Effects of Carbaryl and Dieldrin on the Growth, Protein Content, and Phospholipid Content of HeLa Cells

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Monolayer cultures of HeLa cells were used to monitor the effects of two commonly used insecticides on cell numbers, protein content, and phospholipid content. Analytical grades of carbaryl (a carbamate insecticide) and dieldrin (an organocholorine insecticide) were used in the study. HeLa cells were incubated for 48 hr with different concentrations (1, 2, 4, and 8 ppm) of the selected chemicals. Both carbaryl and dieldrin were found to stimulate cell division at 1 and 2 ppm and to inhibit growth at 4 and 8 ppm. Carbaryl was found to be more toxic than dieldrin at 4 and 8 ppm. A decrease in cellular protein was noted with carbaryl at 4 ppm and with dieldrin at 4 and 8 ppm. Increasing concentrations of carbaryl caused subsequent increases in sphingomyelin content of the phospholipid fractions of exposed cells. Both carbaryl and dieldrin elicited decreases in the amounts of phosphatidylcholine, and a slight increase in lysophosphatidylcholine was noted in cells exposed to carbaryl. The results indicate that carbaryl and dieldrin cause general metabolic changes in HeLa cells. The variations in phospholipid content of HeLa cells caused by carbaryl and dieldrin suggest an alteration of the structure of cellular membranes.

Studies involving insecticide toxicity on mammalian cell cultures were reported first when Gabliks and Friedman (1965) determined the growth inhibition doses (ID_{10} and ID_{50}) of 11 different insecticides on HeLa cells (a malignant human cell line) and Chang liver cells (a nonmalignant human cell line). Gabliks et al. (1967) investigated the effect of organophosphorus insecticides on mouse liver cell cultures. Their results of the response of cells to the insecticides did not correlate with the data obtained for human strain Chang liver cells. These findings strongly supported the idea that cells of different origins respond differently to the same chemical.

Gabliks and Friedman (1969) reported that specific environmental chemicals altered the susceptibility of Chang liver cells to infection by polio virus (an RNA virus) and vaccinia (a DNA virus). The exposure of HeLa cells to DDT decreased the yield of vaccinia virus and increased the yield of polio virus in cells infected with the respective virions. These findings suggested that the chemicals were causing changes in the physiology of the cells.

Additional studies have given increased evidence of changes in cellular metabolism elicited by environmental chemicals, especially insecticides. Chung et al. (1967) exposed HeLa cells to either DDT or dieldrin at concentrations of 5, 10, or 50 ppm and found that these insecticides had the ability to reduce or stimulate depending on the concentrations used, the synthesis of nucleic acids and protein in the cells (Litterst et al., 1969). Studies were made (Litterst et al., 1969; Litterst and Lichtenstein, 1971) on the response of HeLa cells and human skin fibroblasts to various environmental toxicants including the insecticides carbaryl, dieldrin, DDT, parathion, and several insecticide metabolites. The results indicated that smaller concentrations (10 ppm) of DDT and dieldrin stimulated cell growth as well as the production of cellular DNA, RNA, and protein. Also, little difference was noted between the responses of the malignant (HeLa) and the nonmalignant cells to the toxicants, a finding identical with that of Gabliks et al. (1967).

Little information was found regarding the response of cellular lipid production to insecticide exposure, although other lipid studies have been performed on mammalian cells of different origins (Guttler and Clauser, 1969; Weinstein et al., 1969).

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In this investigation the effects of lethal and nonlethal concentrations of carbaryl (a carbamate) and dieldrin (an organochlorine) on the growth (in reference to cell numbers) of HeLa cells were determined. In addition, the quantitative effects of the two insecticides on total protein and specific phospholipid content of HeLa cells were determined. Little difference has been noted between the response of malignant and nonmalignant human cells to carbamate and chlorinated hydrocarbon insecticides (Litterst et al., 1969; Litterst and Lichtenstein, 1971). The mode of action of carbaryl involves the inhibition of acetylcholinesterase, an enzyme required for synaptic transmission in the nervous systems of higher animals as well as insects (Reiner and Simeon-Rudolf, 1966). The mechanism of inhibition, once believed to be competitive and reversible, has been shown to involve an actual carbamylation of the enzyme with freeing of the naphthalene ring. The carbamylated enzyme complex then undergoes slow hydrolysis with the eventual recovery of enzyme activity (Chiu et al., 1973; Wilson et al., 1961). Inhibition of the enzyme by a given concentration of carbaryl is not influenced by the concentration of the normal substrate, acetylcholine (Reiner and Simeon-Rudolf, 1966).

In mammals, carbaryl undergoes hydroxylation at the *N*-methyl group via the action of microsomal enzymes (Boyd, 1972; Hassall, 1969). The resulting compound, an *N*-hydroxy-*N*-methylcarbamate, has been shown to be a potent competitive reversible inhibitor of acetylcholinesterase with the rate of enzyme inhibition being dependent upon the concentration of acetylcholine (Chiu et al., 1973).

A study of Sakai and Matsumura (1968) has given evidence that the action of carbaryl is not limited to acetylcholinesterase, but may inhibit other esterases as well. In their investigation, it was found that a number of esterases isolated from mouse brain were capable of hydrolyzing both carbamate and organophosphorus compounds. This finding points out the possibility that carbaryl might have an effect on the phospholipid metabolism in mammalian cells due to the presence in these compounds of several ester bonds which are susceptible to hydrolysis by esterases (White et al., 1968).

Dieldrin was introduced in 1948 as a substitute for DDT (Mellanby, 1967). Although its use has greatly diminished since that time, its high chemical stability has enabled it to remain in the environment in soil and water (Hassall, 1969). Due to the high lipid solubility of dieldrin, it is present in a variety of foods and in animal tissue today

(Cotner et al., 1968; Duggan et al., 1966). The specific mode of action of this compound remains unknown, but a recent study indicates that, in the insect at least, dieldrin acts at presynaptic membranes of cholinergic junctions, causing excessive and spontaneous release of presynaptic stores of acetylcholine (Shankland and Schroeder, 1973). Dieldrin and other chlorinated hydrocarbon insecticides are inducers of microsomal epoxidation (Gillett and Chan, 1968) and are also established as inducers of microsomal oxidases (Hassall, 1969). No information was found regarding possible effects of dieldrin upon phospholipid metabolism in mammalian cells.

METHODS

The growth medium of choice was Eagle's minimal essential medium (MEM) with Earle's salts and glutamate. The final medium contained 100 units/ml of penicillin, 100×10^{-6} g/ml each of streptomycin and neomycin, 2.5×10^{-6} g/ml of fungizone, and 5% calf serum. The final pH of the medium was 7.2–7.3.

Stock cultures of HeLa cells were grown in monolayers in 250-ml disposable Falcon culture flasks. Full monolayers were formed after incubation at 37° for approximately 24 hr.

Quantitation of Cell Numbers. Cells were counted on a Neubauer standard hemacytometer. The cells were harvested from the floor of the flask by the addition of a 0.25% trypsin solution.

Determination of the Effects of Insecticide Exposure on Cell Numbers. Flasks of cells in full monolayers were harvested and resuspended in 10 ml of MEM/flask. Total cell counts were made on each flask, and additional MEM was added to adjust the cell counts to 5×10^5 cells/ml. Two MEM stock solutions were prepared, one containing 16 ppm of carbaryl, and the other containing 16 ppm of dieldrin. The insecticides were added to 100 ml of medium as a dry powder, and were dispersed throughout the medium by vigorous shaking. Although both compounds are insoluble in water, this method did not result in a significant variation of experimental data. This is indicated by the high reproducibility of results obtained in the growth study. Disposable petri plates (60 mm diameter) were inoculated with 5×10^5 cells in 1 ml of MEM. To each plate was added a mixture of MEM and MEM containing 16 ppm of the respective insecticide in proportions that resulted in the desired insecticide concentration.

The final volume of medium in each plate was 4 ml. The following series of plates was prepared: a control plate containing normal MEM; plates containing MEM + carbaryl at 1, 2, 4, and 8 ppm; and plates containing MEM + dieldrin at 1, 2, 4, and 8 ppm. Triplicate plates were made for each experimental condition, and each experiment was repeated four times. The plates were incubated at 37° in an atmosphere of 10% CO_2 for a period of 48 hr. At the end of the incubation period, the medium was removed from the plates with a Pasteur pipet. The cells were washed with PBS and were harvested by exposure to trypsin and were resuspended in 3 ml of PBS, after which a total cell count was made. The percentage of inhibition or stimulation of cell growth for each insecticide dose was determined by using the following formula: % inhibition or % stimulation = $-\{[$(increase in the number of cells in the control) − (increase in the number of cells in the experimental)$]/$(increase in the number of cells in the control)$\} \times 100$. Actual cell growth figures (the increase in cell numbers above the original inoculum) were determined and placed into the formula. The original inoculum number of 500,000 was subtracted from all cell counts before calculations of inhibition or stimulation were made.

Cultivation of Cells for Protein and Phospholipid Quantitation. HeLa cell monolayers were grown in roller bottles (700 cm^2 total growth area). Six roller cultures were prepared for each control group as well as for each experimental group. The control groups included six cultures grown in normal MEM, and one culture each of cells grown in MEM containing 1, 2, 4, and 8 ppm of PBS. Seven other groups were tested with cells exposed to 1, 2, and 4 ppm of carbaryl and to 1, 2, 4, and 8 ppm of dieldrin. One liter of MEM was prepared for each experimental group. The insecticides, in the appropriate concentrations (e.g., 1 ppm of dieldrin = 1 mg/l. of MEM), were added to their respective medium as a dry powder, and were suspended evenly throughout the media by vigorous shaking before their addition to the inoculum or to the roller bottles. For each roller bottle, two stock culture flasks were trypsinized and combined in 10 ml of the appropriate medium. This suspension was added to a sterile roller bottle containing 90 ml of medium identical with that in the inoculum flask (final volume = 100 ml). Prior to inoculation, the pH of the medium in the bottle was adjusted to approximately 7.3 by bubbling a mixture of 10% CO_2 and 90% air through the medium for about 10 sec.

The cultures were incubated for 48 hr at 37°. The initial speed of rotation of the roller bottles was adjusted to 0.2 rpm to allow for the attachment of the cells in an even layer on the inside of the bottles. After 24 hr the speed was increased to 0.4 rpm for the remainder of the incubation period. The pH of the medium was tested at 24 hr and at the end of incubation to ensure proper growth conditions (Eagle, 1973). The pH was found to remain relatively stable over the entire incubation period. At the end of 48 hr the total cells were collected and the tube containing the cells was filled with nitrogen gas and sealed. The cell pellets obtained for each sample were frozen at −70°.

Preparation of Frozen Samples for Protein and Phospholipid Extractions. The frozen cell samples were opened and lyophilized in a VirTis freeze-drying unit under a vacuum of 50 μ and at a condenser temperature of −50°. Immediately before protein and lipid extraction, the cell samples were weighed and were transferred to clean, labeled tubes.

Extraction of Total Cellular Protein and Lipids. Cellular proteins and lipids were extracted following a modified procedure by Weinstein et al. (1969). To precipitate cellular protein, 0.5 ml of distilled methanol was added to each cell sample and allowed to stand for 15 min. This was followed by the addition to the cell sample of 0.5 ml of distilled chloroform. The chloroform–methanol mixture was allowed to stand for 15 additional min with occasional agitation of the tube. The extraction procedure was repeated five times: twice at 0°, twice at room temperature, and once at 45° under nitrogen. Following each extraction, the protein precipitate was spun down by centrifugation at 1200*g* for 10 min. The supernatant, containing the lipids dissolved in the chloroform–methanol mixture, was drawn off with a tuberculin syringe equipped with a no. 28 needle and was transferred to a clean no. 16 screw-cap test tube. The final volume of a total lipid extract was approximately 5 ml. The lipid extracts were stored under nitrogen in a desiccator jar at −20° until time for assay. The protein precipitate was dried under a stream of nitrogen and saved for quantitation.

Quantitation of Total Cellular Protein. A micro-biuret protein assay was employed (Itzhaki and Gill, 1964). The protein content of each sample was calculated by the use of a standard curve. The absorbance was measured on bovine serum albumin standards containing 400, 800, 1200, and 1600×10^{-6} g of protein in 2 ml of 30% NaOH. All of the samples and standards were read against a blank containing 1 ml of the $CuSO_4$ solution and 2 ml of 30% NaOH. The total amount of protein in a sample was calculated by multiplying the number of micrograms of protein in the 1-ml aliquot (obtained directly from the

Table I. Percent[a] Inhibition or Stimulation of HeLa Cell Growth[b] Obtained from Different Concentrations of Carbaryl and Dieldrin following Exposure for 48 hr

Compound	Concn, ppm[c]	% inhibition (–) or stimulation (+)
Control Cell Count = 4.9×10^5 Cells ± 2%		
Carbaryl	1.0	+9.44 ± 0.60
	2.0	+3.78 ± 0.17
	4.0	−11.32 ± 0.94
	8.0	−33.96 ± 2.61
Dieldrin	1.0	+7.55 ± 0.63
	2.0	+4.68 ± 0.47
	4.0	−5.85 ± 0.20
	8.0	−12.28 ± 0.71

[a] Percent represents change in cell number of treated cultures relative to a control group. [b] Each given value represents the mean of four triplicate samples with standard deviations given for each value. [c] Parts per million (ppm) of carbaryl and dieldrin are in 10^{-6} gram/milliliter.

standard curve) by the total number of milliliters in the sample. Finally, the percent protein in a cell sample was calculated by dividing the total protein value by the weight of the original cell sample.

Separation of Phospholipids. Phospholipids from each sample were separated by a two-dimensional TLC method (Rouser et al., 1970). The volume of a sample spotted was dependent on the weight of the original cell sample (0.20 ml was spotted for samples weighing less than 20 mg and 0.15 ml was spotted for samples weighing 20 mg or greater). The following solvent systems were employed: chamber 1, chloroform–methanol–ammonium hydroxide (65:25:5); chamber 2, chloroform–acetone–methanol–acetic acid–water (3:4:1:1:0.5).

Following development in the second solvent, the plates were spotted in the upper right region with an amount of the lipid sample equal to the volume of the same sample that was spotted for separation. This spot was used to determine the total phospholipid content of the sample.

The phosphorus content of each phospholipid was determined by measuring the amount of inorganic phosphorus liberated during the oxidative destruction of the lipid phosphatides. The phosphorus assay employed was a modification of the procedures described by Scott (1969) and Tietz (1970). The method was dependent on the formation of phosphomolybdate ions reduced to form a blue complex. The reductant employed was *p*-semidine (*N*-phenyl-*p*-phenylenediamine) because it produces maximal color intensity, which is quite stable, in a short time. When the reductant is oxidized, it also forms a blue complex which increases the overall sensitivity of the method. The separated phospholipids and the reference spot were scraped from the plate, and the material was placed in individual tubes. An unused area of the plate approximating the size of the reference spot was scraped, and the material was placed in a tube to serve as a blank. The tubes were placed on a Kjeldahl digestion rack which had been heated to 195°. The phospholipids were digested by the addition of 0.4 ml of 70% perchloric acid to each tube. Following digestion, the tubes were removed from the rack and were allowed to cool. Two milliliters of 12% perchloric acid, 0.2 ml of water, 1.0 ml of a 2.5% ammonium molybdate solution, and 2.0 ml of semidine reagent (50 mg of *p*-semidine hydrochloride in 100 ml of 1% $NaHSO_3$) were added to each tube. The tubes were allowed to stand for 10 min to allow for color development, and the thin-layer adsorbant was spun down by centrifugation at 900*g* for 10 min. The supernatant was transferred to a cuvette, and the absorbance of each solution was measured against the blank solution at 700 nm in a spectrophotometer. The total amount of phosphorus in each sample was read directly off of a standard curve. Inorganic phosphorus standards containing 5, 10, 15, and 20×10^{-6} g of phosphorus were used in the standardization procedure.

Standardization Procedure for the Thin-Layer Chromatography of Phospholipids. Standard solutions containing known amounts of specific phospholipids were separated by TLC in order to determine their respective migrations, separation patterns, and phosphorus contents. Three standard solutions were employed containing 1.0 mg/ml each of the following phospholipids: (1) phosphatidylcholine and sphingomyelin, (2) phosphatidylethanolamine and lysophosphatidylcholine, and (3) phosphatidylcholine, lysophosphatidylcholine, phosphatidylethanolamine, and sphingomyelin. Triplicate plates were run for each standard solution. A 0.1-ml aliquot of each standard was spotted, resulting in a total of 100×10^{-6} g of a specific phospholipid on the plate. The plates were developed as described previously, and the separated phospholipids were located by spraying the plates with the following reagents.

Standard No. 1. Phosphatidylcholine was detected with Dragendorff's reagent (Stahl, 1969). Sphingomyelin was detected with Phospray reagent (Supelco, Inc.).

Standard No. 2. Phosphatidylethanolamine was detected with ninhydrin reagent (Dittmer and Lester, 1964). Lysophosphatidylcholine was detected with Dragendorff's reagent.

Standard No. 3. All four of the phospholipids were detected with Phospray and were identified by comparing the plates with standards 1 and 2.

Other phospholipids (phosphatidic acid, phosphatidylserine, and phosphatidylinositol) in the cell extracts were located by comparing the thin-layer separations with a sample plate illustration supplied by Supelco, Inc. A phosphorus assay was performed on the phospholipids in standard No. 3 immediately after thin-layer separation in order to determine the ratio of lipid phosphorus to phospholipid (10^{-6} g of $P_i/10^{-6}$ g of lipid) for each phospholipid. This value was determined by dividing the number of 10^{-6} grams of inorganic phosphorus liberated during the digestion of the phospholipid spot by 100×10^{-6} g. The mean of the P_i/lipid ratios of the four phospholipid standards was used as the P_i/lipid value for phosphatidic acid, phosphatidylserine, and phosphatidylinositol.

Quantitative Analysis of Specific Phospholipids. The weight of individual phospholipids in 10^{-6} gram was determined by dividing the number of 10^{-6} gram of phosphorus in the specific phospholipid by its P_i/lipid ratio. The relative percentages of individual phospholipids in the cell samples were determined by dividing the weight in 10^{-6} gram of the specific phospholipid by the sum weight in 10^{-6} gram of all of the separated phospholipids.

RESULTS

Effects of Carbaryl and Dieldrin on HeLa Cell Growth. The effect of carbaryl and dieldrin on actively growing HeLa cells in vitro is summarized in Table I. The recorded values represent the mean of four samples done in triplicate. To obtain this value, the mean value of four separate controls was set to equal 100%. The experimental mean values were determined and then compared to the corresponding control mean value. The percent difference between the control value and each experimental value ± the standard deviation are the figures recorded in Table I.

Carbaryl was found to stimulate cell division at 1 and 2 ppm (+9.44 and +3.78%, respectively) and to inhibit cell division at 4 and 8 ppm (−11.32 and −33.96%, respectively). Similar results were observed with dieldrin (+7.55, +4.68, −5.85, and −12.28% for 1, 2, 4, and 8 ppm, respectively). Carbaryl was found to inhibit cell growth (possibly by cell death and/or by inhibition of cell division) to a greater degree than dieldrin at concentrations of 4 and 8 ppm.

Table II. Effect[a] of Carbaryl and Dieldrin on Total Protein Content of HeLa Cells[b] following Exposure for 48 hr

Compd	Concn,[c] ppm	Total protein, %	% inhibition (−) or stimulation (+) of cellular protein[d]
Control		56.25 ± 4.39	
Carbaryl	1.0	59.70 ± 4.28	+6.24 ± 0.93
	2.0	57.00 ± 5.22	+1.33 ± 0.23
	4.0	49.95 ± 5.01	−11.20 ± 1.99
Dieldrin	1.0	55.43 ± 6.58	−1.65 ± 0.32
	2.0	53.39 ± 6.90	−5.08 ± 1.05
	4.0	51.15 ± 5.55	−9.07 ± 1.69
	8.0	47.32 ± 4.43	−15.83 ± 2.72

[a] Each given value represents the mean of six samples with standard deviations given for each value. [b] Total protein = milligrams of protein/milligram dry weight of cells in percent. [c] Parts per million (ppm) of carbaryl and dieldrin are in 10^{-6} gram/milliliter. [d] Percent represents change in cellular protein of treated sample relative to the control.

Effects of Carbaryl and Dieldrin on Protein in HeLa Cell Cultures. The effect of carbaryl and dieldrin on the protein content of HeLa cells is summarized in Table II. The recorded values represent the mean of six samples. Mean values for percentage of protein (milligrams of protein/milligram dry weight of cells) in the control and experimental groups were calculated followed by the standard deviation from the mean for each group. The percent of inhibition or stimulation of cellular protein for each experimental group relative to the control group was calculated from the total protein values for each group. Standard deviations for these values represent the sum of the variations found within control group and the corresponding experimental group from the total protein calculations.

There was no significant change in the protein composition of HeLa cells treated with 1 and 2 ppm of either carbaryl or dieldrin. However, a slight decrease in total protein was noted with carbaryl at 4 ppm (−11.20%) and with dieldrin at 8 ppm (−15.83%).

Effects of Carbaryl and Dieldrin on Specific Phospholipid Content in HeLa Cell Cultures. The effect of carbaryl and dieldrin on specific phospholipid content of HeLa cells is summarized in Table III. Each recorded value represents the mean of six samples. (Duplicate thin-layer chromatography separations were performed on each of three lipid extracts from the control group and from each experimental group.) The values given for each phospholipid are in percentage of the total phospholipid content of the particular group (control or experimental). Standard deviations from the mean of the six determinations follow each value.

Carbaryl at concentrations of 2 and 4 ppm caused an increase in the sphingomyelin level of HeLa cells. The percentage of sphingomyelin in the total phospholipid fraction increased from a control value of 6.50% to 8.29 and 9.70% for 2 and 4 ppm. These figures represent respective increases in the total sphingomyelin level of 27.53 and 49.20%. An increase in the level of lysophosphatidylcholine was noted in cells exposed to 2 and 4 ppm of carbaryl, where the percentage of the total phospholipid fraction was increased from a control value of 6.54% to 9.72 and 8.98% (representing respective increases in the total lysophosphatidylcholine level of 48.60 and 37.31%). Finally, a slight decrease in the level of phosphatidylcholine was noted in cells exposed to 2 and 4 ppm of carbaryl, where the percentage of the total phospholipid fraction decreased from a control value of 42.32% to 36.78 and 38.97% (representing respective decreases in the total phosphatidylcholine level of 13.00 and 9.21%).

Dieldrin was found to cause no significant changes in the phospholipid content of HeLa cells, with the exception of a decrease in the level of phosphatidylcholine in cells exposed to 8 ppm of the compound. This value for phosphatidylcholine decreased from a control value of 42.32% of total fraction to 35.17% (representing a decrease in total phosphatidylcholine of 16.92%).

DISCUSSION

The cytotoxicity of analytical grades of carbaryl and dieldrin on HeLa cells exposed to different concentrations of these insecticides for 48 hr was found to be dependent on the concentrations used. Stimulation of cell division at lower concentration (1 and 2 ppm) of both insecticides followed by increasing toxicity on the cells at higher concentrations (4 and 8 ppm) supports the findings of Litterst et al. (1969). However, the toxicity of both chemicals was found to be much greater in this study. For instance, the studies by Litterst showed stimulation of growth of HeLa cells by both carbaryl and dieldrin at 10 ppm. Doses of both chemicals that produced 50% inhibition in cell division were found to be 250 ppm for carbaryl and 30 ppm for dieldrin. Their results compare to the onset of inhibition of cellular division at concentrations above 2 ppm for both carbaryl and dieldrin that were observed in this study. A concentration of 8 ppm of carbaryl and dieldrin produced inhibitions of −33.96 and −12.88%, respectively.

The greater degrees of toxicity observed in this study appear to be dependent on the different method of exposure of cells to the insecticides. Cells were exposed to the various concentrations of carbaryl or dieldrin at the time of inoculation and were allowed to incubate for 48 hr prior to analysis. In the experiment performed by Litterst the cells were allowed to incubate for 48 hr prior to the introduction of the insecticides. The cells were analyzed at 72 hr. In both procedures the original inoculum consisted of 5×10^5 cells. The procedure employed in this study was preferred because the cells were exposed to the insecticides throughout a period of active cell division. It has been noted in this laboratory that full monolayers are formed at the end of 48 hr. Therefore, the rate of cell division is not as rapid after this time. This decreased rate of division, coupled with a general decrease in metabolic processes in the cells, might be responsible for the lower toxicity observed for carbaryl and dieldrin.

In this study, there was no evidence of a significant increase in cellular protein in cells exposed to lower concentrations (1 and 2 ppm) of carbaryl or dieldrin. These results are not consistent with the findings of Chung et al. (1967), who noted a stimulation of cellular protein at the lower insecticide doses. At higher concentrations, however, both compounds caused a decrease in cellular protein as previously reported. Once again, these conflicting results may be due to differing methods of exposure of the cells to insecticides as was the case in the growth study. In addition, the protein assay used in this study was merely a measurement of the total protein content of the cells, rather than a direct measurement of protein synthesis by the uptake of radioactive precursers.

Both carbaryl and dieldrin are known inhibitors of esterases and oxidative enzymes in the microsomal fraction of mammalian cells (Gillett and Chan, 1968; Sakai and Matsumura, 1968). Such changes in enzyme activity could be a proposed mechanism for toxicity to cells of different origins. Carbaryl and dieldrin are both known to be metabolized to form more toxic compounds in certain types of mammalian cells (Chiu et al., 1973) resulting in effects on cell division and/or death of the cell.

The changes that were observed in the phospholipid content of exposed HeLa cells offer further evidence of alterations of cellular metabolism by carbaryl and dieldrin. The changes that were observed in the specific constitution of the phospholipid fraction appear to be highly sig-

Table III. Effect[a] of Carbaryl and Dieldrin on Specific Phospholipid Content of HeLa Cells[b] following Exposure for 48 hr

Phospholipid	Control, %	1 ppm[c] of carbaryl, %	2 ppm[c] of carbaryl, %	4 ppm[c] of carbaryl, %
Lysophosphatidylcholine	6.54 ± 0.94	5.61 ± 1.65	9.72 ± 1.52	8.98 ± 1.94
Sphingomyelin	6.50 ± 1.01	7.29 ± 1.76	8.29 ± 0.91	9.70 ± 2.84
Phosphatidylcholine	42.32 ± 1.43	40.68 ± 3.52	36.78 ± 1.13	38.97 ± 1.39
Phosphatidylethanolamine	28.62 ± 0.75	29.95 ± 2.80	25.94 ± 2.82	27.25 ± 1.56
Phosphatidic acid	6.35 ± 1.44	6.98 ± 0.92	8.28 ± 1.21	6.61 ± 1.38
Phosphatidylserine, phosphatidylinositol, and others	9.53 ± 0.67	9.64 ± 1.39	11.01 ± 0.43	8.55 ± 1.99

Phospholipid	1 ppm[c] of dieldrin, %	2 ppm[c] of dieldrin, %	4 ppm[c] of dieldrin, %	8 ppm[c] of dieldrin, %
Lysophosphatidylcholine	8.50 ± 0.92	6.18 ± 2.91	7.50 ± 1.09	8.45 ± 0.46
Sphingomyelin	7.11 ± 1.14	7.83 ± 2.78	7.92 ± 3.03	6.77 ± 2.38
Phosphatidylcholine	32.58 ± 3.33	41.36 ± 2.07	39.58 ± 1.32	35.17 ± 1.27
Phosphatidylethanolamine	32.83 ± 0.89	29.96 ± 2.92	26.95 ± 1.18	31.11 ± 4.23
Phosphatidic acid	5.24 ± 1.34	7.14 ± 3.80	7.37 ± 0.85	6.83 ± 1.76
Phosphatidylserine, phosphatidylinositol, and others	13.85 ± 0.23	7.35 ± 2.28	10.67 ± 1.95	11.67 ± 2.12

[a] Each value represents the mean of six samples with standard deviations given to each value. [b] Each value represents the percentage of total phospholipid content in the respective sample for the specific phospholipid. [c] Parts per million (ppm) of carbaryl and dieldrin are in 10^{-6} gram/milliliter.

nificant. The increase in sphingomyelin content observed in cells exposed to carbaryl (Table III) suggests the inhibition of sphingomyelinase, an esterase essential for the catabolism of sphingomyelin in man and other mammals. According to White et al. (1968), sphingomyelinase catalyzes the hydrolysis of sphingomyelin or dihydrosphingomyelin to form ceremide and phosphorylcholine. The action of the enzyme appears to be highly specific.

The alteration of sphingomyelin metabolism further suggests effects on the total organism as well as on the single cell. It has been hypothesized that sphingomyelin and phosphatidylcholine are the major components of the outer half of the lipid bilayer in human erythrocytes and in other mammalian cells (Bretscher, 1972). Sphingomyelin and other sphingolipids are found in high quantities in the membranes of nervous tissue where a rapid turnover rate for phospholipids has been observed (White et al., 1968). The absence of or markedly lowered presence of sphingomyelinase has been noted in the spleens of patients with Neimann-Pick disease, a metabolic disorder characterized by the accumulation of unusual amounts of sphingomyelin (particularly in the spleen and liver) (White et al., 1968).

Both carbaryl and dieldrin were found to have an effect on phosphatidylcholine, which is a primary constituent of biological membranes (Robertson, 1972; Bretscher, 1972). The most significant change in phospholipid constitution of HeLa cells was noted with phosphatidylcholine in cells exposed to dieldrin (Table III). Similar results were noted with carbaryl, but to a lesser degree than with dieldrin.

Although the results observed in this study do not offer explanations regarding the exact modes of action of carbaryl and dieldrin on phospholipid metabolism, they do supply information regarding the implications of such alterations on the membrane structures in cells of human origin. Such structural changes could be of importance in regard to the toxicity of these insecticides to mammalian cells.

Of equal importance, however, are the effects that phospholipid alterations could have on an entire organism, especially on the nervous system. The modes of action of carbaryl and dieldrin in the insect involve a breakdown in nervous function (described previously). It is known that the proper transmission of nerve impulses is dependent to a degree on the relative permeability of the nerve fiber membrane to ions of potassium, sodium, and chlorine (K^+, Na^+, and Cl^-) (Eccles, 1973). A change in the structure of this membrane could have an effect on the normal transmission of nerve impulses in organisms exposed to sufficient amounts of carbaryl, dieldrin, and other insecticides as well.

Metabolic alterations in HeLa cells exposed to carbaryl and dieldrin suggest that insecticides from different chemical groups (such as carbamate and organochlorine insecticides) are nonspecific in their action upon mammalian cells in vitro. The changes in phospholipid composition elicited by both compounds indicate that certain environmental chemicals may have damaging effects on the total organism as well as in the single cell. No specific possibility discussed here can be preferred on the basis of the data resulting from this study. However, the results appear to necessitate the need for more investigation in this area.

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Metabolism of *o,p'*-DDT in Chickens

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The following metabolites were identified in chicken excreta after dosing with *o,p'*-DDT-^{14}C: *o,p'*-DDD; *o,p'*-DDT; 3-hydroxy-2,4'-DDD; 3-hydroxy-2,4'-DDT; 4-hydroxy-3-methoxy-2,4'-DDD; 4-hydroxy-3-methoxy-2,4'-DDT; *o,p'*-DDA; 3-hydroxy-2,4'-DDA; 4-hydroxy-3-methoxy-2,4'-DDA; *o,p'*-DDE; 3-hydroxy-2,4'-DDE; 4-hydroxy-3-methoxy-2,4'-DDE; 4-hydroxy-2,4'-DDE; the methyl ester of *o,p'*-DDA; the methyl ester of a methoxy-2,4'-DDA (probably the 3 isomer); and 4-hydroxy-2,4'-DDD. A significant species difference was found in the metabolism of *o,p'*-DDT; chickens metabolized *o,p'*-DDT to four DDE-type compounds, whereas rats did not.

Our study of the metabolism of *o,p'*-DDT in rats revealed an extensive metabolic breakdown, with the formation of at least 13 metabolites (Feil et al., 1973); however, *o,p'*-DDE-type compounds were, surprisingly, not formed. Dehydrochlorination and hydroxylation at the vacant para position to yield 4-hydroxy-2,4'-DDE were postulated as an explanation for the greater estrogenicity of the ortho, para' isomer. Because responses of different species to technical DDT have been observed to vary with respect to several biological activities (Sell and Davison, 1973; Davison and Sell, 1974), we studied the metabolism of *o,p'*-DDT in chickens. The isolation and characterization of 16 metabolites are presented in this report.

EXPERIMENTAL SECTION

Apparatus. Some of the mass spectra were taken with a Varian CH-5DF mass spectrometer. Other equipment used was as previously described (Feil et al., 1973).

***o,p'*-DDT-*ring*-U-^{14}C.** Crude 1,1,1-trichloro-2-(*o*-chlorophenyl-U-^{14}C)-2-(*p*-chlorophenyl-U-^{14}C)ethane was obtained from Amersham/Searle Corp., Arlington Heights, Ill., and purified as previously reported (Feil et al., 1973).

Animal Experiments. Two mature Leghorn hens, surgically modified to facilitate collection of urine and feces (Paulson, 1969), were given a single oral dose of *o,p'*-DDT-*ring*-U-^{14}C (0.3 μCi/mg) corresponding to 150 mg/kg. Urine and feces were collected for 4 days. The hens had free access to water and to a commercial 16% protein laying mash for several weeks prior to and throughout the experiment.

A 2.1-kg Leghorn hen was given 10 mg (3 μCi) of *o,p'*-DDT-*ring*-U-^{14}C per day for 25 days. Excreta were collected for 31 days.

Purification of Urinary Metabolites. Urine was extracted, first with hexane, then with ethyl acetate, to yield three fractions (hexane soluble, ethyl acetate soluble, and water soluble) from which metabolites were isolated.

Metabolites **15** and **19** were isolated from the hexane-soluble fraction by gas chromatography without preliminary cleanup (6 ft, 3% OV-1, Gas-Chrom Q, 150–250° at 5°/min).

Ethyl acetate extracts were chromatographed on LH-20 with methanol to effect a partial separation of radioactive compounds. A large radioactive peak was eluted first, followed by a broad intermediate peak, then a small peak. After treatment with diazomethane, the material in the large peak was chromatographed on LH-20 with methanol and then on silica gel with ethyl acetate–ethanol. The methyl ester of metabolite **7** was purified finally by gas chromatography (6 ft, 3% OV-1, Gas-Chrom Q, 170–270° at 5°/min). Several other radioactive compounds were also present, but these could not be obtained in sufficient purity for identification. In like manner, the methyl ester of metabolite **7** was also isolated from the intermediate peak, again with evidence of other metabolites that could not be purified adequately for identification. No identifications were made on the activity in the small peak.

The water-soluble fraction was hydrolyzed by refluxing with either 2.5 N hydrochloric acid or 2.5 N sodium hydroxide for 24 hr. After acidification of the basic hydrolysis, the work-up procedures were similar for the two hydrolysates. The hydrolysates were extracted with ether, and the ether extracts in turn were extracted with sodium bicarbonate. The bicarbonate extracts were acidified, extracted with ether, and chromatographed on LH-20 with methanol. The radioactive fraction was then derivatized with diazomethane and purified by gas chromatography [6 ft, 3% OV-1, Gas-Chrom Q, 170–270° at 5°/min (retention times, 5.8 and 10.6 min)]; the methyl ester of metabolite **7** and the methyl ether–methyl ester of metabolite **9** were obtained in this manner after both acid and base hydrolyses.

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