Binding of ^{36}Cl-Dieldrin to Suspected Target and Non-Target Proteins *In Vitro*[1]

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The mode of action of dieldrin and other cyclodiene insecticides is not known, although it is generally believed they disrupt the nervous system. Since the suggestion by MULLINS (1955) that chlorinated hydrocarbon insecticides might exert their action by precise binding to nerves, various workers with the cyclodienes have attempted to substantiate this suggestion (MATSUMURA and HAYASHI 1969a, 1969b); (TELFORD and MATSUMURA 1970, 1971); (SELLERS and GUTHRIE 1971).

Dialysis has been employed to demonstrate binding of muscarone to particular fractions of homogenized Torpedo electroplax (O'BRIEN et al 1970), and of nicotine to extracts of housefly heads (ELDEFRAWI et al 1970). It appeared that this technique could be used to indicate quantitative relationships and also information about tissue specificity, and thus might shed some light on the mode of action of dieldrin.

METHODS and MATERIALS

American cockroaches (Periplaneta americana L.) were reared on a mixture of honey, glycerin, Gaines Dry Dog Chow (2:2:6, v/v), and an ample water supply. Albino rats, Rattus rattus (Sprague / Dawley strain), were fed Purina Lab Chow and given an ample supply of water. Both species were maintained at a constant temperature of 70± 5°F and low humidity (50± 10%) with a light regime of 8L:16D.

Dissection and Homogenization of Tissues. The spinal columns of the laboratory rats were excised, then cut so as to remove the spinal cord from just posterior of the medulla to slightly posterior of the lumbrosacral enlargement. Following excision, three spinal cords were washed with saline (YAMASAKI and NARAHASHI 1959),

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placed in a glass mortar with Teflon pestle , and homogenized over ice in 7 ml of saline for ten minutes. The crude homogenate was centrifuged for 30 seconds at 8000 rpm on a Sorval RC2-B refrigerated centrifuge to remove debris, after which the supernatant was decanted and quantitatively assayed for protein content (LOWRY et al 1951).

The same procedure was followed for the homogenization of the ventral nerve cord of P. americana. Each nerve cord was excised from the level of the second thoracic ganglion down to and including the sixth abdominal ganglion. Approximately 80 nerve cords were then homogenized, centrifuged, and assayed for protein.

Dialysis Technique. Proteins were diluted in saline to a variety of concentrations; this minimized any Donnan effects. The following proteins were studied:

1. Standard albumin-globulin (59%-albumin, 41% globulin) Mayer and Myles Laboratories, Allentown, Pennsylvania.

2. Bovine Serum Albumin - Sigma Chemical Co., St. Louis, Missouri.
   a. Fraction V. - plasma protein precipitated with ethanol.
   b. Fraction V. - same as above, but pretreated with ether-hexane (1:1, v/v) before dialysis.

3. Tissue Homogenates
   a. Rat spinal cord supernatant from 8000 rpm centrifugation (3 cords/7 ml).
   b. Cockroach ventral nerve cord supernatant from 8000 rpm centrifugation (80 cords/7 ml).

Dialysis tubing (Union Carbide) was cut into strips, prepared according to the method of HUGHES and KLOTZ (1956), and soaked in saline for several hours. Five ml of sample protein solution were placed in the dialysis bag and the top sealed off. The filled dialysis tubes were placed into 70 ml of saline containing ^{36}Cl-dieldrin (0.1622 uCi) at 10^{-5} molar concentration; the system was allowed to dialyze for thirty-six hours at 22°C with occasional shaking. Controls containing no dissolved protein in the dialysate were run simultaneously. At the end of 36 hours, samples from both the dialysis tube and the bathing saline were taken, counted in a liquid scintillation system (8 g/l of butyl PBD and 0.5 g/l of PBBO dissolved in analytical grade toluene), and averaged. Counts per minute from the controls were subtracted from the gross counts of the dialysis tube contents (dialysate), yielding net counts per minute as a measure of apparent dieldrin binding. All experiments were duplicated.

Protein Precipitation. To demonstrate that binding was occurring to protein and also to provide a clue to the nature of binding, the protein was extracted from the dialysates. Additional 1-ml samples were taken from the dialysis bag. One ml 10% TCA (trichloroacetic acid) was added to precipitate the protein. Controls showed that dieldrin in saline was not precipitated by

TCA. Each sample was centrifuged for 10 minutes at 1000 rpm and the supernatants assayed for protein content. All supernatants and precipitates were then extracted with ether-hexane (1:1, v/v). After evaporation of the organic solvents, the samples were prepared for scintillation counting (NEWMAN 1968). Samples were counted in a Nuclear-Chicago liquid scintillation counting system, model 6822, at an efficiency of 66% for the isotope ^{36}Cl. A confidence level of 90% or better for counts was maintained by counting the samples for twenty minutes.

RESULTS

Counts per minute of the control runs were consistent throughout the entire series of experiments (75 ± 10 cpm). Dieldrin recovered in the bathing saline was $61 \pm 7\%$ of the total amount added.

Albumin-globulin Dialysis. Binding of radio-labelled dieldrin could be plotted in a direct linear relationship against concentration of protein. When concentrations of the standard albumin-globulin were varied over one order of magnitude (2 - 20 mg/ml), dieldrin was bound to protein in the ratio of 0.534 nM of dieldrin per milligram of protein (Fig. 1). Lower concentrations of albumin-globulin (0.5 - 1.5 mg/ml) did not produce significant cpm above the controls.

Fraction V. Dialysis. Binding of dieldrin to the Fraction V. serum proteins (Fig. 1) was similar to that observed with albumin-globulin. The ratio of binding was calculated to be 0.511 nM of dieldrin per milligram of protein. The Fraction V. protein treated before dialysis with ether-hexane to remove any free lipids showed a similarity to the initial Fraction V. experiments. The ratio was calculated to be 0.422 nM of dieldrin per milligram of protein.

A statistical analysis was performed to fit regression lines to the experimental values and to test for goodness of fit among the lines. The Fraction V. proteins (non-treated and pretreated with ether-hexane) had slopes of 0.4226 and 0.5112, respectively, and both could be represented by one line having a slope of 0.4506 at the 95% confidence level. The regression line for albumin-globulin was found to be a separate and distinct line.

Neural Protein Dialysis. Dialysis of the neural homogenates produced a pronounced increase in binding over that observed with the albumin-globulin standard and the Fraction V. protein. With the centrifuged rat spinal cord homogenate, the same linear relationship between binding and protein concentration was noted except that dieldrin was bound to a greater extent (Fig. 2). An increase of 3.3 times (9.41 nM/mg protein) in binding was observed (Table 1). Because only a single concentration was run with the American cockroach nerve cord homogenate, a linear relationship could not be obtained. However, the two points were found to lie very near the plot obtained with the rat spinal cord homogenate. An average of 9.547 nM dieldrin were bound per mg protein cockroach nerve.

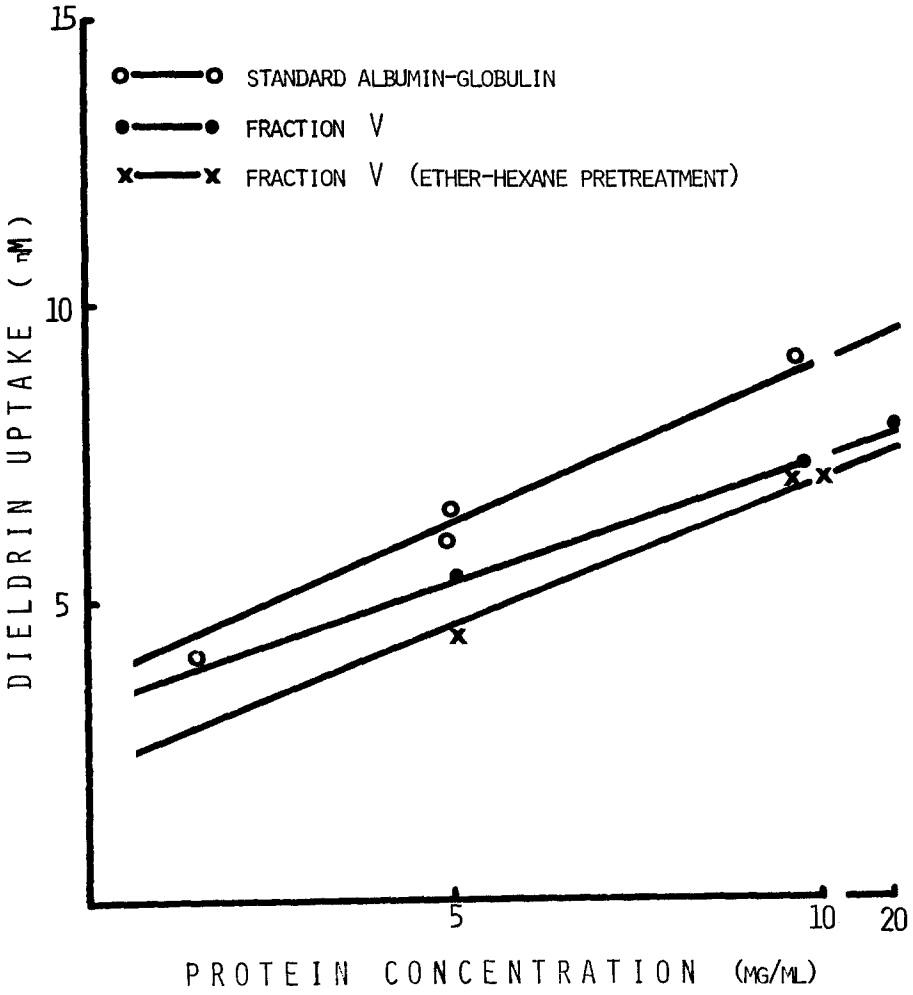

Figure 1. In vitro uptake of dieldrin onto bovine serum proteins as a function of protein concentration per 1 ml sample. Each point represents the average of six values from duplicate experiments. The lines were fitted by regression analysis.

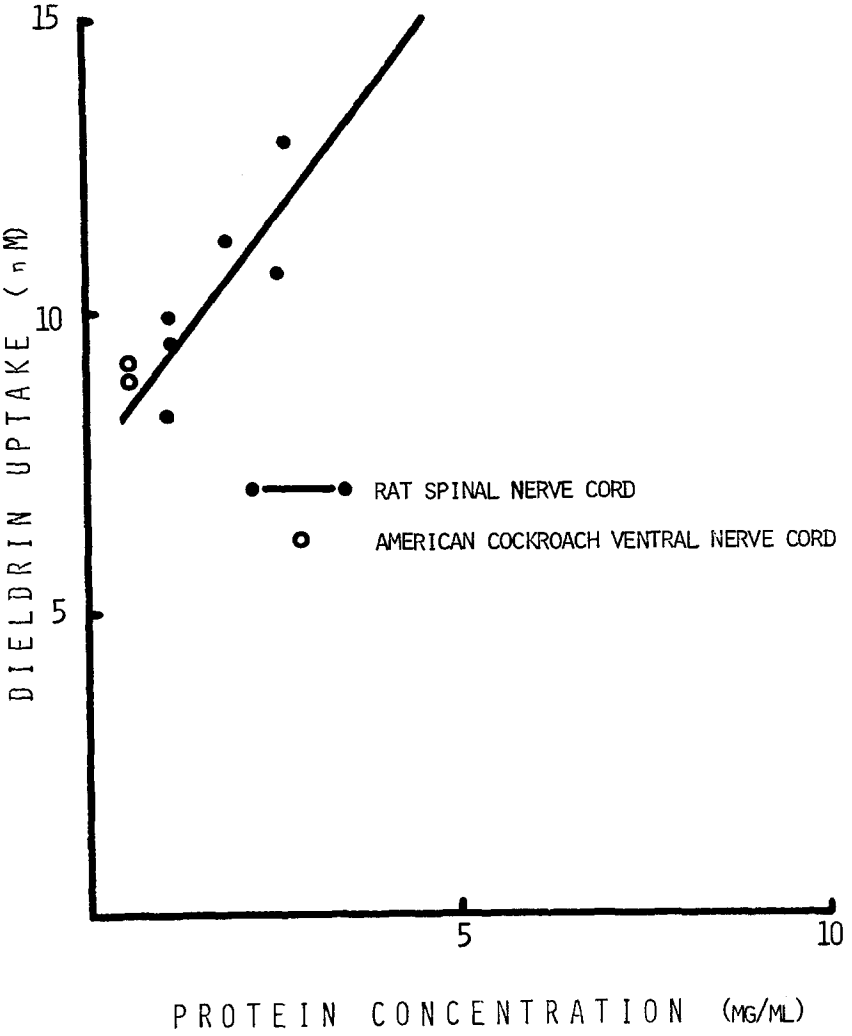

Figure 2. In vitro uptake of dieldrin onto nerve issue homogenates by dialysis as a function of protein concentration per 1 ml sample. Each point represents the average of six values from duplicate experiments. The line was fitted by regression analysis.

TABLE 1

Uptake of dieldrin as a function of protein concentration. Nanomoles of dieldrin bound per milligram of protein is calculated from $y = a + bx$, where $x = 1$ milligram of protein per ml of solution.

	Albumin-globulin	Fraction V.	Fraction V. (ether-hexane)	Rat Spinal Cord	Cockroach Nerve Cord
Slope	0.5340[a]	0.4226[a]	0.5112[a]	1.678	------
Variance about x	1.07	1.50	0.81	2.02	------
nM dieldrin per mg protein @ 1 mg/ml	4.20	3.50	2.53	9.41	9.55[b]

a No significant difference at the 95% confidence level
b Determined from an average of two replicates

Precipitation with TCA. The concentration of protein remaining in the supernatant after TCA precipitation of the nerve homogenates averaged 0.337 mg/ml. This value represents 8% of the total protein not precipitated with TCA, i.e., 92% of the total protein was precipitated by TCA.

Table 2 shows recovery of dieldrin activity following precipitation of protein with TCA, where the average percent recovery ranged from 72.3% to 104.2% of total activity of dieldrin. Little activity was found in the TCA supernatant. Activity was limited, for the most part, to the precipitated protein fraction. Further treatment of the TCA-precipitated protein with ether-hexane resulted in extraction of dieldrin activity, although the extraction was not complete.

DISCUSSION

These data indicate that dieldrin binds to protein alone; extraction of possible free lipids in the Fraction V. protein with ether-hexane and subsequent dialysis revealed that binding was similar to that observed with untreated Fraction V. and albumin-globulin protein. Futhermore, the similarity in slopes of dieldrin binding to both the albumin-globulin and Fraction V. serum proteins might suggest that similar binding sites are available in each type of protein per milligram of weight. It must be pointed out, however, that the lines represented by each type of protein are similar but statistically different. Fraction V. serum protein is

composed of 95% albumin and 5% globulins, while the albumin-globulin standard was composed of 59% albumin and 41% globulin. This would suggest that dieldrin is binding with the albumin, globulins apparently having little to do with binding.

TABLE 2

TCA precipitation of the various proteins and homogenates following dialysis with ^{36}Cl-dieldrin.

		Average % Recovery of Dieldrin[a]				
	Replicates	Protein Precipitate	Ether-hexane extraction of protein precipitate	Supernatant	Ether-hexane extraction of supernatant	Total % Recovery
Albumin - globulin proteins	12	34.0	48.0	2.4	4.1	88.5
Fraction V. serum proteins	20	12.2	59.0	0	1.1	72.3
Fraction V. serum proteins (pre-treated with ether-hexane)	16	9.4	92.0	0.7	2.1	104.2
Rat spinal cord homogenate	12	3.0	67.0	2.1	2.5	74.6
Cockroach nerve cord homogenate	8	7.2	69.0	1.0	5.2	82.4

a Average % recovery of dieldrin based upon total bound dieldrin.

The uptake of dieldrin by the nerve cord homogenates was approximately three times greater than that observed with the serum proteins. Because the spinal cord is composed of large quantities of lipid and lipoprotein the possibility exists that dieldrin binds to the lipid or a lipid moiety of lipoprotein.

Precipitation of proteins with TCA was undertaken to determine

first, whether binding was occurring and, second, to obtain some knowledge as to the nature of the binding. Table 2 reveals that in vitro binding was occurring with protein. The possibility, however, that dieldrin became bound to the lipid moiety of lipoprotein was not eliminated.

Subsequent treatment with organic solvents revealed that whatever bonds are formed appear not to be covalent. Since the dieldrin was recovered mostly in the organic solvent portion, it appears likely that binding is a function of the presence of hydrophobic end groups on the protein (ADAMS 1971). Further work is necessary to determine the nature of these bonds.

By employing a variety of dieldrin concentrations in the preceding manner, it is hoped to find binding constant(s), K, and compare them for neural and non-neural tissue. In this manner, information concerning tissue specificity and, thus, the mode of action of cyclodiene insecticides, might be generated.

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