The Apparent Binding of DDT to Tissue Components

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Sephadex chromatography of tissue homogenates incubated with C^{14}-DDT provided apparent evidence of binding of DDT with cockroach nerve cord and rat liver, muscle, and brain. Binding was not localized in any particular subcellular fraction of brain. The nontoxic analogs DDE and 1,1,1,2-tetrachloro - 2,2 - bis(*p* - chlorophenyl)ethane were equally effective in such binding. Triton X-100 was more effective than tissue homogenates in permitting passage of DDT through Sephadex. Electrophoresis of brain preparations showed no migration of DDT with any fraction. Apparently, evidence for binding based on Sephadex is inadequate in the case of DDT and related compounds.

Previous studies on DDT (Matsumura and O'Brien, 1966a, b) and dieldrin (Matsumura and Hayashi, 1966) have suggested that these compounds can form complexes with components of insect nerve, thereby raising the possibility that this complexing is related to the toxic action of the compounds. A part of the evidence in the above studies was that, after incubation of DDT with nerve tissue homogenates, the DDT would pass through a Sephadex column. The present study shows that such passage can also be obtained by incubation with nonnervous tissues and with detergent and is found with nontoxic analogs of DDT. Electrophoretic studies show no evidence for complex formation. Consequently, the plausibility of a connection between complex formation and toxicity requires re-examination.

MATERIALS AND METHODS

The following tissues were used: the nerve cord, excluding the first thoracic and last abdominal ganglia, of adult male cockroaches, *Periplaneta americana*, and the liver, femoral muscle, and brain of female Holtzman rats. For brain, a vertical slice of about 200 mg. was removed, the rostral cut being just behind the olfactory bulb and the caudal cut being at the middle of the cerebrum.

Method I. Tissue was weighed on an analytical balance and homogenized in 100 times its weight of a solution prepared by adding 1 ml. of $1.05 \times 10^{-3}M$ C^{14}-DDT (4.93 mc. per mmole) in 95% ethanol to 99 ml. of Ringer solution (Matsumura and O'Brien, 1966a). The homogenizer, a Teflon and glass Potter-Elvejhem type, was cooled in ice water during homogenization. The homogenate was held at 25° C. for 10 minutes, then 2 ml. were added to a 30 × 1 cm. column of Sephadex G-50 coarse grade and washed in with three 1-ml. rinsings from the homogenizer. The column was then eluted with 25 ml. of 0.9% NaCl at a flow rate of 1 ml. per 45 seconds followed by 25 ml. of ethanol. One milliliter fractions were collected and 0.5 ml. of each was added to 10 ml. of dioxane counting solution (Hayes, 1962) and counted in a Tri-Carb scintillation counter. In early experiments, the other 0.5 ml. was diluted with 2.5 ml. of water and absorption measured at 280 mμ for organic matter.

The recovery of added DDT was not measured. Extensive extraction with alcohol was needed to remove free DDT, so there would be no assurance that one was not removing both free DDT and other forms incapable of passing the column in aqueous phase. Furthermore, the amount left on the column was extremely large compared with that which could be bound to tissue components, so that apparently complete recoveries would not be very significant—i.e., would not give assurance that there were no column-adsorbed fractions other than free DDT.

Method II. This method was used when homogenization in C^{14}-DDT was not possible—e.g., when subcellular fractionation was to be performed. A 1% homogenate in the above Ringer solution was made, then 1.8 ml. were mixed with 0.2 ml. of a solution prepared by adding 1 ml. of $1.05 \times 10^{-3}M$ C^{14}-DDT in 95% ethanol to 9 ml. of Ringer solution. These 2 ml. were rehomogenized at 0° C., then incubated at 25° C. for 10 minutes, passed through Sephadex as above, and collected either as 1-ml. fractions of which 0.5 ml. was counted (Method IIa) or as a single 20-ml. portion combining fraction 1 through 20 (Method IIb), or as a single portion, combining fractions 9 through 14, which proved to be the single radioactive peak eluted by NaCl (Method IIc). In Methods IIb and IIc, 1-ml. portions were counted.

Cell Fractionation. A slice of rat brain, taken as described above, was homogenized with 100 volumes of $0.32M$ sucrose in $0.067M$ phosphate buffer (pH 7.2). The whole homogenate or the fractions prepared from it were studied by Method IIa. Forty milliliters of the whole homogenate were centrifuged in the cold (Spinco L2) at 1000 × G for 10 minutes and the precipitate ("nuclear fraction") was suspended in the original volume of buffered sucrose. By further centrifugation, the following were prepared: "mitochondrial fraction" at 18,000 × G for 30 minutes, "microsomal fraction" at 100,000 × G for 60 minutes; the "soluble fraction" was the supernatant from this final centrifugation. The first centrifugation step was carried out in 45-ml. tubes, the subsequent in 13-ml. tubes.

Electrophoresis. Some early experiments, which were performed in an uncooled paper-electrophoretic apparatus (Kobayashi, 1954) gave artifactual results, so that even uncharged materials migrated, owing to buffer movement by evaporative loss. The EC 401 water-cooled horizontal electrophoresis apparatus was then used with paper. However, a serious problem was encountered in that the Teflon separating sheets adsorbed large amounts of C^{14}-

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DDT, leading to losses of up to 80% of applied radioactivity; a part of the radioactivity could be washed from the Teflon with acetone. The problem was solved by replacing the Teflon with two sheets of window glass. However, resolution of proteins in brain homogenates or commercial purified protein preparations was extremely poor. Gel electrophoresis using Cyanogum 41 acrylamide gel in an EC 401 horizontal pressure plate apparatus also gave very poor resolution of brain proteins.

Vertical gel electrophoresis was successful in resolving these proteins: The EC 470 vertical electrophoresis apparatus was used with the above gel; the buffer at pH 9.33 contained 3.46% tris(hydroxymethylamino)methane and 0.53% glycine; the applied field of 300 volts at about 7° C. gave a current of from 90 to 120 ma. A solution of 1% pure protein (solubilized where necessary with 10% NaCl) or 20% brain homogenates in $10^{-4}M$ DDT in Ringer was saturated with sucrose, and 20 µl. were applied. In any run, eight such samples were used, and to two a trace of bromphenol blue was added as a marker. After electrophoresis, the gel was sliced. One slice was cut into 1-cm. portions, placed in the dioxane counting solution, let stand for 2 days, then counted. Recovery of added radioactivity was 65%. The remainder of the gel was stained with amido black and destained for several days with 7% acetic acid to determine the extent of migration of the various components.

Synthesis. C^{14}-DDE. A vigorously stirred solution of 1 mg. of C^{14}-DDT (Nuclear Chicago, specific activity 4.93 mc. per mm.) in 10 ml. of 0.5*N* ethanolic KOH was heated for 3 hours at 85° C. in a 25-ml. microware flask fitted with a reflux condenser and drying tube. After removal of the ethanol by evaporation, the residue was taken up in 0.1 ml. of hexane, and C^{14}-DDE separated from C^{14}-DDT by thin-layer chromatography according to the method of Walker and Beroza (1963). The R_f values were 0.88 and 0.77, respectively, and corresponded to those obtained for unlabeled *p,p'*-DDE and *p,p'*-DDT. The appropriate spots were scraped off and extracted with $CHCl_3$. Filtration and evaporation of the extract gave a 73% yield of C^{14}-DDE of about the expected specific activity.

C^{14}-Labeled Cl-DDT [1,1,1,2-Tetrachloro-2,2-bis(*p*-chlorophenyl)ethane]. Chlorine gas was passed through a refluxing mixture of C^{14}-DDT in 2 ml. of CCl_4 and 0.1 ml. of PCl_3, while the reaction was irradiated with a 150-watt light placed 8 inches from the reaction flask. At the end of 1.5 hours, the mixture was cooled and the CCl_4 removed under reduced pressure. The residue was taken up in 0.1 ml. of $CHCl_3$, and Cl-DDT separated from unreacted DDT and by-products as in the preparation of C^{14}-DDE. In this case, DDT and Cl-DDT were detected by ultraviolet light and showed R_f values of 0.73 and 0.83, respectively. Extraction of the Cl-DDT with $CHCl_3$ and subsequent evaporation gave a 60% yield of the desired product with a specific activity close to theoretical.

RESULTS

Tissue Specificity. The binding of DDT was investigated by Sephadex Method I, in which tissue homogenate incubated with C^{14}-DDT was chromatographed. As previously reported for cockroach nerve cord (Matsumura and O'Brien, 1966a), for all tissues with NaCl elution, a single peak of radioactivity was eluted; subsequent elution with ethanol gave a second peak, shown to correspond to free DDT. Also in accordance with the above report with cockroach nerve cord, NaCl eluted an organic matter peak (or group of small peaks) with the radioactivity, and a subsequent larger broken peak of organic matter without radioactivity. With rat liver, a similar effect was seen except that the two organic peaks were of comparable size. With rat brain and muscle, clear organic peaks were not obtained, but instead numerous peaks (of dubious significance) were eluted throughout the NaCl elution.

Table I shows that the binding of DDT to tissues is not restricted to nervous tissue. Roughly comparable binding was found in cockroach nerve cord and in rat brain, liver, and muscle. Table I also demonstrates the large variation encountered in binding to rat brain preparations.

Subcellular fractionation was then applied to the rat brain homogenate, and each cell fraction was incubated with C^{14}-DDT and chromatographed (Method IIa). The subcellular fractions were very uniform in their response; duplicate runs gave binding (in nanograms of DDT per 20 mg. of tissue) of 59.0 and 59.0 for the whole homogenate, 14.2 and 13.7 for the nuclear fraction, 10.9 and 10.4 for mitochondria, 14.2 and 14.8 for microsomes, and 12.6 and 12.9 for soluble. The sum of the binding by the fractions was 88 and 87% of that found in the whole homogenate.

Compound Specificity. It would be evidence in favor of a crucial role of complex formation in toxicity if one could show that only DDT and toxic analogs bound to nervous tissue. Such was not the case; the nontoxic compounds Cl-DDT [1,1,1,2-tetrachloro-2,2-bis(*p*-chlorophenyl)ethane] and DDE complexed with rat brain as well or better than DDT, as judged by Sephadex fractionation (Table II). Once again, substantial variation between replicates was encountered and will be discussed below.

Use of Emulsifier. At this stage of the investigation, the variability encountered with brain homogenates became a concern. The day-by-day variation, though large, was

Table I. Binding of DDT to Homogenates of Various Tissues[a]

Tissue	Peak of Radioactivity Fraction No.	DDT in Peak Fraction, Ng.	Total DDT[b] Bound, Ng. per 20 Mg. Tissue
Cockroach nerve cord	11	29.5	106.0
	11	21.0	108.2
Rat liver	10	65.0	132.2
	10	47.5	130.0
	10	45.4	144.3
Rat muscle	10	14.2	30.6
	10	10.9	26.2
Rat brain	11	10.9	25.1
	11	10.0	19.7
	11	31.1	84.2
	11	33.9	84.2
	11	23.0	53.6
	11	33.9	84.2

[a] Binding was determined by Method I.
[b] Sum of all fractions in the single peak which was eluted from the column. DDT added to column was 7440 ng. and tissue was 20 mg.

Table II. Binding of DDT and Analogs to Rat Brain

Compound	Total Amount Bound, Ng. per 20 Mg. Tissue		
	Method I[a]	Method IIa	Method IIb
DDT	95.2, 66.8	48, 67, 63, 67	92.3, 93.7, 93.9, 93.6
Cl-DDT	212.8, 167.3	44, 65, 98, 157	.....
DDE	...	.....	86.0, 90.6, 90.6, 90.5

[a] The change in method was for simplification. In I and IIa, each fraction was counted; in IIb, the total radioactive peak was pooled and counted in duplicate. This accounts for the difference in number of decimal places. Comparisons between methods are not fully permissible because of variations in counting efficiency.

perhaps tolerable, but the variation over many months was very large. A similar finding was reported by Matsumura and Hayashi (1966) for dieldrin under similar conditions. A contributing factor might be that even in the presence of tissue, only about 1% of the added radioactivity is eluted. However, since virtually none is eluted in the absence of tissue, variability is not attributable to an inconstant background effect.

One possibility was that the variation was due to differences in brain composition of different rats. The standard slices were therefore halved, and right and left halves compared by Method IIb. The results (Table III) show that variation was large between animals and also between half-brains, but that excellent agreement was found when samples from the same homogenate were chromatographed, even on two different days. In fact, all the variation was probably due to minor variations in preparing the homogenate, for it is unlikely that brain halves really differed much in composition. One possibility was that there were variations in the degree of dispersion of the homogenates, and therefore the effect of adding a neutral emulsifier, Triton X-100 (a mixed alkyl aryl polyether alcohol) was studied. Addition of 1% Triton X-100 to the homogenate, followed by measurement of binding according to Method IIb, showed enormous increases in apparent binding. In three brain homogenates without Triton X-100, binding (expressed in all cases as nanograms of DDT per 20 mg. of tissue) averaged 180 (range 129 to 247). With 1% Triton X, the values of seven rats averaged 1098 (range 1024 to 1175). Lesser amounts of Triton X-100 were also effective, the averages and ranges on three animals each being 950 (927 to 972) for 0.5% and 883 (850 to 928) for 0.2% Triton X-100.

These large increases raised the possibility that emulsification might, even in the absence of brain, cause passage of DDT through the column. One could imagine that the original DDT "solution" normally contained aggregates too large for passage, but that emulsification might reduce them to a size small enough to pass but not large enough to be absorbed—as small molecules in true solution—on the Sephadex. And, indeed, 1% Triton X-100 alone could produce a large peak of C^{14}-DDT in the fractions normally containing the complex. Addition of 3124 ng. in 1% Triton X-100 produced a peak containing 149 ng. of DDT. These experiments also indicated that a potential source of variation was the age of the aqueous DDT solution. In an extreme case, Triton X-100 caused elution of 28% of a freshly prepared solution added at 21.3 μg. per column; but only 0.05% of a 120-day solution under the same conditions and concentrations.

Table III. Variation between Individuals and Half-Slices of Rat Brain[a]

Rat No.	Body Wt., G.	Half-Slice	Half-Slice Weight, Mg.	Ng., DDT Bound, Expt. 1	Ng. per 20 Mg., Expt. 2
1	261	Left	148	33	33
		Right	151	58	59
2	277	Left	120	40	40
		Right	116	28	26
3	244	Left	98	23	23
		Right	79	39	38
4	244	Left	118	78	81
		Right	100	46	46

[a] Method IIb was used.

Electrophoresis. The above findings on Triton X-100 raised the possibility that the phenomenon called "binding" above was an artifact of DDT emulsification. It was desirable to test independently whether or not complex formation truly occurred. A good criterion would seem to be that if DDT was bound to an ionizable macromolecule, then DDT should migrate with the macromolecule in an electric field. Experiments were therefore performed in which C^{14}-DDT was incubated with purified materials (α-globulin, β-globulins, lipoprotein, albumin, hemoglobin, and cholesterol, all from human blood) or horse serum or rat brain homogenate, and then submitted to electrophoresis.

Early studies with a simple paper electrophoresis device (see *Methods*) seemed at first to indicate that DDT could migrate with all of the above preparations, but only if 1% Triton X-100 were included. However, this was later shown to be an artifact, for migration was seen even in the absence of an electric field. With vertical gel electrophoresis, true migration of all the preparations was obtained, with excellent resolution of the multiple components, and in every case, DDT remained on the origin, whether or not Triton X-100 was included.

DISCUSSION

The primary outcome of this study is to throw doubt upon the validity of the Sephadex method for demonstrating that DDT forms stable complexes. Possibly Sephadex acts as a DDT filter, and emulsifiers, including natural tissue components such as lecithins, and also synthetic materials such as Triton X-100, serve to produce a DDT micelle small enough to pass this filter. This possibility is as plausible an explanation for apparent complex formation by dieldrin (Matsumura and Hayashi, 1966) as it is for DDT. The evidence for the filtration hypothesis is: that much variation occurs in different preparations of

essentially identical tissues, such as two halves of one brain; that Triton X-100 greatly increases the amount of DDT that passes through the column; that Triton X-100 by itself can mimic the effect of the tissues; that the tissue effects are not specific to any tissue nor to any compound; that DDT does not bind firmly to brain components or other miscellaneous macromolecules which migrate in an electric field; and that surfactants, both synthetic and natural (lecithin, taurocholate, digitonin, and egg-yolk lipoprotein) have the effect of "solubilizing" DDT and related compounds—i.e., dispersing them so that they are far more available for enzymic and nonenzymic reactions in aqueous phase (Lipke and Kearns, 1960).

Although the amount of emulsifier in different tissues undoubtedly varies, the radioactivity is eluted with a constant peak—i.e., always in fractions 10 and 11. This is probably because Sephadex columns do not serve to fractionate macromolecules above a certain limit, about 10,000 mol. wt. for Sephadex G50. Consequently, all micelles of 10,000-mol. wt. aggregate or more will emerge in one peak, corresponding to the void volume of the column. DDT has a mol. wt. of 354, so that the above aggregate corresponds to about 30 molecules of DDT, neglecting the contribution of the emulsifier to the micellar molecular weight.

True complex formation has not been actually disproved by the above experiments. The behavior on Sephadex could be caused both by emulsification and by complex formation, and this complex could perhaps not migrate in an electric field, either because it dissociates or because it is neutral. This possibility seems rather implausible on the basis of the data presented above, for the content of natural emulsifiers in the tissues is probably ample to account for the observed behavior on Sephadex. Nevertheless, the demonstration (Matsumura and O'Brien, 1966b) that Sephadex eluates of DDT–nerve cord mixtures can be subfractionated on DEAE–cellulose into two fully separated peaks deserves confirmation, because it seems unlikely (but possible) that there are two discrete families of emulsified DDT.

Other, quite different lines of evidence favor the existence of DDT binding. Equilibrium studies on whole cockroach nerve cords with DDT (Matsumura and O'Brien, 1966a) and on cockroach nerve homogenates with dieldrin (Matsumura and Hayashi, 1966) give results compatible with some form of binding. Addition of DDT to nerve cord homogenates gives a shift in the ultraviolet spectrum and also gives rise to new fluorescent emission bands (Matsumura and O'Brien, 1966b). Studies along such lines will probably be more useful in evaluating complex formation than will attempts to achieve physical separation by Sephadex or electrophoresis. The requirement for such physical separations to be successful is that either the dissociation constant of the complex be extraordinarily low, or that the dissociation step be extraordinarily slow. In all other cases, the complex will dissociate in the course of the physical separation, unless the separation is done with great speed.

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