Binding of 1,1,1-Trichloro-2,2-di-*p*-chlorophenylethane (DDT) with Subcellular Fractions of Rat Brain

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DDT (1,1,1-trichloro-2,2-di-*p*-chlorophenylethane) and DDE (1,1-dichloro-2,2-di-*p*-chlorophenylethylene, a non-insecticidal analogue of DDT) were found to bind with various nerve components of rat brain. The amount of DDT binding exceeded that of DDE only in the fraction containing mainly the nerve endings. Among various components in the nerve-ending fraction, a subfraction containing mainly the pre- and post-synaptic complexes had the highest affinity for DDT in comparison with that for DDE. By using an 'acetone-powdering' technique on brain preparations, the Sephadex-column method was shown to provide reliable results for studies on the binding of DDT or DDE with soluble proteins in the nerve tissues. From these results it was concluded that DDE had a higher affinity for soluble components of the rat brain than did DDT.

The actual poisoning mechanisms of DDT* in animals are not fully understood. It is known that it attacks the central nervous system of animals and it disrupts the ion-transport mechanism by simultaneously blocking the systems involving Na⁺ efflux and K⁺ influx in nerve cells (Narahashi & Haas, 1967; Matsumura & O'Brien, 1966a).

It appears to be the intact DDT molecule itself that reacts with the nervous system to cause poisoning, for researchers have recovered only unchanged DDT from the nervous system of poisoned insects (Matsumura & O'Brien, 1966a; Eaton & Sternburg, 1967b).

The chlorinated hydrocarbon insecticides belong to the unique group of chemically unreactive insecticides, this being attested by the fact that they are so persistent in various environments. The extreme stability of such compounds makes it difficult to explain their biochemical modes of action. A common characteristic of these chlorinated insecticidal compounds is that they have high affinities for electrons (O'Brien & Matsumura, 1964; Soloway, 1965).

In view of the chemical properties of chlorinated hydrocarbon insecticides, an effort has been made (O'Brien & Matsumura, 1964; Matsumura & O'Brien, 1966a,b) to relate the insecticidal ability to their abilities to form complexes with neural

materials by using the American cockroach (*Periplaneta americana*). These authors have experimentally demonstrated that: (1) DDT becomes associated with various nerve components; (2) apparent bound DDT can be eluted with a protein peak from Sephadex G-50 columns; (3) apparent binding involved a bathochromic shift in the u.v. spectrum.

Subsequent studies by Hatanaka, Hilton & O'Brien (1967) could not, however, confirm the presence of DDT-protein complexes in the supernatant from the nerve homogenates of the rat and the cockroach. The problems encountered during the purification attempts were: (1) the quantitative reproducibility of Sephadex G-50 columns was low, possibly owing to the presence of natural detergents; (2) electrophoresis attempts failed to cause any of the DDT-protein complexes to migrate; (3) DDE, a non-insecticidal analogue of DDT, also can form apparent complexes with protein.

Problems (1) and (2) do not actually deny the possibility of the formation of true DDT-protein complexes, since neutral proteins in the presence of large amounts of lipids would be expected to behave in such a manner. Problem (3) was a rather difficult one to reconcile, since, if such a protein plays an important role in DDT poisoning, it should clearly bind with DDT but not so much with its non-insecticidal analogue DDE.

The purpose of this paper is to report progress made in our laboratory on this subject by using rat brain preparations.

* Abbreviations: DDT, 1,1,1-trichloro-2,2-di-*p*-chlorophenylethane; DDE, 1,1-dichloro-2,2-di-*p*-chlorophenylethylene.

EXPERIMENTAL

Fractionation procedures. The initial centrifugal technique (Table 1) adopted to separate subcellular components was that of Marchbanks, Rosenblatt & O'Brien (1964), who modified the method of Whittaker (1959).

In a typical experiment one half of a male rat brain (the cerebral hemisphere and hypothalamus regions only) was homogenized with 8 ml. of 0.32 M-sucrose at 0° in a Potter-Elvehjem-type homogenizer with a Teflon pestle (clearance 0.064 mm. at 25°). A 5 ml. portion of the homogenate was directly incubated at 24° for 10 min. with 25 nmol of [¹⁴C]DDT (1,1,1-trichloro-2,2-di-*p*-chloro[¹⁴C]phenylethane) (approx. 200 000 c.p.m.), which was added to the homogenate in 25 μ l. of acetone to make the final concentration 5 μ M. Similarly the remaining half of the same brain was homogenized and incubated with the same amount of [¹⁴C]DDE (1,1-dichloro-2,2-di-*p*-chloro[¹⁴C]phenylethylene). The homogenates were cooled to 0° and centrifuged for 10 min. at 1000 *g* to obtain the crude nuclear fraction. The supernatant portions were further centrifuged at 18 000 *g* for 30 min. to obtain the crude mitochondrial fraction from which the mitochondria, the nerve-ending particles and the myelin fragment were separated by the discontinuous-sucrose-density-gradient centrifugation technique described by Marchbanks *et al.* (1964). The supernatant portion of the crude mitochondrial fraction was further centrifuged at 30 000 *g* for 90 min. to obtain the cell-membrane fraction and the supernatant fraction. The particulate components from the density-gradient experiments were diluted with water to bring the final concentration of sucrose to approx. 0.32 M. The suspensions were centrifuged again and the sediments were resuspended in 5 ml. of 0.32 M-sucrose. A 0.5 ml. sample from each fraction was transferred to a 20 ml. counting vial with 10 ml. of liquid-scintillation phosphor, which was prepared by dissolving 5.5 g. of 2,5-diphenyloxazole and 300 mg. of 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene in a solvent mixture of 500 ml. of toluene and 500 ml. of ethylene glycol monomethyl ether. Quenching effects caused by the presence of the organic matter were corrected when necessary by the addition of known amounts of [¹⁴C]DDT to identical preparations of subcellular fractions and comparing the radioactivity with the standard radioactivity in 10 ml. of liquid phosphor with 0.5 ml. of 0.32 M-sucrose solution. The total recoveries were checked for each experiment to ascertain the validity of the comparison between DDT and DDE experiments (Matsumura & Hayashi, 1967).

To fractionate further the components of the nerve-ending particles, the method developed by De Robertis and his associates (see De Robertis, 1967) was adopted. The crude mitochondrial fraction was first subjected to osmotic-shock treatment (Rodriguez de Lores Arnaiz, Alberici & De Robertis, 1967). Only the fractions that sedimented between 0.9 M- and 1.2 M-sucrose ($M_{1.0}$ and $M_{1.2}$ fractions) were collected, and these were further treated with Triton X-100 (Fischer & De Robertis, 1967) to obtain a fraction containing the pre- and the post-synaptic complexes. All fractions thus obtained were examined under an electron microscope to ensure the identities of the preparations. To study the binding phenomena of DDT and DDE in the nerve-ending fractions (Table 2, last three lines) the compounds were added directly to each purified nerve-ending fraction as follows: the combined $M_{1.0}$ and $M_{1.2}$ fractions

from two whole rat brains were diluted threefold with water to lower the sucrose concentration to approx. 0.32 M and the suspension was centrifuged at 20 000 *g* for 1 hr. The sediment was resuspended in 6.0 ml. of 0.32 M-sucrose in a Potter-Elvehjem homogenizer (on average, the protein content became 0.5 mg./ml.). A 1 ml. portion of this suspension was incubated with each radioactive insecticide (5 nmol each, added with 5 μ l. of acetone) at 24° for 10 min. in a 5 ml. glass centrifuge tube. The system was immediately centrifuged at 20 000 *g* for 1 hr. at -3°. The supernatant was saved and the precipitate was further suspended in 2 ml. of 0.32 M-sucrose. The suspension was centrifuged again and the precipitate was resuspended in 2 ml. of 0.32 M-sucrose. Both supernatants were combined, and 0.5 ml. portions of the combined supernatant and the suspension of the precipitate were sampled for radioassay. The protein contents of the samples were monitored throughout by the use of a Folin-Ciocalteu method. The acetone-dried-powder preparations were obtained from the whole brain or from $M_{1.0}$ and $M_{1.2}$ fractions. The former preparation was made from one half of a rat brain by using reagent-grade acetone at -15° and the powder was extracted with 6 ml. of saline solution (Yamasaki & Narahashi, 1960). Similarly the acetone-dried-powder was prepared from $M_{1.0}$ and $M_{1.2}$ fractions (four rat brains yielded about 200 mg. of dry powder). To study the binding of insecticides to the insoluble portion (precipitate, Table 2) of the acetone-dried-powder suspension, acetone-dried-powder was suspended in 0.32 M-sucrose at a concentration of approx. 10 mg./ml. in a small Potter-Elvehjem homogenizer. A 1 ml. portion of this suspension was incubated at 24° for 10 min. with 5 μ M-[¹⁴C]DDT or 5 μ M-[¹⁴C]DDE (5 nmol each, added with 5 μ l. of acetone). The centrifugal methods used to collect the precipitate and to assay the amount of the insecticide bound were identical with those employed for the nerve-ending fractions.

The [¹⁴C]DDT (specific radioactivity 4.5 mc/m-mole) was obtained from The Radiochemical Centre, Amersham, Bucks. The [¹⁴C]DDE was prepared from the above [¹⁴C]DDT (Hatanaka *et al.* 1967) by refluxing it in ethanolic 0.5 M-KOH for 20 min. Sephadex G-50 and G-200 were obtained from Pharmacia Co., Uppsala, Sweden. The Sephadex-column separation method for bound DDT was similar to that of Matsumura & O'Brien (1966b). In a typical run 20-30 mg. of acetone-dried-powder (either from the whole brain or from the nerve-ending fraction) was suspended in saline solution and homogenized for 3 min. at 0° in a small Potter-Elvehjem (Teflon-glass) homogenizer. The homogenate was centrifuged at 20 000 *g* for 30 min. A 1 ml. portion of the resulting supernatant was incubated with (final concentration) 10 μ M-[¹⁴C]DDT or 10 μ M-[¹⁴C]DDE at 24° for 10 min. The sample was then quantitatively transferred to the desired Sephadex column by rinsing the reaction tube repeatedly with small amounts of saline solution immediately after the initial transfer of the sample. The samples were then eluted first from Sephadex G-50 columns (30 cm. $\times$ 1 cm.; two identically prepared columns for DDT and DDE) with saline solution at 4° (in a cold-room); 2 ml. eluate fractions were collected. The u.v. absorption and radioactivity of the eluate fractions were recorded. The portions that contained radioactive material were pooled, concentrated by the aid of Sephadex G-50 (coarse grade) (batch method) to yield 3-4 ml. of sample solutions and further analysed on Sephadex G-200 columns (90 cm. $\times$ 1.2 cm., other chromatographic conditions as

Table 1. *Distribution of [¹⁴C]DDT and [¹⁴C]DDE among the subcellular fractions of rat brain*

The results are expressed as nmoles of [¹⁴C]DDT or [¹⁴C]DDE absorbed/mg. of protein, and are given as means $\pm$ s.e.m. of four independent experiments. Each insecticide (25 nmoles) was incubated for 10 min. at 24° with 5 ml. of brain homogenate containing one-third of a male rat brain. The subcellular fractions were separated by centrifugal methods (Marchbanks *et al.* 1964; Whittaker, 1959) and the specific binding of the insecticide to each component was assayed. Total recoveries: 101.1% for DDT, 100.2% for DDE.

Fraction	Absorption (nmole/mg. of protein)		
	DDT	DDE	Difference (DDT - DDE)
Supernatant	0.168 $\pm$ 0.042	0.197 $\pm$ 0.043	-0.029
Cell membrane	0.394 $\pm$ 0.064	0.424 $\pm$ 0.095	-0.030
Myelin fragment	0.536 $\pm$ 0.062	0.506 $\pm$ 0.078	0.030
Nerve-ending particles	0.605 $\pm$ 0.054	0.537 $\pm$ 0.021	0.068
Mitochondria	0.197 $\pm$ 0.126	0.263 $\pm$ 0.051	-0.066
Nucleus	0.569 $\pm$ 0.053	0.622 $\pm$ 0.053	-0.053

Table 2. *Binding of [¹⁴C]DDT and [¹⁴C]DDE to the precipitate components from various nerve fractions of rat brain*

For binding tests a 1 ml. portion of each nerve component in 0.32 M-sucrose was incubated with 5 nmoles of radioactive DDT or DDE at 24° for 10 min. It was then centrifuged at 20000 g for 1 hr. The precipitate was washed once with the same medium, recentrifuged and assayed for the amounts of bound [¹⁴C]DDT or [¹⁴C]DDE. The results are expressed as nmoles of DDT or DDE recovered in the precipitate portions and are given as means $\pm$ s.e.m. of the numbers of experiments indicated.

Source	Treatment	Amount (nmoles) absorbed			No. of experiments
		[¹⁴ C]DDT	[¹⁴ C]DDE	Difference (DDT - DDE)	
Whole brain	Acetone-dried-powder suspension	4.62 $\pm$ 0.01	4.52 $\pm$ 0.03	0.10	4
Nerve endings	Acetone-dried-powder suspension	4.55 $\pm$ 0.05	4.44 $\pm$ 0.04	0.11	4
Nerve endings	Osmotic-shock precipitate	4.54 $\pm$ 0.02	4.23 $\pm$ 0.07	0.31	8
	Triton X-100 precipitate	3.93 $\pm$ 0.07	3.37 $\pm$ 0.16	0.56	8
	Triton X-100 precipitate, DDE-DDT-pretreated*	3.99 $\pm$ 0.04	3.30 $\pm$ 0.21	0.69	8

* Pretreated with non-radioactive 5 μ M-DDT or 5 μ M-DDE (10 min. preincubation) and then incubated with 5 μ M-[¹⁴C]DDE (DDT-pretreated tube) or 5 μ M-[¹⁴C]DDT (DDE-pretreated tube) for an additional 10 min. at 24°.

before). The same Sephadex experiments were also applied on the neural materials from the American cockroach.

RESULTS

Table 1 is a brief summary of the results from matched DDT-DDE comparison tests (i.e. each matched test for DDT and DDE was on the same brain) under identical experimental conditions. The amount of DDT binding to the supernatant fraction was no more than that of DDE, whereas more DDT than DDE was found in the nerve-ending fraction. The above experiment revealed two important facts: first, the soluble fraction, which has been studied by both groups (Matsumura & O'Brien, 1966b; Hatanaka *et al.* 1967), may not contain the target substance; secondly, DDT is preferentially absorbed by material(s) that is present in the fraction containing largely the nerve-ending particles.

An attempt was made first to solubilize this material from the whole brain or from the nerve-ending fraction by preparing an acetone-dried powder from these neural materials and extracting it into saline solution. The results shown in Table 2 indicate that the acetone-powdering technique did not solubilize the specific DDT-binding substance from either material used. The difference in binding between DDT and DDE remained constant in these two sets of experiments.

To relate the above findings to those obtained by Hatanaka *et al.* (1967) Sephadex-column experiments were conducted on acetone-dried-powder extracts (soluble portions only). Sephadex G-50-column experiments with the acetone-dried-powder extract from the whole brain indicated clearly that: (1) there was only one radioactive peak for each insecticide at an identical peak position (retention volume 40 ml.) that matched with the major protein

peak; (2) in the supernatant fraction DDE binding/mg. of organic matter was approx. 17% higher than that of DDT. The reproducibility of the Sephadex G-50-column separation on the acetone-dried-powder preparations was good, possibly owing to the removal of naturally occurring detergents and lipids by acetone washing. For example, the results of four independent Sephadex G-50 experiments indicated that the standard errors for the specific binding of DDT and DDE (i.e. radioactivity eluted/ μ g. of protein) did not exceed 13% of the corresponding mean values (6.0 ± 0.7 and 7.0 ± 0.9 c.p.m./ μ g. of protein respectively). The eluates corresponding to proteins bound to radioactive DDT from the whole brain were combined and further examined on a Sephadex G-200 column. The resulting chromatogram (Fig. 1) indicated that DDT was bound only to the major protein peak. The same experiment was repeated with acetone-dried-powder extract prepared from 20 heads and 20 nerve cords of the American cockroach to relate the above findings to those obtained previously (Fig. 2). Although the insect nerve preparation appeared to contain more non-specific proteinaceous contaminants, the patterns of binding in these two preparations were remarkably similar.

Attempts were made to locate the sites of DDT binding within the precipitate of nerve-ending fractions by using an ultracentrifugal method developed recently (De Robertis, 1967; Rodriguez de Lores Arnaiz *et al.* 1967) to separate various neural components of the nerve-ending fraction. In practice both $M_{1.0}$ and $M_{1.2}$ fractions were combined to obtain the osmotic-shock-treated fraction of nerve-ending particles (synaptic complexes without synaptic vesicles). The same fraction was further treated with Triton X-100 to obtain a fraction con-

taining the pre- and post-synaptic regions of the complexes.

The results shown in Table 2 indicated that these treatments decreased the actual amounts of DDT (by 12.6%) and DDE (by 9.2%) bound to the precipitate, but increased the difference between DDT and DDE, possibly owing to the elimination of materials causing non-specific absorption of DDT and DDE. Since this treatment by Triton X-100, on the average, decreased the protein content of the precipitate by 65% and acetylcholinesterase by 50%, there was an increase in the amount of DDT and DDE bound/mg. of protein.

To study the specificity of the binding of DDT and DDE, two batches of the Triton X-100-treated fractions were pretreated with non-radioactive $5 \mu\text{M}$ -DDT or $5 \mu\text{M}$ -DDE. After 10 min. $5 \mu\text{M}$ - ^{14}C DDT was added to the batch pretreated with non-radioactive DDE, and similarly $5 \mu\text{M}$ - ^{14}C DDE was added to the batch pretreated with non-radioactive DDT. The result of this competition test (Table 2) indicated that the difference in binding of DDT and DDE had been affected only slightly by the above treatment. When the same preparation was pretreated with non-radioactive DDT (5 nmoles/ml.) instead of DDE and then incubated further with ^{14}C DDT (5 nmoles/ml.), the amount of DDT binding was 3.69 ± 0.19 nmoles (six experiments) compared with 3.99 ± 0.04 nmoles for the DDE-pretreated samples (Table 2, last line), showing that at least a small portion of the binding sites for ^{14}C DDT (approx. 6% of given ^{14}C DDT) had already been occupied by non-radioactive DDT. Even when the amount of non-radioactive DDT was increased to 15 nmoles/ml. the amount of subsequent ^{14}C DDT binding to the precipitate remained constant at 3.69 nmoles, indicating that the excess of DDT occupied only the non-competitive sites. A similar increase in the amount of DDT during

Fig. 1. Sephadex G-200 elution pattern of ^{14}C DDT-protein complex from the acetone-dried-powder extract of rat brain. The sample was precleansed through a Sephadex G-50 column before Sephadex G-200 analysis. $\circ$, Radioactivity; $\bullet$, protein.

Fig. 2. Sephadex G-200 elution pattern of ^{14}C DDT-protein complex from the acetone-dried-powder extract of American-cockroach nervous system. Conditions are identical with those shown in Fig. 1. $\circ$, Radioactivity; $\bullet$, protein.

pretreatment (15nmol/ml.) did not influence the amount of subsequent [^{14}C]DDE binding. It appeared that the binding sites for DDT and DDE were different from each other or that they were largely non-specific or both.

All the above experiments indicate that the pre- and the post-synaptic regions of the nervous system play an important role in DDT binding, and that the difference in the amounts of DDT and DDE binding increase with treatments that attempt to purify this synaptic region.

DISCUSSION

Electrophysiological observations supporting the importance of synaptic junctions in the phenomenon of DDT poisoning have been reported by Eaton & Sternburg (1967a), who found that: (1) there was a direct relationship between stage of poisoning and the degree of synaptic impairment in the thoracic ganglia of the American cockroach; (2) the magnitude of the effect of low temperature on synaptic disruption by DDT coincided with the 'negative temperature coefficient' of DDT poisoning. All other neurophysiological symptoms, such as effects on the sensory endings, appeared to be correlated positively with the external temperature, suggesting that they were not vitally related to the mode of DDT poisoning.

Further evidence in support of the importance of the synaptic junctions for the process of DDT poisoning is the accumulation of acetylcholine during DDT poisoning (Colhoun, 1960) and the finding that cholinesterase is protected from organophosphate inhibition in DDT-poisoned insects (Sternburg & Hewitt, 1962).

The following interpretation is given to the competition tests. It must be first pointed out that the DDT concentration used in this experiment (5 μM) is just above the minimum threshold concentration to elicit a typical electrophysiological poisoning symptom of DDT in the intact central nerve cord of the cockroach, and that an increase of DDT concentration beyond this point up to 50 μM does not actually intensify the poisoning symptom, nor does it shorten the time required to trigger the initial electrophysiological symptom (Narahashi & Yamasaki, 1960; C. M. Wang & F. Matsumura, unpublished work). Undoubtedly a large portion of this amount (5nmol/ml.) of DDT would be absorbed by non-specific neural matter other than the target site *in vivo*, and therefore it can be assumed that only a small portion of DDT is required to occupy the actual target site. Matsumura & O'Brien (1966b) already indicated that the pattern of DDT binding, in terms of relationship between the given DDT concentrations and the amounts of DDT absorbed by the isolated central nerve cords

and by the homogenate of the nerve cords, showed two plateaus, one at 0.3–3 μM and the other at 20–50 μM . In view of the above electrophysiological evidence, it is possible to estimate that it is the former plateau that is related to the binding phenomenon with the actual target site (about 0.3nmol/ml. or 6% of 5nmol/ml.). The results of the competition experiments in this study at least demonstrated that pretreatment of the synaptic complex[†] (Triton X-100-treated) with non-radioactive DDT decreased the extent of subsequent [^{14}C]DDT absorption by about 6%, whereas the same pretreatment by non-radioactive DDE did not cause such a decrease in the [^{14}C]DDT absorption, indicating that the site of binding of DDE may not be the same.

The nature of the specific binding substance(s) is not known at this stage. Since acetone extraction during the preparation of acetone-dried powder did not remove this binding substance, the possibility of it being a simple free lipid or cholesterol is ruled out. The substance appears to be proteinaceous and it is tightly bound to the structural protein of the pre- and post-synaptic region of the nerve cells.

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