. First-order reaction of excess DDP with representative esterases. pH = 7.4, 25° C.

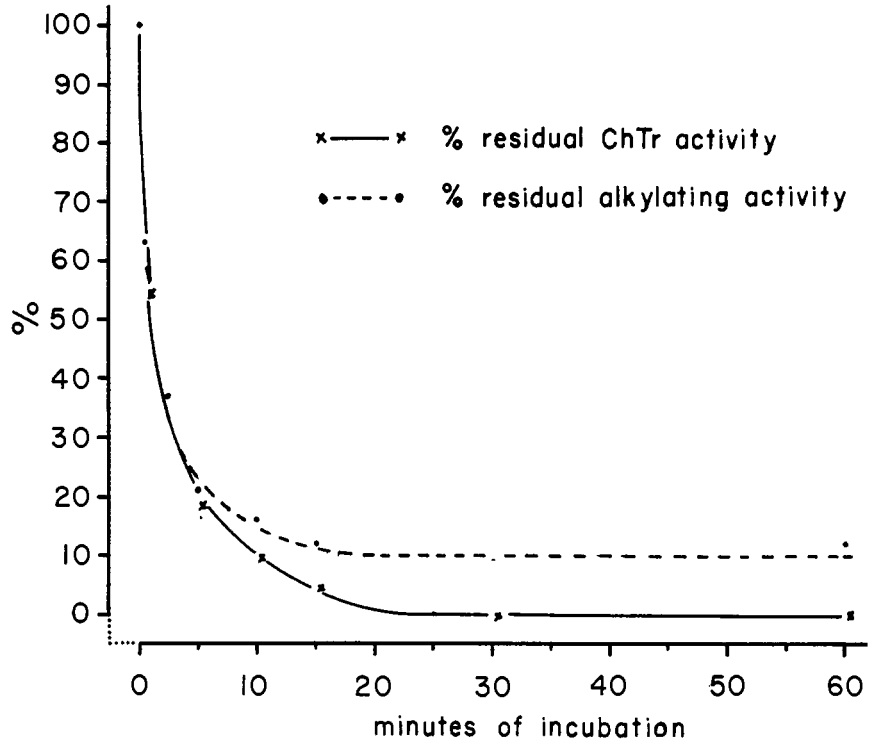


Figure 2. Loss of proteolytic activity and alkylating activity during reaction of DDP with chymotrypsin (5×10^{-4}M final concentration for both reactants) at pH 7.4, 25° C. Results are expressed in % of each activity at zero time

other phosphorus-containing antiesterases:

$$\text{Enzyme—H} + (RO)_2P(O)—X \rightarrow \text{Enzyme—P(O)}(OR)_2 + HX$$

The analogous reaction for DDP would be:

$$\text{Enzyme—H} + (PrO)_2P(O)—OCH{:}CCl_2 \rightarrow \text{Enzyme—P(O)}(OPr)_2 + O{:}CH—CHCl_2$$

Preparation and Analysis of Inhibited Enzyme The reaction product between chymotrypsin and DDP was prepared as follows: Chymotrypsin (270 mg., 1.08×10^{-5} mole) was dissolved in 20 ml. of 10^{-3} *M* DDP, and 2 ml. of *M* phosphate, pH 7.4, were added. The mixture, after standing at room temperature for 30 minutes, was mixed with 2 ml. of 0.1*N* sulfuric acid and sufficient solid ammonium sulfate to precipitate all protein. After centrifuging, the precipitate was taken up in 2 ml. of 0.01*N* sulfuric acid, freed of a small amount of denatured protein by centrifugation, and treated with saturated ammonium sulfate solution to first turbidity. On standing overnight at room temperature, the protein crystallized in long tapered needles. Recrystallization from ammonium sulfate resulted in plates of the form characteristic of chymotrypsin or of diisopropyl phosphoryl-chymotrypsin (*19*). (The two crystal forms, which appear on the first and on all subsequent crystallizations, respectively, were found consistently in three preparations of inhibited enzyme.) The product, after two to four further recrystallizations, was dialyzed against running distilled water in the cold and lyophilized. Analysis produced the following results: P, 0.12% (theoretical for 1P/25,000 grams, 0.124%); Cl, 0.05% (theoretical for 2 Cl, 0.284%). The enzyme has therefore combined, during the process of inhibition, with 1 gram-atom of phosphorus, but no stoichiometric amount of the DDP-derived chlorine, a finding which fits the above theory.

When the inhibited enzyme is incubated with phosphorus-32–labeled DFP, no appreciable radioactivity (less than 0.05 eq. of phosphorus per mole) becomes protein bound under conditions where stoichiometric uptake of 1 eq. of phosphorus per mole of active chymotrypsin (recrystallized, dialyzed, and lyophilized as a control to the above preparation) can be readily demonstrated. This indicates that DFP and DDP react either at the same site, or at closely adjacent sites, of the enzyme molecule.

Acid Production during Phosphorylation The reaction between chymotrypsin and phosphofluoridates leads to the liberation of 1 mole of acid—namely, hydrofluoric acid—per mole of enzyme if it is carried to complete inhibition; on the contrary, the proposed reaction mechanism demands that no acid be produced in the analogous reaction with DDP. Table V demonstrates that this is indeed so, by contrasting the acid production (carbon dioxide liberation in bicarbonate buffer) in the reaction of chymotrypsin with sarin, which amounts to close to 1 mole, with that with DDP, where no acid is produced. That a reaction has taken place at all is shown by the loss of proteolytic activity at the end of incubation with either inhibitor.

The procedure consisted of reaction of 2 μmoles of ChTr with 6 μmoles of sarin or DDP in 0.025*M* sodium bicarbonate at 25° C.; total volume = 2.4 ml.; gas phase, 5% carbon dioxide to 95% nitrogen. At the end of reaction, aliquots of reaction mixtures were used for determination of proteolytic activity. All manometric results were corrected for carbon dioxide retention.

Loss of Alkylating Power during Reaction. A reagent [γ(4-nitrobenzyl)-pyridine] which appears to be generally reactive with actively alkylating groups, has recently been described by Epstein, Rosenthal, and Ess (*10*). The general reactivity of dihalovinyl compounds suggested that DDP should yield a positive test with this reagent; the postulated product, dichloroacetaldehyde, should yield a negative result in this test. This has been confirmed; in addition, it may be seen from Figure 2 that during the reaction of approximately equimolar amounts of chymotrypsin and DDP, the loss of reactivity of the solution with this reagent parallels the loss of enzymatic activity of the reaction mixture.

Identification of Split Product. When DDP is allowed to react with a stoichiometric excess of chymotrypsin and the mixture is subsequently deproteinized, a chlorine-containing moiety of DDP is found in the protein-free filtrate (Table VI) with simultaneous loss of free DDP.

DDP (1 ml. containing 2 μmoles of DDP = 4 μeq. of Cl) was incubated with chymotrypsin (1 ml. containing 3 μmoles in 0.025*M* phosphate, pH 7.4) for 30 minutes at 27° C. The mixture was deproteinized with 2 ml. of barium hydroxide and 2 ml. of zinc sulfate, centrifuged, and filtered through Whatman No. 41 paper. Controls (4 micromoles of sodium chloride)

Table V. Acid Liberation during Reaction of Chymotrypsin with Sarin and DDP

Manometric	ChTr	ChTr + Sarin	ChTr + DDP
CO_2, μl.	14.0	56.0	11.5
Corr. for control		42.0	0.0
Moles acid/mole ChTr		0.94	0.0
Proteolytic activity (R_{280} per mg. protein)	27.4	0.04	0.0

and appropriate blanks were run simultaneously. Unreacted DDP in the protein-free filtrate was determined by the test for "alkylating power."

Essentially all the chlorine found in the supernatant can be accounted for as alkali-labile chlorine (a small amount of inorganic chloride appears to be present as impurity in either the chymotrypsin or the reagents used). Recovery of total chlorine in these experiments was poor, apparently owing to adsorption on the precipitating mixture, since 95% or better recovery (in terms of total or inorganic chloride or of DDP) was found in simultaneously run controls containing no protein, and water instead of the precipitating reagents. Nevertheless, the recovery is consistent and in conjunction with the analytical results on the inhibited enzyme, supports the hypothesis that the reaction between chymotrypsin and DDP is accompanied by the stoichiometric liberation of chlorine in organic, alkali-labile form.

Reaction of Split Product with 2,4-Dinitrophenylhydrazine. A mixture of 18 μmoles of chymotrypsin and 12 μmoles of DDP was made to react in a total volume of 10 ml. at 25°, pH 7.4, for 30 minutes and then deproteinized. The filtrate was evaporated almost to dryness, transferred with small quantities of water to a centrifuge tube, and diluted to 1.0 ml. After amorphous material had been centrifuged off, the supernatant was brought to 1.5 ml. with methanol and treated with 1.0 ml. of acid 2,4-dinitrophenylhydrazine reagent (500 mg. in 50 ml. of methanol + 0.75 ml. of concentrated hydrochloric acid). The yellow amorphous precipitate was centrifuged off, taken up in 2 ml. of methanol plus 1 drop of 12*N* hydrochloric acid, and centrifuged. The precipitate was washed once more with acid methanol and then three times with 2-ml. portions of methanol without added hydrochloric acid. The washed precipitate was extracted twice with 0.3-ml. portions of hot methyl Cellosolve, leaving a small amount of nearly white residue which was discarded. The combined supernatants were allowed to stand at 3° C. overnight with occasional scratching. The resulting yellow-orange needles were washed four times in the centrifuge with 0.5-ml. volumes of diethyl ether and dried. Melting point (uncorr.) was 313–314° C. with decomposition; mixed melting points with authentic samples of glyoxal 2,4-dinitrophenylosazone, prepared as above but on a larger scale from glyoxal or from dichloroacetaldehyde, were 313° C. with decomposition and 312° C. with decomposition, respectively.

Reaction of Split Product with Dimedone [adapted from Horning and Horning (*13*)]. A deproteinized mixture was prepared as described in the preceding paragraph, the filtrate was evaporated to dryness and extracted with 0.5 ml. of ethyl alcohol. The supernatant was transferred to a small reaction flask and was treated with 0.4 ml. of water; 50 mg. of dimedone (5,5-dimethyldihydroresorcinol) and 0.1 ml. of a solution of one drop of piperidine in 1 ml. of 50% ethyl alcohol was added. After standing at room temperature for a few hours a heavy crystalline precipitate developed which was recrystallized from methanol-water; melting point 145° C., mixed melting point with authentic sample 144° C. Analysis: Calculated for $C_{18}H_{24}O_4Cl_2$: C, 57.6; H, 6.40. Found: C, 57.5; H, 6.39. Melting point 145° C. Because of the similarity of this melting point with that of dimedone itself (147–149° C.) a mixed melting point was taken and found to be 128° C.

Table VI. Liberation of Organically Bound Chlorine during Reaction of DDP with Chymotrypsin

Incubation Mixture	Inorganic Cl μeq.	Inorganic Cl Recovery, %	Alkali-Labile Cl μeq.	Alkali-Labile Cl Recovery, %	Unreacted DDP μmoles	Unreacted DDP Recovery, %
ChTr + DDP	0.42	..	3.06	77	0.0	0
ChTr	0.44	..	0.10	..	...	..
Control[a]	0.48	..	2.94	74	1.43	72
DDP	0.00	..	2.96	74	1.38	69
ChTr + NaCl	3.38	74[b]	0.11	..	...	..

[a] DDP added after addition of deproteinizing reagents.
[b] Corrected for inorganic Cl in ChTr.

Discussion

The identification of dichloroacetaldehyde supports the previously proposed mechanism of the reaction between DDP and chymotrypsin, which consists of a stoichiometric phosphorylation of the protein with simultaneous liberation of dichloroacetaldehyde. The lack of acid production during the reaction, as well as the loss of alkylating property of the dichlorovinyl moiety of DDP upon reaction with esterase, is further evidence for the postulated reaction mechanism. It would, therefore, appear that chymotrypsin reacts with DDP in a manner entirely analogous to its reaction with other phosphorus - containing antiesterases. Furthermore, on the basis of similarity of rate constants of DDP and other antiesterases with various esterases one may assume that cholinesterases react with DDP in a similar manner also.

At present there is no ready explanation for the considerable reactivity of DDP with enzymes, and its stability to nonenzymatic hydrolysis. In the series of phosphoro- and phosphonohalidates the stability of the P—*X* bond toward nucleophilic reagents (hydroxyl, catecholate, hydroxamate ion) is believed to result from the polarizing effect of the *X* group on the phosphoryl moiety. The reaction has been pictured as a bimolecular one of the S_N2 type involving an unstable transition compound (*8*, *9*). An analogous mechanism has been postulated for the reaction with enzymes (*33*). The dichlorovinyl group undoubtedly confers polarizing properties on the rest of the DDP molecule, as evidenced by the reactivity of the intact molecule in a test system specific for alkylating compounds. The experimental evidence suggests that the activation energy necessary to disrupt the P—OCH: CCl_2 bond by nucleophilic reagents is far higher than is the case for the corresponding bond in phosphohalidates. The analogous reaction with esterases would require an activation energy low enough to be available for either type of compound. So far the few available thermochemical data having a bearing on this problem are insufficient as evidence for or against this conclusion.

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Rate constants for compounds other than DDP are original observations by Harry O. Michel of this laboratory (*16*). The author is indebted to Dr. Michel for permission to use these data.

The use of the procedure of Epstein, Rosenthal, and Ess (*10*) for determination of alkylating power was suggested to the author by W. H. Summerson.

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SYNERGISM IN PESTICIDES

Comparative Synergistic Effects of Synthetic 3,4-Methylenedioxyphenoxy Compounds in Pyrethrum and Allethrin Fly Sprays

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The comparative value of 63 synthetic 3,4-methylenedioxyphenoxy derivatives and two related compounds as synergists with either pyrethrins or allethrin was estimated in tests against the housefly, *Musca domestica* L., by the turntable method. Forty-three compounds were demonstrated to be synergistic with each insecticide. Certain ethers, acetals, and esters of aromatic sulfonic acids had strong effect, whereas esters of carboxylic and carbamic acids had little or no effect. The intensity of synergism was so high for 18 compounds—toxicity was raised to at least six times that expected for pyrethrins alone or three times that expected for allethrin alone—that further work was recommended.

THE BEST SYNERGISTS for pyrethrins and allethrin contain the 3,4-methylenedioxyphenyl group in the molecule, although the presence of this group does not in itself assure synergistic effect (*6, 8, 12*). It has recently been shown that sesamolin, obtained from sesame oil and differing structurally from sesamin in containing a 3,4-methylenedioxyphenoxy group in place of one of the 3,4-methylenedioxyphenyl groups (*2, 3*), is a much more effective synergist with pyrethrins against houseflies, *Musca domestica* L., by the turntable method than is sesamin (*7*). It was, therefore, desirable to prepare other 3,4-methylenedioxyphenoxy derivatives and evaluate them as candidate synergists. The preparation of 66 such compounds has been reported (*1*). The present paper reports the results of tests with 63 of these compounds designed to evaluate them separately in mixtures with pyrethrins or allethrin against the housefly. The purpose of this study is to select the most promising synergists for future, more precise comparison. Similar tests with two compounds that are not 3,4-methylenedioxyphenoxy derivatives are also reported for comparison with those of closely related compounds—1-allyl-3,4-methylenedioxybenzene (known as safrole) and 2-(*p*-methoxyphenoxy)tetrahydropyran.

Purified samples of the synthetic compounds were used. The sample of pyrethrins was the complex contained in the extractives from pyrethrum flowers not further processed for the removal of