K^+ absorption (*9*). We measured the rate of K^+ absorption by corn roots (*10*) at several concentrations of La^{3+} and found an inhibition similar to that reported for Ca^{2+} (for example, at 0, 0.05, 0.1, 0.25, and 0.5 m*M* $La(NO_3)_3$, K^+ absorption was 2.43, 1.81, 1.44, 1.31, and 1.26 μmole per gram fresh weight per hour, respectively). In the presence of Ca^{2+}, K^+ absorption was inhibited (0.25 m*M* Ca^{2+}; 1.03 μmole per gram fresh weight per hour) and addition of La^{3+} had no further inhibitory effect. These results are consistent with the proposal that the sites of La^{3+} deposition on membranes are, at least in part, sites which also bind Ca^{2+}.

G. NAGAHASHI
W. W. THOMSON
Department of Biological Sciences, University of California, Riverside 92502

R. T. LEONARD
Department of Plant Sciences, University of California, Riverside

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5. Corn seedlings (*Zea mays* L., WF 9 X M 14) were obtained by allowing seeds to germinate on paper towels saturated with 0.1 m*M* $CaCl_2$ at 26°C for 4 days in the dark [R. T. Leonard and J. B. Hanson, *Plant Physiol.* **49**, 430 (1972)]. The primary roots were excised and rinsed three times in cold distilled water. Roots were incubated for 1 hour with aeration at 30°C in 0.25 m*M* KCl plus 1 percent $La(NO_3)_3$ such that the "opened" end of the excised root did not come into contact with the incubation medium. After incubation, the apical 2 cm of the root was excised and fixed for 1.5 hours in 2.5 percent glutaraldehyde in 0.1*M* KH_2PO_4 adjusted to *p*H 7.5 with NaOH. The tissue was then fixed in 2 percent OsO_4 for 1 hour, and then dehydrated in a graded acetone series before being embedded in Spurr's epoxy plastic [A. R. Spurr, *J. Ultrastruct. Res.* **26**, 31 (1969)]. Thin sections were cut 1.5 cm from the root apex on a Porter-Blum MT-2 microtome and were either stained in 1 percent aqueous uranyl acetate and lead citrate [E. S. Reynolds, *J. Cell Biol.* **17**, 208 (1963)] or viewed unstained at 60 kv with a Philips 300 electron microscope.
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10. Primary roots were excised, rinsed three times in cold distilled water, and cut into 2-cm sections. Batches of 16 sections (a random selection of 4 apical and 12 basal, about 250 mg fresh weight) were incubated with aeration at 30°C in 35 ml of 0.25 m*M* KCl labeled with ^{86}Rb. After 10 minutes, the tissue was removed from the solution, rinsed with 25 ml of 1 m*M* KCl plus 0.25 m*M* $CaCl_2$, and then placed for 5 minutes in the same solution for exchange at 22°C. Lanthanum nitrate and $CaCl_2$ were added as indicated in the text. Blotted and weighed sections were counted in scintillation fluid [G. S. Bruno and J. E. Christian, *Anal. Chem.* **33**, 1216 (1961)] containing a thixotrophic gel.
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Diphenylhydantoin: Effects on Calcium Metabolism in the Chick

Abstract. *Rickets, hypocalcemia, decreased duodenal calcium transport, and reduction of calcium binding protein have been produced in chicks treated with diphenylhydantoin. These effects are directly related to diphenylhydantoin dose and inversely related to the intake of vitamin D_3 (cholecalciferol).*

Hypocalcemia, rickets, and osteomalacia develop with variable frequency in patients on long-term anticonvulsant therapy (*1*). Both phenobarbital (PB) and diphenylhydantoin (DPH) have been implicated; several reports suggest that these agents, by altering calciferol metabolism, may produce a state of functional vitamin D deficiency. Our studies in chicks demonstrate that DPH causes a decrease in intestinal calcium absorption, intestinal calcium binding protein (CaBP), serum calcium, and bone ash. Radiographic and histologic evidence of rickets was also seen. These effects were directly related to the DPH dose and inversely related to the amount of cholecalciferol, that is, vitamin D_3 (D_3), given concomitantly.

Cholecalciferol is hydroxylated in the liver to 25-dihydroxycholecalciferol [25-(OH)D_3] and in the kidney to 1,25-$(OH)_2$-dihydroxycholecalciferol [1,25-$(OH)_2D_3$] (*2*). Each step increases polarity and biological activity. The same sequence obtains for ergocalciferol (D_2), which is the form commonly used for dietary supplementation. Hahn *et al.* (*3*) found decreased serum concentrations of 25-(OH)D_3 in patients receiving either PB or DPH. Concentrations of 25-(OH)D_3 correlated directly with serum calcium and with calciferol intake. Hahn *et al.* (*4*) also reported that the half-life of D_3 in the plasma was shortened in subjects receiving PB and described increased conversion of D_3 and 25-(OH)D_3 to more polar metabolites by hepatic microsomes from PB-treated rats.

Tolman *et al.* (*5*) reported accelerated plasma disappearance rates and increased urinary excretion of both D_3 and 25-(OH)D_3 in patients who received either PB or DPH. In contrast, Schaefer *et al.* (*6*) and Silver *et al.* (*7*) found increased serum 25-(OH)D_3 under similar circumstances. Healing of bone lesions has been reported after administration of large doses of D_3 (*1*) or small doses of 25-(OH)D_3 (*8*). These findings have led to the view that anticonvulsants act as calciferol antagonists by inducing liver enzymes that convert D_3 to inactive or rapidly excreted metabolites (or both).

In the rat, both Koch *et al.* (*9*) and Caspary (*10*) found that absorption of calcium was depressed by DPH and PB. Neither found any significant change in intestinal calcium binding activity, and concluded that the synthesis of the CaBP was not affected. Since CaBP has an absolute dependence on vitamin D (or metabolite or vitamin D active steroid) for its synthesis and maintenance (*11*), the negative findings of Koch and Caspary would have important implications with respect to the pathogenesis of osteomalacia and rickets accompanying anticonvulsant therapy, if verified. Also, Clark *et al.* (*12*) saw no radiographic evidence of rickets in DPH-treated rats.

White Leghorn cockerels (1 day old) were offered a calciferol-free but otherwise adequate diet (*13*). After 5 days they were divided into seven groups whose D_3 and DPH intakes are given in Table 1. Crystalline D_3 was dissolved in sesame oil to give 3 or 6 international units (I.U.) per 0.1 ml, which was administered orally. Group 1 received sesame oil alone. The DPH (diphenylhydantoin, Eastman) was added to the diet to give 1 or 2.5 g per kilogram of diet, and DPH intake was calculated from weekly averages of food consumption and body weight (*W*). The body weight was converted to surface area

(M^2) by the formula $(M^2) = 9.2 \times 10^{-4} \times W^{2/3}$. The calculated intakes were higher than those used therapeutically in humans. At 28 days the duodenal absorption of ^{47}Ca was assessed, by in situ ligated loop procedures (*13*). The duodenal mucosa was then scraped free of the underlying muscle layers and homogenized in a buffered solution (119 m*M* NaCl, 4.74 m*M* KCl, 13.7 m*M* tris-HCl, *p*H 7.4) with the use of a Potter-Elvehjem homogenizer and Teflon pestle. After centrifugation at 38,000*g* for 20 minutes in the cold, the supernatant was treated at 60°C for 10 minutes, cooled, and centrifuged; its calcium binding activity was measured by the Chelex 100 ion exchange binding assay (*14*). The absolute amount of CaBP in the supernatant was also determined by a specific radioimmunoassay (centrifugal diffusion in gel containing antiserum to CaBP), with the use of a highly purified CaBP as the standard (*15*). Protein concentrations were measured by the Lowry method (*16*). Serum calcium was determined by the procedure of Bett and Fraser (*17*). Serums were then pooled, and the DPH content was measured (*18*). The ash content of the dried tibiae that were extracted with ether was also estimated. At 30 days representative chicks were subjected to radiography.

Table 1. Effect of diphenylhydantoin on body weight, serum calcium concentration, and tibia ash of chicks on two levels of vitamin D_3.

Group*	Treatment		Terminal body weight (mg)	Serum calcium (mg/100 ml)	Tibia ash (%)‡
	D_3† (I.U./day)	DPH (mg/m²)			
1	0	0	193 ± 11	6.4 ± 1.2	26 ± 1
2	3	0	280 ± 14	9.7 ± 0.1	39 ± 2
3	3	760	258 ± 10	7.9 ± 1.1	34 ± 1
4	3	1800	198 ± 9	6.2 ± 0.2	29 ± 2
5	6	0	305 ± 13	10.0 ± 0.2	41 ± 1
6	6	760	293 ± 6	9.7 ± 0.2	42 ± 1
7	6	1800	260 ± 12	8.5 ± 0.3	36 ± 1

* Six chicks per group; values represent means ± standard error of the means. † Vitamin D_3 given orally. ‡ Percentage of fat-free dry weight.

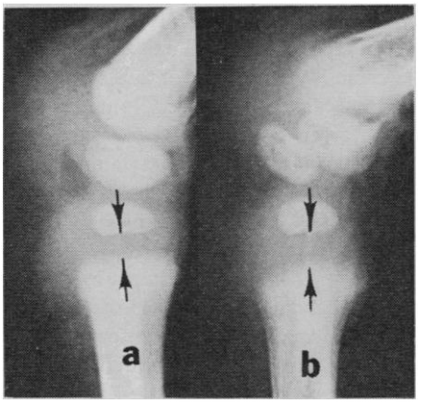

Fig. 1. Tarsometatarsal joints. (a) Group 2, control diet. The chicks were given 3 I.U. of D_3 per day. (b) Group 3, DPH (1 g per kilogram of diet provided 760 mg per square meter per day) was added to the diet, in addition to the 3 I.U. of D_3 given daily.

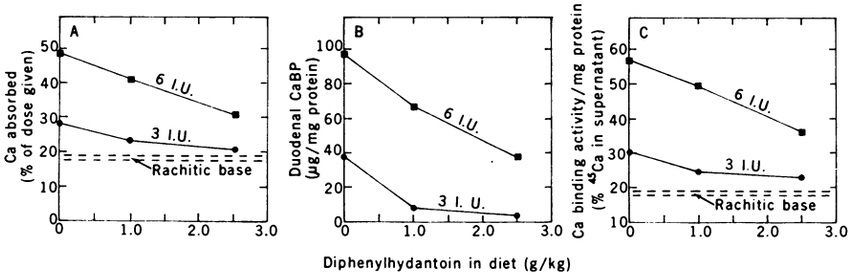

Fig. 2. Effect of diphenylhydantoin and D_3 on calcium absorption, CaBp, and calcium binding activity. Six chicks per group were used; the values represent the means ± standard error of the means. (A) Duodenal absorption of calcium was determined by the in situ ligated loop technique (*13*). (B) Calcium binding protein was determined by the radioimmunoassay (*15*). (C) Solid circles indicate a D_3 dosage of 6 I.U. per day and open circles indicate a D_3 dosage of 3 I.U. per day. Calcium binding activity was determined by the ion exchange resin (Chelex) assay (*14*).

Serum DPH was 2.0 mg per 100 ml on the lower DPH intake and 4.7 mg per 100 ml on the higher. The radiographic appearance and the effects of DPH on the skeleton were visualized (Fig. 1). Direct measurements of the width of the physes (indicated by the arrows, Fig. 1) show a 50 percent increase in the DPH-treated chicks. Histologic examination of sections taken longitudinally through the epiphyseometaphyseal junction and stained with hematoxylin and eosin showed the characteristic changes of rickets. Irregularity and disarray of all cellular components were present in all of the cellular components of the widened cartilaginous physeal plate; the vacuolating and degenerating cells gave no evidence of mineral depostion; excessive amounts of osteoid were present as broad "seams" without calcification; and those bony trabeculae present in both the epiphysis and metaphysis were thin and sparse. The last-mentioned resulted in compromised capacity of the bone to withstand the stress of weight bearing, leading to "flaring" of the juxtaphyseal regions on x-ray. Severe rickets was produced at 30 days by DPH in the chicks on the lower level of vitamin D_3. The greatest effect was observed at the higher DPH intake, being a combination of severe rickets with stunting of overall growth (group 4). In the chicks receiving the higher dose of D_3 radiologic evidence of early rickets was seen in only one animal on the higher intake of DPH (group 7) Radiologic evidence of severe rickets was present in the group 1 chicks at 23 days. The only other group showing abnormality at this time was group 4, which showed evidence of early rickets.

The terminal body weight, serum calcium concentration, and degree of bone mineralization (ratio of ash weight to dry weight) of the various groups are shown in Table 1. The chicks receiving the lower dose of vitamin D_3 (groups 2, 3, and 4) were most severely affected by DPH. Group 4 chicks receiving 2.5 g of DPH per kilogram of diet showed growth rates, serum calcium concentration, and bone ash values (Table 1) indistinguishable from those chicks raised in the absence of D_3 (group 1). Chicks of group 3 yielded values intermediate between those of group 4 (high DPH) and group 2 (controls). A similar situation occurred with respect to calcium absorption and the vitamin D–induced CaBP (Fig. 2).

DPH decreased the duodenal absorption of the test dose of ^{47}Ca and duodenal CaBP to near zero (comparable to that in rickets) in a dose-related fashion. A depression in calcium binding activity, which reflects CaBP, was also noted.

Chicks on the higher dosage of D_3 (groups 5, 6, and 7) were affected by DPH but less so than those receiving 3 I.U. of D_3 per day. The ingestion of 2.5 g of DPH per kilogram of diet (group 7) resulted in a significant reduction in growth ($P < .03$), serum calcium ($P < .01$), and tibia ash ($P < .01$) (Table 1). Little or no effect of the lower level of DPH on these parameters was noted. Similarly, group 7 (6 I.U. of D_3; 2.5 g of DPH per kilogram of diet) displayed a reduction in ^{47}Ca absorption (Fig. 2A), vitamin D–induced CaBP (Fig. 2B), and calcium binding activity (Fig. 2C). At the lower intake of DPH (1 g per kilogram of diet), the only significant effect observed was a reduction in CaBP (Fig. 2).

Group 7 chicks maintained calcium absorption and CaBP levels equal to those of group 2, but were hypocalcemic. Bone analysis showed no significant difference between group 2 and group 7 ($P = .25$) (Table 1 and Fig. 2). Hypocalcemia without bone involvement has been noted clinically (*1*); no explanation is apparent at the present time.

Our data showing a depression in CaBP by DPH treatment is in disagreement with the reports of Koch *et al.* (*9*) and Caspary (*10*). These investigators assessed CaBP by a nonspecific calcium binding assay, using the ion exchange resin technique (*14*), which measures total calcium binding activity and not that due exclusively to CaBP. In our study, both the nonspecific ion exchange resin procedure and the specific radioimmunoassay indicated a depression in intestinal CaBP (Fig. 2). Since Koch *et al.* (*9*) and Caspary (*10*) used rats, there is a possibility of a species difference in responsiveness to DPH.

It is apparent that, in chicks, DPH administration causes readily demonstrable effects on calcium metabolism which provides support for clinical observations on epileptic patients given anticonvulsant therapy. The effects were dose dependent and related inversely to the amount of calciferol given. The fact that the vitamin D–induced CaBP also changed in proportion to the change in calcium absorption provides further support for the contention that the DPH-dependent defect is related in some way to the abnormal metabolism of or responsiveness to D_3. There is no evidence for a direct effect of DPH on the calcium absorption mechanisms per se, as thought to pertain to cortisone treatment (*19*). Our findings support suggestions that close attention should be paid to calciferol intake in patients requiring seizure therapy. They also indicate that the chick may be a useful model for testing drugs suspected of being calciferol antagonists.

M. VILLAREALE
Department of Biochemistry, Upstate Medical Center, Syracuse, New York 13210
L. V. GOULD
Department of Radiology, Upstate Medicine Center
R. H. WASSERMAN, A. BARR*
New York State Veterinary College, Cornell University, Ithaca
R. T. CHIROFF
Department of Orthopaedic Surgery, Upstate Medical Center
W. H. BERGSTROM
Department of Pediatrics, Upstate Medical Center

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* Permanent address: Volcani Institute, Rehovoth, Israel.

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DDE: Its Presence in Peregrine Eggs in 1948

Abstract. *DDE has been eluted from the dried membranes of peregrine eggshells collected in California from 1948 to 1950, and identified by gas-liquid chromatography.*

The question of the involvement of DDT [1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane] in the sudden decrease of eggshell thickness in many species of predatory birds starting around 1947 has been hotly debated (*1–4*). Ratcliffe (*1*) marshalled the evidence to show that enough DDT could have been present by 1947 to cause eggshell thinning, while Gunn (*4*) came to the opposite conclusion. It appears that the information available on the use of DDT at that time is not adequate to support a definite conclusion. Gunn (*4*) concluded by saying that "an effect occurring before its cause is utterly unacceptable." It is certainly correct that the decrease in eggshell thickness was abrupt and occurred very soon after the introduction of DDT as an insecticide in 1945. In Great Britain the annual mean shell thickness for the peregrine (*Falco peregrinus*), calculated from the data of Ratcliffe (*1*), decreased in 1946 and was significantly lower by 1947. Hickey and Anderson (*5*) found that peregrine eggshells from California were markedly lighter in weight from 1947 on. In recent years it has been possible to demonstrate eggshell thinning caused by DDT and its principal metabolite DDE [1,1-dichloro-