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Effect of Pentachloronitrobenzene upon Egg Production, Hatchability, and Residue Accumulation in the Tissues of White Leghorn Hens[1]

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Effect of Pentachloronitrobenzene upon Egg Production, Hatchability, and Residue Accumulation in the Tissues of White Leghorn Hens. DUNN, J. S., BUSH, P. B., BOOTH, N. H., FARRELL, R. L., THOMASON, D. M., AND GOETSCH, D. D. (1979). *Toxicol. Appl. Pharmacol.* 48, 425–433. Individual groups of White Leghorn female chickens were fed graded concentrations (0, 10, 50, 100, and 1000 ppm) of pentachloronitrobenzene (PCNB), containing hexachlorobenzene (HCB), pentachlorobenzene (PCB), and tetrachloronitrobenzene (TCNB) as contaminants, from 1 day through 35 weeks of age. No concentration of PCNB caused toxic effects or death. Histopathologic examination of 10 organs, including ovaries, failed to reveal abnormalities in either control or treated groups. Onset of egg production was delayed for 1 month, and hatchability of eggs was significantly lower in hens at 1000 ppm PCNB. No embryonic abnormalities were observed. Only small amounts of PCNB and metabolites, pentachloroaniline (PCA) and pentachlorophenylmethylsulfide (PCMS), were found in eggs. Conversely, HCB and PCB were readily transmitted into eggs. TCNB either was not detected or was present in only trace or finite amounts in eggs. PCNB, contaminates of PCNB (HCB, PCB, and TCNB), and metabolites of PCNB (PCA and PCMS) occurred in the highest concentration in adipose tissue. PCNB was not detectable (< 0.05 ppm) in adipose tissue by 6 days following its withdrawal from the diet. In egg, the half-life of PCNB was not determined since only trace amounts were detected. Depletion half-lives of HCB and PCB in eggs were as high as 5.1 and 3.6 weeks, respectively.

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Pentachloronitrobenzene (PCNB) is an agricultural chemical used primarily as a fungicidical agent to prevent root rot diseases in the production of cotton, cereal, and vegetable crops. This chemical, along with hexachlorobenzene (HCB), a contaminant of PCNB, is of concern because of the possibility that entry into the food chain of animals and of humans may occur.

Tissue residues from PCNB have been determined in Hubbard–Hubbard broilers fed up to 20 ppm of the chemical (Reed *et al.*,

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1977), in White Leghorn chickens fed up to 1000 ppm through 8 weeks of age (Dunn, J. S., Bush, P. B., Booth, N. H., Farrell, R. L., Thomason, D. M., and Goetsch, D. D., unpublished observations), and in White Leghorn cockerels (Dunn *et al.*, 1978). The longest period of dietary exposure to PCNB studied was 16 weeks, in Comet Red and White Leghorn chickens (McCoy *et al.*, 1976). PCNB, along with impurities and metabolites, accumulated to the greatest extent in adipose tissue, followed by bile, liver, egg, skeletal muscle, and blood, but actual tissue concentrations were not reported, nor was the effect of PCNB on fertility or hatchability of eggs studied. The effects of PCNB over the life cycle, including chick, adult, and egg phases, have not been studied.

The objectives of the present study were: (1) to determine the effect of PCNB on egg production and hatchability of eggs; (2) to determine the extent of transmission of PCNB, trace contaminants of PCNB [i.e., HCB, pentachlorobenzene (PCB), and tetrachloronitrobenzene (TCNB)], and metabolites [pentachloroaniline (PCA) and pentachlorophenylmethylsulfide (PCMS)] of PCNB into eggs; and (3) to determine the distribution, storage, and depletion rate of PCNB including its impurities (trace contaminants) in the organs and tissues of laying chickens.

METHODS

White Leghorn pullets that had received 0, 10, 50, 100, or 1000 ppm PCNB in feed from 1 day of age were removed from caged batteries and placed in floor pens at 8 weeks of age. They were continued for 27 weeks more on these same concentrations of PCNB in a formulated ration (Bush *et al.*, 1977) fed *ad libitum*. These groups, which comprised 43, 37, 40, 37, and 29 pullets, respectively, were each divided into two nearly equal replicates. The number of pullets in each group differed since the sex at 1 day of age was undetermined when the study was initiated. In addition, birds were killed periodically up through 8 weeks of age in another study (Dunn, J. S., Bush, P. B., Booth, N. H., Farrell, R. L., Thomason, D. M., and Goetsch, D. D., unpublished observations), which accounts for the uneven number of females. Replicate groups received the same treatment a were located at opposite ends in the broiler house ensure that location did not alter the effect of light and other environmental factors. At 23 weeks, sexually mature White Leghorn roosters were pla in each of the 10 pens.

The Terraclor[4] used in this study was analyzed b gas–liquid chromatography (glc) method reported Reed *et al.* (1977) and found to contain 75% PC 0.6% HCB, 0.022% PCB, and 0.08% TCNB.

Shell strength and hatchability. Daily egg p duction during the 25th through the 35th weeks w recorded. At 33 weeks, 25 eggs per replication w collected for determination of the force (expressed kg/total egg shell) required to break the shell, using Instron Quasistatic loader.[5] Hatchability of e (approximately 36 per dose concentration per 33rd, and 34th weeks) placed in a Jamesway In bator[6] was determined. In additon, unhatched e were examined for embryonic abnormalities.

Collection of tissues. At 35 weeks, three birds dose concentration were killed in a CO_2 chamber. liver, kidney, heart, gizzard, leg muscle, br muscle, and abdominal adipose tissue were ta from each. Three eggs per dose level were collec These eggs and tissues were frozen immediately stored at −17°C for residue analysis. Tissues brain, liver, pancreas, small intestine, gizz spleen, kidney, lung, heart, and gonads were lected and fixed in 10% buffered formalin for hi pathological examination. Paraffin sections 6 μm thickness were prepared and stained with hematox and eosin. Examination of the tissues was conduc by a veterinary pathologist (R.L.F.).

Withdrawal of PCNB from diet. At 35 weeks of all hens were transferred to control feed for 7 we they were not removed from their original litter p During this period, at weekly intervals, three b from each dose concentration were killed and br muscle, leg muscle, heart, kidney, liver, gizzard, abdominal adipose tissue were collected, frozen, stored. Also, eggs were collected weekly for 6 we All tissues and eggs were analyzed for PCNB, H PCB, TCNB, PCA, and PCMS.

[4] Terraclor is the proprietary name of pentachl nitrobenzene (PCNB) manufactured by Olin C Agricultural Division, Little Rock, Arkansas. batch number of the preparation used in this s is LR 380E-OSV 6124875, EPA Reg. No. 1258- AA. Seventy-five and twenty-five percent of the paration was labeled as PCNB and inert ingredi respectively; contaminants of the batch are not cated on the manufacturer's label.

[5] Instron Corp., 2500 Washington Street, Car Massachusetts.

[6] James Manufacturing Co., Inc., Fort Atkin Wisconsin.

ed analysis. Samples of feed at each dose level e analyzed to ensure that there was adequate ng and no previous contamination from PCNB pesticides. The feed was processed and extracted d upon the method of sample cleanup described Reed *et al.* (1977).

mple clean-up and extraction. Tissue samples were essed and extracted as reported by Reed *et al.* 77). In preparation for analysis, eggs (three each) e broken and beaten together. A 20-g aliquot of roughly mixed yolks and whites, 200 ml of ethyl tate, and 50 g of Na_2SO_4 were blended for 5 min a high-speed blender jar. The homogenate was red with suction through a Buchner funnel fitted h a Reeves Angel glass-fiber filter paper. The rate was evaporated to dryness with the aid of a ary evaporator (60°C) and the residue made to ml with ethyl acetate : toluene (3 : 1). Fat was re- ved by gel permeation chromatography (Johnson l., 1976).

To provide further clean-up, 25 g (60- to 100-mesh) risil in a glass column was prewashed with 50 ml ane and the egg sample transferred to it with ml hexane. The column was eluted sequentially h 200 ml 6% diethyl ether in hexane and 200 ml % diethyl ether in hexane. Each fraction was lected separately and evaporated to dryness with aid of a rotary evaporator (60°C). The residue s dissolved in 5 ml hexane and the solution stored a capped tube for analysis.

Analytical procedures. Tissues and eggs were yzed by the glc procedure of Reed *et al.* (1977). NB, HCB, and PCA were each quantitated by nparison of the sample peak height with that of a wn spiked standard, thus compensating for vary- degrees of recovery. PCB, TCNB, and PCMS e quantitated by comparison of the sample peak ght with that of standard compounds since cient quantities of standard compounds were not able for recovery studies. Standards of PCNB, ding those of metabolites and impurities, for glc sis were provided by the U.S. Food and Drug ministration. Average percentage recoveries found ggs were PCNB, 76.7%; HCB, 90.4%; and PCA, %. For other tissues they were PCNB, 69%; B, 71%; and PCA, 82%. A reagent blank and a ed sample were included for each set of analyses.

All data were analyzed by one-way analysis of ance and Duncan's multiple range test (Steel and rie, 1960) with $p < 0.05$ set as the level of statistical icance. The SAS computer program package *et al.*, 1976) was utilized for analysis of the data.

RESULTS

Feeding PCNB up to 1000 ppm did not se toxic signs or death in White Leghorn hens in this study. Histopathological examination of liver, small intestine, pancreas, gizzard, spleen, kidney, lung, heart, ovary, and brain from 35-week-old hens in either control or treated groups revealed no lesions. Grossly, the size and color of organs from treated birds did not appear to differ from those of the control group.

Bioaccumulation equations for PCNB, HCB, PCB, and PCA in adipose tissue are listed in Table 1. Birds receiving 1000 ppm PCNB including its impurities (HCB, PCB, and TCNB) tended to accumulate the most in adipose tissue compared to other tissues (Tables 2–5). Next in order of PCNB concentration were gizzard, followed by heart, leg muscle, and kidney; only trace amounts of PCNB were found in liver and breast muscle (Table 5).

Metabolites of PCNB were present in adipose tissue and in other tissues of birds that had received 100 and 1000 ppm PCNB in the diet (Tables 2 and 5). Only small amounts (< 1% of feeding level) of PCNB, PCA, and PCMS were found in the egg (Table 6). Conversely, HCB and PCB, the trace contaminants present in commercial PCNB, are readily transmitted into the egg (Fig. 1). TCNB was not detected in eggs in birds fed 0, 10, and 50 ppm of PCNB.

TABLE 1

BIOACCUMULATION EQUATIONS OF PCNB, HCB, PCB, AND PCA IN ADIPOSE TISSUE OF 35 WEEK-OLD WHITE LEGHORN HENS FED CONTINUOUSLY 10, 50, 100, AND 1000 ppm PCNB FROM 1 DAY OF AGE

Residue	Equation[a]	R^{2b}
PCNB	$\hat{y} = 0.219 + 0.003x$	0.83
HCB	$\hat{y} = 0.492\,x^{1.089}$	0.97
PCB	$\hat{y} = 0.123\,x^{0.806}$	0.96
PCA	$\hat{y} = 0.056\,x^{0.513}$	0.71

[a] $\hat{y}$ = Predicted concentration of residue in adipose tissue; x = ppm of PCNB in diet. PCNB was contaminated with 0.6% HCB and 0.022% PCB.

[b] R^2 is the relative amount of variation in the dependent variable ($\hat{y}$) explained by the variation in the independent variable (x).

TABLE 2

RESIDUES (IN ppm) OF PCNB AND METABOLITES IN ADIPOSE TISSUE OF 35-WEEK-OLD WHITE LEGHORN HENS

Dietary PCNB (ppm)	PCNB $\bar{x} \pm \sigma$	PCA $\bar{x} \pm \sigma$	PCMS $\bar{x} \pm \sigma$
0	0.15 ± 0.02	—[a]	—[a]
10	0.23 ± 0.20	0.18 ± 0.07	0.16 ± 0.08
50	0.23 ± 0.09	0.31 ± 0.01	0.51 ± 0.17
100	0.74 ± 0.06	1.24 ± 0.47	0.93 ± 0.14
1000	2.94 ± 0.71	1.60 ± 0.45	1.68 ± 0.22

[a] Trace, < 0.05 ppm.

TABLE 3

RESIDUES (IN ppm) OF CONTAMINANTS OF AGRICULTURAL GRADE PCNB IN ADIPOSE TISSUE OF 35-WEEK-OLD WHITE LEGHORN HENS

Dietary PCNB (ppm)	HCB $\bar{x} \pm \sigma$	PCB $\bar{x} \pm \sigma$	TCNB $\bar{x} \pm \sigma$
0	0.72 ± 0.28	—[a]	ND[b]
10	7.41 ± 1.85	0.77 ± 0.04	—[a]
50	38.38 ± 0.94	3.19 ± 0.69	0.13 ± 0.01
100	50.30 ± 2.07	5.49 ± 1.80	0.31 ± 0.01
1000	1132.18 ± 266.15	33.10 ± 6.23	1.01 ± 0.20

[a] Trace, < 0.05 ppm.
[b] Nondetectable at 0.005 ppm.

However, a trace (< 0.05 ppm) of TCNB was detected following the feeding of 100 ppm PCNB for 35 weeks. Following the feeding of 1000 ppm PCNB, TCNB was detected at 0.06 ± 0.01 (mean ppm ± SE).

After transfer to a basal diet containing no detectable PCNB, HCB, and PCB, birds previously fed 1000, 100, 50, and 10 ppm PCNB showed depletion half-lives of HCB in adipose tissue equal to 15.4, 17.0, 20.0, and 38.4 days, respectively, during a 7-week withdrawal period. Tissue samples were analyzed weekly during this period. Equations of the depletion rate of HCB in adipose tissue are provided in Table 7. The half-life of PCB was 29.25 days in birds at 1000 ppm PCNB; the equation of the depletion rate of PCB from adipose tissue is shown in Table 8. PCNB became nondetectable by 6 days and remained so during the 7-week withdrawal period. Depletion half-lives of HCB in eggs were 3.1, 5.1, 2.3, and 4.1 weeks, respectively, for birds fed 10, 50, 100, or 1000 ppm PCNB; Table 7 shows the equations of the depletion rate of HCB from eggs. The half-life of PCB in eggs was 3.6 weeks for birds fed 1000 ppm PCNB. For PCNB in eggs, the half-life was not determined since it accumulated there in trace amounts only.

Egg production (during the 25th–35th weeks) was not affected significantly ($p > 0.05$) PCNB in the diet (Table 9). However, onset of production was delayed for 1 month, and the number of chicks hatched was significantly lower in hens on 1000 ppm (Table 9). No embryonic abnormalities were observed in this study. Shell strength of eggs from hens on 100 or 1000 ppm was not significantly altered (Table 9).

TABLE 4

RESIDUES (IN ppm) OF CONTAMINANTS IN TISSUES OF 35-WEEK-OLD LEGHORN HENS FED 1000 ppm PCNB

Tissue	HCB $\bar{x} \pm \sigma$	PCB $\bar{x} \pm \sigma$	TCNB $\bar{x} \pm \sigma$
Heart	58.5 ± 20.0	6.6 ± 1.5	0.1 ± 0.1
Liver	17.1 ± 8.1	1.4 ± 0.6	—[a]
Gizzard	11.9 ± 1.6	1.4 ± 0.5	—[a]
Kidney	12.6 ± 3.0	0.8 ± 0.7	—[a]
Leg muscle	9.3 ± 4.0	0.7 ± 0.4	—[a]
Breast muscle	1.9 ± 0.3	0.1 ± 0.08	—[a]

[a] Trace, < 0.05 ppm.

TABLE 5

TISSUE RESIDUES (IN ppm) OF PCNB AND METABOLITES IN SPECIFIC TISSUES OF 35-WEEK-OLD LEGHORN HENS

Tissue	Dietary PCNB[a] (ppm)	PCNB $\bar{x} \pm \sigma$	PCA $\bar{x} \pm \sigma$	PCMS $\bar{x} \pm \sigma$
Heart	100	0.20 ± 0.04	0.21 ± 0.07	0.21 ± 0.16
	1000	0.38 ± 0.25	0.21 ± 0.06	0.13 ± 0.08
Liver	100	—[b]	0.12 ± 0.05	—[b]
	1000	—[b]	0.12 ± 0.04	0.16 ± 0.04
Gizzard	100	0.43 ± 0.26	0.09 ± 0.02	0.12 ± 0.07
	1000	0.84 ± 0.48	0.11 ± 0.02	—[b]
Kidney	100	0.07 ± 0.04	0.11 ± 0.05	—[b]
	1000	—[b]	0.12 ± 0.01	0.07 ± 0.06
Leg muscle	100	0.14 ± 0.11	—[b]	—[b]
	1000	0.06 ± 0.06	0.06 ± 0.02	—[b]
Breast muscle	100	—[b]	—[b]	—[b]
	1000	—[b]	—[b]	—[b]

[a] Dietary PCNB treatment levels not shown (0, 10, and 50 ppm) contained nondetectable or trace amounts of PCNB and metabolites.
[b] Trace, < 0.05 ppm.

TABLE 6

RESIDUES OF PCNB AND METABOLITES IN EGGS OF 35-WEEK-OLD WHITE LEGHORN HENS

	Residue (ppm ± SE) in eggs		
Dietary PCNB (ppm)	PCNB	PCA	PCMS
0	ND[a]	ND	ND
10	ND	—[b]	—[b]
50	—[b]	0.06 ± 0.02	0.08 ± 0.02
100	ND	0.14 ± 0.04	0.12 ± 0.03
1000	—[b]	0.31 ± 0.08	0.42 ± 0.13

[a] Nondectectable at 0.005 ppm.
[b] Trace, < 0.05 ppm.

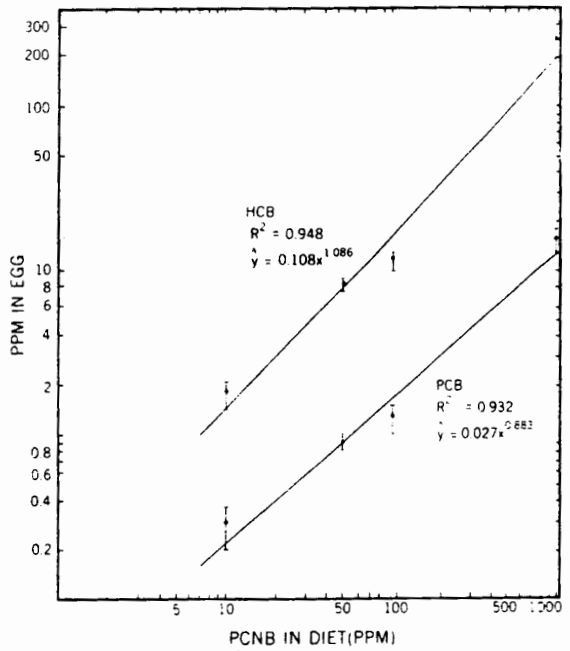

FIG. 1. HCB and PCB accumulation in eggs from 35-week-old White Leghorn hens fed 10, 50, 100, and 1000 ppm PCNB from 1 day of age; the concentrations of the HCB and PCB contaminants were 0.6 and 0.022%, respectively.

DISCUSSION

Unlike PCNB or its metabolites, the impurities (HCB and PCB) accumulate in tissue to a significant degree. White leghorn hens in our study had high concentrations of HCB in adipose tissue and tended to have the lowest concentrations in skeletal muscle. These findings are similar to those reported by Koss and Koransky (1975) for rats. Moreover, our data indicate that HCB accumulates in all tissues examined and at all concentrations fed. Studies in swine have also revealed that HCB accumulated in all of the tissues examined and at all dosages (den Tonkelaar, 1978). A significant linear relationship exists between concentrations of PCNB in adipose tissue and in the diet, described by $\hat{y} = 0.219 + 0.003x$. According to this equation, 94 ppm PCNB in the diet would produce illegal or violative levels of PCNB (tolerance 0.5 ppm in U.S.A.) in adipose tissue; whereas Dunn *et al.* (unpublish observations) estimated that 500 ppm PC in the diet of 8-week-old Leghorns would required.

Two PCNB metabolites, PCA and PCM tended to accumulate to the greatest exte in adipose tissue, with low concentratio found in other body organs. A detectat concentration of TCNB (impurity of PCN was found in adipose tissue but little w found in other tissues. Reed *et al.* (19 found traces of PCA in body organs but n in adipose tissues of 8-week-old broile Dunn *et al.* (unpublished observations) ported trace amounts (<0.05 ppm) of PC and PCMS in adipose tissue and bo organs of 8-week-old Leghorns fed up 1000 ppm PCNB.

In some species (Levin, 1975), includi humans (Peters, 1976), HCB induces p phyria following prolonged or chronic posure. It is not known whether HCB capable of inducing porphyria in chick or if they fail to develop porphyria similar that reported for the beagle dog (Gralla *et* 1977). In our study, the livers of hens w not subjected to ultraviolet light to determ whether or not hepatic fluoresence existed to the presence of porphyrin. Moreover, b chemical determinations to detect porphy were not made.

Little accumulation of PCNB and metabolites, PCA and PCMS, occurred eggs. McCoy *et al.* (1976) found less PC (concentrations unreported) in eggs than fat and liver, but more than in kidney breast muscle of hens fed up to 300 pp residues of PCNB in eggs reached a plat within 1 week of its inclusion in the he diet. Only trace amounts of PCNB w detected in eggs of hens, even those h receiving 1000 ppm.

The concentration of HCB in eggs found to be 19 to 41 times that in the (there was 0.6% HCB in the PCNB us The level of HCB in the egg was 21.6 that in fat, which is similar to the 22. reported by Avrahami and Steele (19

TABLE 7

EQUATIONS OF THE DEPLETION RATE OF HCB FROM ADIPOSE TISSUE AND EGGS OF WHITE LEGHORN HENS FED PCNB FOR 35 WEEKS

Dietary PCNB (ppm)	Tissue	Equation[a]	R^2
10	Adipose	$\hat{y} = e^{1.570-0.018x}$	0.27
50		$\hat{y} = e^{3.062-0.035x}$	0.31
100		$\hat{y} = e^{3.668-0.021x}$	0.19
1000		$\hat{y} = e^{6.497-0.045x}$	0.60
10	Egg	$\hat{y} = e^{0.336-0.167x}$	0.43
50		$\hat{y} = e^{1.979-0.302x}$	0.72
100		$\hat{y} = e^{2.331-0.137x}$	0.60
1000		$\hat{y} = e^{5.435-0.229x}$	0.71

[a] $\hat{y}$ = Predicted ppm HCB; x = time in days.

TABLE 8

EQUATIONS OF THE DEPLETION RATE OF PCB FROM EGGS AND ADIPOSE TISSUE OF WHITE LEGHORN HENS FED PCNB FOR 35 WEEKS

Dietary PCNB (ppm)	Tissue	Equation[a]	R^2
10	Egg	$\hat{y} = e^{-1.395-0.116x}$	0.28
50		$\hat{y} = e^{-0.164-0.230x}$	0.40
100		$\hat{y} = e^{-0.142-0.001x}$	0.18
1000		$\hat{y} = e^{2.779-0.190x}$	0.58
1000	Adipose	$\hat{y} = {}^{31.667-0.536x}$	0.44

[a] $\hat{y}$ = Predicted ppm PCB; x = time in weeks.

TABLE 9

EFFECT OF PCNB IN THE DIET ON EGG PRODUCTION, HATCHABILITY, AND SHELL STRENGTH[a]

Dietary PCNB (ppm)	Eggs per hen/day	% Hatchability	Shell strength[b]
0	0.535 a[c]	91 a	5.31 a
10	0.428 a	90 a	4.00 b
50	0.536 a	90 a	3.85 b
100	0.556 a	85 a	4.85 a
1000	0.464 a	69 b	4.98 a

[a] White Leghorns were continuously fed graded levels of PCNB for 35 weeks. Starting with the 25th week, daily egg production records were kept. The percentage hatchability was determined by three trials using eggs collected during weeks 31, 33, and 34.

[b] Expressed in kilograms of breaking force of the total egg shell.

[c] Treatment means in a column not followed by the same letter are not significantly different at the 0.05 level according to Duncan's multiple range test.

The latter also observed HCB residues in eggs up to 25 weeks after treatment was terminated. Six weeks after removal of PCNB from the hens' diet, HCB residues in eggs were above the interim tolerance level (0.5 ppm) established for adipose tissue.

Variation in the depletion half-life values with dietary concentrations observed for HCB in eggs is unexplainable. In adipose tissue, the depletion half-life values of HCB decreased progressively from 38 to 15 days with increase in the concentration of the chemical in the diet. Since HCB is known to induce the hepatic microsomal enzyme system in rats (Stonard and Nenov, 1974; Turner and Green, 1974), the progressive decrease in the depletion half-life observed in the present study may be related to a greater biotransformation rate. Also, an increase or decrease in deposition of body fat would vary the depletion half-life of HCB. An increase would enhance the apparent depletion rate as a result of dilution, while a loss in weight would have an opposite effect. Moreover, the available routes of elimination from an animal appear to influence the depletion rate of HCB. In nonlactating animals (steers) the depletion rate is longer for HCB (Dingle and Palmer, 1977) than in lactating cows (Fries and Marrow, 1976). Interestingly, a longer depletion rate for HCB in adipose tissue of nonlaying chickens (cockerels) has been observed by Dunn *et al.* (1978) than in the laying hens of the present study; a depletion half-life up to 95 days has been found in cockerels.

Although in this study, egg production began 1 month later in hens receiving 1000 ppm PCNB, PCNB did not affect the number of eggs produced during the period measured. McCoy *et al.* (1976) also found no effect on egg production of PCNB (up to 300 ppm) in the hens' diet.

Absence of abnormalities in embryos, as well as in chicks hatched, is consistent with previous studies. In rats, daily oral dosage of up to 1563 ppm PCNB during the period of embryogenesis was not teratogenic (Jordan and Borzelleca, 1973). In an unpublish study cited by Villeneuve and Khera (197 dosages of up to 200 mg/kg PCNB caused fetotoxic effects, nor were they associat with teratological anomalies in the rat.

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