24-Hydroxylation of 25-Hydroxyvitamin D_3: Is It Required for Embryonic Development in Chicks?

Abstract. *As shown previously, laying hens given 1,25-dihydroxyvitamin D_3 as their sole source of vitamin D produce fertile eggs having normal shells, but only 35 to 55 percent of the embryos are normal. Giving these hens additional 25-hydroxyvitamin D_3, 24,25-dihydroxyvitamin D_3, or 24,24-difluoro-25-hydroxyvitamin D_3 at 1.25 nanomoles per day resulted in 90 to 100 percent normal embryos, and hence, hatchability. Since 24,24-difluoro-25-hydroxyvitamin D_3 cannot be 24-hydroxylated, 24-hydroxylation is not required for this function of 25-hydroxyvitamin D_3.*

Although it is well accepted that vitamin D must be converted to 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] to stimulate intestinal calcium transport, intestinal phosphate transport, and bone calcium mobilization (*1–3*), there have been suggestions that conversion of vitamin D to 24*R*,25-dihydroxyvitamin D_3 [24*R*,25$(OH)_2D_3$] is required for some functions (*4–8*). The experiments of Henry and Norman (*9*) suggested that 24*R*,25$(OH)_2D_3$ may be required for the normal development, and hence the normal hatching, of chick embryos. We had found earlier that 1,25$(OH)_2D_3$ provided to laying hens fails to support this function (*10*). However, improvement in embryonic development in the eggs from such hens was observed when the eggs were injected with various forms of vitamin D, including 1,25$(OH)_2D_3$; this finding suggested that 1,25$(OH)_2D_3$ may not be transferred efficiently from hen to egg (*10*). With the chemical synthesis of 24,24-difluoro-25-hydroxyvitamin D_3 (24,24-F_2-25-OH-D_3) (*11, 12*), a compound that does not undergo 24-hydroxylation, came the possibility of testing the alleged requirement for 24*R*,25-$(OH)_2D_3$ for chick embryonic development (*9*).

Table 1. Composition of the diet. The calculated analysis was protein, 16.00 percent; metabolizable energy, 2766 kcal/kg; calcium, 2.51 percent; and phosphorus, 0.65 percent.

Ingredient	Percent
Corn	65.50
Soybean meal (44 percent)	23.30
Dicalcium phosphate	1.50
Calcium carbonate	5.80
Celufil T.M.*	3.00
Iodized salt	0.50
Vitamin and mineral mix†	0.40
Total	100.00

*Nonnutritive bulk obtained from United States Biochemical Corporation, Cleveland, Ohio 44128. †Provides (per kilogram of diet): vitamin A, 5000 I.U.; vitamin E, 5 I.U.; vitamin K, as menadione sodium bisulfite, 0.5 mg; calcium pantothenate, 10 mg; vitamin B_{12}, 0.01 mg; choline chloride, 444 mg; MnO, 70 mg; $ZnCO_3$, 90 mg; and 1,25$(OH)_2D_3$, 3 μg. Other vitamins and minerals are provided by the corn and soybean meal components.

Single-comb White Leghorn hens from the University of Wisconsin flock were housed in individual cages. For 55 weeks, they were given free access to a diet containing 3 μg of 1,25$(OH)_2D_3$ per kilogram (Table 1). The hens had free access to water, and incandescent bulbs provided 14 hours of light per day. All of the hens were artificially inseminated once a week with pooled semen from New Hampshire males. All collected eggs were incubated weekly, and the percentages of fertility, embryonic mortality, and survival through hatching were recorded. At the end of the 55th week, the hens were separated into four groups of four hens each. The experimental groups received oral doses of 24,24-F_2-25-OH-D_3, 24*R*,25$(OH)_2D_3$, or 25-OH-D_3 dissolved in corn oil (1.25 nmole/ml per hen per day) for a 2-week period. The hens in the control group received corn oil (1 ml per hen per day). At the end of the experimental 2-week period, all of the hens were again placed on the control diet for four additional weeks.

No significant differences were observed in percent fertility among treatment and control groups (Table 2). Hens on the control diet produced eggs showing only 35 to 55 percent normal embryos, in agreement with previous results (*9, 10*). The percentage of normal embryos, resulting in normal hatching, was significantly ($P < .001$) increased to 90 to 100 percent with the supplements of 24,24-F_2-25-OH-D_3, 24*R*,25$(OH)_2D_3$, or 25-OH-D_3 (Fig. 1). The improvement persisted in all three groups for more than 1 week after treatment was withdrawn. After this point the percentage of normal embryos returned to 35 to 55 percent. No significant differences were observed in percent hatchability among the three groups by the end of the experimental period. The eggs from hens receiving the unsupplemented control diet containing 3 μg of 1,25$(OH)_2D_3$ per kilogram of diet produced embryos that failed to hatch and having embryonic mortality during the third week of incubation. Analysis of blood (*13*) from hens given 24,24-F_2-25-OH-D_3 revealed no detectable 24,25-$(OH)_2D_3$.

These results demonstrate that

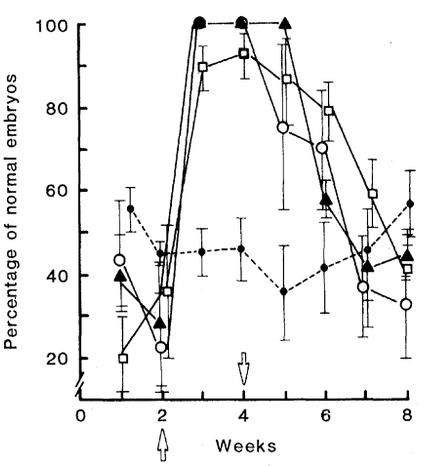

Fig. 1. Restoration of normal embryonic development and hence hatchability by analogs of 25-OH-D_3. The arrow at 2 weeks indicates beginning of supplementation with analog at 1.25 nmole/day, and the arrow at 4 weeks indicates withdrawal of supplementation. (○) 24,24-F_2-25-OH-D_3, (▲) 24*R*,25$(OH)_2D_3$, (□) 25-OH-D_3, and (●) control. Experimental groups differed significantly from the control group in percentage of normal embryos ($P < .001$).

Table 2. Fertility of eggs gathered from all groups. Values are means ± standard deviation of percentages of total eggs collected for four hens in each group.

Treatment	Fertility at weeks indicated (%)								
	1	2	3	4	5	6	7	8	Average
Control	95.8 ± 4	100 ± 0	100 ± 0	100 ± 0	76.6 ± 14	85.4 ± 8	100 ± 0	90.9 ± 10	93.4 ± 8
24,24-F_2-25-OH-D_3	90.1 ± 6	78.3 ± 10	100 ± 0	86.1 ± 7	100 ± 0	93.7 ± 6	96.4 ± 3	83.3 ± 9	90.9 ± 7
24*R*,25$(OH)_2D_3$	81.2 ± 11	81.6 ± 6	93.7 ± 6	87.5 ± 12	88.8 ± 11	95.0 ± 5	93.7 ± 6	91.6 ± 8	89.1 ± 5
25-OH-D_3	80.0 ± 2	87.5 ± 7	100 ± 0	93.3 ± 6	82.5 ± 6	95.0 ± 5	93.3 ± 6	93.3 ± 6	90.6 ± 6

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$1,25(OH)_2D_3$ given to hens by itself cannot support normal embryonic development and hatchability in chicks. However, our results also show that 25-OH-D_3 by itself is fully capable of carrying out this function. Furthermore, the results with 24,24-F_2-25-OH-D_3 provide strong evidence that 24-hydroxylation of 25-OH-D_3 is not required as well. Thus, 25-OH-D_3 itself or some other unknown metabolite must be normally responsible for this function.

It is not clear why the embryos do not survive if their mothers do not have 25-OH-D_3. It may be that $1,25(OH)_2D_3$ is not transferred or stored in egg yolk. The requirement may be specifically for 25-OH-D_3 and may represent a phylogenetic indication of a function for 25-OH-D_3 itself. Which of these possibilities is correct will undoubtedly become apparent on continued investigation.

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14. 24*R*,25$(OH)_2D_3$, $1,25(OH)_2D_3$, and 25-OH-D_3 were gifts from the Hoffmann–La Roche Company, Nutley, N.J. This study was supported by a program project grant (AM-14881) from the National Institutes of Health, by U.S.–Japan cooperative grant INT-8016902, and by the Harry Steenbock Research Fund of the Wisconsin Alumni Research Foundation.

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Cysteamine: A Potent and Specific Depletor of Pituitary Prolactin

Abstract. *Cysteamine rapidly reduces the concentration of prolactin in pituitary tissue in vivo and in vitro. The effect is dose-dependent, reversible, and cannot be accounted for by prolactin release. Cysteamine does not appear to exert its effect through dopamine receptors and does not alter lactotrope morphology, as determined by electron microscopy.*

Cysteamine (2-aminoethanethiol) is a sulfhydryl drug with both clinical and experimental applications. Clinically, cysteamine has been used in the treatment of paracetamol (acetaminophen) poisoning (*1*) and nephropathic cystinosis (*2*). Experimentally, it has been shown to be radioprotective (*3*) and to be a duodenal ulcerogen in rats (*4*).

Szabo and Reichlin (*5*) found that cysteamine, administered orally to rats, rapidly and selectively depletes the gastrointestinal tract, hypothalamus, and plasma of immunoreactive somatostatin. We have since shown that cysteamine (300 mg/kg, subcutaneously) reduces the concentration of somatostatin throughout the central nervous system, especially in the hypothalamus (*6*). This effect is reversible, with the level of somatostatin returning to normal within 1 week.

Since somatostatin plays an important role in neuroendocrine regulation as a physiological inhibitor of the secretion of growth hormone (GH) and thyroid-stimulating hormone (TSH) (*7*), we investigated the effects of cysteamine administration on neuroendocrine function. We now report that the most striking effect of cysteamine on the neuroendocrine axis is on prolactin secretion.

Male Long-Evans hooded rats (Charles River) weighing 275 to 300 g were injected with cysteamine (300 mg/kg, subcutaneously) between 900 and 1000 hours and were killed at the intervals indicated in Table 1 (*8*). Within 2 hours of drug administration, prolactin concentrations in the anterior pituitary and serum (*9*) were reduced to less than 10 and 2 percent of control values, respectively. Prolactin levels in the serum and anterior pituitary were decreased for 8 hours, with serum levels returning to normal within 24 hours and pituitary levels within 72 hours. Cysteamine had no significant effect on the content of GH or TSH in the anterior pituitary.

Table 1. Time course of the effects of cysteamine (300 mg/kg, subcutaneously) on the concentration of prolactin in rat anterior pituitary and serum. Values are means ± standard errors for six animals per group. Control animals received 0.1*M* NaCl.

Group	Prolactin in anterior pituitary (μg/mg protein)	Prolactin in serum (ng/ml)
	2 hours	
Control	4.80 ± 0.77	55.9 ± 20.1
Cysteamine	0.57 ± 0.03*	1.1 ± 0.1*
	4 hours	
Control	8.67 ± 1.87	64.4 ± 16.5
Cysteamine	0.69 ± 0.08*	1.1 ± 0.1*
	8 hours	
Control	11.68 ± 2.25	78.8 ± 21.9
Cysteamine	0.82 ± 0.06*	1.2 ± 0.2*
	24 hours	
Control	7.23 ± 0.93	59.2 ± 21.6
Cysteamine	4.31 ± 0.50*	33.9 ± 8.7
	72 hours	
Control	10.15 ± 1.43	83.1 ± 14.3
Cysteamine	10.63 ± 2.30	49.5 ± 11.1
	1 week	
Control	5.45 ± 1.00	42.8 ± 11.7
Cysteamine	5.90 ± 0.50	43.3 ± 7.3

*Significantly different from corresponding control value at $P < .05$ (*9*).

Table 2 shows the response of prolactin to different doses of cysteamine. The drug produced a dose-dependent decrease in the concentration of prolactin in the anterior pituitary, with maximal effects observed at doses of 90 and 300 mg/kg. The median effective dose was approximately 30 mg/kg. The content of GH or TSH in the anterior pituitary was not affected by any dose of cysteamine.

These data indicate that cysteamine is a potent depletor of prolactin in vivo and that its effects occur rapidly and are reversible. In a previous study (*10*) we showed that luteinizing hormone but not follicle-stimulating hormone was slightly reduced in the anterior pituitary of animals given cysteamine subcutaneously at a dose of 300 mg/kg. However, lower doses of cysteamine (less than 200 mg/kg) did not affect the concentration of luteinizing hormone in the anterior pituitary. Thus, prolactin appears to be the anterior pituitary hormone most sensitive to the effects of cysteamine.

We next investigated the effect of cysteamine on dispersed anterior pituitary cells in culture (*11*). Pituitaries were harvested from male CD rats (Charles River) weighing 225 to 250 g; all tests were done on day 4 or 5 in culture. Cysteamine caused a dose-dependent decrease

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