
AN AUTORADIOGRAPHIC STUDY OF INVERTEBRATE UPTAKE OF DDT-CL³⁶¹

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ABSTRACT

This research sought to locate autoradiographically DDT-Cl³⁶ in tissues of leeches, amphipods, and copepods three months after their marsh habitat was treated with the amount of insecticide routinely used for mosquito control. Isotope DDT or its metabolite was found in cytoplasm of nerve cell bodies, gut mucosa, and vascular tissue of leeches. No isotope DDT was detected in the tissue of amphipods and copepods.

INTRODUCTION

On July 7, 1964, DDT-Cl³⁶ was applied to a four-acre marsh in western Sandusky Bay, Ohio, to determine the fate of DDT in this natural environment. The plan included the collection of plants and animals at various post-application intervals for quantitative analysis. Because the plan did not include an experimental design for determining exact sites of the labeled compound within any invertebrates, arrangements were made to collect and process some of these organisms, with the objective of locating the isotope. Of several radiochemical techniques available, only autoradiography can be used to locate cellular and sub-cellular sites of isotope deposition in animal tissue.

The purposes of this particular investigation were to determine: (1) whether the isotope was taken up by leeches, amphipods, and copepods, (2) whether the isotope was evenly distributed throughout the bodies of these animals, or relatively concentrated in specific tissues and organs, and (3) the effectiveness of autoradiography in a field study of this type.

METHODS AND MATERIALS

In preparation for the application of this insecticide to the experimental marsh, technical DDT (0.8 lb.) and chlorine-36-labeled DDT (3.0 millicuries, $E_{\max} = 0.71$ mev.) in xylene solution were mixed with 400 lbs. of 20-40 mesh "AA" RVM "Attaclay" granules. The granules were applied to the four-acre marsh at the

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rate of 100 lbs. per acre (0.2 lb. technical DDT per acre) the amount of insecticide normally used for mosquito control, with a helicopter using an Amchem applicator. The operation took place on a calm morning. A control marsh, located adjacent to the experimental marsh, was not sprayed with DDT-Cl³⁶ and was isolated by a water-tight dike.

Approximately three months post-application, leeches, amphipods, and certain copepods were collected and fixed. Four fixative solutions with properties which seemed suitable for this project were selected. These were: (1) potassium dichromate solution, a strong oxidizing agent, (2) Bouin's solution, containing acetic acid, a very rapid penetrating substance, (3) glutaraldehyde, which has a remarkable reputation for good fixation, and (4) formalin.

Relaxation of the leeches and amphipods prior to fixation was accomplished by adding dilute ethyl alcohol, a drop at a time, to the native water in a stender dish containing the animals. The final concentration of alcohol was not determined, but is estimated to have amounted to about 5 per cent or less of the total. Preparation and use of the four fixative solutions used in this experiment are included here.

Modified Bouin's fixation

Stock Bouin's fixative was prepared according to McManus and Mowry (1960). As soon as the isolated animals relaxed from the effects of the alcohol, Bouin's fixative was added to the stender dish. The volume of fixative used was equal to that of the marsh water which contained the animals to be fixed. The specimens were stored in the fixative until further processing which was preparatory to paraffin embedding. Specimens were stored for from three to twelve months, which, admittedly, is too long.

Glutaraldehyde fixation

Commercially prepared 25 per cent glutaraldehyde was mixed with an equal volume of Sorenson's buffer (pH 7.4). In the field, this 12.5 per cent mixture was added to an equal volume of habitat water containing specimens. This brought the final concentration to 6.25 per cent. The animals in this solution were fixed for 24 hours before transfer to Sorenson's buffer for storage.

Potassium dichromate fixation

Five grams of potassium dichromate and 1 gram of calcium chloride (anhydrous) were dissolved in 100 ml of water. In the marsh, this solution was added to an equal volume of water containing the collected specimens. The animals remained in this solution until further histological processing.

Formalin fixation

Twenty per cent formalin, prepared from commercial formalin, was used with an equal volume of native water to fix leeches. Similarly, when fixing amphipods, 10 per cent formalin was added to marsh water containing the animals. Copepods were concentrated in a vial with a relatively small volume of marsh water and an equal volume of 10 per cent formalin. All formalin-fixed specimens were stored in this solution until further histological processing.

Another complete set of the same kinds of invertebrates was collected from the control marsh. These controls were fixed by the same methods.

Selection and identification of specimens

In preparation for this study, a total of 17 leeches of the species *Erpobdella punctata* (Leidy) were selected from those collected from the experimental marsh. Six other leeches of the same species, collected from a marsh not sprayed with DDT-Cl³⁶, were used as controls.

Four amphipods, *Hyallela* sp., and 4 controls of the same species were prepared for autoradiography in this study. Two groups of experimental copepods were also included. One group contained 6 specimens of *Cyclops bicuspidatus* (Claus) and the other contained 8 specimens of *Diaptomus organensis* (Lillj). Sections of specimens used in autoradiography were kept in contact with the autoradiographic emulsion for a number of days; thirty to forty days exposure yielded the best results.

Dehydration and paraffin embedding

Each experimental animal and its control within any given group (based on type of fixative) were handled as nearly alike as possible. After remaining in fixation solution the prescribed period, the animals were washed in running tap water for at least 6 hours and then processed through an alcohol dehydration series to xylene. The specimens were left one hour in each change of alcohol in the dehydration series. All animals were then embedded in paraffin for sectioning.

Some concern has been expressed that the DDT might be leached out in this processing. In the pilot study for this work, the alcohols left over after histological dehydration were analyzed by an extraction-scintillation technique and no isotope was found in these solutions. This was interpreted to indicate that these alcohols were not leaching out the Cl^{36} compound. Neither the fixative nor the xylene solutions were so analyzed.

The extreme anterior portion of 4 sets of leeches (4 experimentals and 4 controls) were microtomed in cross-section. The remaining portions of these leeches were later parasagittally sectioned. All other erpobdellids were cut parasagittally ($6\ \mu$). All crustaceans were histologically prepared in a manner similar to that of the leeches.

Selected sections were floated out on water containing a pinch of powdered gelatin and mounted on clean microscope slides that had been previously dipped in a subbing solution. Five ml of 5 per cent aqueous chrome alum and 25 ml of 10 per cent gelatin solution were mixed and enough distilled water added to make 500 ml of subbing solution. Sixty ml of this solution in a coplin jar were used to dip 40 to 50 slides before the subbing solution was replaced. Subbed slides were then placed in 100-capacity slide boxes with absorbent paper in the bottom. Tissues mounted on these subbed slides were dried in a dust-free atmosphere at room temperature. They were then deparaffinized with xylene and hydrated through an ethyl alcohol series to water, and transferred into a darkroom for the application of nuclear emulsion. This proceeded according to a dipping technique of autoradiography which is described by Jofte (1959). This method was preferred to freeze-drying because the latter was considered to be too difficult for secure attachment of the tissue section to the glass slide, in preparation for dipping autoradiography. Incubation (exposure to the isotope) was carried out at $4-5^{\circ}C$ for 30 to 40 days.

Staining of autoradiographs

Most autoradiographs were stained with basic fuchsin according to Bergeron (1958). These were then run individually through an ethyl alcohol dehydration series to xylene and cover-slipped with piccolyte. Some of the autoradiographs were stained with Delafield's hematoxylin and counterstained with eosin for better histological detail. A standard light microscope was used to analyze the autoradiographs.

RESULTS

As a result of this work, over 370 autoradiographs containing more than 6,000 tissue sections were produced. None of the control autoradiographs of any of

the control invertebrates contained silver grain deposition, appreciably above background, indicating the absence of labeled pesticide in these animals.

Of the 17 experimental leeches, seven showed evidence of isotope activity in the autoradiographs. The autoradiographs indicated that radioactive molecules

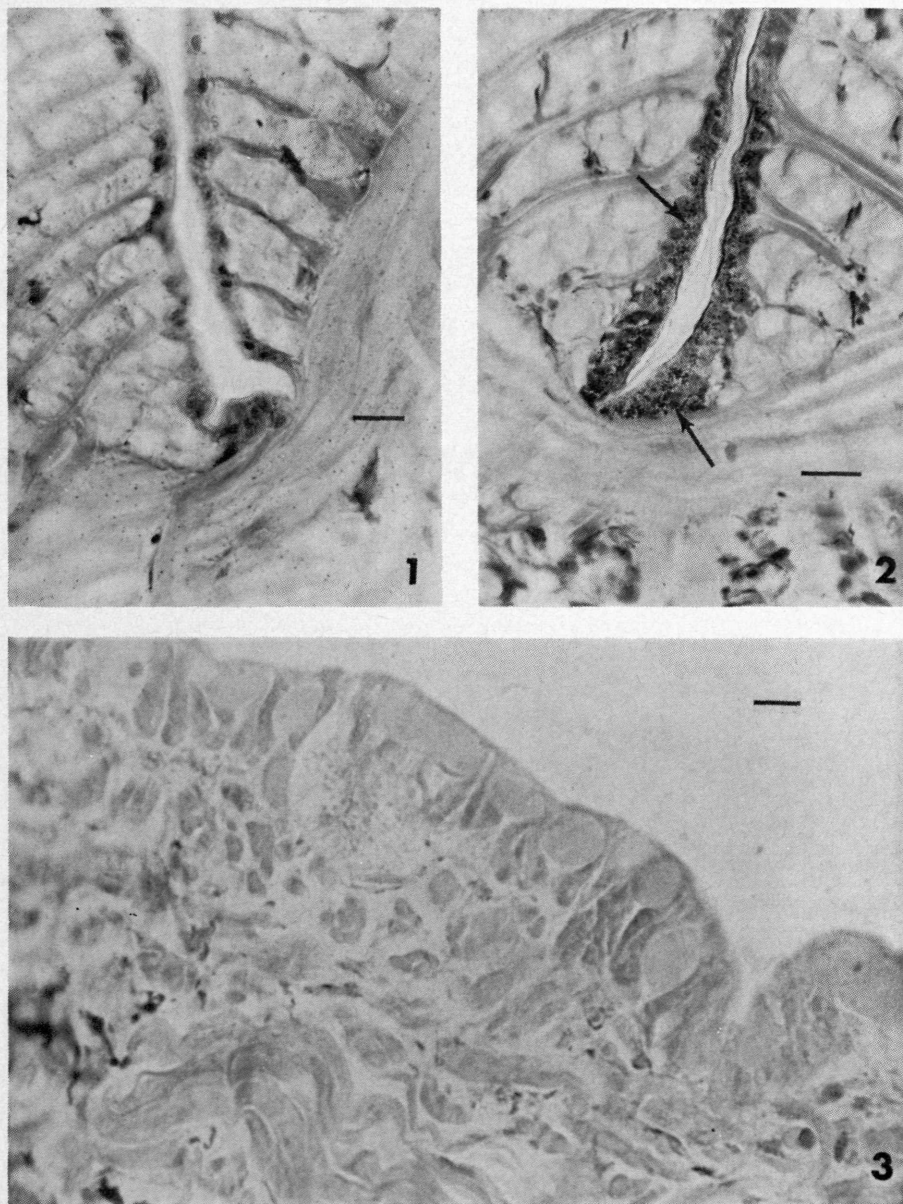


FIGURE 1. Section through pharynx of a control leech $\times 430$. (Bar equals 20 microns.)

FIGURE 2. Section through pharynx of an experimental leech. Arrow indicates localization of DDT-Cl³⁶ or a metabolite $\times 430$. (Bar equals 20 microns.)

FIGURE 3. Section of epidermis, dermis and underlying muscle layers. All are free of label. $\times 430$. (Bar equals 10 microns.)

were not distributed evenly throughout the entire body of the leech, but were concentrated in specific cells and tissues. No isotope activity occurred in any microtome sections from experimental animals fixed in Bouin's solution.

Autoradiographs of all 7 positive leeches showed localization in the mucosa of the foregut (fig. 2). No evidence of DDT- Cl^{36} was observed in the pharyngeal tissue of the controls (fig. 1). Isotope activity was found in the midgut and hindgut of only one of these leeches. Figure 4 shows the epithelium of the midgut from an experimental animal free of labeled insecticide. No autoradiographs contained definite silver-grain deposition in the emulsion associated with the food or fecal material in the alimentary canal of any leeches. The transition zone between the epithelial lining of the buccal cavity and the outer epidermis shows a transition from labeled to unlabeled tissue (fig. 5). No cells associated with dermis, epidermis, or muscular system were found to be labeled (fig. 3).

The hemolymph contained no detectable quantity of the isotope, but some blood cells of the experimental leeches did contain enough to cause grain deposition significantly above background (fig. 7). This localization was confined primarily to the cytoplasm of the cells, but in a few cases grains were also found over nuclei.

Neurons of the central nervous system, including the suprapharyngeal ganglia, subpharyngeal ganglia, and ventral cord ganglia, were found to have taken up DDT or a Cl^{36} compound in varying amounts (fig. 6). Some neuron cell bodies of a given ganglion were free of the isotope, while some adjacent cell bodies were labeled. Tissues of the reproductive organs were also negative. Occasionally a few cells of these tissues would appear positive for isotope uptake, but this was so inconsistent and infrequent that I believe it to be artifact.

DISCUSSION

Amphipods and Copepods

No emulsion-grain deposition above background was observed in any of the autoradiographs prepared with tissue sections of amphipods and copepods, indicating a lack of DDT uptake. There was no evidence derived from this work to indicate why no label was found. It might be possible that, if these crustaceans had hatched during a time when DDT- Cl^{36} was still available in the water and had then assimilated the labeled compound, it could have been eliminated by normal metabolism of growth and development.

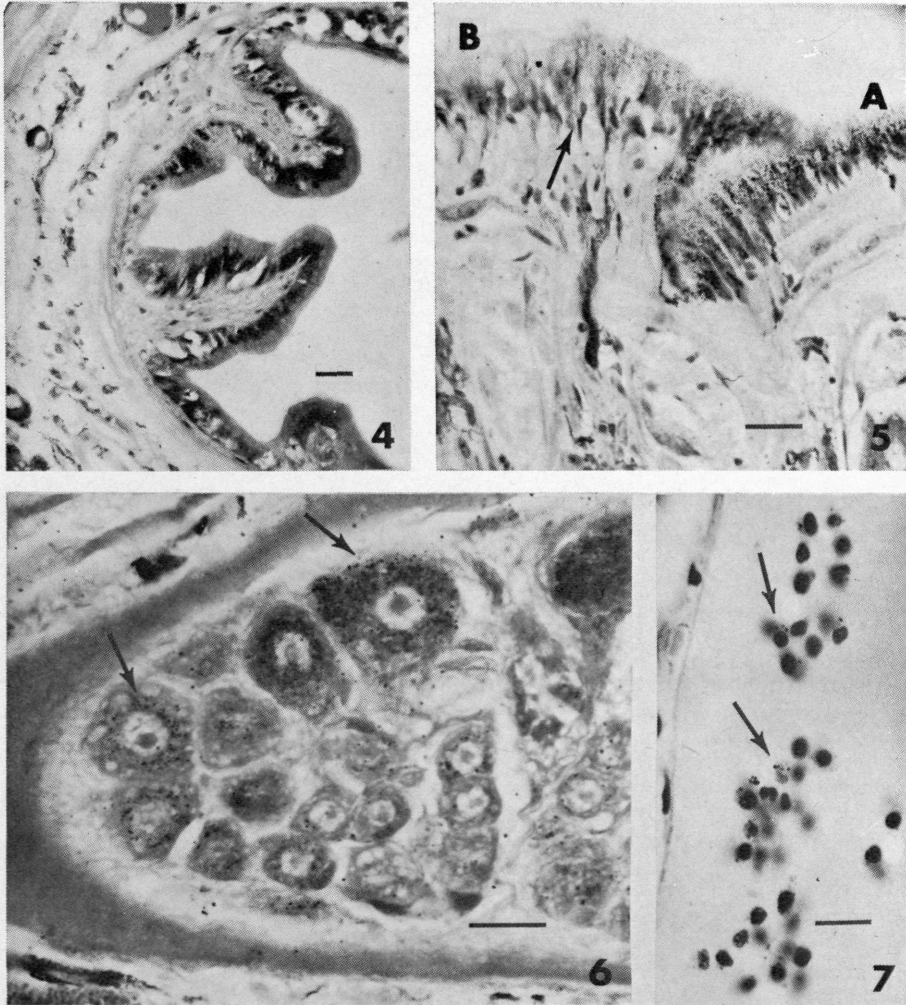
Leeches

In view of the localization of Cl^{36} in the mucosa of the foregut of 7 leeches, and in even the midgut and hindgut of one of the 7, one might speculate that these animals had fed shortly before they were taken from the marsh. This is known not to be the case because some leeches were found to have very little food material in the gut while others had a greater quantity. In addition in a report by Butler (1964), it was stated that, though DDT was not detectable in bottom- or surface-water samples after 14 days, DDT residues in vegetation and sediments reached a maximum between 3 and 6 weeks post-treatment. According to Peterle and Meeks (verbal communication), radiochemical analysis of this marsh water and the contained free-living forms, such as small insect larva and plankters, indicated no detectable amount of isotope activity after 7 days post-application.

The leeches in this study were collected 3 months post-application, a time when the water and possibly even the bottom sediments were free of the insecticide. This may indicate one of two things. First, it might mean that they had been feeding on labeled organisms in subsurface bottom sediments that were not reached by the researchers, though in no case was there consistent evidence of DDT in the gut contents. Second, it might mean that they had fed during the time when free-swimming prey was labeled and that the isotope was stored in

their bodies by the mucosal cells. It is also possible that both events occurred in some cases.

In view of all the factors which might affect the localization of the Cl³⁶ compound in the leech foregut, no conclusion about how or when the parent compound arrived there can be reached from the available data at this time. There is conclusive evidence that DDT passes through the integument of other animals (Patton, 1963). However, had this been the route to the mucosal lining of these leeches, one would expect to find isotope localization in the dermis and epidermis. No indication of DDT was found in these parts of these leeches (fig. 3).



- FIGURE 4. Cross-section of experimental leech midgut with no DDT localization in the mucosa $\times 250$. (Bar equals 25 microns.)
 FIGURE 5. Section from the buccal cavity. Arrow indicates transition zone from labeled (A) to unlabeled (B) epithelium $\times 250$. (Bar equals 25 microns.)
 FIGURE 6. Section through ventral nerve cord and ganglion showing nerve cell bodies, some labeled and some not labeled. $\times 1000$. (Bar equals 10 microns.)
 FIGURE 7. Autoradiographs of coelomocytes. Arrows indicate label in cytoplasm $\times 1000$. (Bar equals 10 microns.)

Fats from other organisms could have been the vehicle for the introduction of DDT-lipid. There is conclusive evidence for the accumulation of DDT at sites of lipid metabolism and storage (Gilmour, 1965; Pillmore and Wilson, 1964; Hickey and Keith, 1964; Patton, 1963). Based on the work of Eisner (1955) and Treherne (1958), O'Brien and Wolfe (1964) presumed that the foregut of some insects absorbs fat and stores it. If this is also the case in leeches, then this might account for the DDT or Cl^{36} -compound in the gut mucosa. These leeches could have been the receptors of the DDT metabolite from scavenged bottom materials, thereby gradually accumulating and storing the isotope over a considerable period of time.

DDT in coelomocytes

The fact that a significant number of free coelomocytes were also labeled suggests that the circulatory system is involved in the transport of the insecticide. In some insects the distribution of DDT is brought about by the hemolymph system (O'Brien and Wolfe, 1964; Patton, 1963). In leeches, coelomocytes engulf foreign substances in the coelomic sinuses. All labeled experimental leeches in this study contained free coelomocytes which contained DDT or a Cl^{36} -bearing compound. Relatively few coelomocytes were labeled in any one animal and this number varied from animal to animal. The deposition of silver grains in the emulsion corresponded to the location of the cytoplasm of the cells (fig. 7). A few cells had grain located over the nuclei, but this is probably due to a lack of autoradiographic resolution and/or due to labeled cytoplasmic structure above the nucleus in the tissue section.

DDT in the central nervous system

DDT or a Cl^{36} -compound has been found in association with the nervous system of various animals and has been reported by other investigators (Patton, 1963; Gilmour, 1965; Pillmore and Wilson, 1964; O'Brien, 1964). In these erpobdellids, DDT or a Cl^{36} -bearing compound accumulated in the cytoplasm of neuron cell bodies in the ganglia of the ventral nerve cord (fig. 6). No evidence was found which indicated an appreciable amount of neural localization of DDT in any place other than the central nervous system.

Some leeches contained more labeled neurons than others, but there seemed to be no correlation with the number of labeled coelomocytes in the sinuses. This can not be accepted as any particular evidence for the location of the action site of this insecticide. The fact that the leeches survived might indicate that these neurons were not sites of action. It could also mean that the DDT might have been converted to innocuous DDE.

SUMMARY

Liquid-emulsion autoradiography was effectively utilized to determine uptake and localization of DDT- Cl^{36} in some marsh invertebrates.

Leeches, *Erpobdella punctata* (Leidy), contained DDT- Cl^{36} or a Cl^{36} -compound of this insecticide three months after their natural habitat was treated.

Amphipods (*Hyalella* sp.) and copepods (*Diaptomus organensis* (Lillj) and *Cyclops bicuspidatus* (Claus) removed from the same marsh at the same time did not contain autoradiographically detectable amounts of DDT- Cl^{36} .

Autoradiographs prepared with leech-tissue sections indicated that radioactive molecules were not evenly distributed throughout the entire body of the leech, but rather were localized in the pharyngeal mucosa, coelomocytes of the circulatory system, and neurons of the central nervous system. No cells associated with dermis, epidermis, reproductive system, or excretory system were found to contain a detectable amount of DDT- Cl^{36} . All localization was limited to the cytoplasm; no conclusive nuclear localization was observed. Localization

in nerve cells of ganglia was further restricted to the cell body and did not extend into the nerve processes or to the synapses.

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