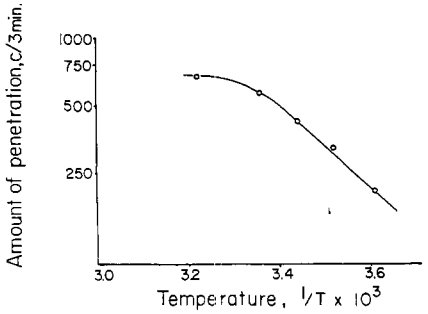

Figure 7. DDT uptake by nerve cord as a function of temperature

represents the whole complex or dissociated DDT produced by the treatment with these organic solvents. The fraction reacted with Folin-Ciocalteu's reagent indicating a presence of 20 to 30 μg. per ml. of protein equivalent, based on calibration with crystalline bovine albumin. Judging from the result of the Sephadex column experiment, the complex has a relatively large molecular size.

Effect of Temperature on Rate of DDT Uptake by the Nerve Cord. DDT has a negative temperature coefficient—i.e., its toxicity is enhanced when the temperature is lowered. To investigate whether this phenomena is related to the gross amount of DDT uptake by the nerve cord, the absorption of $10^{-5}M$ DDT was tested on the intact cords. The relation between temperature and the rate of diffusion can be expressed in a linear fashion when the reciprocal of the absolute temperature is plotted against the logarithms of the amount penetrated (*5*). The result (Figure 7) indicates that the rate of DDT penetration steadily increases from 4° to 25° C. to reach a plateau at 37° C.

Discussion

The question of DDT solubility raises several problems. Saturated solutions in water have been reported as being as high as 1000 p.p.b. ($2.8 \times 10^{-9}M$) (*9*), and probably more correctly as 1.2 p.p.b. ($3.4 \times 10^{-12}M$) or less (*7*). The value can be increased to $1.2 \times 10^{-2}M$ by a lipoprotein (*6*). In the present work, the values given are based on the assumption of true solution, and the DDT behaves as if this were the case.

The rate of DDT outflow from the nerve cord indicated that a portion (approx. 30%) of DDT is rather loosely associated, and the rest is tightly bound to the cord. Apart from this absorption phenomenon, the rate of inward and outward diffusion with respect to time closely follows first-order kinetics. The rate of total DDT penetration also proportionally increases with the external DDT concentrations higher than $5 \times 10^{-5}M$. These facts indicate that the gross behavior of DDT toward the nerve cord is in accord with a simple law of diffusion.

No serious effort was made to identify the binding substance(s), for the amount involved was too small to permit any chemical analysis at present. The most obvious candidates for the binding substances are the lipids of the nerve cord, as DDT is one of the most apolar compounds so far known. The difficulty is that the DDT complex is not easily extracted by ether, though this does not exclude the possibility that a water-soluble lipoprotein is involved in the reaction. However, this is only speculation, and further studies are needed to draw any decisive conclusion.

DDT potency for insects decreases with increasing temperature. Total uptake into nerve cords actually increased with temperature, and total uptake is, therefore not simply correlated with potency. However, the α, β, and γ components were jointly studied in the temperature experiment, and it is entirely possible that the individual components might respond differently.

In conclusion, it has been shown that DDT does indeed complex with components of insect nerve, and these complexes have been isolated. Whether these complexes are of the charge-transfer type (*10*) and whether they play a role in poisoning are matters requiring further study.

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INSECTICIDE REACTION WITH NERVE

Interactions of DDT with Components of American Cockroach Nerve

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THE ACTUAL TARGET of DDT in insects and mammals is considered to be the central nervous system, as judged by symptomological observations (*2*). Electrophysiological evidence (*7*) suggests that DDT blocks the transport of cations (particularly potassium) across the nerve membrane. The mechanism of this blockade is not known, although several hypotheses to explain the interaction of DDT with the nerve membrane have appeared. For instance, Mullins (*6*) has hypothesized that DDT fits into an intermolecular lattice, and Gunther *et al.* (*3*) have postulated binding of DDT to proteins in the nervous system. All of these theories suggest the entry and fixation of the DDT molecule to the nerve components, but do not answer the basic problem of the way in which DDT, thus fixed, can block ion transport.

The authors have suggested (*8*) that DDT acts by forming a charge-transfer complex with a component of the axon, thus destabilizing it. (Complexes of this type may form between two sterically matched molecules, one of which is a good electron donor, the other a good electron acceptor; complex for-

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The nature and consequences of binding of DDT to components of the cockroach nerve cord were examined. The butanol extract of a DDT-treated nerve homogenate showed a pronounced bathochromic shift in ultraviolet spectrum in comparison with DDT alone. The absorption spectrum of an unextracted DDT-treated homogenate showed a new shoulder at 240 to 270 mμ; the fluorescent spectrum on activation at 310 mμ showed a new peak at about 420 mμ. A complex, possibly of the charge-transfer type, was therefore suggested between DDT and a component of nerve. Nerve cords from DDT-treated cockroaches showed large increases in their ability to take up sodium or lose potassium, whereas ability to lose sodium or take up potassium was unchanged. The relation among complex formation, changes in ion permeability, and toxicity is not yet established.

mation may give rise to new spectral absorption bands and paramagnetism.) The destabilization might involve induction of localized semiconductivity, for a series of alternating layers of electron-donating and -accepting materials can form a semiconducting membrane. As the first step in confirming the above hypothesis, the interaction of DDT with the nerve components has been studied, and it has been shown that DDT bound itself to at least two components of the nerve cord of the American cockroach (*5*). The complexes were partially purified and found to be very stable, being able to withstand treatments such as diffusion, column chromatography, and gel filtration.

Two important matters are relevant to the validity of the above suggestion: demonstration that the complexes which DDT forms are indeed of the charge-transfer type, and that the complex formation is related to the physiological interference with cation transports. In the present paper, spectral evidence is provided on the nature of the complex, and it is shown that DDT treatment leads to severe interferences with cation permeability, as measured directly with radioactive cations.

Materials and Methods

The isolated cockroach nerve cord was prepared as before (*5*). C^{14}-DDT (specific activity 4.93 mc. per mmole) was added to the isolated nerve cord or injected into a living male cockroach with ethanol (final concentration of ethanol, 1%) followed by isolation of the nerve cord.

When ion-transport through the nerve membrane was studied with radioactive cations, nonradioactive DDT was used in place of C^{14}-DDT. To study the effect of DDT in vivo, 0.3 ml. of $1 \times 10^{-3}M$ DDT (200 μg. per gram) was injected into a male roach and then, after 15 minutes, radioactive ions (either K^{42} or Na^{24}) were also injected. After 1 hour, the nerve cord was removed, and the rate of ion discharge into fresh saline was studied. To study the effect of DDT on the isolated nervous system, the isolated nerve cords were first allowed to stand at 25° C. in saline solution containing radioactive ions, with or without DDT ($1 \times 10^{-5}M$) for various time intervals. They were then taken out and washed briefly with fresh saline, and the excess moisture was removed with a filter paper. The nerve cords were then either directly extracted with ethanol and dissolved in 1 ml. of hot Hyamine (hydroxide form, $1M$ in methanol), or transferred into fresh saline, with or without DDT, to study the rate of ion exchange thereafter. Radioactive samples in 1 ml. were added with 9 ml. of dioxane counting solution (*4*) to a counting vial, and the radioactivity was measured in a Packard liquid scintillation spectrometer. The radioactive salts used were $Na^{24}Cl$ (100 mc. per gram), $K^{42}Cl$ (20 mc. per gram) and $Ca^{45}Cl_2$ (500 mc. per gram).

Ultraviolet absorption was studied with the Bausch and Lomb 505, or Beckman Model DU spectrophotometer. The former model had the advantage of compensating the absorption of the carrier substances. Fluorescence spectra were studied by means of an Aminco-Bowman spectrophotofluorometer.

Results

Changes in Absorption and Fluorescent Spectra. Changes in absorption spectra can be a good indication of formation of a charge-transfer complex (*7*). Examples of charge-transfer complex formation with appearance of new bands in absorption spectra are also described by Szent-Györgyi (*9*). In the following experiments, the absorption spectrum due to the homogenate or other macromolecules was compensated for by using the reference solution with a Bausch and Lomb spectronic 505. The complex in aqueous phase was first extracted into isobutyl alcohol before the measurement of spectra. Addition of DDT to the nerve homogenate (Figure 1) resulted in change in the peak position as well as the shape. The absorption spectrum for the DDT-homogenate complex, when left in the original aqueous solution, changed with time by decreasing its absorbance to nearly 1/3 in 3 hours at 25° C., possibly because of the combined effect of slow reversal and enzymic breakdown of DDT. To examine more fully the characteristics of each complex, purified and unpurified, the absorption spectra against saline were studied in the DU spectrophotometer (Figure 2). The purified samples of DDT-nerve component complexes—i.e., sephadex fraction and DEAE-fraction—had a shoulder of absorption at 240 to 270 mμ. The procedures of purifying the complex(es) on sephadex G-50 and DEAE-cellulose columns to yield above fractions have been described previously (*5*).

Formation of a complex could also be demonstrated by fluorometry. Appearance of a new fluorescent peak at 420 mμ immediately after mixing DDT and sephadex fraction was observed at an activation wavelength of 260 mμ and scanning the fluorescent spectrum (Figure 3). The addition of DDT to the denatured fraction (denatured at 100° C. for 5 minutes) did not significantly alter the spectrum of the fraction itself, indicating that DDT by itself does not show any native fluorescence.

The identity of the bound radioactivity was studied; the complex with whole homogenate was first treated with 1% trichloroacetic acid and was extracted with an equal volume of ether five times (either fraction). The aqueous phase was further treated with concentrated H_2SO_4 (10 to 1) and was extracted with cyclohexane. The remaining aqueous solution showed no radioactivity. The recovered radioactive materials were examined by thin-layer and paper chromatographic techniques and identified as DDT itself (Figure 4). The reversibility of the DDT binding under mild conditions, such as simple dialysis against saline solution, was negligible. No detectable amount of radioactivity was recovered from the external medium after 48 hours of continuous dialysis at 25° C.

Effect of DDT on Rate of Ion Movement in Nerve Cord. The above experiments indicated the formation of DDT complexes, possibly of the charge-transfer type. The possibility that this process can disrupt the ion-transport system was investigated. The rate of ion flux through the nerve mem-

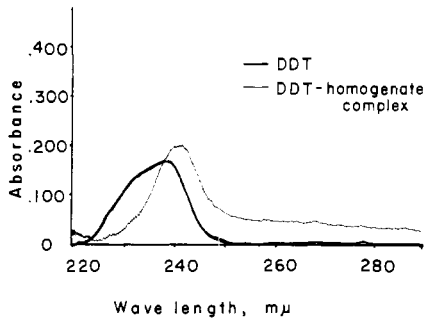

Figure 1. Effect of DDT binding to nerve components on ultraviolet absorption spectra

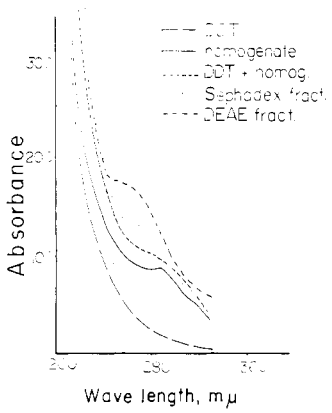

Figure 2. Ultraviolet absorption spectra of free and bound DDT

Each aqueous fraction was measured directly with a Beckman DU spectrophotometer against saline solution blank. "DDT + Homog": calculated by adding separate spectra. "Sephadex fraction": radiooctive eluate from Sephadex fractionations of C^{14} DDT bound to nerve homogenate. "DEAE fraction" radioactive eluate from further fractionation of the Sephadex fraction on diethylaminoethyl cellulose (5)

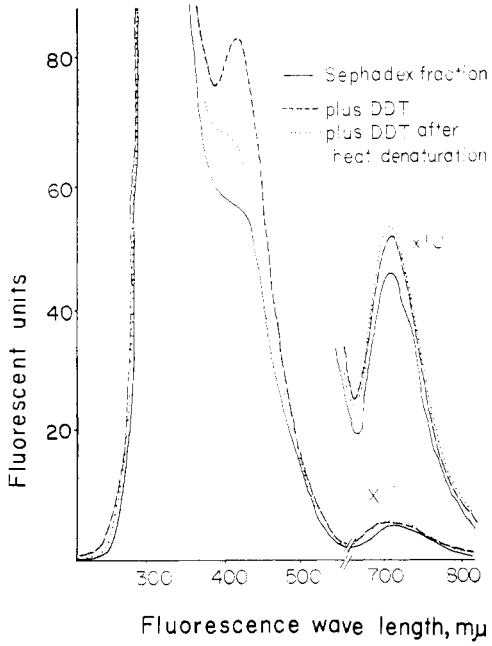

Figure 3. Fluorescence spectra of Sephadex-bound and free DDT

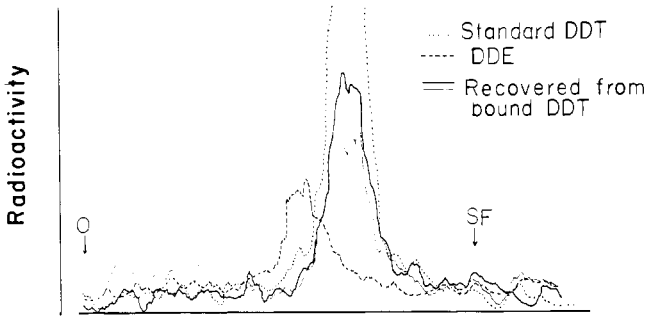

Figure 4. Paper chromatogram of DDT recovered from the bound form

Measured by rodiotracer scanning method. O: origin. SF: Solvent front

brane was first studied by using the isolated nerve cord (*5, 10*). The nerve cords were allowed to stand in saline solution with radioactive ions for 1 hour, washed briefly, and then transferred into fresh saline with or without $10^{-5}M$ DDT. The results (Figure 5) indicate that the rates of efflux of these cations from the nerve cord under these conditions were not drastically altered by the presence of $1 \times 10^{-5}M$ DDT. The rate of radioactive sodium ion efflux in the presence of DDT seems to be slightly lower than that of the control.

To study the effect of DDT poisoning in vivo, male cockroaches were injected with 200 μg. per gram of nonradioactive DDT and then with one of the following: 26 mμmole per roach of K^+ (2 μg. of KCl); 67.5 mμmole of Na^+ (4 μg. of NaCl); or 34.5 mμmole of Ca^{+2} (4 μg. of $CaCl_2$). The insects started to show hyperexcitability from 15 to 20 minutes after the injection. The first tremors started at about 20 minutes, and in 40 minutes, the insects were completely prostrated with occasional convulsions. The nerve cords removed from poisoned insects showed a marked increase in K^+ ion efflux in the first 20-minute period as compared with the control (Figure 6*A*). The effect was not so striking with sodium ions (Figure 6*B*); and the tendency was reversed, though again not drastically, in the experiments with calcium ions (Figure 6*C*). In examining the figures, it should be borne in mind that the logarithimic scale used exaggerates the variation between the results for different individuals, particularly for low values of "per cent remaining"; furthermore, in DDT-treated insects, considerable variation arises because cords were removed at a fixed time after treatment, and the degree of poisoning, therefore, varied between individuals. To affirm the validity of the difference between treated and untreated insects, a number of additional individuals were examined 30 minutes after treatment. The mean values (± standard error) for the amounts of K^+ ions remaining in the treated and untreated nerve cords were 9 ± 3% and 39 ± 7%, respectively.

The total uptake of radioactive ions by the abdominal nerve cord and the initial outward diffusion in the above experiments (see Figures 6*A* and *B*) are summarized in terms of milliequivalents per gram in Table I. Cords from DDT-poisoned roaches took up twice as much Na^+ as those from the untreated, but lost Na^+ at an almost equal rate as the untreated. The cords from poisoned insects also took up just as much radioactive K^+ as the untreated ones, but unlike the control, lost most of the radioactive K^+ in the first 20-minute period.

The above experiments revealed much faster K^+ efflux in the treated nerve cord than the untreated one, but this could mean either the K^+ ions were exchanged (inward as well as outward) much faster in the DDT-treated insect's nerve cord, or only the outward ion movement was accelerated without affecting the inward movement of K^+ ions. To examine these alternatives, cation uptake was measured in an in vitro preparation. Cords were removed from untreated

Table I. Uptake and Efflux of Potassium and Sodium Ions in Abdominal Nerve Cords of the Cockroach Treated with DDT[a] in Vivo

	Total[b] Uptake, Meq./Gram	Efflux in Initial 20 Min.[c] Meq./gram	Efflux in Initial 20 Min.[c] % of total
	K^{42}		
Untreated cockroach	0.170	0.095	56
DDT-treated	0.174	0.145	83
	Na^{24}		
Untreated cockroach	31.4	22.1	70
DDT-treated	60.6	49.5	82

[a] Injection with 200 μg./gram of DDT and 2 μg. or 4 μg./roach of K^{42} or Na^{24} equivalents 1 hour prior to assay.

[b] All results expressed in terms of movement of total injected (nonradioactive plus radioactive) ion.

[c] At 30 minutes, K^{42} efflux for untreated 61 ± 7%, DDT-treated 91 ± 3%; Na^{24} efflux for untreated 80 ± 2%, and DDT-treated 87 ± 5% as based on four independent measurements.

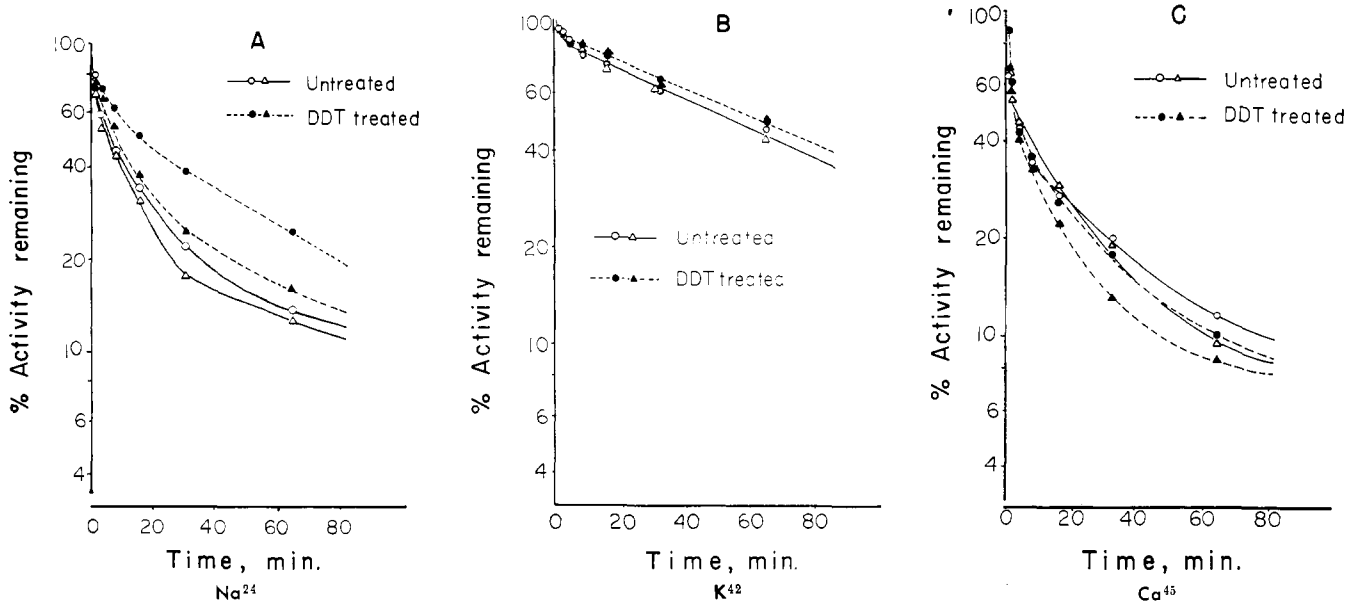

Figure 5. Efflux of ions from isolated nerve cords in the presence and absence of DDT in the external medium

Results were calculated on the basis that total radioactivity recovered from the external medium and from the nerve cord was 100%. Each line represents a single individual

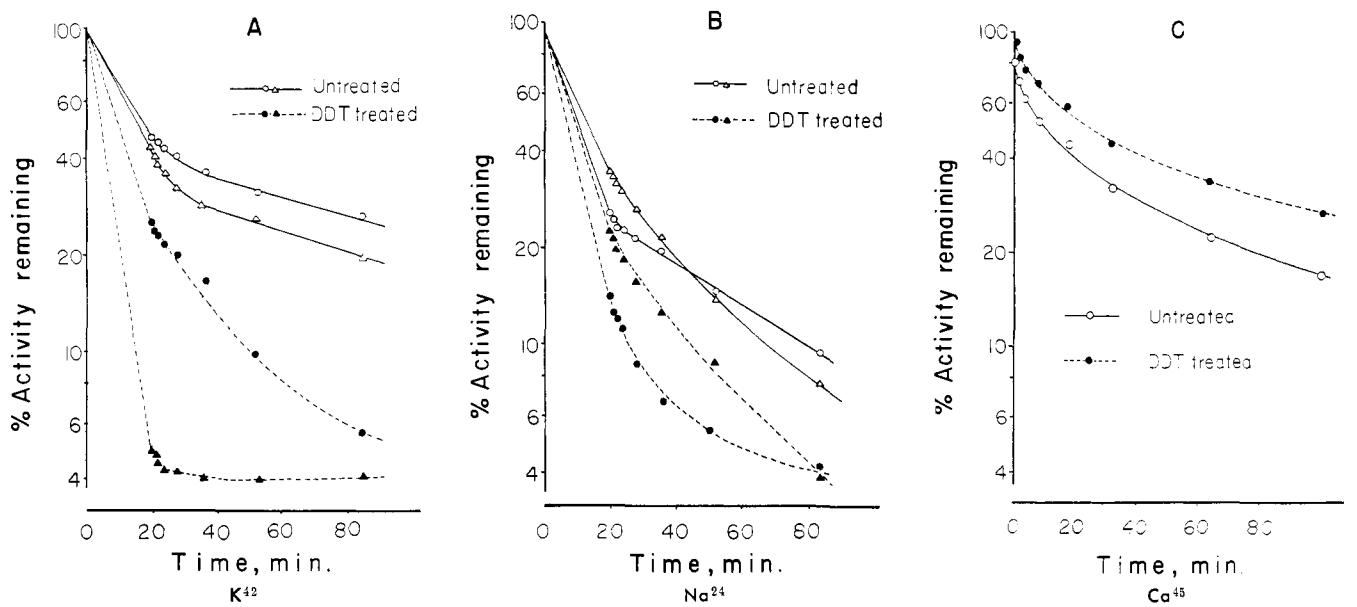

Figure 6. Efflux of ions from nerve cords of DDT-poisoned and untreated cockroaches

Legend as in Figure 5

insects, and incubated with DDT for 15 minutes at 25° C., then transferred to fresh insect saline at 25° C. containing K^{42} or Na^{24}. After 30 minutes, the cords were taken out and washed with fresh insect saline, and the excess saline was removed with filter paper. The total uptake of K^{42} was somewhat higher with DDT treatment (0.802 meq. per gram *vs.* 0.672 meq. per gram for untreated), and the Na^{24} was little (13 meq. per gram *vs.* 12.6 meq. per gram, respectively). It is likely, therefore, that the DDT treatment causes depletion of K^+ ions by accelerating the efflux rate without affecting the influx rate.

Discussion

DDT can bind tightly to several components of the abdominal nerve cord of the American cockroach (*5*). The results obtained in the present work indicate that this binding results in a bathochromic shift in the ultraviolet spectrum following addition of DDT to nerve homogenates or even bovine serum albumin.

DDT itself has little native fluorescence (*11*), though proteins do fluoresce. Appearance of a new peak in the fluorescence spectrum beyond the sum of these two components could be interpreted to indicate the formation of new DDT-complex(es) or production of other compounds which could be secondarily produced by DDT poisoning. Another possibility is that DDT can be metabolized by the nerve to yield a metabolite which happens to possess native fluorescence. Inspection of the structure of DDT does not suggest that there can be such fluorescent metabolites, and also it is not likely that such compounds could be enzymatically produced in sufficient quantity within the short period required for the assessment of the spectra, for they were recorded immediately after mixing DDT and the nerve components.

Furthermore, the chromatographic evidence suggests that DDT is unaltered. Though the finding of fluorescence could not prove, alone, the presence of the newly formed DDT complex(es) in the mixture, the rapidity of the peak appearance upon the addition of DDT to the nerve components—e.g., Sephadex fractions—is in harmony with the authors' previous view that the DDT molecule must have combined with a nerve component to form a new complex, for such complexes should form almost instantly.

Although the results of the cation transport experiments clearly indicate that DDT indeed interrupts normal ion transport in vivo, its implications should be considered with utmost caution. The effect of DDT in vivo appeared to be most significant in the potassium transport system. In the first 20-minute period of diffusion, 16% of radioactive K^+ remained in the nerve cord of the DDT-poisoned cockroaches compared with 45% in the untreated ones. At the end of the initial one-hour period, the figure became 6 and 25%, respectively. This could mean that the DDT-poisoned nerve exchanged more K^+, or else that the poisoned nerve discharged more K^+ without taking up nonradioactive K^+. The results of in vitro experiments indicate that the DDT-treated nerve cord took up as much K^+ as the untreated controls. The balance of evidence, therefore, indicates that DDT-treatment caused a drastic increase of K^+ efflux and no change in K^+ influx. As a whole, the results with isolated nerve cords were much less clear-cut than those in vivo, for the K^+ exchange of isolated nerve cords in the presence or absence of DDT was not markedly different. The cause of the difference may be that the isolated nerve cords in this particular experiment were DDT-treated simultaneously with measurement of ion movement, whereas in the in vivo experiments the intact cord was exposed to DDT before ion measurement. Similar differences were seen in Na^+ transport. The DDT-poisoned nerve cord took up twice as much Na^+ as the untreated in vivo, though the DDT-treated isolated nerve cord behaved in an identical manner to the untreated. Possibly the DDT-treatments with isolated nerve cords were milder ($1 \times 10^{-5} M$, simultaneous with recording, or 15 minutes prior to recording) than that in vivo ($3 \times 10^{-4} M$, assuming weight of whole body in grams equal to volume in milliliters, for 1 hour preinhibition).

Conclusions

DDT combines with components of cockroach nerve; ultraviolet and fluorescent data suggest that the combination involves formation of a charge-transfer complex and simultaneous profound interference with K^+ efflux. It remains to be proved that these processes are causally related, or are directly related to disruption of nervous activity observed in DDT poisoning.

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ANTHELMINTIC RESIDUES

A Photofluorometric Method for the Determination of Maretin (*N*-Hydroxynaphthalimide Diethyl Phosphate) Residues in Animal Tissues

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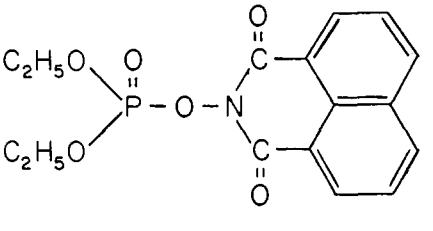
A sensitive method has been developed for the estimation of microgram quantities of Maretin (formerly B 9002, *N*-hydroxynaphthalimide diethyl phosphate) in animal tissues. The pesticide is treated with alkali to give a product which is measured fluorometrically. The sensitivity of the method, based on the observed values for untreated controls, is 0.1 p.p.m.

MARETIN, *N*-hydroxynaphthalimide diethyl phosphate, is an anthelmintic developed by Farbenfabriken Bayer A.G. of Leverkusen, Germany. The purified compound, a yellow crystalline powder melting at 179° to 181° C., is quite unstable to alkaline hydrolysis. Its structural formula is as follows:



Giang (*7*) has a fluorometric method for Bayer 22408 (the sulfur analog of Maretin). The fluorescence was measured following addition of methanolic sodium hydroxide and a dilute solution of hydrogen peroxide in dioxane. Because of the similarity of the two compounds, the same type of behavior was