(*12*). We looked for histopathologic changes in the unoperated testis of unilaterally vasoligated guinea pigs; the presence of these sorts of lesions has been reported by others (*5, 6*), suggesting that systemic rather than local pathogenetic mechanisms are involved.

Lesions of allergic orchitis were adoptively transferred to normal recipients by peritoneal exudate cells from vasoligated syngeneic donors. Mineral oil–induced peritoneal exudates in the guinea pigs consist of more than 90 percent macrophages or neutrophils (or both), 7 percent T lymphocytes, and only a few B lymphocytes (*13*). Peritoneal cell mixtures from guinea pigs immunized with testicular antigen in complete Freund's adjuvant have been shown to adoptively transfer allergic orchitis in inbred guinea pigs (*8*); the adoptive transfer is antigen-specific, requires donor T lymphocytes, and is dependent on the cell dose (*8, 13*).

Evidence is now available in support of allergic orchitis as a possible sequel of vasoligation in the guinea pig and the rabbit. In rabbits, orchitis after vasoligation has been associated with immune complex formation (*7*); allergic orchitis, with an indistinguishable immunopathologic picture, was also induced in rabbits by immunization with heterologous guinea pig testicular antigen in complete Freund's adjuvant (*14*). The immunopathology of orchitis after vasectomy is different in rabbits and guinea pigs; however, each correlates well with experimental allergic orchitis in the corresponding species. The results from vasectomized animals suggest that cell-mediated immune reaction is an important pathogenetic mechanism in the testicular lesions of guinea pigs. In the rabbit, the formation an immune complex is probably an important pathogenetic factor, although the role of cell-mediated immunity in the initiation of the disease cannot be ruled out. Since half a million men in the United States undergo vasoligation annually (*15*), a critical evaluation of both their humoral and cellular immune responses to purified testicular antigens should be studied in vitro.

KENNETH S. K. TUNG
Department of Pathology, University of New Mexico, School of Medicine, Albuquerque 87131

References and Notes

1. G. A. Voisin, A. Delaunay, M. Barber, *Ann. Inst. Pasteur Paris* **81**, 48 (1951); J. Freund *et al.*, *J. Exp. Med.* **97**, 711 (1953).
2. M. H. Johnson, *Adv. Reprod. Physiol.* **6**, 279 (1973); K. S. K. Tung and N. J. Alexander, in *The Testis: Advances in Physiology, Biochemistry and Function*, A. D. Johnson and W. R. Gomes, Eds. (Academic Press, New York, 1977), p. 491.
3. E. Zappi, U. Ahmed, J. Davis, S. Shulman, *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **29**, 374 (1970); R. Ansbacher, *Fertil. Steril.* **22**, 629 (1971); K. S. K. Tung, *Clin. Exp. Immunol.* **20**, 93 (1975); P. E. Bigazzi, L. L. Kosuda, L. L. Harnick, *Science* **197**, 1282 (1977).
4. J. M. Bedford, *Biol. Reprod.* **14**, 118 (1976); N. J. Alexander and K. S. K. Tung, *Anat. Rec.* **188**, 339 (1977).
5. N. J. Alexander, *Contraception* **8**, 147 (1973).
6. K. S. K. Tung and N. J. Alexander, *Biol. Reprod.* **17**, 241 (1977).
7. P. E. Bigazzi, L. L. Kosuda, K. C. Hu, G. A. Andres, *J. Exp. Med.* **143**, 382 (1976).
8. K. S. K. Tung, E. R. Unanue, F. J. Dixon, *J. Immunol.* **106**, 1453 (1971); G. L. Kantor and F. J. Dixon, *ibid.* **108**, 329 (1972).
9. K. S. K. Tung, E. R. Unanue, F. J. Dixon, *Am. J. Pathol.* **60**, 313 (1970).
10. B. H. Waksman, *J. Exp. Med.* **109**, 311 (1959).
11. K. S. K. Tung, personal observation.
12. T. S. Leeson and C. R. Leeson, *Invest. Urol.* **8**, 127 (1970); A. K. Chowdhury and E. Steinberger, *J. Reprod. Fertil.* **22**, 205 (1970); M. W. H. Bishop, *J. Reprod. Fertil. Suppl.* **12**, 65 (1970); S. A. Gunn and T. C. Gould, in *The Testis*, A. D. Johnson, W. R. Gomes, N. L. VanDemark, Eds. (Academic Press, New York, 1970), p. 377.
13. K. S. K. Tung, C. Leong, T. McCarty, *J. Immunol.* **118**, 1774 (1977).
14. K. S. K. Tung and A. J. Woodroffe, *ibid.* **120**, 320 (1978).
15. B. J. Wilson, N. J. Alexander, G. Porter, D. K. Fulgham, *Fertil. Steril.* **28**, 1349 (1977).
16. Supported by NIH contract HDO 32717 and grant HDO 07591.

9 March 1978; revised 26 May 1978

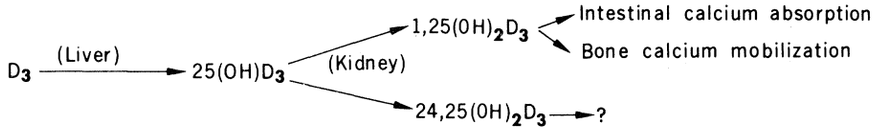
Vitamin D: Two Dihydroxylated Metabolites Are Required for Normal Chicken Egg Hatchability

Abstract. *When hens are raised to sexual maturity from hatching with 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] as their sole source of cholecalciferol (vitamin D_3), fertile eggs appear to develop normally but fail to hatch. When hens receive a combination of 1,25$(OH)_2D_3$ and 24R,25-dihydroxyvitamin D_3 [24,25$(OH)_2D_3$], hatchability equivalent to that with hens given vitamin D_3 is obtained. These results suggest a biological role for 24,25$(OH)_2D_3$ not previously recognized.*

The secosteroid vitamin D_3, or cholecalciferol, is now known to undergo a series of hydroxylations prior to exerting its biological effects on the intestine (to promote absorption of dietary calcium and phosphorus) and the bone (to stimulate mobilization of calcium) (*1*). The first of these hydroxylations is at the C-25 position on the side chain and takes place in the liver; 25-hydroxyvitamin D_3 [25$(OH)D_3$], the major circulating form of the vitamin D steroids, is further hydroxylated in the kidney resulting in the renal production of either 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] or 24R,25-dihydroxyvitamin D_3 [24,25-$(OH)_2D_3$].

time periods (3 to 4 weeks) early in life which are short relative to the life-span of the animal. Virtually nothing is known about the ability of 1,25$(OH)_2D_3$ to support all the biological functions of the parent vitamin D throughout growth, development, and sexual maturation.

In contrast to the accumulated knowledge regarding the short-term actions of 1,25$(OH)_2D_3$, little is known of the biological function of 24,25$(OH)_2D_3$. It is known that 24,25$(OH)_2D_3$ is considerably less active than 1,25$(OH)_2D_3$ in stimulating the classical vitamin D responses of intestinal calcium transport and bone mineral mobilization (*5*). That 24,25$(OH)_2D_3$ is of biological importance



After the initial discovery of the biological activity of 1,25$(OH)_2D_3$ (*2*) many investigations of the biological and biochemical actions of this steroid were carried out (*3, 4*). It is now widely accepted that 1,25$(OH)_2D_3$ generates its biological response in the intestine through a cytosol-nuclear receptor system in a manner analogous to that of other steroid hormones (*3*). Further, it has been shown that 1,25$(OH)_2D_3$ is produced in a regulated fashion by the kidney in response to an increased requirement for calcium (*4*). The study of the biological actions of 1,25$(OH)_2D_3$, while quite thorough in some details, has been limited in experimental animals to acute situations or to

was suggested to us by a series of experiments in which the regression of chicken parathyroid glands, which had undergone hypertrophy and hyperplasia due to vitamin D deficiency (*6*), was measured in response to various vitamin D metabolites. Parathyroid gland regression resulted from short-term treatment with vitamin D_3 but not from the separate administration of 1,25$(OH)_2D_3$ or 24,25-$(OH)_2D_3$; however when the dihydroxylated vitamin D steroids were administered simultaneously, the parathyroid gland regressed promptly in a fashion identical to the response to vitamin D administration (*7*).

The lack of information on the role of

0036-8075/78/0901-0835$00.50/0

Table 1. Effect of daily doses of vitamin D_3 and its dihydroxylated metabolites on adult hen weight and egg production. Vitamin D and the dihydroxylated metabolites were given orally daily from hatching in 0.2 ml of Wesson oil.

Dose (nmole kg^{-1} day^{-1})			Body weight at 34 weeks* (g)	Eggs per group per week†	Eggshell thickness‡ (mm × 10^2)	Age at established egg laying§ (weeks)
Vitamin D_3	1,25-$(Oh)_2$-D_3	24,25-$(OH)_2$-D_3				
10‖	0	0	1680 ± 100	30	39.3 ± 2.6	26
0	0.24	0	1240¶ ± 100	12	31.0 ± 5.2	29
0	1.2	0	1670 ± 80	27	35.4 ± 4.9	27
0	0	16.0	1140¶ ± 40	5	34.5 ± 2.7	30
0	0.24	3.2	1250¶ ± 130	11	35.9 ± 5.5	29
0	0.24	16.0	1540 ± 100	31	36.0 ± 4.3	29
0	1.2	3.2	1590 ± 180	28	38.2 ± 3.1	27
0	1.2	16.0	1540 ± 130	30	38.3 ± 2.4	27

*Body weights are the mean of five hens per group ± the standard deviation. †The total number of eggs produced by a group of five hens during a representative week following established egg hatching. ‡The thickness of each egg shell was measured and the average and standard deviation for all the eggs produced by the group in a week is given. §The age at which the hens in each group reached a plateau of egg production. ‖Ten nanomoles of vitamin D_3 per kilogram of body weight per day is equivalent to 154 I.U. per kilogram per day; 1.0 I.U. of vitamin D_3 is 65 pmole or 25 ng. ¶Significantly different from the control group that received vitamin D_3 ($P < .001$).

$1,25(OH)_2D_3$ in the full course of growth, development, and reproduction, the poor understanding of the biological significance of $24,25(OH)_2D_3$, and the possibility that both metabolites might be required for completely normal calcium metabolism led us to investigate in detail the ability of these two dihydroxylated metabolites of vitamin D_3 to support growth and reproduction in the chick. As a part of these studies we are reporting the requirement of hens for both dihydroxylated metabolites for production of embryos capable of hatching.

Female White Leghorn chicks obtained at hatching were raised under our usual conditions on a nutritionally adequate diet devoid of vitamin D (*8*). Groups of five chicks were given daily oral doses of vitamin D or the dihydroxylated metabolites, singly or in combination (see Table 1).

Table 1 shows that the hens receiving the higher dose of $1,25(OH)_2D_3$ reached the same adult weight as those hens given the parent vitamin D_3; this indicates that no obvious growth retardation occurs in the absence of $24,25(OH)_2D_3$.

Egg laying was initiated between 24 and 30 weeks (*9*). Distinct differences were noted in the time required for establishment of steady-state egg production between the various groups (see Table 1). Hens receiving vitamin D_3 or the combination doses of high levels of $24,25(OH)_2D_3$ and $1,25(OH)_2D_3$ initiated egg production at weeks 26 to 27 while hens receiving the high dose of $24,25(OH)_2D_3$ or low dose of $1,25(OH)_2D_3$ alone were delayed to week 29 to 30. Steady-state egg production was established in all groups by week 30. At the beginning of week 34, and again a week later, hens were artificially inseminated with a pool of semen collected from 15 normal White Leghorn roosters. Eggs were collected and stored at 12°C until the end of the 2-week period of fertility.

Seven days after initiation of incubation, fertility was determined by candling. As shown in Fig. 1 (shaded bars), no marked differences in fertility were observed for any groups; the percentage of fertility ranged from 70 to 85 percent.

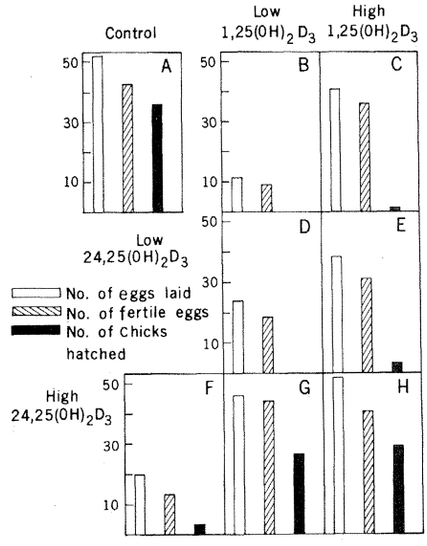


Fig. 1. Influence of vitamin D or its dihydroxylated metabolites on egg production, fertility, and hatchability. Hens were artificially inseminated at the beginning of weeks 34 and 35. The open bars indicate the number of eggs collected per group during the 2-week period of fertility (1 week following each insemination). The center shaded bar in (A) to (H) is the number of eggs judged to be fertile 1 week after incubation. The solid bars represent the number of chicks that hatched. The control group (A) received vitamin D_3.

There were marked differences, however, in total egg production for all groups during the 2-week period of collection. Essentially equivalent numbers of eggs were produced in the 2-week interval by the groups receiving D_3, high $1,25(OH)_2D_3$, and high $24,25(OH)_2D_3$ with high or low doses of $1,25(OH)_2D_3$. All other groups produced significantly fewer eggs, particularly those hens receiving only high $24,25(OH)_2D_3$ or only low doses of $1,25(OH)_2D_3$.

Striking differences were observed in the hatchability of the fertile eggs from the various groups. The eggs from hens that received only the high dose of $1,25(OH)_2D_3$ were virtually incapable of hatching (Fig. 1C). Similarly, fertile eggs from hens that received only the high dose of $24,25(OH)_2D_3$ also had a low level of hatchability (Fig. 1F). The addition of the lower dose of $24,25(OH)_2D_3$ to either level of $1,25(OH)_2D_3$ (Fig. 1, D and E) had only a slight effect, but when the higher dose of $24,25(OH)_2D_3$ was given with $1,25(OH)_2D_3$ (Fig. 1, G and H) normal hatchability was obtained.

These differences in hatchability cannot be explained simply on the basis of either early embryo mortality (*10*) or inappropriate shell thickness (see Table 1). While the eggshells of some groups were thinner than those obtained from the hens receiving vitamin D_3, none were thicker.

Little specific information is available on whether vitamin D or its metabolites is transmitted to the egg to support calcium metabolism and homeostasis during embryo development. It has been reported (*11*) that both vitamin D_3 and, to a much lesser extent, $25(OH)D_3$ are incorporated into the chicken egg yolk. From the results in Fig. 1 it must be concluded that both $24,25(OH)_2D_3$ and $1,25(OH)_2D_3$ are capable of being transferred to the egg. Only in the groups of hens receiving both metabolites were significant levels of hatchability observed. If a problem in transfer of either dihydroxylated metabolite to the egg yolk occurred, hatching would not have been improved by the simultaneous administration to hens of both vitamin D metabolites.

Our data demonstrate that (i) administration of $1,25(OH)_2D_3$ alone is not capable of providing all the biological responses that the parent vitamin D supports, and (ii) the metabolite $24,25(OH)_2D_3$, when administered in combination with $1,25(OH)_2D_3$, is capable of supporting normal growth, development, and reproduction. This clearly shows that $24,25(OH)_2D_3$ has a biological function; heretofore it was proposed that $24,25(OH)_2D_3$ existed only on a shunt

pathway of catabolism (*12*). However, the precise molecular role of 24,25-$(OH)_2D_3$ remains to be elucidated.

These results are consistent with several inferential reports of positive biological contributions of 24,25$(OH)_2D_3$. Miravet *et al.* (*13*) found that 24,25-$(OH)_2D_3$ administration to phosphate-depleted, vitamin D–deficient rats resulted in a decrease in serum calcium levels. Also, Bordier *et al.* (*14*) reported that simultaneous oral administration of 1,25-$(OH)_2D_3$ and 24,25$(OH)_2D_3$ to human sugjects with vitamin D–deficient osteomalacia was more effective than 25-$(OH)_2D_3$ or 1,25$(OH)_2D_3$ alone in stimulating the rate of bone mineralization. Similarly Kanis *et al.* (*15*) reported preliminary evidence in a limited number of normal subjects and patients with chronic renal failure that short-term oral administration of 24,25$(OH)_2D_3$ stimulated intestinal calcium absorption and reduced serum alkaline phosphate levels; this would be consistent with an effect of 24,25$(OH)_2D_3$ on bone mineralization. When these reports (*13–15*) are taken in concert with our previous report concerning the effectiveness of simultaneous dosing of 24,25$(OH)_2D_3$ and 1,25$(OH)_2D_3$ on parathyroid gland reduction (*7*) and our present data, it seems clear that 24,25$(OH)_2D_3$ is an integral component, along with 1,25$(OH)_2D_3$, of the vitamin D endocrine system.

HELEN L. HENRY
ANTHONY W. NORMAN
Department of Biochemistry, University of California, Riverside 92521

References and Notes

1. A. W. Norman and H. L. Henry, *Recent Prog. Horm. Res.* **30**, 431 (1974); H. F. DeLuca and H. K. Schnoes, *Annu. Rev. Biochem.* **45**, 631 (1976).
2. J. F. Myrtle, M. R. Haussler, A. W. Norman, *J. Biol. Chem.* **245**, 1190 (1970); J. F. Myrtle and A. W. Norman, *Science* **171**, 78 (1971); A. W. Norman and R. G. Wong, *J. Nutr.* **102**, 1709 (1972).
3. A. W. Norman, *Biol. Rev.* **43**, 97 (1968); *Vitam. Horm.* **32**, 325 (1975); ——— and W. R. Wecksler, *Receptors and Hormone Action*, B. W. O'Malley and L. Birnbaumer, Eds. (Academic Press, New York, 1977), pp. 533.
4. E. J. Friedlander, H. L. Henry, A. W. Norman, *J. Biol. Chem.* **252**, 8677 (1977); H. L. Henry, R. J. Midgett, A. W. Norman, *ibid.* **249**, 7584 (1974); D. R. Fraser and E. Kodicek, *Nature (London)* **241**, 163 (1973); N. Horiuchi, T. Suda, S. Sasaki, I. Izawa, Y. Sana, E. Ogata, *FEBS Lett.* **43**, 353 (1974); L. Galante, K. W. Colston, I. M. A. Evans, P. G. H. Byfield, E. W. Matthews, I. MacIntyre, *Nature (London)* **244**, 438 (1973); I. T. Boyle, R. W. Gray, H. F. DeLuca, *Proc. Natl. Acad. Sci. U.S.A.* **68**, 2131 (1971).
5. H. L. Henry, A. W. Norman, A. N. Taylor, D. L. Hartenbower, J. W. Coburn, *J. Nutr.* **106**, 724 (1976); H. Y. Lam, H. K. Schnoes, H. F. DeLuca, T. C. Chen, *Biochemistry* **12**, 4851 (1973).
6. A characteristic feature of the chronic hypocalcemia resulting from vitamin D deficiency is hypertrophy and hyperplasia of the parathyroid glands [J. F. Nonidez and H. D. Goodale, *Am. J. Anat.* **38**, 319 (1927); W. Y. Au and L. G. Raisz, *Am. J. Physiol.* **209**, 637 (1965)]. We have shown that administration of vitamin D_3 for 4 to 7 days to vitamin D–deficient chicks reduces by 50 percent not only parathyroid gland weight but also gland content of DNA and protein (*7*).
7. H. L. Henry, A. N. Taylor, A. W. Norman, *J. Nutr.* **107**, 1918 (1977).
8. Chicks were housed in brooders in a room devoid of sunlight and fluorescent lighting and fed a standard rachitogenic diet [Norman and Wong (2)] with 1.2 percent calcium and 1.0 percent phosphorus.
9. After the age of 22 weeks the hens were housed in individual cages designed for egg collection. Dietary calcium was increased to 3.0 percent and the lighting cycle was changed to 12 hours of light and 12 hours of darkness (from 9 and 15 hours, respectively).
10. Hatching of viable chicks normally occurs after 21 to 22 days of incubation. The chicks in this experiment hatched between 21 and 23 days. After 23 days, chicks in eggs that had not hatched were assumed to have died from lack of air and the eggs were frozen for later analysis. These chicks showed no gross structural abnormalities that could explain their failure to hatch. When candled at 20 days of incubation these chicks all appeared to be normal and alive and could not be distinguished from those chicks which subsequently hatched successfully. It is interesting to note that failure to hatch was absolute in that no pipping (initial shell breakage) occurred. Whether these chicks died prior to pipping or were unable to pip is not clear.
11. D. R. Fraser and J. S. Emtage, *Biochem. J.* **160**, 671 (1976).
12. J. C. Knutson and H. F. DeLuca, *Biochemistry* **13**, 1543 (1974).
13. L. Miravet, J. Redel, M. Carre, M. L. Queille, P. Bordier, *Calcif. Tissue Res.* **21**, 145 (1976).
14. P. Bordier, A. Ryckwaert, P. Marie, L. Miravet, A. W. Norman, H. Rasmussen, in *Vitamin D: Biochemical, Chemical and Clinical Aspects Related to Calcium Metabolism*, A. W. Norman, K. Schaefer, J. W. Colburn, H. F. DeLuca, D. Fraser, H. G. Grigoleit, Eds. (de Gruyter, New York, 1977), p. 897.
15. J. A. Kanis, G. Heynen, R. G. G. Russel, R. Smith, R. J. Walton, G. T. Warner, in *ibid.*, p. 793.
16. We thank T. Okamoto, B. Thuman, H. Ochoa, and J. Bishop for exceptionally conscientious technical assistance; A. Baumstock for the rooster semen; and M. Swanson of the University of California, Riverside, and W. Rooney of the University of California Agricultural Extension Service for helpful advice. This work was supported by grants from Hoffman-LaRoche and by PHS grants AM-09012 and AM-21398 and NSF grant PCM 76-24445.

17 May 1978

Phenylalanine Depletion for the Management of Phenylketonuria: Use of Enzyme Reactors with Immobilized Enzymes

Abstract. *Multitubular enzyme reactors with immobilized phenylalanine ammonia lyase were tested in vitro and in vivo for depletion of phenylalanine in circulating blood. Sustained reduction of phenylalanine was achieved in less than 30 minutes. A 50 percent decrease of phenylalanine was obtained with a 2-hour application of enzyme reactors and was maintained for more than 2 days. Similar enzyme reactors have therapeutic potential for temporary management of phenylketonuric patients when their circulating phenylalanine becomes exceedingly high because of infection, fever, or pregnancy.*

A genetic deficiency of phenylalanine hydroxylase, an enzyme that degrades phenylalanine, is responsible for the clinical entity of phenylketonuria (PKU), a syndrome characterized by high levels of circulating phenylalanine. The degree of neuropsychiatric disorders, such as mental retardation, confusion, and irritability, correlate with the amount of phenylalanine in the blood (*1*). In addition, injection of phenylalanine into normal volunteers decreases their mental acuity and coordination (*2*). Even when symptoms in PKU patients are controlled by a phenylalanine-poor diet, phenylalanine in their blood rises during episodes of fever and infection (*3*). Increase in circulating phenylalanine has also been reported during pregnancy, resulting in mental retardation in genetically normal children because of their exposure in utero to the high concentrations of phenylalanine (*4*).

Treatment of PKU patients with replacement of phenylalanine hydroxylase does not seem practical because of the

Table 1. Metabolism of L-phenylalanine by phenylalanine ammonia lyase reactors, measured in 120 ml of blood circulating in vitro at a flow rate of 100 ml per minute at 37°C. Blood phenylalanine was assayed enzymatically (*8*) by measuring the rate of degradation by a standard amount of lyase reactor. Results are expressed as the percent reduction of phenylalanine in times given. Base line determinations were made before the addition of 30 mg phenylalanine per 100 ml of blood. The unit of activity of the lyase reactors is the number of micromoles of phenylalanine metabolized per minute when a 1 m*M* phenylalanine solution in 50 m*M* borate buffer, *p*H 8.5, is circulated through the reactor at a flow rate of 50 ml/min at 25°C.

Reactor		Percentage of circulating phenylalanine metabolized			
Series	Units of activity	Base line at 10 minutes	After phenylalanine added		
			5 minutes	10 minutes	2 hours
HP-303	100	22	78	88	91
HP-304	10	33	83	84	90
HP-311	90	25	67	84	86
HP-312	180	19	83	83	86

0036-8075/78/0901-0837$00.50/0