Inhibition of Biological Activity of Cholecalciferol (Vitamin D₃) by
\( o,p'\)-DDT or \( p,p'\)-DDT in Rachitic Cockerel

Henry G. Nowicki, Richard G. Wong, James F. Myrtle, and Anthony W. Norman*

Considerable evidence suggests that chlorinated hydrocarbons cause thin eggshell formation, apparently in part through an altered calcium metabolism of adult birds and thus reproductive failure in raptors. This report concerns a comparative study of some of the known biological effects of cholecalciferol (vitamin D₃), a major steroid regulator of \( Ca^{2+} \) metabolism, in \( o,p'\)-DDT [1,1,1-trichloro-2-(o-chlorophenyl)-2-(p-chlorophenyl)ethane], or \( p,p'\)-DDT [1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane] treated and untreated rachitic chicks.

It was found that \( o,p'\)-DDT or \( p,p'\)-DDT treatment decreased the normal cholecalciferol (CC) mediated increase in intestinal calcium absorption, when assayed \textit{in vivo} or \textit{in vitro}, bone calcium resorption, and intestinal alkaline phosphatase activity. It is suggested that \( o,p'\)-DDT or \( p,p'\)-DDT interference in some of the biological responses to CC may furnish a partial explanation for the thin eggshell formation and thus reproductive failure of some raptors such as the peregrine falcons, brown pelicans, bald eagles, and sparrow hawks.

A number of recent reports in the technical literature (Ratcliffe, 1967; Porter and Wiemeyer, 1969; French and Jefferies, 1969; Hickey, 1969; Robinson, 1970; Edwards, 1970) and the public news media (Bengelsdorf, 1969; Moser, 1971) have focused attention on some of the ecological hazards that result from the widespread occurrence of chlorinated hydrocarbons, such as DDT and its major metabolite DDE, in the biosphere. The insecticide commonly used is technical grade with a composition of 79% of \( p,p'\)-DDT [1,1,1-trichloro-2-bis(p-chlorophenyl)ethane] and 20% of \( o,p'\)-DDT [1,1,1-trichloro-2-(p-chlorophenyl)-2-(o-chlorophenyl)ethane]. These compounds have been suggested as the possible cause for altered calcium metabolism and subsequent avian reproductive failures. It has also been shown that dietary dieldrin and/or DDT presence in a variety of avian species leads to avian reproductive failures (Edwards, 1970).

Raptors in Britain, including the peregrine (\textit{Falco peregrinus}), sparrow hawk (\textit{Accipiter nisus}), and golden eagle (\textit{Aquila chrysaetos}), have reproduced less successfully with the increased use of chlorinated hydrocarbons (Ratcliffe, 1958, 1960, 1967) and egg breakage in the nest seems to be a major part of the phenomenon. Contamination by dieldrin has been suspected of causing increased egg breakage in \textit{Phalacrocorax aristotelis} populations in Britain (Potts, 1968).

In the United States, declining populations of bald eagles (\textit{Haliaetus leucocephalus}) and ospreys (\textit{Pandion haliaetus}) and extirpated populations of peregrines have been related to decreases in eggshell weight (Hickey and Anderson, 1968). High levels of DDT and its metabolites in the eggs of herring gulls (\textit{Larus argentatus}) have also been correlated with decreased egg cracking (Ludwig and Tomoff, 1966) and decreased eggshell thickness (Hickey and Anderson, 1968).

A captive population of American sparrow hawks (\textit{Falco sparverius}), given a diet containing both dieldrin and DDT,
showed increased egg disappearance, increased egg destruction by parent birds, and reduced eggshell thickness (Porter and Wiemeyer, 1969). A captive population of mallard ducks (<i>Anas platyrhynchos</i>), given a diet of sublethal dielcurin, similarly showed a decrease in shell thickness (Lehner and Egbert, 1969).

The incorporation of calcium into an eggshell is the end result of a complex pathway of calcium metabolism (Simkiss, 1967; Marshall, 1960). Dietary calcium is absorbed from the intestine into the blood and then is deposited in the medullary bone, which is comprised primarily of the femur, tibia, and fibula. The medullary bone functions as a temporary depot for the calcium. When the female is not producing an egg, the serum Ca<sup>2+</sup> is 9 mg%; during egg production it can approach 30 mg%. The final step in the process involves transport of calcium across the eggshell gland and deposition in the shell as CaCO<sub>3</sub>

The fluxes of calcium involved in eggshell formation can be summarized as follows: dietary Ca<sup>2+</sup> → intestinal calcium absorption, MBCD → medullary bone calcium deposition, MBCR → medullary bone calcium resorption, and EGCA → eggshell gland calcium absorption. The calcium in the eggshell largely comes from the storage form in the medullary bone (Simkiss, 1967). The mobilization of medullary bone calcium is largely under control by a synergistic interaction between estrogens and androgens (Simkiss, 1967). The eggshell calcium is in combination with carbonate, which is formed from carbon dioxide and water via carbonic anhydrase.

In view of this multi-step process, it is apparent that there are many potential sites and steps for the deleterious interaction of the chlorinated hydrocarbon. Since the formation and resorption of medullary bone is largely under the synergistic control of estrogens and androgens, it has been hypothesized (Kuntzman et al., 1966) that DDT treatment produces a sex hormone imbalance. This hypothesis has been documented experimentally. Peakall (1967) and Nowicki and Norman (1971a) have shown that dietary DDT induces an increase in the hepatic metabolism of estrogens and androgens. However, a direct link between this finding and the calcification process has not been established.

Cholecalciferol (vitamin D<sub>3</sub>) is recognized as one of the prime regulators of calcium metabolism in the intestine (Nicolaysen, 1937), skeleton (Carlsson, 1952), and possibly in the eggshell gland of laying birds (Norman, 1968).

Another attractive hypothesis for the thin eggshell syndrome is that DDT may interfere with the function and/or metabolism of vitamin D. It is well recognized that vitamin D or its metabolites are intimately involved in many of the calcium fluxes which are essential for normal eggshell formation. Therefore, it seemed appropriate to examine whether chlorinated hydrocarbons would impair any of the known biological responses to vitamin D treatment. It is the primary purpose of this paper to assess the effect of o,p<sup>-</sup>DDT or o,p<sup>-</sup>DDT pretreatment upon the response to cholecalciferol administration to the rachitic chick at two of the steroids primary target tissues, namely, the small intestine and the skeleton.

**MATERIALS AND METHODS**

**Chickens.** White Leghorn Cockerels, generously provided by H & N of California, Inc., were employed in all experiments. 1-Day-old chicks were raised for 3 weeks on a 2% CaHPO<sub>4</sub>, vitamin D-deficient diet. Its composition has been previously described (Hibberd and Norman, 1969). All chicks were utilized during the fourth week, at which time they had become rachitic (-CC), showed a leveled growth rate (100-110 g), and had serum calcium levels of 6.3 mg/100 ml of serum and a bone ash percentage of 28%. Vitamin D-treated chicks (+CC) (with or without organochlorine hydrocarbon pretreatment) were prepared by oral administration of 5-500 IU (0.325-32.5 nmol) of cholecalciferol dissolved in 0.20 ml of 1,2-propanediol to rachitic chicks 24 hr prior to assay. One IU (International Unit) of cholecalciferol (vitamin D<sub>3</sub>) is equivalent to 0.025 μg (65 pmol). Hibberd and Norman (1969) have determined the minimum daily requirement for cholecalciferol in the chick to be 0.65 to 1.30 nmol (10 to 20 IU). Pesticide-treated chicks received either p,p<sup>-</sup>DDT or o,p<sup>-</sup>DDT at a level of 1 mg/day for 7 consecutive days prior to comparative biological assay. Control chicks received equal amounts of vehicle, 1,2-propanediol.

**CALCULUM TRANSPORT METHODS**

**Studies in vivo**. Intestinal calcium absorption, <i>in vivo</i>, was determined by the procedure of Coates and Holdsworth (1961), as modified by Hibberd and Norman (1969). In this procedure chicks were anesthetized with ether. Following an abdominal incision, a 0.20-ml aqueous solution of calcium containing 2 mg of 45Ca<sup>2+</sup>-45Ca<sup>2+</sup> (6 μCi) was injected into the exposed duodenal loop. The chicks were sacrificed by decapitation 30 min later and blood was collected in the presence of heparin to prevent clotting. Red cells were removed by centrifugation and a 0.20-ml aliquot of the plasma was placed onto a planchet for determination of 45Ca<sup>2+</sup> radioactivity.

**Studies in vitro**. For intestinal calcium transport through segments of chick ileum, <i>in vitro</i>, the tissue was mounted in the Ussing-transport apparatus (Ussing, 1949), as described by Forte et al. (1967), and modified by Adams and Norman (1970). The device consisted of two 15-ml compartments separated by a lucite diaphragm in which was mounted a 3-cm segment of ileal tissue. The surface area through which transport occurs was 0.60 cm<sup>2</sup>. Each compartment was filled with 15 ml of a modified Krebs-Ringer bicarbonate buffer. Its composition was 0.123 M NaCl, 0.026 M NaHCO<sub>3</sub>, 0.005 M KCl, 0.02 M fructose, and 0.10 mM CaCl<sub>2</sub>. The pH was adjusted to 7.4 after saturation with 95% O<sub>2</sub>-5% CO<sub>2</sub>. All experiments were run at room temperature with the solutions aerated constantly by bubbling 95% O<sub>2</sub>-5% CO<sub>2</sub> in each compartment. Normally 100 μCi of 45Ca<sup>2+</sup> carrier-free was added to the compartment bathing the mucosal surface of the ileal segment. After a 30-min period, 0.10-ml aliquots were removed at 5-min intervals for 40 min from the compartment bathing the serosal surface of the ileal segment. The 0.10-ml aliquots were placed onto planchets for determination of 45Ca<sup>2+</sup> radioactivity.

The level of replenishment, in Table II, with cholecalciferol (CC) was 100 IU 24 hr before assay of intestinal calcium transport <i>in vitro</i>. Chicks were pretreated for 7 days with organochlorine insecticide and vehicle, respectively, before testing the ability of the vitamin to stimulate intestinal calcium transport.

**Radioactivity Determination.** The activity of 45Ca<sup>2+</sup> was determined by counting to 2% error in a Model 1042 Nuclear Chicago planchet counter. Rates of calcium transport, or flux, J, in <i>in vitro</i>, in nmol/hr/cm<sup>2</sup> were calculated by knowing the specific activity of 45Ca<sup>2+</sup> in the mucosal compartment.

J. AGR. FOOD CHEM., VOL. 20, NO. 2, 1972 377
After equilibrating 2.0 mM zinc acetate, 1.0 mM Eicholz (1969). Mucosal tissue was scraped from duodenal volume of enzyme solution was added to a cuvette containing enzized in 30 vol of (w/v)buffer.

Serum was collected as described under calcium transport studies. Serum calcium analysis was performed on 0.50-ml aliquots with a Perkin-Elmer Model 303 atomic absorption unit under conditions recommended for calcium analysis by Perkin-Elmer.

The experiments were performed in the Ussing type apparatus as described under Methods. p < 0.01. Comparing +CC and -CC in the presence of pesticide, p < 0.05. Comparing -CC and +CC treated with +CC, +p,p'-DDT treated with -CC, p < 0.2. Comparing +CC and p,p'-DDT treated with +CC, +p,p'-DDT treated with -CC, 2.00 ± 0.42 (3) 2.12 ± 0.08 (4). Comparing +CC and p,p'-DDT treated with +CC, +o,p'-DDT treated with -CC, +o,p'-DDT treated with -CC, p < 0.05.

The values shown are the average cpm ± the standard deviation contained in 0.20 ml of serum from each chick. Each group consisted of six individual chicks.

Table II. The Effect of Organochlorine Insecticide on Calcium Flux, J_{Ca}\text{in vivo}, in ileal Segments from Vitamin D_{3}-Deficient and Vitamin D_{3}-Treated Chicks

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Calcium flux J_{Ca}, nmol/hr/cm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-CC</td>
</tr>
<tr>
<td>o,p'-DDT</td>
<td>1.50 ± 0.43 (11) 3.02 ± 1.05 (8a)</td>
</tr>
<tr>
<td>p,p'-DDT</td>
<td>1.62 ± 0.53 (7) 1.64 ± 0.50 (8b)</td>
</tr>
<tr>
<td>*</td>
<td></td>
</tr>
</tbody>
</table>

The values are the average ± standard deviation of the indicated number of chicks.

Table I. Comparative Biological Effect of Cholecalciferol (Vitamin D_{3}) on Intestinal Calcium Absorption in vivo in Control and Organochlorine Insecticide-Treated Chicks

<table>
<thead>
<tr>
<th>Assay no.</th>
<th>Cholecalciferol Dose IU</th>
<th>0</th>
<th>10</th>
<th>20</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Control</td>
<td>Control 71 ± 4e 255 ± 97 581 ± 160</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2. Control</td>
<td>29 ± 10 149 ± 60 197 ± 7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3. Control</td>
<td>119 ± 34 234 ± 27 517 ± 121 582 ± 186</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4. Control</td>
<td>118 ± 45 290 ± 51 288 ± 73 717 ± 93</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5. Control</td>
<td>119 ± 31 275 ± 40 323 ± 42 710 ± 139</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6. Control</td>
<td>173 ± 27 210 ± 29 241 ± 29 504 ± 168</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The values shown are the average cpm ± the standard deviation contained in 0.20 ml of serum from each chick. Each group consisted of six individual chicks.

and by a linear regression analysis (Steel and Torrie, 1960) of the rate of appearance of 44Ca\textsuperscript{2+} in the serosal compartment.

Bone Calcium Resorption, in vivo. Bone calcium resorption, in vivo, was determined by the method of Carlson and Lindquist (1955), as modified by Hibberd and Norman (1969). Chicks were raised for 3 weeks on the standard vitamin D deficient diet. Some were treated with chlorinated hydrocarbon starting in the second week. They were next ferred for 3 days to a vitamin D-deficient diet which lacked added CaHPO\textsubscript{4}. (K\textsubscript{2}HPO\textsubscript{4} was substituted in equimolar amounts), while still receiving chlorinated hydrocarbon treatment. Each group of chlorinated hydrocarbon-treated and untreated chicks then received one oral dose of cholecalciferol. Each group consisted of six individual chicks

Effect of Organochlorine Insecticides on Calcium Transport. Undoubtedly, vitamin D is the most important single factor affecting the absorption of calcium from the intestinal tract. The effect of organochlorine insecticides on intestinal calcium transport in vivo are summarized in Table I. It was noted that insecticide pretreatment had a moderate effect on increasing the intestinal calcium transport in vivo of rachitic (cholecalciferol-deficient) chicks. When chicks were pretreated with p,p'-DDT (1 mg) daily for a week before calcium transport was measured in vivo, they showed an increased transport rate for calcium of 41% over that of untreated rachitic controls.

Effect of Organochlorine Insecticides on Calcium Transport. Undoubtedly, vitamin D is the most important single factor affecting the absorption of calcium from the intestinal tract.
cholecalciferol stimulated calcium transport flux level J (mucosal-to-serosal) to approximately the same extent. There was an 89% inhibition of "cholecalciferol-stimulated calcium transport" due to p,p'-DDT pretreatment and a 99% inhibition due to o,p'-DDT pretreatment. In both the studies in vivo and in vitro it was observed that o,p'-DDT inhibited cholecalciferol-mediated calcium transport better than p,p'-DDT.

Another intestinal response that is characteristic of vitamin D or CC treatment is the elevation of the enzyme alkaline phosphatase. Norman et al. (1970) has previously shown that vitamin D administration to a rachitic chick results in a three-fold increase in the level of alkaline phosphatase in the brush border region of the intestine. This increase occurs over a 60-hr period and closely parallels the time-dependent increase in calcium transport.

Table III shows the effect of pesticide pretreatment on the cholecalciferol-mediated increase in duodenal alkaline phosphatase activity. In experiment 1 the effect of o,p'-DDT on enhancement of duodenal alkaline phosphatase activity was determined on a pooled homogenate fraction obtained from intestines which were used for the calcium transport studies in vitro (Table II). Cholecalciferol-mediated alkaline phosphatase activity was found to be inhibited 39% by o,p'-DDT. p,p'-DDT also inhibited the cholecalciferol-mediated increase. It was found that p,p'-DDT was a more effective inhibitor of the alkaline phosphatase response than o,p'-DDT (74% vs. 39%).

The data contained in Tables I and II clearly demonstrate that p,p'-DDT or o,p'-DDT adversely affect the ability of the rachitic chick intestine to produce a functional calcium transport system. Thus, the decrease in vitamin D-mediated intestinal calcium absorption by pretreatment with o,p'-DDT or p,p'-DDT may be a partial explanation for the thin eggshell syndrome in various raptors, who accumulate these persistent pesticides in their body. Clearly this extrapolation to the bird in the field is premature and needs to be tested experimentally. It appears that cholecalciferol mediates de novo synthesis of intestinal alkaline phosphatase (Norman et al., 1970). Therefore, the observation that pesticide (Table III) decreases the CC-mediated increase in this gene product suggests that the pesticide has interfered with transcription, translation, translocation of the protein to the brush border, or that the chlorinated hydrocarbon may act directly on the enzyme to form a deleterious complex.

Recently, Pocker et al. (1971) have reported that DDE (a stable metabolite of DDT) and dieldrin form a complex with carbonic anhydrase which results in loss of enzyme activity. Carbonic anhydrase is responsible for catalyzing the hydration of carbon dioxide to bicarbonate, thereby making carbonate available for eggshell formation in the oviduct of the bird. The complex (DDE-carbonic anhydrase) results in an insoluble form of the enzyme. It appears that the chlorinated hydrocarbon does not inactivate existing levels of intestinal alkaline phosphatase, as the specific activity in the control and pesticide-treated chicks were similar.

In a preliminary experiment (Midgett, 1971) o,p'-DDT or p,p'-DDT decreased the CC stimulation in intestinal mucosa template activity. Norman (1966) has shown that o,p'-DDD [1-(2-chlorophenyl)-1-(4-chlorophenyl)-2,2-dichloroethane] decreased the incorporation of 3H-uridine into rapidly labeled RNA of intestinal mucosa stimulated by CC.

Effect of p,p'-DDT on Bone Resorption. Serum calcium levels normally are an average measurement of cholecalciferol-mediated responses of intestinal calcium absorption (ICA) and bone calcium resorption (BCR). A precise estimation of the effect of chlorinated hydrocarbon on the BCR system was determined by utilizing rachitic chicks raised for several days on a diet devoid of calcium. This technique was originally utilized by Carlsson and Lindquist (1955). Under these conditions any increase in total serum calcium could be only due to a response of the bone calcium resorption system. As shown in Table IV, pretreatment of chicks with p,p'-DDT blocked the ability of cholecalciferol to mobilize bone calcium. Bone calcium resorption was blocked at cholecalciferol doses from 3.25-32.5 nmol (50 to 500 IU).

These studies showed that o,p'-DDT or p,p'-DDT decreases the biological activity of CC-mediated ICA and BCR systems. It is suggested that DDT interference in some of the biological responses to CC may furnish a partial explanation for the thin eggshell formation and thus reproductive failure of some raptors such as the peregrine falcons, brown pelicans, bald eagles, and the sparrow hawks.

In view of these collective results demonstrating that o,p'-DDT or p,p'-DDT treatment blocked the response to cholecalciferol (vitamin D$_3$) it seemed appropriate to examine whether the pesticide treatment altered the metabolism of vitamin D$_3$. Previous work has conclusively established that vitamin D$_3$ must first be metabolized prior to its mediating intestinal calcium transport. The next paper is concerned with the effects of p,p'-DDT on the metabolism of cholecalciferol.

ACKNOWLEDGMENT

Organochlorine insecticides were the generous gift of Robert March.

**Table III. Comparative Biological Effect of Cholecalciferol and Organochlorine Insecticides on Duodenal Alkaline Phosphatase Specific Activity**

<table>
<thead>
<tr>
<th>Dose CC, IU</th>
<th>Alkaline phosphatase specific activity (μmol/min per mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>0</td>
<td>1.95 (7)</td>
</tr>
<tr>
<td>100</td>
<td>3.02 (8)</td>
</tr>
</tbody>
</table>

**Table IV. Comparative Biological Effect of Cholecalciferol on Bone Calcium Resorption in vivo in Vitamin D$_3$-Deficient and Organochlorine Insecticide-Treated Chicks**

<table>
<thead>
<tr>
<th>Dose CC, IU</th>
<th>Serum Ca$^{2+}$ level (mg Ca$^{2+}$/100 ml serum)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>0</td>
<td>4.50 ± 0.63</td>
</tr>
<tr>
<td>50</td>
<td>4.96 ± 0.30</td>
</tr>
<tr>
<td>100</td>
<td>6.23 ± 0.31</td>
</tr>
<tr>
<td>500</td>
<td>7.26 ± 0.41</td>
</tr>
</tbody>
</table>

* p < 0.001.
LITERATURE CITED

Midgett, R. J., unpublished observations (1971).

Effects of Organochlorine Insecticides on Metabolism of Cholecalciferol (Vitamin D₃) in Rachitic Cockerel

Henry G. Nowicki, James F. Myrtle, and Anthony W. Norman

The metabolic pathway involved in the conversion of cholecalciferol (CC) to its biologically active form is: CC → 25-OH-CC → 1,25-dioH-CC. The most predominant form of the steroid in the target intestine is 1,25-dioH-CC. It is known that CC must undergo at least these two conversions prior to stimulating intestinal calcium transport. Using the blood levels of 25-OH-CC as an indication of liver function, it was found that organochlorine pesticide treatment did not influence this hydroxylation step, whereas the amount of 1,25-dioH-CC in intestine of chicks exposed to pesticide was slightly more than in untreated chicks. 1,25-dioH-CC was shown to be homogenous and the same in control and organochlorine insecticide-treated intestines by both sensitive silicic acid and Celite column chromatography. It is concluded from this data that cholecalciferol is converted to its biologically active forms in sufficient quantity in the presence of organochlorine insecticides to maintain normal calcium metabolism in the chick. These results do not then explain how organochlorine insecticides impair the biological responses to cholecalciferol.

Pro- and anti-DDT scientific papers have proliferated since Rachel Carson’s book Silent Spring (1962), e.g., some 50,000 papers have appeared. The insecticide DDT commonly used is technical grade with a composition of 79% of p,p'-DDT [1,1,1-trichloro-2,2-bist(p-chlorophenyl)ethane] and 20% of p,p'-DDT [1,1,1-trichloro-2-(o-chlorophenyl)-2-(p-chlorophenyl)ethane]. Perhaps the most widely agreed upon ecological hazard of DDT is the decline in the population of several raptorial birds (Ratcliffe, 1967; Porter and Wiemeyer, 1969; French and Jefferies, 1969; Hickey, 1969; Bengelsdorf, 1969; Edwards, 1970; Robinson, 1970; Moser, 1971). These failures in reproduction have been shown to result from a lesion in calcium metabolism, and thus thinning of the eggshell. As presented in the previous paper, a possible partial explanation for the thin eggshell syndrome may be that DDT interferes in the known biological activities of cholecalciferol (vitamin D₃), a major steriod regulator of calcium metabolism.

The concept has been developed in a number of laboratories (Haussler et al., 1968; Lawson et al., 1969; Myrtle et al., 1970; Cousins et al., 1970) that cholecalciferol (CC) must undergo at least two metabolic conversions prior to stimulating intestinal calcium transport. It has been conclusively...