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BIOLOGIC EFFECTS OF SHORT-TERM PHENOBARBITAL TREATMENT ON THE RESPONSE TO VITAMIN D AND ITS METABOLITES IN THE CHICK

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Abstract—The action of cholecalciferol (vitamin D_3) (D_3) and two of its active metabolites, 25-hydroxycholecalciferol (25-OH-D_3) and 1,25-dihydroxycholecalciferol (1,25-$(OH)_2$-D_3), was studied in phenobarbital-treated and untreated rachitic chicks by means of a bioassay for intestinal calcium absorption and bone resorption. Phenobarbital treatment (7 days) reduced the ability of single doses (0·65 to 3·25 nmoles) of D_3 and 25-OH-D_3 to stimulate intestinal calcium absorption and bone resorption. The impaired biological responses to these moderate doses of D_3 could be overcome by increasing the dose (32·5 nmoles). In contrast, administration of small doses of 1,25-$(OH)_2$-D_3 (0·26 nmole) stimulated both the intestinal calcium transport and bone resorption responses in phenobarbital-treated chicks. Moreover, phenobarbital treatment appeared to enhance the calcemic response of bone to 1,25-$(OH)_2$-D_3. The metabolism of radioactive D_3 was also studied in control rachitic chicks and rachitic chicks treated with phenobarbital for 7 days. Sephadex LH-20 column chromatography of lipid-soluble extracts of blood, intestinal mucosa and bone was carried out 24 hr after administration of a single dose of 4-^{14}C-vitamin D_3. The anticonvulsant treatment resulted in markedly lower quantities of 1,25-$(OH)_2$-D_3 in the bone, but with no major changes in the amount of this steroid present in the intestine. The drug treatment also resulted in increased amounts of D_3 and 25-OH-D_3 in the blood and intestinal mucosa. Seven-day anticonvulsant treatment of the rachitic chick clearly impairs the biological responses to D_3 and also alters its metabolism. It is possible that the reported high incidence of osteomalacia occurring in patients on long-term anticonvulsant therapy may possibly be caused by derangements in the conversion of D_3 to its biologically active metabolites and in their association with the target tissues. This may provide some explanation for the disruption of normal calcium metabolism which occurs after chronic administration of phenobarbital and other related drugs.

Osteomalacia and rickets have been diagnosed in a large number of epileptic patients on long-term anticonvulsant drug therapy [1]. A positive correlation of hypocalcemia with anticonvulsant drug therapy was observed in another study [2].

Dent *et al.* [3] have previously reported that osteomalicic and rachitic patients on long-term anticonvulsant drug therapy responded exceptionally well to vitamin D (calciferol) supplementation. Dietary intake of vitamin D in these patients was observed to be minimal, and their exposure to ultraviolet radiation was low. In a different study, von Herrath *et al.* [4] determined that phenobarbital treatment had a greater effect on vitamin D metabolism and its action on calcium metabolism than did another commonly employed anticonvulsant, diphenylhydantoin. Hahn *et al.* [5] provided evidence of a definite alteration of vitamin D metabolism and a lowered circulating concentration of plasma 25-hydroxycholecalciferol [6] in patients on long-term anticonvulsant drug therapy and in otherwise normal patients on short-term phenobarbital treatment. Evidence for a phenobarbital-induced increase in the hepatic conversion, *in vitro*, in rats of cholecalciferol (D_3),* and 25-hydroxycholecalciferol to more polar metabolites was also reported by this group [5–7]. Recently Livingston *et al.* [8] advanced the view that long-term anticonvulsant drug therapy is not necessarily related to osteomalacia or rickets. They felt it is mere coincidence that these bone diseases sometimes appear in patients on long-term anticonvulsant drug therapy.

The present report provides evidence for: (a) a reduced ability of D_3 and its metabolite 25-OH-D_3 to stimulate intestinal calcium transport in chicks on short-term phenobarbital treatment; (b) an altered effect of D_3, 25-OH-D_3 and 1,25-$(OH)_2$-D_3 on bone calcium resorption; and (c) a reduced accumulation of 1,25-$(OH)_2$-D_3 by bone but not intestine.

METHODS

One-day-old White Leghorn cockerels (H & N, Inc.) were employed in five separate experiments. The chicks were raised on a calciferol-deficient, rachitogenic diet [9] from the day of hatching. They were utilized when they had become severely rachitic in their fourth week. Phenobarbital dissolved in saline (0·9% NaCl) was administered intraperitoneally at a dose level of 10 mg/0·5 ml/100 g of body weight to randomly selected groups of chicks. Treatment with phenobarbital in all instances was carried out for a 7-day period beginning 21 days after hatching. A second group of control chicks was given 0·9% NaCl at a dose level of 0·5 ml/100 g of body weight for

* Abbreviations used in this paper are: D_3 = cholecalciferol (vitamin D_3); 25-OH-D_3 = 25-hydroxycholecalciferol; and 1,25-$(OH)_2$-D_3 = 1,25-dihydroxycholecalciferol.

7 days. In experiments concerning the biological responses, groups of control rachitic chicks or phenobarbital-treated rachitic chicks received intracardial doses of D_3 (5–500 I.U.) or 25-OH-D_3 (5–15 U.) 24 hr before death and 1,25-$(OH)_2$-D_3 (2–6 U.) 10 hr before death.

Intestinal $^{45}Ca^{2+}$ absorption and bone calcium resorption were measured in groups of ten chicks by the bioassay procedures described by Hibberd and Norman [10]. For these assays, all chicks were switched from the standard rachitogenic diet containing 0·6% calcium to a rachitogenic diet containing "0%" added calcium 3 days before assay. Three to 5 μCi $^{45}Ca^{2+}$ and 4·0 mg $^{40}Ca^{2+}$/0·2 ml saline were placed in an exposed segment of the duodenum 30 min before death. At death, 2–3 ml of blood was collected. Intestinal calcium absorption was assayed by determination of the $^{45}Ca^{2+}$ content of 0·20-ml aliquots of plasma dried on planchets. Bone calcium resorption was assessed via determination of the serum Ca^{2+} by atomic absorption spectrometry.

The metabolism of cholecalciferol was assessed at the same time that the biological responses to the steroid were determined (Tables 1 and 2). For the experiment in Table 1, one group of four chicks was given phenobarbital for days 21–28 and another group of four chicks was given saline. The phenobarbital-treated chicks were then given intraperitoneal doses of 20 I.U. of 4-^{14}C-cholecalciferol (Philips Duphar, Amsterdam, The Netherlands), sp. act. 4900 dis./min/I.U. 24 hr before death. The controls received intraperitoneal doses of 20 I.U. 1,2-^{3}H-cholecalciferol (Amersham–Searle), sp. act. 20,000 dis./min/I.U. 24 hr prior to sacrifice. The blood, small intestine, liver, kidneys, tibia-fibulae and femora were removed from each chick at the time of sacrifice. Each sample was individually extracted in a Waring blender by the method of Bligh and Dyer [11]. The chloroform and H_2O–methanol layers were collected and dried in air. The radioactivity present in these two phases was determined on a Beckman liquid scintillation counter model CPM 233 (Beckman Instruments, Inc., Fullerton, Calif.).

In the experiments concerning D_3 metabolism reported in Table 2, two groups of four chicks were randomly selected, one group from the phenobarbital-treated chicks and the other from the controls. Both groups received oral doses of 50 I.U.* or intraperitoneal doses of 20 I.U. 4-^{14}C-cholecalciferol, sp. act. 4900 dis./min/I.U. on day 7 of phenobarbital treatment. All steroid doses were dissolved in 0·2 ml of 1,2-propanediol. The chicks were killed 24 hr later.

The blood, intestinal mucosa and bone samples were taken. The bones taken included the right and left tibia-fibula and femora, all with the marrow removed. Individual samples were pooled in groups and extracted according to the method of Bligh and Dyer [11].

The chloroform layers from the lipid extractions from experiments 1 and 2 were dried under nitrogen and redissolved in 4 ml chloroform–hexane (65:35, v/v). Two ml of these samples was then applied to an 80 cm × 1·5 cm Sephadex LH-20 column. The chromatographic procedures employed were those described by Holick and DeLuca [14]. Fractions obtained from these columns were poured into 20-ml liquid scintillation vials and air dried. Ten ml of PBD toluene counting solution was then added to each sample. Finally the samples were counted on a Beckman CPM-200 liquid scintillation counter to 5 per cent error. The data were analyzed by a computer program utilizing the external standardization technique for efficiency determination. The statistical analyses indicated in Figs. 1–4 were carried out according to Student's t-test.

RESULTS

The effects in the rachitic chick of short-term phenobarbital treatment on cholecalciferol-stimulated intestinal calcium absorption are summarized in Figs. 1 and 2. It is evident that the normal effects of moderate physiological doses (5–50 I.U.) of D_3 given 24 hr prior to sacrifice are markedly inhibited by the anticonvulsant treatment. Phenobarbital treatment also inhibited the normal effect of moderate (5–15 U.) doses of 25-OH-D_3 in terms of stimulating intestinal calcium absorption (Fig. 2). In contrast, the intestinal calcium absorption response mediated by large doses (500 I.U.) of cholecalciferol was not inhibited by treatment with phenobarbital. In chicks receiving as little as 2–6 U. of 1,25-$(OH)_2$-D_3 9 hr prior to sacrifice, little or no impairment of intestinal calcium absorption was apparent in the phenobarbital-treated chicks compared to saline-injected control birds. These results suggest that the biologic action mediated directly by 1,25-$(OH)_2$-D_3 is not impaired by short-term phenobarbital treatment.

The alterations in bone resorption due to phenobarbital treatment as assayed *in vivo* are presented in Figs. 3 and 4. In both experiments, phenobarbital

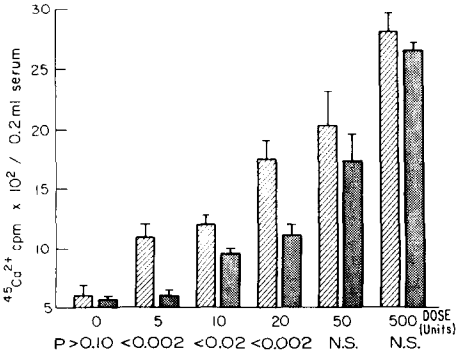

Fig. 1. Effect of phenobarbital treatment on cholecalciferol-stimulated intestinal calcium absorption. This experiment was conducted as described in Methods. Slashed bars = control chicks; cross-hatched bars = phenobarbital-treated chicks. Each group consisted of ten birds. Brackets indicate the standard deviation for the data.

* One international unit (I.U.) of cholecalciferol (vitamin D_3) is equivalent to 0·025 μg or 0·065 nmole [12]. The minimum daily requirement for cholecalciferol for the chick is 10–20 I.U. [10]. No formal definition of units has been formulated for 25-OH-D_3 or 1,25-$(OH)_2$-D_3. For this report, 1·0 unit (U.) of each of these compounds is defined as 0·065 nmole. See Ref. 13 for a discussion of these problems.

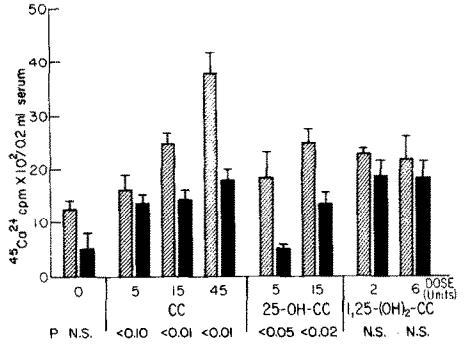

Fig. 2. Effects of phenobarbital treatment on cholecalciferol and its metabolites on intestinal calcium absorption. This experiment was conducted as described in Methods. Slashed bars = control chicks; cross-hatched bars = phenobarbital-treated chicks. Each group consisted of ten birds. Brackets indicate the standard deviation for the data.

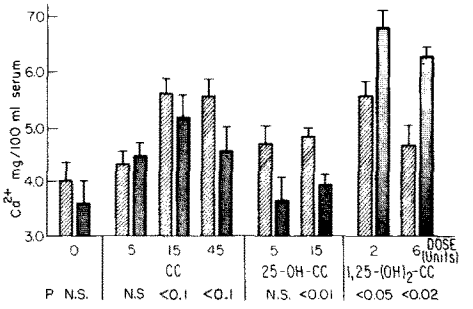

Fig. 4. Effect of phenobarbital treatment on cholecalciferol and its metabolites on bone resorption. This experiment was conducted as described in Methods. Slashed bars = control chicks; cross-hatched bars = phenobarbital-treated chicks. Each group consisted of ten birds. Brackets indicate the standard deviation for the data.

treatment impaired the ability of moderate doses (5–50 I.U.) of D_3 to elevate serum calcium via bone mobilization. However, this impaired response could be overcome by administration of 500 I.U. of D_3 (Fig. 3). Phenobarbital treatment also blocked the bone mobilization response to moderate doses (5–15 U.) of 25-OH-D_3. In marked contrast were the results obtained after administration of moderate (2–6 U.) doses of $1,25-(OH)_2$-D_3. There was no evidence of inhibition, but instead a small enhancement of serum calcium elevation in the birds treated with phenobarbital.

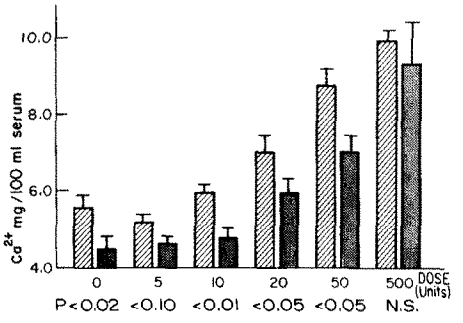

Fig. 3. Effect of phenobarbital treatment on cholecalciferol-stimulated bone resorption. This experiment was conducted as described in Methods. Slashed bars = control chicks; cross-hatched bars = phenobarbital-treated chicks. Each group consisted of ten birds. Brackets indicate the standard deviation for the data.

The amount of more polar water-soluble metabolites of cholecalciferol as determined by Bligh and Dyer [11] for lipid extractions of various tissues was not significantly altered by treatment with the anticonvulsant (Table 1). Although the relative difference in amounts of water-soluble radioactivity between the controls and the phenobarbital-treated chicks was small, it is of interest to note that the highest relative proportion of aqueous metabolites appeared in the H_2O–methanol extracts of the liver and small intestine, while the lowest percentage was recovered from the bone.

The effects of phenobarbital treatment on cholecalciferol metabolism as determined by LH-20 column chromatography are shown in Table 2. Phenobarbital treatment resulted in an average of 36 per cent (range 15–16 per cent) of the pmoles of total steroid present in all the tissues examined. There was, however, in the intestine a percentage reduction of $1,25-(OH)_2$-D_3 (41·9 to 22·3 per cent, Expt. 1; 62·0 to 38·0 per cent, Expt. 2) as a consequence of the anticonvulsant treatment. But this largely reflects a higher amount of D_3 and 25-OH-D_3, rather than a reduction in the absolute amount of $1,25-(OH)_2$-D_3. In both experiments,

Table 1. Effect of phenobarbital treatment on the conversion of labeled vitamin D_3 to more polar water-soluble metabolites*

	Phenobarbital-treated chicks			Control chicks		
	Total activity (pmoles)	% Soluble in		Total activity (pmoles)	% Soluble in	
		$CHCl_3$	H_2O		$CHCl_3$	H_2O
Blood	10·3	99·4	6·6	8·6	91·1	8·9
Intestine	6·9	79·7	20·3	6·2	85·9	14·1
Liver	17·1	89·0	11·0	13·3	84·4	15·6
Kidney	5·4	93·1	6·9	4·9	90·5	9·5
Bone	7·4	98·7	1·3	4·5	100·0	0·0

* Tissue samples were taken 24 hr after intraperitoneal injections of 20 I.U. of ^{3}H-D_3 (control) or ^{14}C-D_3 (phenobarbital-treated). The samples were extracted by the lipid extraction technique of Bligh and Dyer [11]. Radioactivity was determined from the resulting chloroform and H_2O–methanol layers. Values represent the average of four determinations; the standard deviation in all instances was less than 8 per cent of the total pmoles present.

Table 2. Distribution of lipid-soluble radioactivity as determined by LH-20 column chromatography*

	Total activity of steroid (pmoles)		Activity (pmoles recovered/peak)					
			D_3 (% of total)		25-OH-D_3 (% of total)		1,25-$(OH)_2$-D_3† (% of total)	
	C	P	C	P	C	P	C	P
Experiment 1								
Blood	166	197	3·6 (2·1)	48·0 (24·4)	144 (87·0)	129 (65·8)	12·6 (7·6)	7·8 (4·0)
Intestine	28	37	10·1 (35·7)	21·7 (58·8)	3·9 (13·9)	3·9 (10·5)	11·9 (41·9)	8·2 (22·3)
Experiment 2								
Blood	15	39·0	1·2 (8·2)	5·1 (12·9)	11·1 (76·4)	30·6 (78·6)	0·6 (5·0)	2·1 (5·3)
Intestine	3·8	8·6	0·7 (17·9)	3·0 (35·0)	0·3 (6·6)	1·6 (19·0)	2·4 (62·0)	3·3 (38·0)
Bone	2·9	3·9	0·8 (27·6)	1·1 (28·2)	1·3 (44·8)	2·7 (69·2)	0·8 (27·6)	0·1 (2·6)

* The chicks in Exp. 1 were given oral doses of 50 I.U. 4-^{14}C-cholecalciferol 24 hr before death. Those in Exp. 2 were given intraperitoneal doses of 20 I.U. 4-^{14}C-cholecalciferol 24 hr before death. The amount of blood collected is assumed to be one-third of the total volume in the chick. C = control rachitic chicks; P = phenobarbital-treated chicks. The total pmoles steroid/tissue is slightly less than the sum of the total pmoles for the three metabolites; the difference represents small amounts of other unidentified metabolites.

† In the blood, this peak is a composite of 1,25-$(OH)_2$-D_3 and 25,26-$(OH)_2$-cholecalciferol.

there were also higher amounts of D_3 circulating in the plasma of the phenobarbital-treated birds.

The most striking effects of treatment with phenobarbital were apparent in the bone. Here the anticonvulsant caused a marked reduction in the amount of 1,25-$(OH)_2$-D_3 and an increase in the quantity of 25-OH-D_3 present.

DISCUSSIONS

The results of the present studies clearly demonstrate that phenobarbital treatment of rachitic chicks can impair two classical vitamin D responses, that of enhancing the intestinal absorption of calcium and that of mobilizing bone calcium. This report represents the first unequivocal demonstration of this deleterious action of phenobarbital and strongly supports the concept that anticonvulsant therapy can ultimately produce clinical effects analogous to that of vitamin D deficiency [1–7]. Of some interest is the observation that the biological responses of both D_3 and its metabolite 25-OH-D_3 were impaired, while that of 1,25-$(OH)_2$-D_3 was not.

1,25-$(OH)_2$-D_3 is currently believed to be the biologically active form of vitamin D [14–17]. When given on a daily basis to chicks, it is able to carry out all the physiological responses mediated by D_3 and thus produce chicks with a normal calcium metabolism [9]. Further, 1,25-$(OH)_2$-D_3 has been shown to be biologically active in the chick [9], rat [9], dog [18] and man [19]. D_3 is first metabolized by the liver to give 25-OH-D_3 [20], which is in turn metabolized by the kidney to 1,25-$(OH)_2$-D_3 [21, 22]. This complex metabolic process in two separate organs may render the metabolic pathway for production of the hormonal form of D_3, i.e. 1,25-$(OH)_2$-D_3, particularly vulnerable to agents which may potentially alter steroid metabolism.

Conney and coworkers [23, 24] have postulated that both steroids and drugs may be substrates for the same hepatic microsomal P-450 enzyme systems. Specifically, they have observed [24] that phenobarbital treatment alters the metabolism and action of estradiol-17β. Thus, it is not inconceivable that phenobarbital treatment may cause parallel alterations in the metabolism of the steroid calciferol which could lead to an altered production of 1,25-$(OH)_2$-D_3. Also, it is known that the hepatic 25-hydroxylase activity is found in the microsomal fraction [25]. But in view of the present observation (Figs. 2 and 4) that the biological responses of 25-OH-D_2 are also impaired, it seems possible that the phenobarbital-induced sterol metabolism may have other sites of action in addition to the liver.

The basis for the differential actions of 1,25-$(OH)_2$-D_3 in the intestine (Fig. 2) vs the bone (Fig. 4), where a significant enhancement of response in the phenobarbital-treated birds over that of the controls was observed, is not known. The bone has a slightly higher affinity for 1,25-$(OH)_2$-D_3 than does the intestinal mucosa.* Thus, when limiting amounts of 1,25-$(OH)_2$-D_3 are made available e.g. 2–6 U. (Figs. 2 and 4), the bone may accumulate the 1,25-$(OH)_2$-D_3 more effectively than the intestine.

The present data (Tables 1 and 2) concerning the metabolism of D_3 in the phenobarbital-treated birds

* H. C. Tsai and A. W. Norman (manuscript in preparation) have found that after an intracardial dose of ^{3}H-1,25-$(OH)_2$-D_3 to rachitic chicks the skeletal system has a maximum uptake and localization of 1,25-$(OH)_2$-D_3 within 2 hr, whereas 4 hr is required for this to occur in the other target organ, the intestinal mucosa.

are somewhat difficult to interpret in relation to the striking impairment of biological responses to D_3 and 25-OH-D_3. Since only severely rachitic birds were employed in these studies, there was not present any nonradioactive endogenous pools of calciferol plus metabolites to complicate the interpretation. Clearly, when a single dose of radioactive D_3 is given to a phenobarbital-treated rachitic chick, there is no major distortion in the metabolism of the steroids. This potentially might be reflected in an increase in "H_2O-soluble" radioactivity due to multiple hydroxylation. It may be that such gross effects become apparent only after chronic or daily administration of the parent vitamin. Hahn *et al.* [5, 6] observed phenobarbital-mediated alterations of calciferol metabolism in both normal man and normal D-treated rats, which was consistent with their view that the drug enhanced the conversion of the steroid to more polar metabolites. Von Herrath *et al.* [4] noted similar effects again in normal D-treated rats.

The present study clearly show that phenobarbital treatment has changed the metabolism and tissue localization of calciferol plus metabolites. The obvious changes are a reduced accumulation of 1,25-$(OH)_2$-D_3 by the bone and an increased association of D_3 and also of 25-OH-D_3 in both the target intestine and bone. Clearly, further work is required to delineate the exact relationship between the impaired biological response and metabolism of calciferol.

In a separate but related study, Norman *et al.* have shown that chronic dietary feeding of the chlorinated hydrocarbons DDT* and PCB* to rachitic chicks produces results exactly analogous with the results reported in this paper. Chronic DDT or PCB feeding impairs the intestinal or skeletal responses to single doses of CC [28]. Yet these impaired biological responses occurred without major alterations in D_3 metabolism [29]. Both PCB and DDT treatment are known to increase the hepatic metabolism of estrogens and androgens [30]. Thus, our understanding of the effects of both phenobarbital treatment and chlorinated hydrocarbon treatment on the metabolism of D_3 and the biological action of its metabolites is that they are similar.† Also, with chronic renal failure, there have been suggestions and evidence of an impaired metabolism of CC [31, 32] which leads to lowered biological responses. Thus in each instance, in phenobarbital or DDT treatment and in chronic renal failure, there is a possibility of an impairment of both D_3 metabolism and the end organ action of its metabolites. In each instance, the possible consequences of a deficiency of 1,25-$(OH)_2$-D_3 or the end organ impairment can be largely overcome by administration directly of small quantities of 1,25-$(OH)_2$-D_3 (Figs. 2 and 4, Refs. 19, 33).

The immediate clinical implication of these results clearly is that anticonvulsant treatment may lead in some circumstances to the appearance of an impaired response to vitamin D. Such patients should be carefully evaluated for this development. It is possible that amelioration of the problem can be achieved by increasing the dietary intake of D_3. The results obtained for the 500-I.U. dose of D_3 (Figs. 1 and 3) suggest that the phenobarbital-impaired response could be overcome in rachitic chicks by this protocol. However, further work should be carried out to validate this observation more generally before it is adopted for clinical management of patients.

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* DDT [1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane] and PCB (polychlorinated biphenyl mixtures manufactured in the United States) are marketed by Monsanto Co., St. Louis, under the trade name Aroclor. The Aroclors, designated by four-digit numbers, the last two digits of which define the percentage of chlorine content by weight (e.g. Aroclor 1254 contains 54% chlorine), have been proposed to disrupt calcium metabolism in birds, and may lead to development of the "thin eggshell" syndrome [26, 27]. In certain wild birds of prey which are particularly vulnerable, this could lead to extinction of the species.

† It is interesting to note that molecular models of phenobarbital and DDT are unusually similar. This suggests a possible reason for the similar biological actions of these agents.

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