PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 153, 220–224 (1976)

Inhibition of Vitamin D-Stimulated Active Transport of Calcium of Rat Intestine by Diphenylhydantoin–Phenobarbital Treatment (39514)

HELEN C. HARRISON AND HAROLD E. HARRISON

Departments of Pediatrics, Johns Hopkins University – School of Medicine, Baltimore, Maryland 21205, and Baltimore City Hospitals, Baltimore, Maryland 21224

The significantly increased incidence of biochemical and radiologic evidence of rickets and osteomalacia in patients receiving anticonvulsants for prolonged periods has been adequately documented (1–3) since the first reports of Schmid (4) and Kruse (5). Lifshitz and MacLaren (6) in an epidemiological study in an institution for severely retarded patients have shown that the patients on anticonvulsant drugs had lower serum calcium and phosphate concentrations and higher alkaline phosphatase activities than those with comparable motor disabilities who were not receiving anticonvulstants. This effect was seen primarily in patients receiving both phenobarbital and diphenylhydantoin. Inasmuch as phenobarbital is known to induce the liver microsomal enzymes of the cytochrome *P*-450 system which hydroxylates steroids which are then glycuronidated (7), the possibility that anticonvulsant medication has an anti-vitamin D effect through increasing the rate of inactivation of 25-OH-vitamin D has been proposed (8). MacLaren and Lifshitz (9) have treated institutionalized patients with anticonvulsant-associated rickets using physiologic amounts of 25-OH-cholecalciferol and have demonstrated healing in patients who were unresponsive to much larger amounts of ergocalciferol. They suggested that the anticonvulsants interfered with 25-hydroxylation by liver.

Studies in rats have suggested an additional effect of diphenylhydantoin which might contribute to the increased vitamin D requirement of patients on anticonvulsant therapy. No evidence of an anti-vitamin D action of diphenylhydantoin in rats has been demonstrated using skeletal changes of rickets (10) or hypocalcemia as an indicator (unpublished data). However, Caspary (11) and Koch *et al.* (12) have reported impairment of intestinal calcium transport in rats given diphenylhydantoin or diphenylhydantoin plus phenobarbital.

The present studies were designed to examine this effect and to determine whether it could be separated from a deficiency of the active vitamin D metabolite, 1,25-dihydroxyvitamin D, resulting from the action of the anticonvulsants. The design of the experiments was based on the measurement of response of hypocalcemic, vitamin D-depleted rats treated with diphenylhydantoin and phenobarbital to physiological amounts of vitamin D or 25-hydroxy-vitamin D using three criteria of response: serum calcium concentration, active transport of calcium *in vitro* by everted intestinal loops, and diffusibility of calcium across the intestinal wall *in vitro*. The active transport of calcium in the *in vitro* system is indicated by the development of a concentration of calcium in serosal fluid much higher than that in mucosal fluid and is an energy-requiring process. The diffusion of calcium from a higher concentration in the mucosal phase to a lower concentration in the serosal phase occurs at low temperature and in the presence of metabolic inhibitors which block active transport of calcium. Vitamin D increases both diffusibility and active transport of calcium across the intestinal mucosa. If a separation of the effect of vitamin D on these two processes could be demonstrated in the diphenylhydantoin- and phenobarbital-treated rats, the locus of action of the anticonvulsant drugs on calcium transport might be further delineated. The combination of diphenylhydantoin and phenobarbital was used to duplicate more closely the clinical state in man since the increased susceptibility to rickets and osteomalacia is most striking in patients receiving combinations of anticonvulsant drugs usually including diphenylhydantoin (6, 13).

Methods. Weanling male rats of the

Holtzman strain were fed a vitamin D-deficient diet containing 0.6% calcium and 0.5% phosphorus. On this diet, vitamin D depletion is not associated with hypophosphatemic rickets but with hypocalcemia (14). After 3 weeks on the diet the rats were bled from the tail and serum calcium concentrations determined to verify the state of vitamin D depletion. The animals were then divided into groups. Treated rats were given sodium diphenylhydantoin, 8 mg/100 g, and sodium phenobarbital, 5 mg/100 g, daily by intraperitoneal injection divided in two doses, and treatment was continued until the animals were killed. Control rats were given equivalent volumes of 0.85% saline solution intraperitoneally. The food intake and weight gain of treated and control rats were measured and the anticonvulsant drug treatment did not reduce food intake or weight gain. On the fifth day of such injection the rats were given by stomach tube 0.25 μg (625 pmoles) of ergocalciferol, cholecalciferol, or 25-OH-cholecalciferol dissolved in propylene glycol. Groups of treated and control rats given only solvent were also studied. One series of treated and control rats was given a larger dose of cholecalciferol, 2.5 μg (6.25 nmoles). Seventy-two hours after the steroid administration the rats were anesthetized with sodium pentobarbital and bled from the abdominal aorta, and segments of duodenum and ileum were taken for the measurement of active calcium transport *in vitro* by an everted intestinal loop sytem (15). The buffer solutions in the calcium transport system contained 50 mequiv/liter of sodium since at this concentration of sodium both ileal and duodenal transport of calcium are maximal (16).

In another series of experiments the diffusibility of calcium across the intestinal wall was measured, also by use of everted intestinal loops of duodenum or ileum. These determinations were done at room temperature (23°). In mucosal fluid the initial concentration of calcium labeled with calcium-45 was 2.5 m*M*, with no calcium in serosal fluid. The accumulation of calcium-45 in serosal fluid during a 30-min incubation was determined. The effect of vitamin D in increasing net mucosal to serosal movement of calcium under these conditions is not blocked by inhibition of metabolic energy production (15, 17), and this system may measure facilitated diffusion of calcium across the brush border of the mucosal cell. The treatment of the animal was the same as in the first series of experiments. Serum calcium concentrations were measured by atomic absorption spectrophotometry (18) and phosphate concentrations by the Fiske and SubbaRow method (19).

Results. Table I summarizes the response of serum calcium and phosphate concentrations of diphenylhydantoin- and phenobarbital-injected and control vitamin D-depleted rats to treatment with ergocalciferol, cholecalciferol, and 25-OH-cholecalciferol. Dilantin/phenobarbital administration did not alter the concentrations of these ions in the serum of vitamin D-deficient rats nor did it modify the increase of serum calcium concentration following administration of the various forms of vitamin D. Table II

TABLE I. SERUM CALCIUM AND PHOSPHATE CONCENTRATIONS IN CONTROL AND ANTICONVULSANT-TREATED RATS.

Concentration in serum (mg/100 ml)	No vitamin D		EC[a] (0.25 μg)		CC[a] (0.25 μg)		CC (2.5 μg)		25-OHCC[a] (0.25 μg)	
	C[b]	D-P[c]	C	D-P	C	D-P	C	D-P	C	D-P
Calcium	5.3[d] ± 0.20 (6)[e]	5.6 ± 0.19 (5)	7.3 ± 0.56 (6)	7.7 ± 0.40 (7)	7.6 ± 0.30 (5)	7.9 ± 0.13 (9)	9.6 ± 0.12 (6)	9.8 ± 0.19 (5)	7.8 ± 0.27 (5)	8.4 ± 0.23 (9)
Phosphorus	8.7 ± 0.27 (6)[e]	8.4 ± 0.35 (5)	8.1 ± 0.30 (6)	8.2 ± 0.30 (7)	8.2 ± 0.17 (5)	7.8 ± 0.19 (9)	10.8 ± 0.25 (6)	10.4 ± 0.34 (6)	9.0 ± 0.20 (5)	8.4 ± 0.25 (9)

[a] EC, ergocalciferol; CC, cholecalciferol; 25-OHCC, 25-hydroxycholecalciferol.
[b] C, control, saline injected.
[c] D-P, diphenylhydantoin and phenobarbital injected.
[d] Mean ± SEM.
[e] (*n*), Number of rats in group.

TABLE II. EFFECT OF DIPHENYLHYDANTOIN/PHENOBARBITAL ADMINISTRATION ON *in Vitro* TRANSPORT OF CALCIUM BY EVERTED SEGMENTS OF RAT SMALL INTESTINE.[a]

	No vitamin D		EC (0.25 μg)		CC (0.25 μg)		CC (2.5 μg)		25-OHCC(0.25 μg)	
Calcium	C	D-P	C	D-P	C	D-P	C	D-P	C	D-P
Duodenum	1.58 ± 0.18 (6)	1.57 ± 0.20 (4)	4.16 * ± 0.75 (6)	2.20 ± 0.37 (5)	5.61 * ± 1.52 (5)	2.58 ± 0.20 (8)	6.94 * ± 1.64 (5)	1.76 ± 0.18 (6)	5.08 * ± 0.74 (9)	2.29 ± 0.31 (9)
Ileum	1.00 ± 0.03 (6)	1.00 ± 0.09 (4)	4.16 ± 1.27 (6)	2.51 ± 0.36 (7)	3.75 ± 0.61 (5)	2.66 ± 0.45 (9)	10.50 * ± 1.45 (6)	5.73 ± 0.63 (6)	4.77 * ± 0.78 (9)	2.51 ± 0.34 (9)

[a] All of the data represent C_s/C_m values, i.e., the ratio at the end of incubation of the concentration of ion in the serosal fluid to that in mucosal fluid. The degree to which this ratio is increased above 1, the initial state, represents the net mucosal to serosal transport of the ion. The incubation period for the calcium transport studies was 90 min. The values are means ± SEM. An asterisk (*) between a pair of values indicates a significant difference ($P < 0.05$). (n), Number of everted loops on which mean is based.

presents the results of the active calcium transport measurements expressed as C_s/C_m values. The diphenylhydantoin plus phenobarbital injection did not further reduce the low calcium transport rates in duodenum or ileum of vitamin D-depleted rats. This treatment did, however, significantly reduce the effect of 0.25 μg of ergocalciferol, cholecalciferol, and 25-OH-cholecalciferol on calcium transport by duodenum as well as the effect of the larger dose of cholecalciferol, 2.5 μg. Although ileal calcium transport was somewhat less in the treated rats receiving 0.25 μg of ergocalciferol in comparison with controls, the differences were not statistically significant. The decreases were significant in the groups receiving either the larger dose of cholecalciferol, 2.5 μg, or 25-OH-cholecalciferol.

The results of the second type of experiment in which the non-energy-requiring diffusibility of calcium across the intestinal wall was measured are shown in table III. The diffusion of calcium into the serosal compartment was increased by treatment of the vitamin D-depleted rats with 0.25 μg of cholecalciferol, and this effect was not significantly altered by injection of the rats with diphenylhydantoin and phenobarbital. In this group of rats the increase of serum calcium concentration following vitamin D was actually greater in the rats given anticonvulsants than in the saline-injected controls, but this was not found in the other series of experiments.

Discussion. The significant inhibition by diphenylhydantoin/phenobarbital treatment of the vitamin D-induced increase of metabolically dependent calcium transport by rat intestine in these *in vitro* experiments cannot be explained in terms of reduced concentrations of the active vitamin D metabolite unless it is postulated that the effect of vitamin D on active transport of calcium requires higher concentrations of vitamin D than its other effects. There is no inhibition of the serum calcium raising effect of the steroid which also presumably involves the formation of the active metabolite, 1,25-diOH-cholecalciferol. The interaction of the active vitamin D metabolite with the intestinal epithelial cell is apparently not blocked by the anticonvulsant drugs since the vitamin D effect on calcium diffusibility across

TABLE III. EFFECT OF DIPHENYLHYDANTOIN/PHENOBARBITAL TREATMENT ON DIFFUSIBILITY OF CALCIUM ACROSS INTESTINAL WALL *in Vitro*.

Calcium diffusion[a] (μg Ca/30 min/loop)	No vitamin D	CC (0.25 μg)	
	C	C	D-P
Duodenum	3.08 ± 0.17 (11)	8.02 ± 1.00 (14)	6.28 ± 0.54 (14)
Ileum	1.33 ± 0.17 (11)	2.22 ± 0.20 (14)	2.52 ± 0.18 (14)
Serum Ca (mg/100 ml)	5.4 ± 0.16 (11)	7.7 * ± 0.24 (14)	8.6 ± 0.14 (14)

[a] The everted loops were incubated at 23° for 30 min.

the mucosal barrier is not diminished. This supports the observation of Koch *et al.* (12) who found that treatment with diphenylhydantoin reduced rat duodenal calcium transport *in situ* but did not inhibit the vitamin D-induced increase of calcium-binding protein in duodenal mucosa. It is possible that the increase of calcium diffusibility across the intestinal wall is related to the increase of calcium-binding protein following vitamin D treatment. Since these two effects of vitamin D on intestinal epithelium are not blocked by diphenylhydantoin or the diphenylhydantoin/phenobarbital combination it would follow that the reduction of calcium transport by anticonvulsants is at a more distal stage of the calcium transport system. If it is hypothesized that one function of vitamin D-induced calcium-binding protein is to accelerate the flow of calcium from mucosal fluid across the brush border surface of the epithelial cell, the inhibitory action of diphenylhydantoin and phenobarbital could be on some phase of the linkage of metabolic energy production with the energy-dependent ejection of calcium at the basal lateral surfaces of the cell. It is this latter function which increases the concentration of calcium in the fluid in contact with the serosal pole of the cell. How this action of diphenylhydantoin on intestinal calcium transport is related to its pharmacologic actions on the central nervous system or cardiac conduction system is only conjectural.

Addition of diphenylhydantoin *in vitro* to the buffer solution in which the intestinal loops were incubated was made to determine whether a direct inhibitory effect on calcium transport would occur. In these experiments active calcium transport was measured in everted duodenal loops from rats given 2.5 μg of cholecalciferol 72 hr earlier. In the treated loops, diphenylhydantoin was added to the buffer in a final concentration of 0.5 m*M*. The average C_s/C_m ratios following 90 min of incubation were 6.78 and 6.39 for control and treated duodenal loops. It is possible that the diphenylhydantoin did not enter the intestinal mucosal cells during the period of incubation or that the inhibitory effect is not due to this compound but to a metabolic derivative or to alteration of a cellular component following prolonged exposure to the drug.

In man, anticonvulsant combinations such as diphenylhydantoin and phenobarbital do have actions on vitamin D metabolism since there is evidence of reduced concentrations of 25-OH-cholecalciferol in patients treated with these drugs (20). However, Jubiz *et al.* (21) have recently reported that 1,25-diOH-cholecalciferol concentrations in the serum of anticonvulsant-treated patients were not reduced despite lower concentrations of 25-OH-cholecalciferol. They concluded that the disturbances of calcium metabolism of patients treated with anticonvulsants cannot be explained by a deficiency of the active vitamin D metabolite. The possibility that diphenylhydantoin may reduce intestinal calcium absorption in man also by a mechanism analogous to the *in vitro* or *in situ* inhibition found in the rat remains to be determined. If such calcium malabsorption due to diphenylhydantoin does occur, this could explain hypocalcemia and secondary hyperparathyroidism in the presence of normal concentrations of 1,25-diOH-vitamin D. Such a diphenylhydantoin action might also be more significant in the immobile, bedridden patient who seems to be at greater risk for bone disease associated with anticonvulsant medication.

Summary. The effect of combined diphenylhydantoin and phenobarbital administration on the response of vitamin D-depleted rats to ergocalciferol, cholecalciferol, and 25-hydroxycholecalciferol (25-OHCC) was measured by determinations of serum calcium and phosphate concentrations and of intestinal transport of calcium *in vitro* by everted loops of small intestine. The major action of this anticonvulsant drug treatment in the rat was to inhibit the action of the various forms of vitamin D including 25-OHCC in increasing active transport of calcium by the everted intestine. The increase of calcium diffusibility across the intestine by vitamin D was not blocked nor was the action of vitamin D in increasing serum calcium concentrations. A specific inhibitory effect of the anticonvulsant drugs on an energy-dependent calcium transport system in the intestinal mucosa is suggested.

This work was supported by USPHS Grant AM-00668. 25-hydroxy-cholecalciferol was supplied to us

by Dr. John Babcock, the Upjohn Company. We thank Mrs. Evelyn Bull for important technical assistance.

1. Dent, C. E., Richens, A., Rowe, D. J. F., and Stamp, T. C. B., Brit. Med. J. **1,** 69 (1970).
2. Schaefer, K., Flury, W. H., and Von Herrath, D., Schweiz. Med. Wochenschr. **102,** 785 (1972).
3. Borgsted, A., Bryson, M., Young, L., and Forbes, G., J. Pediat. **81,** 9 (1972).
4. Schmid, F., Fortschr. Med. **9,** 381 (1967).
5. Kruse, R., Monatschr. Kinderheilk. **166,** 378 (1968).
6. Lifshitz, F., and MacLaren, N. K., J. Pediat. **83,** 612 (1973).
7. Conney, A. H., Pharmacol. Rev. **19,** 317 (1967).
8. Hahn, T. J., Birge, S. J., Scharp, C. R., and Avioli, L. V., J. Clin. Invest. **51,** 741 (1972).
9. MacLaren, N. K., and Lifshitz, F., Pediat. Res. **7,** 914 (1973).
10. Clark, R. L., Kuhn, J. P., and DeJorne, C. A., Invest. Radiol. **56,** 152 (1971).
11. Caspary, W., Naunyn-Schmiedeberg's Arch. Pharmacol. **274,** 146 (1972).
12. Koch, U. V., Kraft, D., Van Herrath, D., and Schaefer, K., Epilepsia **13,** 829 (1972).
13. Richens, A., and Rowe, D. F. J., Brit. Med. J. **4,** 73 (1970).
14. Harrison, H. E., Harrison, H. C., and Park, E. A., Amer. J. Physiol. **192,** 432 (1958).
15. Harrison, H. E., and Harrison, H. C., Amer. J. Physiol. **199,** 265 (1960).
16. Harrison, H. C., and Harrison, H. E., Amer. J. Physiol. **217,** 121 (1969).
17. Harrison, H. E., and Harrison, H. C., Amer. J. Physiol. **208,** 370 (1965).
18. Zettner, H., and Seligson, D., Clin. Chem. **10,** 869 (1964).
19. Fiske, C. H., and SubbaRow, Y., J. Biol Chem. **81,** 629 (1929).
20. Hahn, T. J., Hendin, B. A., Scharp, C. R., and Avioli, L., N. Engl. J. Med. **287,** 900 (1972).
21. Jubiz, W., Haussler, M. R., Tolman, K. G., and McCain, T. A., Clin. Res. **24,** 255A (1976).

Received January 8, 1976. P.S.E.B.M. 1976, Vol. 153.