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ENVIRONMENTAL POLLUTANTS IN TWO SPECIES OF SNAKES FROM THE PAWNEE SITE

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ABSTRACT

Food chain magnification of environmental pollutants would suggest that a nonmigratory, locally occurring, higher order consumer such as the snake, may prove to be a valuable pollution indicator on the shortgrass prairie. Fat bodies were removed from two male gopher snakes, (*Pituophis catenifer*), and two male prairie rattlesnakes, (*Crotalus viridis*), collected near the Pawnee Site during July 1971. Adipose tissue was analyzed by the Colorado State Department of Health Pesticide Laboratory, Greeley, Colorado, for the presence of 36 different herbicides, pesticides, and organophosphate fertilizers by using electron capture gas chromatographic methods. Samples were also analyzed for PCB's (poly-chlorinated biphenals) and sulfur compounds. Results show that the snakes sampled, which are secondary consumers feeding on small mammals, have low levels of environmental pollutants in their adipose tissues. Gopher snakes were found to contain .20 ppm of p,p'-DDE, .04 ppm of dieldrin, .013 ppm beta benzene hexachloride, and .01 ppm heptachlor epoxide. Prairie rattlesnakes contained .62 ppm p,p'-DDE and .03 ppm dieldrin. Data indicated that further analysis by flame photometric methods was unnecessary. Low levels found in snakes sampled would seem to correlate with agricultural practices on the Pawnee National Grasslands. Artificial fertilization and pesticide spraying are seldom used in this area.

INTRODUCTION

A locally occurring, higher order consumer such as the snake may prove to be a valuable pollution indicator on the shortgrass prairie through food chain magnification of environmental pollutants. In this study, male snakes of two species were analyzed qualitatively and quantitatively for 36 different herbicides, pesticides, and organophosphates, in an ecosystem where these substances have seldom, if ever, been applied. Food chain magnification of these residues by living organisms is the practical result of differential absorption, detoxification, storage, and excretion (Decker 1966; Johnson 1967; Keith and Flickinger 1965; Meeks 1968; Negherborn 1959; Scott, Willis, and Ellis 1959; Westlake and San Antonio 1960; and others). The highest concentrations are generally found to occur in carnivorous secondary and tertiary consumers.

Snakes may prove to be a valuable indicator species in local ecosystems. They are carnivores which feed upon vertebrates or invertebrates, depending upon the species of snake selected. Unlike birds, most snakes are sedentary in nature, and seldom move more than a few miles in their entire lifetime, (King 1968). Thus, there would be little risk of a contaminated individual migrating into an area and providing an inaccurate measure for a specific locality, as would be the case with many birds. Similarities in renal anatomy and protein metabolism may eventually facilitate some extrapolation of pollution research data presently available on birds to certain widespread species of snakes. The rates of storage, excretion, and metabolism of organochlorine compound residues are presently unknown for reptiles.

Thousands of pounds of persistent organochlorine pesticides, herbicides, and organophosphates are used each year in the United States. Statements by the U.S.D.A. show that in 1958-1959, 78 million lb. of DDT and 73 million lb. of chemicals of the aldrin group were used in this country. By 1964-1965, DDT use had declined to 53 million lb., but the use of the more toxic chemicals of the aldrin group had increased to 80.5 million lb. (Stickel 1968).

The effects of these organochlorine chemicals are of a spectrum from direct mortality to delayed mortality long after dosage ceases. Other effects include the stimulation of breakdown of hormones acting directly as estrogens, involvement in embryonic and post embryonic toxicity, interferences with antibody formation, effects on behavior, and interactions with stresses such as nutritional deficiencies or food deprivation (Stickel 1968). However, the total effect of these residues on the environment has not yet been determined.

This study may help to provide a base level of snake pollutants for the Pawnee Site of the U.S. International Biological Program, from which changes can be determined as population growth and modern agricultural practices continue to extend into that locality in future years.

METHODS AND MATERIALS

Two male gopher snakes, (*Pituophis catenifer*), and two male prairie rattlesnakes, (*Crotalus viridis*), were collected near the Intensive Study Site of the International Biological Program, the Pawnee Site, during July 1971 (Table 1). Male snakes were used in order to avoid any adipose variation due to the production of young by females. Two specimens of each species were necessary in order to accumulate enough fat tissue for analysis.

Table 1. Specific data for the two species of snakes captured on the Pawnee Site.

Species	Sex	Weight	Length	Date	Location
Pituophis catenifer	M	534.8 g	1257 mm	July 4	2 miles east of intensive site
Pituophis catenifer	M	231.3 g	862 mm	July 18	3½ miles east of site
Crotalus viridis	M	331.9 g	857 mm	July 21	2 miles east and 1 mile south of site
Crotalus viridis	M	121.5 g	645 mm	July 4	3 miles northeast of site

Abdominal cavities of the snakes were opened by ventral incision, and adipose bodies attached to intestinal connective tissues were removed. The adipose bodies from two snakes of the same species were placed in a beaker and mixed. A 5 g sample from each species was removed for analysis. During the preparation and extraction procedure, the actual percentage of fat present in this 5 g sample was determined.

Analytic processing was done by laboratory technicians at the Colorado Community Pesticide Study facility of the Colorado State Department of Health, Greeley, Colorado. The Modified Mills, Onley, Gaither Procedure (on the analysis of human or animal adipose tissue) for Pesticide Monitoring Laboratories was used for this study. Instrumentation used included Micro Tek MT 220 electron capturing gas chromatographs and accessories. The specific information concerning the gas chromatograph used in this experiment is as follows:

Instrument:	Micro Tek MT 220; Electron capturing
Primary Column:	4% SE 30/ 6% QF-1, on 80-100 Chromosorb, W-H.P., 6 ft × ¼ inch outside diameter
Secondary Column:	OV-17, 1.5% 0017-1.95% QF-1
Nitrogen Flow:	85 cc/minute
Column Temp:	203°C
Detector Temp:	210°C
Detector Source:	Tritium
Inlet Temp:	215°C
Transfer Temp:	225°C
Volts:	10

Attenuation: 10 × 8

Chart Speed: ½ inch/minute

The specific herbicides, pesticides, and organophosphate compounds tested for this study are listed in Table 2.

Table 2. Compounds tested for in this study, and the elution patterns from florisil partitioning.

Compound	6% Eluate	15% Eluate	50% Eluate
α-BHC	X		
β-BHC	X		
γ-BHC	X		
Heptachlor	X		
Hept. Epoxide	X		
Aldrin	X		
o,p'-DDE	X		
p,p'-DDE	X		
Dieldrin		X	
Endrin		X	
o,p'-DDD	X		
p,p'-DDD	X		
o,p'-DDT	X		
p,p'-DDT	X		
Perthane	X		
Methoxychlor	X		
Chlordane	X		
Chlorobenside	X		
Chlorobenzilate		X(80%)	X
2,4,5-T,isooctyl ester		X	
2,4,5-T,isopropyl ester		X	
2,4,5-T,n-butyl ester		X	X
2,4-D,isobutyl ester		X	
2,4-D,isoctyl ester		X(80%)	
2,4-D,isopropyl ester		X	
Tedion		X	
Endosulfan I		X	
Endosulfan II			X
Dilan			X
Ronnel	X		
Diazinon		X	
Methyl Parathion		X	
Ethyl Parathion		X	
Malathion		X	X
		(possible)	
Ethion	X		
Trithion	X		

ANALYSIS OF HUMAN OR ANIMAL ADIPOSE TISSUE[a/]

(MODIFIED MILLS, ONLEY, GAITHER PROCEDURE)

I. EQUIPMENT:

1. Aluminum foil, household type.
2. Beakers, 250 ml stainless steel or heavy duty glass.
3. Beakers, 250 ml Griffin low form.
4. Stirring rods, glass 10 mm.
5. Water bath with temperature adjustment to 75 to 80°C.
6. Filter paper--Whatman No. 1, 15 cm diameter.
7. Funnels, glass, ca. 60 ml diameter.
8. Separatory funnels--125 ml and 1-liter.
9. Chromatographic columns--25 mm o.d. × 30 cm long, with Teflon stopcocks, without fritted glass plates.
10. Filter tubes, 150 × 24 mm, such as Corning #9480.
11. Erlenmeyer flasks--500 ml capacity.
12. Kuderna-Danish concentrator fitted with grad. evaporative concentrator tube. Available from the Kontes Glass Company, each component bearing the following stock numbers:
    a. Flask, 500 ml, stock # K-570001
    b. Snyder column, 3-ball, stock # K-503000
    c. Steel springs, ½ inch, stock # K-662750
    d. Concentrator tubes, 10 ml, size 1025, Stock # K-570050
13. Modified micro-Snyder columns, 19/22, Kontes K-569251.
14. Glass beads, 3 mm plain, Fisher # 11--312 or equivalent.

II. REAGENTS:

1. Hexane--AR grade, redistilled, b.p. 30°-60°C.
2. Diethyl ether--AR grade, peroxide free, Mallinckrodt #0850 or the equivalent. The ether must contain 2% (v/v) absolute ethanol. Some of the AR grade ethers contain 2% ethanol, added as a stabilizer, and it is therefore unnecessary to add ethanol unless peroxides are found and removed.

   NOTE: To determine the absence of peroxides in the ether, add 1 ml of freshly prepared 10% KI solution to 10 ml of ether in a clean 25 ml cylinder previously rinsed with the ether. Shake and let stand 1 minute. A yellow color in either layer indicates the presence of peroxides which must be removed before using. See Misc. Note 4 at end of procedure. The peroxide test should be repeated at weekly intervals on any single bottle or can as it is possible for peroxides to form from repeated opening of the container.

[a/] This method, with appropriate modifications, may be used for the analysis of other tissues if original sample size is adequate.

3. Eluting mixture, 6% (6+94)--purified diethyl ether 60 ml is diluted to 1000 ml with redistilled petroleum ether and anhydrous sodium sulfate (10-25 g) is added to remove moistures.
4. Eluting mixture, 15% (15+85)--purified diethyl ether 150 ml is diluted to 1000 ml with redistilled petroleum ether and dried as described above.

   NOTE: Neither of the eluting mixtures should be held longer than 24 hours after mixing.

5. Florisil, 60/100 mesh, PR grade, to be stored at 130°C until used.

   NOTE: (1) In a high humidity room, the column may pick up enough moisture during packing to influence the elution pattern. To insure uniformity of the Florisil fractionation, it is recommended to those laboratories with sufficiently large drying ovens that the columns be packed ahead of time and held (at least overnight) at 130°C until used.

   (2) Florisil furnished by the Perrine Laboratory on order, has been activated by the manufacturer at 1200°F and is pretested both by Perrine and the manufacturer, and elution pattern data are included with each shipment. However, each laboratory should determine their own pesticide recovery and elution pattern on each new lot received, as environmental conditions in the various laboratories may differ somewhat from that in Perrine. Each new batch should be tested with a mixture of β-BHC, aldrin, heptachlor epoxide, dieldrin, p,p'-DDE, p,p'-DDD and p,p'-DDT, eluting the standard mixture as described in Section E under FLORISIL FRACTIONATION. Dieldrin should elute entirely in the 15% diethyl ether fraction, whereas all other compounds should be in the 6% fraction.

6. Acetonitrile, reagent grade, saturated with hexane.

   NOTE: Occasional lots of CH_3CN are impure and require redistillation. Generally vapors from impure acetonitrile will turn litmus paper blue when the moistened paper is held over the mouth of the bottle.

7. Anhydrous sodium sulfate, reagent grade granular, Mallinckrodt Stock #8024 or the equivalent.

   NOTE: When each new bottle is opened, it should be tested for contaminants that will produce peaks by Electron Capture Gas Liquid Chromatography. This may be done by transferring ca. 10 g to a 125 ml Erlenmeyer flask, adding 50 ml pet. ether, stoppering and shaking vigorously for 1 minute. Decant extract into a 100 ml beaker and evaporate down to ca. 5 ml. Inject 5 µl into the Gas Liquid Chromatograph and observe chromatogram for contaminants. When impurities are found, it is necessary to remove them by extraction. This may be done using hexane in a continuously cycling Soxhlet extraction apparatus or by several successive rinses with hexane in a beaker. The material is then dried in an oven and kept in a glass stoppered container.

8. Sodium chloride solution, 2%, from reagent grade NaCl.

   NOTE: See NOTE for sodium sulfate, Step 7.

9. Sand, quartz, which has been acid washed and extracted with hexane to produce a zero background in the determinative step.
10. MgO-Celite mixture (1:1) weigh equal parts of reagent grade MgO and Celite 545 and mix thoroughly.
11. Hexane, redistilled.

III. SAMPLE PREPARATION & EXTRACTION:

1. On a cupped sheet of light weight aluminum foil, weigh 5 g of the previously minced fat. Transfer entire cup to a 250 ml stainless steel or heavy duty glass beaker.
2. Add ca. 10 g of clean, sharp sand, ca. 10 g of anhydrous Na_2SO_4 and 1.0 ml of hexane solution containing 200 ng of aldrin.

   NOTE: The aldrin is added here for the dual purpose of (1) providing a built-in retention marker for direct peak identification on all chromatograms of the first fraction extract, and (2) as a quantitative recovery check for the procedure. This inoculation should of course not be made if aldrin is suspected to be in the substrate.

3. Grind the mixture with a heavy glass rod and continue adding portions of Na_2SO_4 to give a uniform, dry granular mass.
4. Add 50 ml of pet. ether and warm *carefully* on a water bath with continuous stirring until solvent boils gently.
5. Place Whatman No. 1 filter paper in glass funnel and rinse several times with pet. ether. Place funnel over previously tared 250 ml beaker and transfer extract to funnel by decantation.
6. Extract the contents of the first beaker with two more 50 ml portions of pet. ether as described in steps 4 and 5.
7. Transfer insoluble material to the filter paper and rinse beaker and paper with a final 10 ml of pet. ether.
8. Place beaker on a 40°C water bath and evaporate *just* to dryness under stream of nitrogen. Check odor to be sure all solvent is removed and allow to cool to room temperature in a dessicator.
9. Weigh beaker and record for calculation of percent fat in the sample.
10. Accurately weigh between 2.8 and 3.0 g of the fat obtained in Step 9 into a 125 ml separator. Add 12 ml of pet. ether previously saturated with acetonitrile.

    NOTE: In the case of highly saturated animal fat, it will be necessary to add 17 ml of hexane to the separator. In such a case the amount of acetonitrile used in the partitioning step should be increased to 40 ml.

IV. LIQUID - LIQUID PARTITIONING:

1. Add 30 ml of acetonitrile, previously saturated with hexane. Stopper funnel and shake vigorously for 2 minutes.

2. Allow phases to separate and draw off the acetonitrile layer into a 1-liter separator containing 700 ml of a 2% solution of NaCl and 100 ml of pet. ether.
3. Similarly extract the pet. ether layer in the 125 ml separator three more times with 30 ml portions of acetonitrile, combining all acetonitrile extracts in the 1-liter separator.
4. Stopper, invert 1-liter separator, vent off pressure and mix by shaking for 2 minutes, releasing pressure as required.
5. Allow the layers to separate and drain aqueous layer into a second 1-liter separator.
6. Add 100 ml pet. ether to second separator, and after a 30 second vigorous shaking, discard aqueous phase and transfer hexane phase into first 1-liter separator.
7. Wash pet. ether with two 100 ml portions 2% NaCl and discard the aqueous washings.
8. Prepare a 2-inch column of anhydrous, granular Na_2SO_4 in a 150 × 24 mm filter tube and position over a 500 ml K-D evaporator fitted with a 10 ml grad. concentrator tube containing one glass bead. Dry the pet. ether by filtering through this column. Rinse the separator twice with 10 ml portions of pet. ether and finally rinse down sides of the filter tube with 10 ml pet. ether.
9. Attach a 3-ball Snyder column to the top of the K-D evaporator and place in a 75°-80°C water bath. Approximately 1½ inches of the concentrator tube should be below the surface of the water.
10. Concentrate the extract to ca. 5 ml, rinse down the sides of the K-D evaporator and the ground glass joint with a total of 3 ml hexane. Reconcentrate extract to ca. 5 ml under a gentle stream of nitrogen at room temperature.

V. FLORISIL FRACTIONATION:

1. Prepare a chromatographic column containing 4 inches (after settling) of activated Florisil topped with ½ inch of anhydrous, granular Na_2SO_4. A small wad of glass wool, pre-extracted with hexane, is placed at the bottom of the column to retain the florisil.
2. Place a 500 ml Erlenmeyer flask under the column and pre-wet the packing with hexane (40-50 ml, or a sufficient volume to completely cover the Na_2SO_4 layer).

   NOTE: From this point and through the elution process, the solvent level should never be allowed to go below the top of the Na_2SO_4 layer.

3. Using a 5 ml Mohr or a long disposable pipet, *immediately* transfer the tissue extract (ca. 5 ml) from the evaporator tube onto the column and permit it to percolate through.
4. Rinse tube with two successive 5 ml portions of hexane, carefully transferring each portion to the column with the pipet.

   NOTE: Use of the Mohr or disposable pipet to deliver the extract directly onto the column precludes the need to rinse down sides of the column.

5. Prepare two Kuderna-Danish evaporative assemblies complete with 10 ml graduated evaporative concentrator tubes. Place one glass bead in each concentrator tube.
6. Replace the 500 ml Erlenmeyer flask under each column with a 500 ml Kuderna-Danish assembly and commence elution with 200 ml of 6% diethyl ether in hexane (Fraction I). The elution rate should be ca. 5 ml/minute. When the last of the eluting solvent reaches the top of the Na_2SO_4 layer, place a second 500 ml Kuderna-Danish assembly under the column and continue elution with 200 ml of 15% diethyl ether in hexane (Fraction II).
7. To the second fraction *only*, add 1.0 ml of hexane containing 200 ng of aldrin, place both Kuderna-Danish evaporator assemblies in a water bath and concentrate extract to ca. 2 ml.
8. Remove assemblies from bath, cool and carefully rinse down sides of Kuderna-Danish flask with ca. 6 ml hexane, delivered from a 10 ml Mohr pipet.
9. Disconnect collection tube from Kuderna-Danish flask and carefully rinse joint with 2 ml hexane.
10. Attach modified micro-Snyder column to collection tubes, place tubes back in water bath and concentrate extracts to 1 ml.
11. Remove from bath, cool, disconnect tubes and rinse columns and joints with ca. 3 ml hexane.

    NOTE: The extent of dilution or concentration of the extract at this point is dependent on the pesticide concentration in the substrate being analyzed and the sensitivity and linear range of the Electron Capture Detector being used in the analysis.

12. Should it prove necessary to conduct further cleanup on the 15% fraction, transfer 10 g MgO-Celite mixture to a chromatographic column using vacuum to pack. Pre-wash with ca. 40 ml pet. ether, discard pre-wash and place a Kuderna-Danish receiver under column. Transfer concentrated Florisil eluate to column using small portions of hexane. Force sample and washings into the MgO-Celite mixture by slight air pressure and elute column with 100 ml pet. ether. Concentrate to a suitable volume and proceed with Gas Liquid Chromatography.

VI. ASSESSMENT OF EXTRACT CONCENTRATION:

1. Inject 5 µl of each fraction into the gas chromatograph for the purpose of determining the final dilution. If all peaks are on-scale and quantifiable, it will not be necessary to proceed with any further adjustment in concentration. With human fat, however, it is probable that there will be several sizable on-scale peaks and one or more off-scale peaks in the 6% fraction.
2. If off-scale peaks are obtained in either fraction, it will be necessary to dilute volumetrically with hexane to obtain a concentration that will permit quantitation of those peaks from a 5 µl injection.

NOTE: A 5 ml dilution of a 3.0 g sample containing .01 ppm of a given pesticide will yield 30 pg. of the pesticide per 5 µliter injection. Provided the detector is operating at average sensitivity, it should be possible to obtain quantifiable peaks of most compounds likely to be present at this concentration.

VII. MISCELLANEOUS NOTES:

1. The two fractions from the Florisil column should never be combined for examination by Gas Liquid Chromatography. By so doing, a valuable identification tool is voided.
2. Meticulous cleaning of glassware is absolutely essential for success with this procedure. All reagents and solvents must be pretested to insure that they are free of contamination by electron capturing materials at the highest extract concentration levels. Reagent blanks should be run with each set of samples.
3. The method, as described, is known to be capable of producing recoveries of most of the chlorinated pesticides of from 85 to 100%. Each laboratory should conduct their own recovery studies to make certain of their capability to achieve this recovery range. A clue may be obtained from the recovery of the aldrin spike. The recovery of this compound should not be less than 70%.
4. For the removal of peroxides from the ethyl ether, place an appropriate volume in a separatory funnel and wash it twice with portions of water equal to about ½ the volume of ether. The washed ether is shaken with 50 to 100 ml of saturated NaCl solution and all of the aqueous layer is discarded. The ether is then transferred to a Ƹ flask containing a large excess of anhydrous sodium sulphate and shaken vigorously on a mechanical shaker for 15 minutes. This treatment removes the ethanol as well as the peroxides and it is therefore necessary to replace the 2% ethanol (v/v) before using.
5. If the presence of malathion is suspected it is necessary to pass 200 ml of 50% diethyl ether in pet. ether through the Florisil column into a third K-D evaporator assembly, concentrating the eluate as described for the 6% and 15% eluates.
6. Table 2 gives the elution pattern for a number of common pesticides. On occasion it may be observed that a portion of a given compound may elute into a different fraction than the one given. For example, some operators have difficulty eluting all the dieldrin in the 15% fraction. This is generally due to either moisture in the system or the use of solvents of different polarity than those specified in the reagent list. For example, it is essential that the diethyl ether contain 2% (v/v) ethanol. Ether without the ethanol would expectedly result in an altered elution pattern.

RESULTS

Analysis of a 5 g sample of adipose from each species of snake yielded the following results: prairie rattlesnake (*C. viridis*) fat bodies contained 61.4% fat, gopher snake (*P. catenifer*) fat bodies contained 69.3% fat.

Prairie rattlesnakes (*C. viridis*) were found to contain .62 ppm of p,p' DDE and .03 ppm dieldrin. The aldrin test spike recovery was 86%.

Gopher snakes (*P. catenifer*) were found to have .20 ppm p,p' DDE, .04 ppm dieldrin, .013 ppm beta benzene hexachloride (B-BHC), and .01 ppm heptachlor epoxide. The aldrin test spike recovery was 92%.

Results of tests for all other compounds in these two species of snakes was negative. Minute levels of organophosphates necessitated no further analysis by flame photometric methods. A further 3 g sample was taken from each species and analyzed for PCB's, (poly-chlorinated bipherals), with negative results.

DISCUSSION

This study indicates that if locally occurring gopher snakes, (*P. catenifer*), and prairie rattlesnakes, (*C. viridis*), are used as indicators, the vicinity of the Pawnee Site of the International Biological Program has had little contamination by persistant environmental pollutants. Trace quantities of some organochloride compounds were found to be present in snake adipose tissue, however.

The compounds detected in this study may have reached the Pawnee Grasslands by several methods. The most feasible method would be by wind blown particulate matter. Strong prevailing winds from the northwest blow across

several agricultural areas before passing into the Pawnee Site study area. The city of Laramie, Wyoming, 60 miles away, is situated so that these winds may carry trace amounts of industrial pollutants into the area. General atmospheric contamination may also contribute to these findings.

Except for scattered U.S.D.A. test plots, no herbicides, pesticides, or organophosphate fertilizers are recorded to have been used on the Pawnee National Grasslands. Exceptions may have occurred during the 1920's and early 1930's when some small acreages in the grasslands were used for agriculture for short periods. However, long-term residents of the area testified that, (to the best of their knowledge), none of the land had been farmed where the snakes used in this study were collected.

It will be important to establish original pollutant levels in the carnivores of this region in order to monitor future changes. Dry land farming and irrigation are continuing to replace grazing in this area. Population experts are foreseeing the eventual establishment of a continuous city extending the length of the state of Colorado along the front range. This study should serve as an initial base level for measurement of snakes in this vicinity. Snakes should supply additional supplementary information about local ecosystems, which could support and/or verify regional avian studies.

Future studies should sample large numbers of snakes, and should introduce heavy metal analysis. Studies on the rate of metabolism, storage, and excretion of residues would be invaluable in determining the final role of snakes as pollution indicators.

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