nonequilibrium globin structure in photodissociated Hb_{4PR}^{20xy} apparently exerts no significant transient constraint on the heme structure (at least on the time scale that we are able to observe). Concerning the second point, it has been clearly demonstrated (*12*) that static globin constraints result in no resonance Raman–detectable distortion of the heme group of carp hemoglobin (which can exist in the T or R protein conformation, independent of the ligation state of its hemes). It was conceivable, however, that such an effect of globin constraint might be observed in an experiment sensitive to the dynamics of the structural reorganizations. Our results indicate that no such dynamic effect occurs.

Our results provide new insight into the possible mechanisms of hemoglobin cooperativity. If the stereochemical trigger hypothesis is correct, then the effects of the heme structure change must initially be stored as strain energy exclusively in the globin structure. This strain must, on a longer time scale, trigger the reorganization of the globin tertiary structure. It is entirely plausible that such a temporal sequence could occur, considering the magnitude of the protein reorganizations involved and the likelihood that the globin represents a "weak spring" compared to the heme (*12–15*). Our results are mute concerning the validity of the stereochemical trigger hypothesis; they are equally consistent with its validity or its failure. If, however, the stereochemical trigger is accepted as valid, then our results support the basic premise of the distributed energy model (*15*) of hemoglobin cooperativity, where the free energy of cooperativity is stored as small strains in the globin structure.

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References and Notes

1. B. Alpert, R. Banerjee, L. Lindqvist, *Proc. Natl. Acad. Sci. U.S.A.* **71**, 558 (1974).
2. W. H. Woodruff and S. Farquharson, in *New Applications of Lasers in Chemistry*, G. M. Hieftje, Ed. (American Chemical Society, Washington, D.C., in press); *Anal. Chem.*, in press.
3. D. L. Drabkin, *J. Biol. Chem.* **164**, 703 (1966).
4. J. L. Hoard, in *Hemes and Hemoproteins*, B. Chance, R. W. Estabrook, T. Yonetani, Eds. (Academic Press, New York, 1966), pp. 9–24; J. L. Hoard, M. J. Hamor, T. A. Hamor, W. S. Caughey, *J. Am. Chem. Soc.* **87**, 2312 (1965).
5. M. F. Perutz, *Nature (London)* **228**, 726 (1970).
6. T. G. Spiro and T. M. Loehr, in *Advances in Infrared and Raman Spectroscopy*, R. J. H. Clark and R. E. Hester, Eds. (Heyden, London, 1975), vol. 1, chap. 3.
7. C. V. Shank, E. P. Ippen, R. Bersohn, *Science* **193**, 50 (1976).
8. C. A. Sawicki and Q. H. Gibson, *J. Biol. Chem.* **251**, 1533 (1976).
9. F. A. Ferrone and J. J. Hopfield, *Proc. Natl. Acad. Sci. U.S.A.* **73**, 4497 (1976).
10. E. Antonini and M. Brunori, *Hemoglobin and Myoglobin in Their Reactions with Ligands* (North-Holland, Amsterdam, 1971).
11. T. G. Spiro and T. C. Strekas, *J. Am. Chem. Soc.* **96**, 338 (1974).
12. D. M. Scholler and B. M. Hoffman, in *Porphyrin Chemistry*, F. R. Longo, Ed. (Ann Arbor Sciences Publishers, Ann Arbor, Mich., 1978).
13. T. G. Spiro and J. M. Burke, *J. Am. Chem. Soc.* **98**, 5482 (1976).
14. W. H. Woodruff, D. H. Adams, T. G. Spiro, T. Yonetani, *ibid.* **97**, 1695 (1975).
15. J. J. Hopfield, *J. Mol. Biol.* **77**, 207 (1973).
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Allergic Orchitis Lesions Are Adoptively Transferred From Vasoligated Guinea Pigs to Syngeneic Recipients

Abstract. *Histopathology typical of allergic orchitis developed in testes of inbred guinea pigs 16 months after vasoligation. A similar histopathology was found in unoperated testes after unilateral vasoligation. Peritoneal exudate cells from vasoligated guinea pigs transferred identical lesions to syngeneic recipients. The testicular lesions in long-term vasoligated guinea pigs have an immunologic basis.*

Allergic orchitis, an experimental autoimmune disease of the testis, is readily induced in guinea pigs after immunization with testis tissue or testicular extracts in complete Freund's adjuvant (*1*). The disease is characterized by inflammation in the testis and loss of spermatogenic cells; Leydig cells, Sertoli cells, and spermatogonia are not affected (*2*). Vasoligation of man and experimental animals results in the production of antibodies to sperm (*3–7*), and concomitant histopathologic changes in the epididymis and the testis have been described (*4–7*). According to some investigators the morphology of these testicular lesions is similar to those of allergic orchitis (*5, 6*). However, it is difficult to distinguish immunologic lesions from pathologic changes that might result from the vasoligation procedure. In this report, testicular lesions were adoptively transferred by the injection of peritoneal exudate cells from long-term

Table 1. Incidence of testicular and epididymal pathology in strain 13 guinea pigs 16 months after bilateral or sham vasoligation. Under general anesthesia and with aseptic technique, both testes were exteriorized through a low midline abdominal incision. For bilateral vasoligation, the vas were ligated by two silver clips, and a silk ligature was placed between the clips, and the bladder and the vas were cut between the clips. The testes were carefully returned to the scrota, and the abdominal wall was closed in layers. For sham-vasoligation, a silk ligature was loosely tied around, but did not constrict, the vas. Two silver clips were placed on tissues adjacent to the ligature. The testes were carefully returned to the scrota, and the abdominal wall was closed in layers.

Vasoligation	*N*	Histologic findings in testis	Number of animals with epididymal granuloma
Bilateral	4	Multiple macrophagic-invasive lesions with many atrophic seminiferous tubules	1
Bilateral	4	Focal macrophagic-invasive lesions with focal hypospermatogenic tubules	2
Bilateral	10	Normal	4
Sham	5	Normal	0

Table 2. Testicular changes in strain 13 guinea pigs 16 months after unilateral vasoligation. The vasoligation was performed as for bilateral vasoligation (Table 1), while the nonvasoligated testis was untouched.

Guinea pig	Testicular histopathology	
	Vasoligated	Nonvasoligated
1301	Normal	Normal
1302	Normal	Normal
1303	Normal	Normal
1304	Numerous macrophagic-invasive lesions	Several macrophagic-invasive lesions
1307	Numerous macrophagic-invasive lesions	Occasional macrophagic-invasive lesions
1308	Numerous macrophagic-invasive lesions and aspermatogenesis in 50 percent of seminiferous tubules	Numerous macrophagic-invasive lesions and some hypospermatogenic seminiferous tubules

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vasoligated donors to normal syngeneic guinea pigs, conditions under which only immunologic disease would be transferred.

Twenty-nine strain 13 guinea pigs (Southwestern), were bilaterally vasoligated, unilaterally vasoligated, or bilaterally sham-operated (*6*) (Table 1), and were studied 16 months later. Testes from unilaterally vasoligated guinea pigs were studied only histologically; those from bilateral vasoligated or sham-operated guinea pigs were studied both for histologic changes and by immunofluorescence for the presence of immune complexes. Testes for histologic study were fixed as serial blocks in Zenker's fixative and embedded in paraffin, and 5-μm sections were stained with periodic acid–Schiff stain. For immunofluorescence studies, the entire testis was snap-frozen, and 4-μm (frozen) sections were stained with fluorescein-conjugated antiserums to guinea pig immunoglobulin G and complement component C3. The guinea pigs were also the cell donors for adoptive transfer experiments (*8*). Four days before cell transfer, each guinea pig was injected intraperitoneally with 20 ml of sterile mineral oil. At the time of cell transfer, the donors were exsanguinated, and peritoneal exudate cells were harvested under sterile conditions. Cells from bilaterally and unilaterally vasoligated guinea pigs were pooled. The cells were washed twice in RPMI 1640 medium (Grand Island Biological) by centrifugation at 4°C at 200*g* for 10 minutes, and then suspended to an appropriate cell concentration so that 0.3 ml could be injected into each testis with a 30-gauge needle inserted under the tunica albuginea. To ensure that local transfer of lymphoid cells would not cause nonspecific testicular lesions, ten testes were injected with peritoneal exudate cells from guinea pigs immunized with complete Freund's adjuvant alone. Testes of recipients were studied 6 days after cell transfer for histopathologic changes.

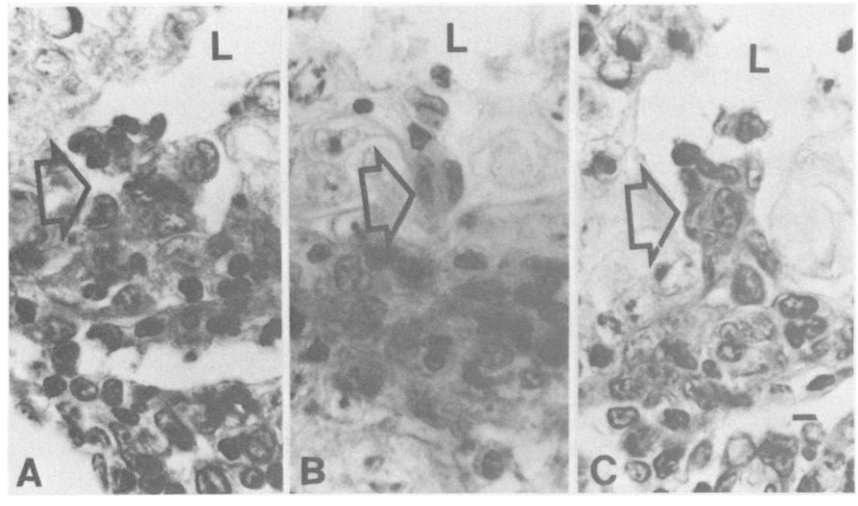

Fig. 1. Histopathologic changes in (A) the right testis of a strain 13 guinea pig (1308, Table 2) unilaterally vasectomized 16 months before; (B) the left, unoperated testis of animal 1308; and (C) the testis of a strain 13 guinea pig that received 10^8 peritoneal exudate cells from syngeneic donors vasectomized 16 months before. The arrows point to the typical macrophagic-lymphocytic lesions invading through the boundary tissue into the lumen (*L*) of the seminiferous tubules (periodic acid Schiff-hematoxylin stain, bar = 7 μm).

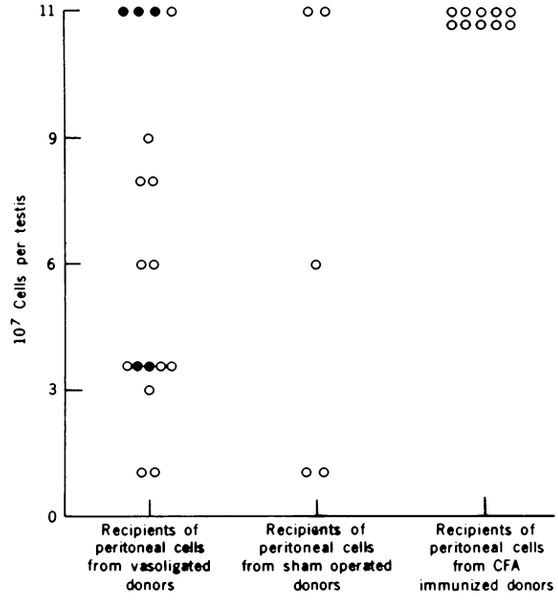

Fig. 2. Incidence of local adoptive transfer of allergic orchitis by peritoneal cells from vasectomized syngeneic guinea pigs. The filled circles represent the 5 of 17 (30 percent) recipients that developed lesions. Most testes that received 1.1×10^8 cells developed lesions. Testes that received peritoneal cells from sham-operated donors or donors immunized 9 days before with complete Freund's adjuvant were normal (open circle denotes a normal testis). Both vasectomized and sham-operated donors were operated on 16 months before.

Although the histopathology of experimental allergic orchitis is complex (*9*), a typical and pathognomonic feature of the disease is the multifocal invasion of the seminiferous tubules by clusters of lymphoid cells and macrophages (*10*). When this lesion was used as the diagnostic criterion of allergic orchitis, testes from 8 of 18 (44 percent) bilaterally vasoligated guinea pigs showed allergic orchitis (Table 1), some testes being more severely affected than others. The presence of testicular lesions was unrelated to the granuloma found in the cauda epididymis, which most likely had resulted from the vasoligation procedure itself (*6*). Although orchitis in vasectomized rabbits reveals sperm antigen-antibody complexes surrounding seminiferous tubules, immunofluorescence studies of vasectomized guinea pig testes show no such immune complexes. Three of six unilaterally vasoligated guinea pigs also developed multiple macrophagic invasive lesions in the vasoligated testis (Table 2); and in these animals, the contralateral, unoperated testes also developed lesions that were indistinguishable from those on the operated side (Fig. 1, A and B). The testes of sham-operated guinea pigs were normal. Macrophagic invasive lesions were also found in some recipients of peritoneal exudate cells from vasoligated donors (Figs. 1C and 2). However, peritoneal cells from sham-operated guinea pigs or from guinea pigs immunized with complete Freund's adjuvant alone did not transfer lesions to recipients.

Inbred strain 13 guinea pigs were used because these animals are more resistant to allergic orchitis induction and require 15 times more testicular antigens than do outbred Hartley guinea pigs (*11*). The criterion for allergic orchitis was the invasion of seminiferous tubules by small clusters of lymphoid cells and macrophages. Other histopathologic changes, such as aspermatogenesis and interstitial inflammation, are less specific since they can be found in other testicular diseases

(*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.

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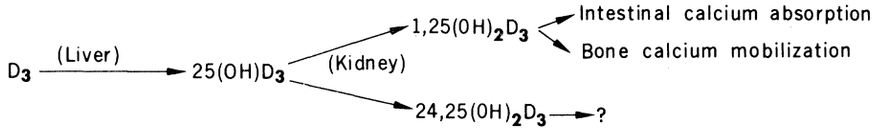
Vitamin D: Two Dihydroxylated Metabolites Are Required for Normal Chicken Egg Hatchability

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-$(OH)_2D_3$].



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 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 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

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