Tissue Residue Studies on Toxaphene in Broiler Chickens

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Low levels of toxaphene (0, 0.22, 0.40, 2.16, and 3.82 ppm) were added to the diets of unsexed broiler chicks from 1 day of age. Each treatment consisted of 90 randomly selected birds (30 birds in each of three replicates). On the sixth week, 15 birds in each replicate were transferred to floor pens and fed toxaphene-free feed. Three birds from each of the replicates were sacrificed at 2-week intervals. Liver, heart, gizzard, kidneys, skeletal muscle, and fat tissue samples were taken for residue analysis. The accumulation of toxaphene in the adipose tissue of 8-week-old broilers was best described by the equation, $\hat{y} = 0.765 + 4.081x$, where $\hat{y}$ = ppm of toxaphene in adipose tissue, x = ppm of toxaphene in the ration (detectability limit = 1.0 ppm). Toxaphene dissipation was characterized by the following equation: $\hat{y} = e^{(-0.280x + 1.97)}$. Histopathological examination of 4- and 8-week-old birds revealed no significant lesions in any of the tissues examined.

The chlorinated insecticide toxaphene is more extensively used than any other pesticide in the Southeastern United States (Andrilenas, 1975). Little information, however, is available concerning the fate of this pesticide in either the environment or the successive phases of the food chain. Studies related to the accumulation and dissipation of toxaphene are, therefore, critically needed to prevent or at least intelligently deal with accidental contamination.

A pesticide monitoring program (Bush et al., 1975) revealed the occasional occurrence of toxaphene in various filler commodities used in feed formulation such as peanut hulls and rice mill feed. The extent of bioaccumulation of various chlorinated hydrocarbons in broilers was reported by deVos et al. (1972) and summarized by Kan and Tuinstra (1975). Little or no information is available in the literature dealing with the bioaccumulation or dissipation of toxaphene residues in broiler chicken tissues.

The objectives of this study were to determine (a) rate of toxaphene accumulation as a consequence of feeding graded levels of toxaphene to day-old chicks for a period of 8 weeks (or until the broiler slaughter stage), (b) tissue distribution and storage pattern of toxaphene residues in the organs and tissues of broiler chickens, and (c) decay profile or depletion rate of toxaphene in the growing broiler chicken. Toxaphene-^{36}Cl was used to obtain lower detectability limits for tissue residues and confirmation of GLC residue data.

MATERIALS AND METHODS

Experimental Design and Procedures. The study of the effect of toxaphene on broilers was undertaken in two phases. In study I, dosages of 0, 0.22, 0.40, and 2.16 ppm toxaphene labeled ^{36}Cl were added to the formulated diet (Bush et al., 1977) of 1-day-old unsexed broiler chicks (Hubbard-Hubbard). In study II, dosages of 0 and 4 ppm nonradioactively labeled toxaphene were added to the diet of 1-day-old broiler chicks.

Study I. A total of 90 randomly selected broiler chicks per treatment group (three replicates of 30 birds each) were individually wing banded and weighed prior to the initiation of the feeding trial. The birds were initially housed in electrically heated brooder batteries in groups of 30 birds each. The birds received continuous incandescent light. Daily observations were made for any overt clinical signs and the birds were individually weighed at weekly intervals to monitor their growth rate. Feed and water were supplied ad libitum.

Prior to the initiation of the feeding trial, the diet was analyzed for initial pesticide residue content and was found to be free of contamination. After the mixing of the treated feed, the diets of each of the four treatment groups were again analyzed for pesticide residue content by SC and GLC and found to contain 0, 0.22, 0.40, and 2.16 ppm toxaphene, respectively. The radiolabeled ^{36}Cl toxaphene (sp act. 98.2 dpm/mg) used to spike the feed was supplied by Hercules, Inc.

On weeks 1, 2, 4, 6, and 8, three birds per replicate (a total of nine birds per treatment) were sacrificed by cervical dislocation, and liver, kidney, heart, skeletal muscle, gizzard, and abdominal fat were collected for residue analysis. The liver, kidneys, heart, and gizzard from each bird were individually weighed. At 4 and 8 weeks of age, tissues (liver, kidney, heart, small intestine, brain, pancreas, spleen, bursa fabricius, proventriculus, and gizzard) were collected and fixed in formalin for histopathological examination. All tissues for residue analysis were frozen and stored until pesticide residue analysis could be completed.

To determine the depletion rate of toxaphene from avian tissue, 15 birds from each of the replicates (45 birds per treatment group) were moved to clean floor pens at the end of 6 weeks and fed toxaphene-free feed. The floor litter consisted of wood chips containing no detectable toxaphene. Three birds from each of the replicates were sacrificed at 6, 8, and 10 weeks of age. Skeletal muscle and abdominal fat were collected from each sacrificed animal and were frozen and stored for residue analysis.

Study II. A total of 180 1-day-old unsexed Hubbard-Hubbard broiler chicks were individually weighed, wing banded, and assigned to two treatment groups of three replicates each (30 chicks per replicate). The birds were housed in standard battery brooders, supplied with feed and water ad libitum, and received continuous incandescent lighting. The birds were individually weighed at weekly intervals for the first 8 weeks of the feeding trial.

The feed used in this part of the study was analyzed for pesticide residue content and found to be free of contamination. Analytical grade toxaphene (Chem Service, Inc., West Chester, Pa.) was added to the feed. Dosages of 0 and 3.82 ppm toxaphene used in this part of the study were verified by residue analysis.

On days 7, 14, 28, 42, and 56, three birds from each replicate were sacrificed by cervical dislocation and tissues collected for residue analysis. These tissues (liver, kidney, heart, gizzard, skeletal muscle, and fat) were frozen as in study I and stored for residue analysis. The heart, gizzard,

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liver, and kidneys of each bird were weighed. At weeks 4 and 8, samples of the above organs as well as samples of the small intestine, brain, pancreas, spleen, bursa fabricius, and proventriculus were fixed in formalin for histopathological examination.

At the end of six weeks, 15 birds from each of the replicates were placed in floor pens and taken off the treated feed in order to monitor the depletion rate of toxaphene during a 10-week withdrawal period. Three birds from each of the replicates were sacrificed at biweekly intervals and fat and skeletal muscle were collected, quick frozen, and stored for pesticide residue analysis.

Feed and tissue samples were extracted for GLC analysis as previously described (Bush et al., 1977). The residue extracts were made to 10 mL with gel permeation chromatography (GPC) solvent (75:25, ethyl acetate–toluene) for fat removal by GPC (Johnson et al., 1976).

Abdominal fat samples were thawed and placed in 16 × 125 mm glass culture tubes with Teflon-lined caps and melted in a hot-water bath. A 1-g sample of the liquified fat was transferred to a graduated culture tube and made to a total volume of 10 mL with ethyl acetate–toluene (3:1). The tubes were thoroughly mixed with a vortex mixer and subsequently centrifuged for 15 min ($^1/_2$ speed) to remove insoluble materials. Fat was removed by GPC (Johnson et al., 1976).

Gel Permeation Chromatography Cleanup. Samples were prepared for quantification of toxaphene residues by GLC through the use of a Gel Permeation Chromatography System (GPC-1001, Analytical Biochemistry Labs, Columbia, Mo.) containing a 2.5 × 27 cm column of SX-3 Bio-Beads (3% divinylbenzene–polystyrene copolymer) and using ethyl acetate–toluene (3:1 v/v) as the eluting solvent and operated under the following conditions: solvent flow rate, 4.5 mL/min; dump time, 20 min; collect time, 20 min; wash time, 10 min. The pesticide containing fraction was collected in a 250-mL boiling flask and reduced to near dryness on a Buchi Rotavapor. Five milliliters of hexane was added to the flask, and the contents were transferred into a 16 × 125 mm glass culture tube with Teflon-lined screw cap for storage until quantification by liquid scintillation counting (LSC) and/or GLC methods could be completed.

Toxaphene Residue Quantification. All tissue samples (studies I and II) were analyzed by gas–liquid chromatography. The use of toxaphene-^{36}Cl in study I permitted the analysis of a split sample by LSC. Gas chromatographic analysis was accomplished through the use of a Tracor gas chromatograph (Model 550, Tracor Inc., Houston, Tex.) equipped with 6 ft × 0.25 in. coiled glass columns and ^{63}Ni electron-capture detectors. The columns were packed with either 3% OV-1 on Anakrom ABS (80/100 mesh) or 2% OV-17 plus 1% OV-210 on Gas-Chrom Q (100/120 mesh) (Supelco, Inc., Bellefonte, Pa.). The operating conditions were: carrier gas, nitrogen; flow rate, 65 cm^3/min; column oven temperature, 215 °C; inlet temperature, 265 °C; a chart speed of 0.5 in./min.

Identification of toxaphene residues present was based on their retention times relative to those of reference toxaphene standard solution. Toxaphene levels were determined by comparison of peak height of sample chromatograms to those of an analytical standard in a spiked sample. The average of the three peak heights (RRT aldrin = 3.40, 4.11, 5.64) was taken as the toxaphene response (Gaul et al., 1966).

A reagent blank and a spiked sample were included with each set of analyses and values were corrected for percent recovery. Samples spiked with radioactive toxaphene-^{36}Cl at the time of homogenization gave average percentage recovery of greater than 70% in feed, muscle, egg, and fat samples.

Table I. Comparison of GLC and Liquid Scintillation Methods of Toxaphene Determination[a]

Treatment	GLC $\bar{X}$, ppm	LSC $\bar{X}$, ppm
Group 5 (0 ppm)	N.D.	N.D.
Group 6 (0.22 ppm)	1.00 ± ---	2.14 ± 0.13
Group 7 (0.40 ppm)	4.40 ± 0.58	3.33 ± 0.10
Group 8 (2.16 ppm)	10.59 ± 1.36	9.85 ± 0.77

[a] Adipose tissue was heated at 100 °C until fat was liquid (connective tissue remains). A 0.2-g aliquot of fat was taken for ^{36}Cl determination and 0.5-g aliquot was cleaned up via GPC and analyzed by GLC.

Table II. Effect of Toxaphene on the Average Body Weight of 8-Week-Old Broilers[a]

Treatment, ppm	Body weight, g (mean)
Study I	
0	1575.4 ± 189.6 abc
0.22	1491.6 ± 162.0 abcd
0.40	1379.9 ± 186.9 cde
2.16	1442.7 ± 113.1 bcde
Study II	
0	1760.5 ± 197.6 a
4	1715.2 ± 245.2 ab

[a] Treatment means in a column not followed by the same letter are significantly different at the 0.05 level according to the Duncan's multiple range test. In study I, Hubbard-Hubbard broiler chicks were fed graded levels (ppm) of toxaphene-^{36}Cl for 8 weeks. In study II, Hubbard-Hubbard broiler chicks were fed a ration fortified with analytical grade toxaphene for a period of 8 weeks.

LSC Analysis. Residue patterns in the tissue samples from study I were determined through the use of a Liquid Scintillation Spectrophotometer (Packard Model 3380, Packard Instruments Inc.). Samples cleaned up by GPC were made to 5 mL with hexane, and the samples were split with 2.5 mL going through gas chromatographic analysis and 2.5 mL of the sample transferred to a glass scintillation vial for ^{36}Cl determination. Ten milliliters of the scintillation cocktail (1.5 L of dioxane, 0.4 g of POPOP) was added to the sample. Each sample was counted, along with a blank for background determination, for 100 min or 10000 counts at the following settings: 4 °C; green channel, preset count 10000, window opening 25–250, 1% gain. The blue or main counting channel settings were: preset count 10000, window opening 50–5000, and 1% gain. After counting for 100 min the samples were spiked with 0.1 mL of an external standard (an 18.6 ppm standard of toxaphene-^{36}Cl in isooctane) and counted for additional 100 min at the same settings.

Values obtained by LSC were significantly lower than those for GLC (Table I). Data obtained from both gas chromatographic analysis and liquid scintillation counting were statistically analyzed using a one-way analysis of variance table and Duncan's multiple range test with a significance level of $P < 0.05$ (Duncan, 1955).

RESULTS AND DISCUSSION

Feeding toxaphene at levels up to 4 ppm did not produce toxicity symptoms in broiler chickens throughout the 8-week study. Mortality was minimal, with all groups including the control group, having less than 5% mortality. No significant reduction in body weight was observed at 8 weeks in either feeding trial (Table II). The birds in the second trial were somewhat larger than those in the

Table III. Effect of Toxaphene on Organ Weights (g) at 8 Weeks[a]

Treatment, ppm	Mean organ weight, g			
	Heart	Liver	Kidney	Gizzard
Study I				
0	7.10 ± 1.69 c	56.47 ± 5.89 ab	13.22 ± 1.61 abc	38.01 ± 7.67 ab
0.22	7.19 ± 0.62 c	49.68 ± 6.45 abcd	12.21 ± 1.39 abcd	36.04 ± 5.71 ab
0.40	6.18 ± 0.83 c	40.48 ± 6.09 d	8.94 ± 1.33 e	29.43 ± 5.99 b
2.16	6.57 ± 1.29 c	58.29 ± 6.42 a	10.92 ± 2.83 cde	38.67 ± 6.47 a
Study II				
0	11.94 ± 3.85 a	53.75 ± 11.68 abc	14.48 ± 2.34 a	37.15 ± 4.72 ab
4	10.93 ± 2.37 ab	43.38 ± 6.86 d	14.19 ± 2.35 a,b	36.14 ± 6.11 ab

[a] Treatment means in a column not followed by the same letter are significantly different at the 0.05 level according to the Duncan's multiple range test. Birds were fed graded levels (ppm) of toxaphene (study I: toxaphene-^{36}Cl; study II: analytical grade toxaphene) for a period of 8 weeks.

Table IV. Effect of Toxaphene on Feed Conversion[a]

Dietary level, ppm	Feed conversion
Control	1.88 a
0.22	1.94 a
0.40	1.87 a
2.16	1.93 a

[a] Treatment means in a column not followed by the same letter are significantly different at the 0.05 level according to the Duncan's multiple range test. Birds were fed graded levels of toxaphene for 6 weeks and the feed conversion was determined.

first trial. There were no significant treatment related changes in the heart weight at 8 weeks (Table III). The birds fed 0.4 ppm in their diet tended to have decreased liver, kidney, and gizzard weights at 8 weeks (Table III). No effect on feed conversion was observed (Table IV).

Histopathological examination of the 4- and 8-week-old birds revealed no significant lesions in any of the tissues examined. Necropsy of 8-week-old birds fed 0.4 and 2.1 ppm toxaphene in the diet revealed deformation of the sternum involving the cartilaginous tissues. In addition, an increase in the growth of cartilage was found in the second feeding trial (4.0 ppm toxaphene). Dietary Ca and P were analyzed and found to be 1.5% Ca and 0.5% P, respectively, in both treated and control rations.

Plotting the level of toxaphene in adipose tissue of birds fed graded levels of toxaphene (Figure 1), an initial rapid rise in tissue residue levels (0–4 weeks) is observed, followed by a somewhat slower increase thereafter. This slower rate of increase in toxaphene levels from 6 to 8 weeks is probably due to the large amount of fat (dilution effect) broilers put on during this period (Edwards et al., 1973).

The level of toxaphene in the excisable adipose tissue of 8-week-old birds increased with increasing dietary toxaphene as described by the equation $y = 0.264 + 4.081x$, where x is the level of toxaphene in the feed (Figure 2). Toxaphene, therefore, has a bioaccumulation factor of 4.9 which is substantially less than the accumulation factor for hexachlorobenzene (12–30) (Reed et al., 1977a,b), heptachlor epoxide (17), and dieldrin (17). The bioaccumulation factor of toxaphene approximates that of lindane (3) (Onley et al. 1975).

Assuming 2.0 lb of feed were fed per pound of weight gain, a 3.5-lb bird would have consumed 7 lb of feed containing 4 ppm of toxaphene or 12 727 μg. If an 8-week-old bird contains approximately 10% fat (Edwards et al., 1973), then a residue level of 400 ppm would be expected in the adipose tissue. Birds fed 4 ppm actually contain 21 ppm toxaphene. Thus, only 5% of consumed toxaphene is accounted for in the adipose tissue of the birds. Toxaphene accumulates to a level of four to five

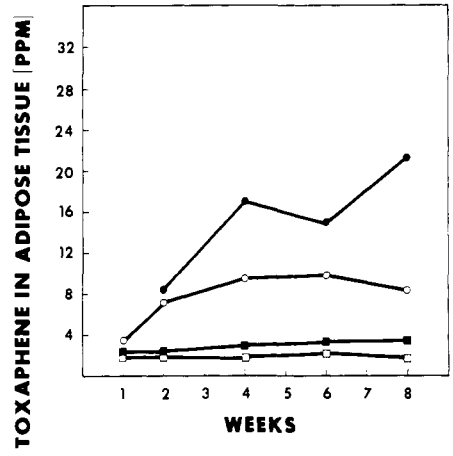

Figure 1. Toxaphene levels in adipose tissue of birds fed graded levels of toxaphene for periods up to 8 weeks. Toxaphene levels were determined by GLC (feeding levels: (□) 0.22 ppm; (■) 0.40 ppm; (○) 2.16 ppm; (●) 3.82 ppm).

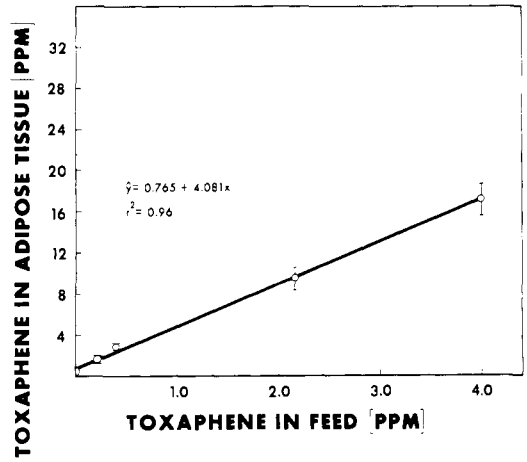

Figure 2. Toxaphene levels in adipose tissues of 8-week-old birds as a function of toxaphene levels in the feed. Confidence limits are ± one standard deviation. Values were determined by GLC.

times that in the feed, but this still accounts for only 5% of the toxaphene consumed as compared to 100% for HCB (Reed et al., 1977a,b) and 50% of the polychlorinated biphenyls (Britton and Charles, 1974).

Heart, gizzard, leg muscle, kidney, breast muscle, and liver tissues were analyzed from birds fed toxaphene for 8 weeks. Accumulation ratios for all tissues were: heart, 0.16; gizzard, 0.16; leg muscle, 0.13; kidney, 0.09; breast muscle, 0.05; and liver, 0.03 (Figures 3 and 4). These values are lower than the accumulation ratios for heptachlor epoxide (0.82) and dieldrin (0.84) reported for broiler liver tissues (Onley et al., 1975). Breast muscle and

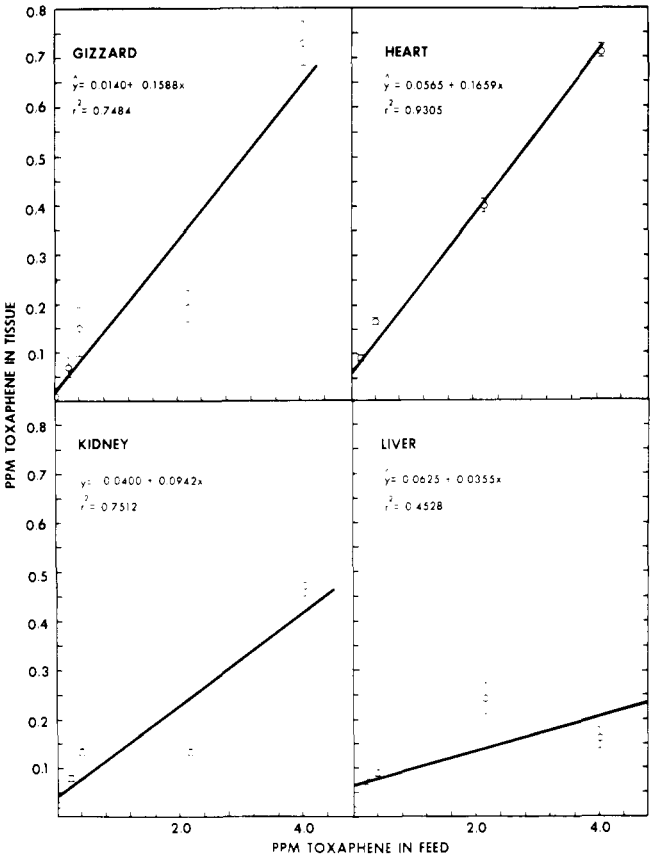

Figure 3. Toxaphene accumulation in gizzard, heart, kidney, and liver tissues of 8-week-old broilers fed graded dietary levels of toxaphene. Residue levels for 3.82 ppm feeding level were determined by LSC. Confidence intervals are ± one standard deviation.

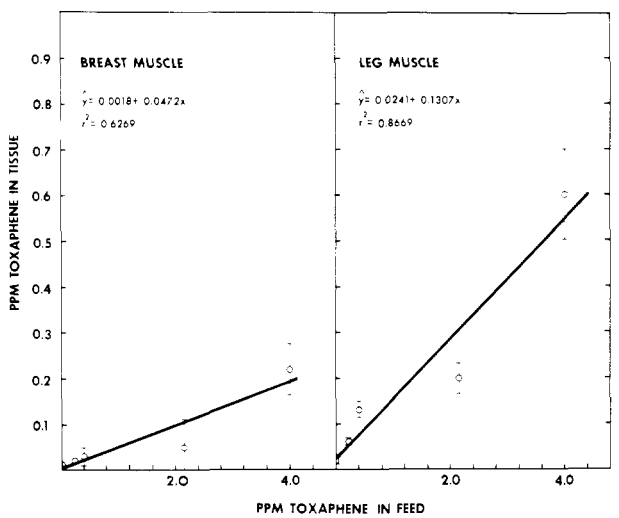

Figure 4. Toxaphene accumulation in leg and breast muscle tissue of 8-week-old broilers fed graded dietary levels of toxaphene. Residue levels for 3.82 ppm were determined by GLC. All other levels were determined by LSC. Confidence intervals are ± one standard deviation.

leg muscle from broilers fed 0.75 ppm endrin for 6 weeks were found to contain 0.24 and 0.3 ppm endrin, thus suggesting a biomagnification factor of 0.32 and 0.40, respectively. Thus toxaphene does not accumulate to a significant extent in edible tissues other than fat, and the relative amount in each tissue is probably related to the amount of fat in the various tissues since chlorinated hydrocarbon accumulation has been found to be directly

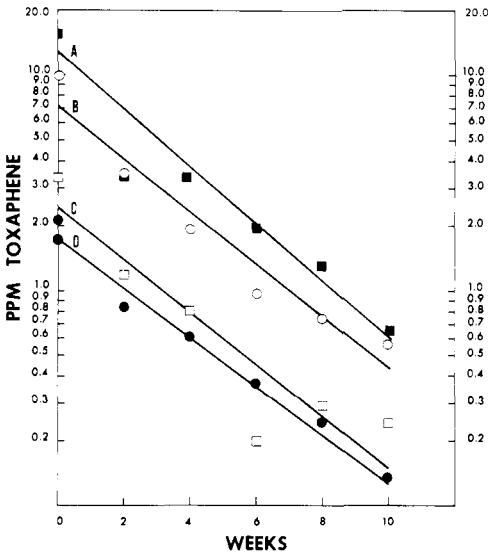

Figure 5. Toxaphene withdrawal study birds were fed graded levels of toxaphene (feeding levels: (●) 0.22 ppm; (□) 0.40 ppm; (○) 2.16 ppm; (■) 3.82 ppm for 6 weeks. At the end of 6 weeks, 15 birds from each of the replicate groups were removed from the toxaphene containing feed. Three of the 15 birds were killed at 2-week intervals. This provided for depletion periods of 2, 4, 6, 8, and 10 weeks, respectively. Residue patterns for 0.22, 0.40, and 2.16 ppm feeding levels were determined by liquid scintillation counting. Toxaphene levels in adipose tissue of the 3.82 feeding level were determined by GLC. (A) $y = e^{(-0.277x + 2.299)}$ with an $r^2 = 0.92$; (B) $y = e^{(-0.280x + 1.97)}$ with an $r^2 = 0.902$; (C) $y = e^{(-0.215x + 0.884)}$ with an $r^2 = 0.917$; (D) $y = e^{(-0.26x + 0.58)}$ with an $r^2 = 0.919$.

related to fat content (Avrahami and Steele, 1972). Plotting the natural logarithm of the tissue concentration vs. toxaphene in the diet or natural logarithm of the feed concentration vs. toxaphene in the tissue did not improve the correlation coefficient.

To determine the length of time necessary for depletion of toxaphene residues from adipose tissue, 15 birds from each of the replicates (45 birds per treatment group) were moved to clean floor pens at the end of 6 weeks and fed toxaphene-free feed. The half-life of toxaphene in adipose tissue was found to be 2.66, 2.76, 2.47, and 2.5 weeks for birds fed 0.22, 0.40, 2.16, and 3.82 ppm toxaphene, respectively (Figure 5). This half-life is considerably shorter than the 6.91-week half-life observed for laying hens fed 5 ppm until they were 32 weeks of age (Bush et al., 1977). This shorter half-life may be due to dilution as the broilers lay down additional body fat.

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Interactions of Kepone with Rabbit Muscle Lactate Dehydrogenase

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The inhibition of rabbit muscle lactate dehydrogenase catalyzed oxidation of lactic acid by the polychlorinated hydrocarbon pesticide, Kepone, was investigated with respect to the documentation of direct interactions of the pesticide with this enzyme in the absence of the coenzyme, NAD. The slow loss of catalytic activity of this dehydrogenase in 20% ethanol is greatly increased by the presence of micromolar concentrations of Kepone, and the rate enhancement observed was proportional to the concentration of Kepone added. The suggestion that the Kepone effect results from a direct interaction with the enzyme, resulting in a destabilization of protein structure, is supported by the protective effects observed with NAD and NADH. Using fluorescence quenching techniques, no evidence was obtained for complex formation between Kepone and 3-aminopyridine adenine dinucleotide. In addition, the properties of the Kepone inhibition of rabbit muscle lactate dehydrogenase did not change when the chemical structure of the pyridinium and purine moieties of the coenzyme were varied. These observations add further support to a proposed direct interaction of Kepone with the enzyme as a mode of inhibition by the pesticide.

The polychlorinated hydrocarbon pesticide, Kepone (decachloro-1,3,4-metheno-1*H*-cyclobuta[6*d*]pentalen-2-one), has been shown in in vitro studies to be an effective inhibitor of certain pyridine nucleotide requiring dehydrogenases. In early studies of the Kepone inhibition of beef liver glutamic dehydrogenase (EC 1.4.1.3) (Freedland and McFarland, 1965), a degree of specificity was attributed to the Kepone inhibition since several other chlorinated hydrocarbons were observed to be ineffective as inhibitors of this enzyme. However, saturated solutions of Kepone were employed and K_i values for Kepone were not determined. More recently an effective inhibition of lactate dehydrogenase (EC 1.1.1.27) by Kepone was observed by Hendrickson and Bowden (1975, 1976a), and evidence was presented (Hendrickson and Bowden, 1976a) to indicate that the structurally related, fully chlorinated pesticide mirex inhibited the lactate dehydrogenase in a similar fashion. Studies of Kepone inhibition of lactate dehydrogenase were extended by Anderson and Noble (1977) to include studies of the effects of the pesticide on homologous lactate dehydrogenase isozymes from rabbit, beef, pig, and chicken. In these studies, Kepone was observed to be an effective inhibitor of several M_4 isozymes while within the limits of solubility, no inhibition of H_4 isozymes was detected. The isozymic preference shown would again argue for a more selective inhibitory process and as in earlier studies, mirex appeared to function in a similar fashion. Although ethanolic solutions are required to solubilize Kepone and mirex, the low concentrations of these pesticides required to obtain the effects observed with lactate dehydrogenases compare favorably with those readily available through tissue accumulation of these compounds, indicating a possible physiological significance to these types of interactions.

Kepone and mirex have also been observed (Anderson et al., 1977) to be effective inhibitors of bovine mitochondrial, porcine mitochondrial, and porcine cytoplasmic malate dehydrogenase (EC 1.1.137). Mixed inhibition with respect to substrates and coenzymes was observed in the reactions catalyzed by these enzymes. In these studies, however, an additional important observation was made in that at concentrations inhibitory to the bovine mitochondrial enzyme, Kepone induced an accelerated rate of inactivation of the enzyme by *N*-ethylmaleimide. The data obtained suggested the possible importance of an enzyme unfolding or dissociation process promoted by the binding of Kepone. Since these effects were obtained in the absence of substrates or coenzymes, a more direct interaction between Kepone and the enzyme was indicated. A different mechanism, one requiring complex formation between mirex and pyridine nucleotides was recently suggested (Hendrickson and Bowden, 1976a) for the inhibition of lactate dehydrogenase. Since this appears not to be the case with Kepone inhibition of malate dehydrogenases (Anderson et al., 1977), additional studies of the Kepone inhibition of lactate dehydrogenases were indicated.

MATERIALS AND METHODS

Crystalline rabbit muscle lactate dehydrogenase, NAD, NADH, nicotinamide hypoxanthine dinucleotide, thionicotinamide adenine dinucleotide, and L(+)-lactic acid, lithium salt were obtained from Sigma Chemical Company. A sample of Kepone was generously supplied by Allied Chemical Company and was recrystallized twice from *n*-hexane prior to use. 3-Aminopyridine adenine dinucleotide was prepared chemically from NAD as described by Fisher et al. (1973). After purification by ion-exchange chromatography on Dowex-1-formate, the dinucleotide was desalted by gel filtration on a Sephadex

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