$H_3{}^{33}PO_4$ from the medium by adult snails and the subsequent incorporation of the phosphorus-33 into AEP, as well as the breakdown of AEP during embryonic development, *Helisoma* appears to be an excellent animal for use in fundamental studies on the metabolism of the naturally occurring alkylphosphonates such as AEP.

We have also obtained ^{31}P NMR spectra from the egg masses of other species of the family Planorbidae, as well as representatives of the families Physidae and Lymnaeidae. In general, these gave rise to ^{31}P NMR spectra similar to that shown in Fig. 1A. Included in these preliminary studies was the planorbid snail *Biomphalaria glabrata*, an obligate intermediate host for the human pathogenic trematode *Schistosoma mansoni*. As is the case for the related *Helisoma* sp., alkylphosphonates represent more than 95 percent of the total phosphorus in freshly laid egg masses of *B. glabrata*.

These studies are of particular interest, especially in *B. glabrata*, because of the potential for control of snail proliferation. Further work may lead to the discovery of specific metabolic sites in which these biochemical processes can be interdicted without harming other life forms, since most life forms, particularly higher vertebrates, have no significant metabolic involvement with alkylphosphonates.

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11. This work is based on a thesis submitted by M.V.M. to the graduate college of the University of Illinois at the Medical Center, Chicago, in partial fulfillment of the requirements for the Ph.D. degree. M.V.M. was the recipient of a Graduate College fellowship. We acknowledge C. T. Burt's contribution during the initial phases of the work.

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1α,25-Dihydroxyvitamin D_3 Nuclear Receptors in the Pituitary

Abstract. *Specific binding of 1α,25-dihydroxyvitamin D_3 was found in nuclear and cytosol fractions of the bovine pituitary. For nuclear binding, the dissociation constant was 0.1 nanomole per liter, and maximum binding was 104 femtomoles per milligram of protein. In competition studies, 25-hydroxyvitamin D_3 was 300 times weaker than 1α,25-dihydroxyvitamin D_3. The existence of high-affinity sites supports a physiologic role for 1α,25-dihydroxyvitamin D_3 in the pituitary.*

Growth hormone (GH) and prolactin influence the synthesis of the active form of vitamin D, 1α,25-dihydroxyvitamin D_3 [1α,25-$(OH)_2D_3$] (*1*). Conversely, vitamin D metabolites may affect the production of hormones in the pituitary. It is moreover established that other steroid hormones have pituitary target sites (*2*). Receptors for 1α,25-$(OH)_2D_3$ have been characterized in small intestine, kidney, parathyroid, pancreas, and bone (*3*–*7*). To assess pituitary vitamin D interactions, we examined the specific binding of tritiated 1α,25-$(OH)_2D_3$ to subcellular fractions of bovine pituitary. Specific binding was observed in both cytosol and nuclear fractions. Binding to crude nuclear fractions is rapid in onset, saturable, and has properties strikingly similar to those observed with cytosolic 1α,25-$(OH)_2D_3$ receptors in a variety of recognized target tissues.

Intact bovine pituitaries and brain areas were excised immediately after the animals were killed. Subsequent tissue preparation was performed at 0° to 5°C. Connective tissue, white matter, and blood were removed, and tissues were minced in a medium containing 0.01*M* tris-HCl (*p*H 7.4 at 25°C), 0.25*M* sucrose, 1.5 m*M* disodium EDTA, and 0.5 m*M* dithiothreitol. Homogenates (1:10, weight to volume) centrifuged at 1200*g* for 10 minutes produced a crude nuclear (P_1) pellet; the mitochondrial pellet (P_2) was prepared by centrifuging the supernatant at 8000*g* for 20 minutes. Further centrifugation at 105,000*g* for 60 minutes yielded a microsomal pellet (P_3) and a supernatant (cytosol) fraction.

For membrane studies, subcellular fractions were resuspended at 1:10 or 1:20 (weight to volume) in 0.05*M* tris-HCl (*p*H 7.4 at 25°C), 1.5 m*M* EDTA, and 0.5 m*M* dithiothreitol, and incubated for 30 minutes at 0°C. The suspensions were then incubated with 1α,25-$(OH)_2$-$[^3H]D_3$ (0.5 to 0.7 n*M*) in the presence or absence of unlabeled steroids (in a total volume of 0.23 ml), for 20 minutes at 25°C, and rapidly filtered under vacuum through Whatman GF/B glass fiber filters. The filters were washed with 12 ml of ice-cold buffer containing 0.01*M* tris-HCl, *p*H 7.8, and 0.1 percent Triton X-100. For studies of binding to cytosol, 1.3 to 4.3 n*M* 1α,25-$(OH)_2$-$[^3H]D_3$ and unlabeled steroids were distributed into borosilicate tubes and dried under nitrogen. The steroids were redissolved in 15 to 20 μl of ethanol before the addition of 150 to 200 μl of cytosol. Samples were incubated for 120 to 180 minutes at 0°C; 50 μl of the total incubation volume was filtered under vacuum through DE81 cellulose filters, which were then rinsed with 5 ml of ice-cold 0.02*M* tris-HCl, *p*H 7.9 to 8.2. Radioactivity on the filters (fraction bound to membranes or cytosolic proteins) was assayed by liquid

Table 1. Specific binding of 1α,25-dihydroxyvitamin-$[^3H]D_3$ in subcellular fractions of bovine pituitary. Subcellular fractions were prepared as described in the text. Data are from two representative experiments. In experiment a, the 1α,25-$(OH)_2$-$[^3H]D_3$ concentration was 0.62 n*M* (110 Ci/mmole; Amersham). In experiment b, the 1α,25-$(OH)_2$-$[^3H]D_3$ concentrations were 0.49 nm (P_1 to P_3 fractions) and 1.0 n*M* (cytosol fraction) (160 Ci/mmole, New England Nuclear). The specific binding of 1α,25-$(OH)_2$-$[^3H]D_3$ in each subcellular fraction in experiment b was expressed as a percentage of the specific binding obtained in all four fractions; thus P_1 percent = $[P_1/(P_1 + P_2 + P_3 + \text{cytosol})] \times 100$ with each value in the equation expressed as femtomoles bound per fraction.

Experiment	Tissue fraction	Amount of 1α,25-$(OH)_2$-$[^3H]D_3$ bound (count/min)				Percent of total tissue binding
		Total	Blank	Specific	Femtomoles per milligram of protein	
a	P_1	1298	271	1027	21.90	
b		705	216	489	7.09	47
a	P_2	176	129	47	0.51	
b		107	84	23	0.18	1
a	P_3	121	86	35	0.64	
b		69	77			
b	Cytosol	2697	2008	689	8.54	52

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scintillation spectrometry at 35 percent efficiency (*4, 8*). Proteins were determined by the method of Lowry *et al.* (*9*).

Specific binding was defined as the binding component inhibited by 1 μM unlabeled 1α,25-$(OH)_2D_3$. Under the above conditions, the ratio of specific to nonspecific binding in the bovine pituitary was approximately 6:1 in the P_1 fraction and 1:4 in cytosol.

Initial studies indicated high levels of specific binding of 1α,25-$(OH)_2$-[^{3}H]D_3 in the crude nuclear fraction (P_1). Specific binding was also detectable in the cytosol fraction, but not in the P_2 or P_3 fractions. Under the present assay conditions, specific binding per milligram of protein was generally lower in the cytosol fraction than in P_1 (Table 1). When tissue preparation and binding studies were performed with 0.3M KCl added, specific binding in the cytosol fraction was doubled, with no change in nonspecific binding. Under these conditions, however, specific binding in P_1 was abolished, and nonspecific binding was substantially increased (data not shown).

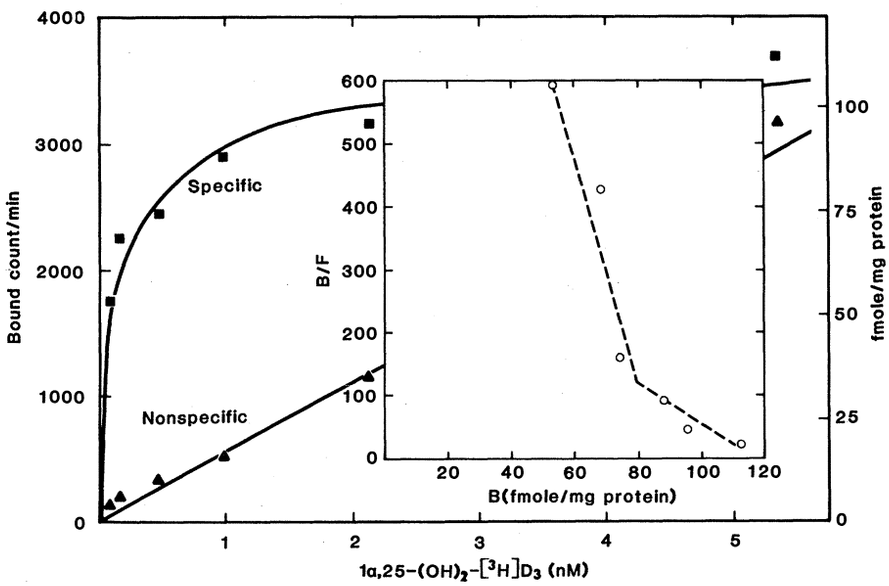

Fig. 1. Binding of 1α,25-$(OH)_2$-[^{3}H]D_3 to bovine pituitary P_1 fraction as a function of increasing concentrations of 1α,25-$(OH)_2$-[^{3}H]D_3. Points were assayed in triplicate, and nonspecific binding was determined in parallel samples which included 1.0 μM nonradioactive 1α,25-$(OH)_2D_3$. Data are from one representative experiment. Inset: Scatchard plot of specific binding. Lines of best fit for each portion of the curve were determined by linear regression analysis. *B*, ligand bound (femtomoles) per milligram of protein; *F*, ligand added (nanomoles) to the assay.

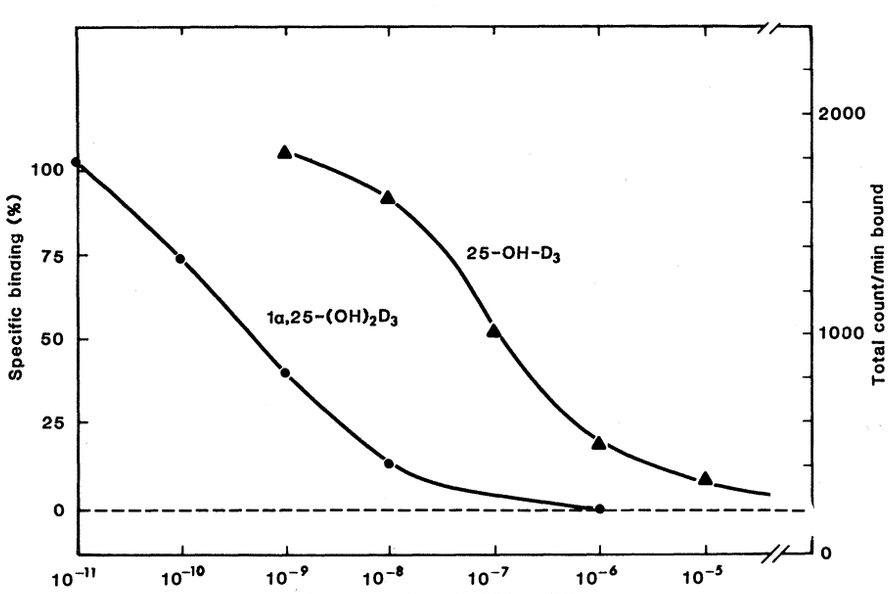

Fig. 2. Inhibition of 1α,25-$(OH)_2$-[^{3}H]D_3 (0.7 nM) binding to bovine pituitary P_1 fraction by vitamin D_3 metabolites. Points were assayed in triplicate, and IC_{50} values were determined by log-probit analysis of inhibition curves, as the concentration of metabolite that inhibited 50 percent of specific binding [binding displaceable by 1.0 μM 1α,25-$(OH)_2D_3$].

Specific binding of 1α,25-$(OH)_2$-[^{3}H]D_3 to crude nuclear fractions at 25°C was rapid, with half-maximal binding reached by 2.5 minutes, and equilibrium by 5 minutes. Specific binding was stable for at least 20 minutes more. Association to nonspecific P_1 sites was almost instantaneous at 25°C. Equilibrium specific binding was therefore characterized at 25°C after 20 minutes of incubation.

At a crude nuclear protein concentration of 2.0 mg/ml, specific binding leveled off between 3 and 5 nM 1α,25-$(OH)_2$-[^{3}H]D_3, with about 100 fmole of ligand bound per milligram of protein (Fig. 1). Binding was half-maximal at about 0.1 nM 1α,25-$(OH)_2$-[^{3}H]D_3. Scatchard (*10*) analysis resulted in a nonlinear plot (inset in Fig. 1). From regression analysis of all points, the dissociation constant (K_d) for 1α,25-$(OH)_2$-[^{3}H]D_3 was 0.10 nM, and the maximum number of nuclear fraction binding sites (B_{max}) was 104 fmole per milligram of protein. Visual inspection of the Scatchard plot suggested the possible existence of two components of binding, one with a K_d of about 0.05 nM, and a second, lower affinity site with a K_d of 0.4 nM.

Both biological and binding studies indicate that at vitamin D target organs in vitro, the 1,25-dihydroxylated metabolite is generally 100 to 1000 times more potent than the precursor compound, 25-hydroxyvitamin D_3 (25-OH-D_3) (*11, 12*). A similar degree of specificity was observed with binding of 1α,25-$(OH)_2$-[^{3}H]D_3 to the pituitary P_1 fraction (Fig. 2). The concentration of unlabeled 1α,25-$(OH)_2D_3$ inhibiting the specific binding of 0.7 nM 1α,25-$(OH)_2$-[^{3}H]D_3 by 50 percent (IC_{50}) was 0.3 nM, in good agreement with the observed K_d value for 1α,25-$(OH)_2$-[^{3}H]D_3 in saturation experiments (Fig. 1) and corresponding well to the potency of 1α,25-$(OH)_2D_3$ in mediating responses in target systems in vitro (*12*). Unlabeled 25-OH-D_3 was approximately 300 times weaker in inhibiting 1α,25-$(OH)_2$-[^{3}H]D_3 binding, with an IC_{50} value of 100 nM.

The action of vitamin D is closely associated with the induction of a calcium binding protein in the small intestine (*13*), and there is evidence of immunological cross-reactivity of calcium binding proteins from the small intestine with those from the hypothalamus and the cerebellum (*14*). We therefore investigated P_1 binding at these bovine brain areas and found negligible specific binding of 1α,25-$(OH)_2$-[^{3}H]D_3 under the con-

ditions used to examine pituitary binding. The specific binding of 1α,25-$(OH)_2$-$[^3H]D_3$ to a bovine pituitary nuclear site has characteristics similar to that previously described for vitamin D receptors in several target tissues (*3–6*). Most of the studies of vitamin D receptors in small intestine and bone have dealt with the interactions of the steroid hormone with cytosolic receptors and the subsequent translocation and binding of the hormone-receptor complex to a nuclear component (*5, 6*). In a report on binding of free 1α,25-$(OH)_2$-$[^3H]D_3$ to nuclear fractions from chick intestine and kidney (*7*), specific binding was a relatively small component of the overall binding. Our studies have revealed high-affinity labeling by free 1α,25-$(OH)_2$-$[^3H]D_3$ of receptors in the nuclear fraction of the bovine pituitary, with specific binding amounting to 85 percent of the total binding. Specific binding of 1α,25-$(OH)_2$-$[^3H]D_3$ to a cytosolic pituitary site in KCl-free medium was less extensive, but with 0.3*M* KCl present, binding to cytosol doubled and nuclear binding was abolished. This contrasts with the observation in the hen oviduct that under high ionic conditions (increased KCl), specific binding of progesterone hormone-receptor complexes to nuclear fractions is greatly increased (*15*). The kinetic constants for 1α,25-$(OH)_2D_3$ binding to the pituitary P_1 fraction are similar to those obtained for cytosolic receptors in rat bone cells (K_d = 0.2 n*M*; B_{max} = 75 fmole per milligram of protein) (*6*). However, in small intestine from vitamin D–deficient chicks, 1α,25-$(OH)_2D_3$ appears to label a much greater number of cytosolic receptors with a tenfold lower affinity (K_d = 2.2 n*M*; $B_{max} \sim$ 1700 fmole per milligram of protein) (*4*).

The present experiments demonstrating binding to the P_1 fraction do not differentiate between the direct interactions of 1α,25-$(OH)_2D_3$ with nuclear constituents and the exchange of 1α,25-$(OH)_2D_3$ with that already bound in hormone-cytosol receptor complexes. It is unlikely that the observed specific binding is due to interactions with plasma vitamin D binding protein retained in the tissue preparation, because at the plasma site 25-OH-D_3 is more potent than 1α,25-$(OH)_2D_3$, and the dissociation constant of 1α,25-$(OH)_2D_3$ is much greater than that found in the present experiments (*16*).

Although calcium-binding protein that is immunologically cross-reactive with the intestinal vitamin D–dependent calcium-binding protein is present in some brain areas, the brain protein does not appear to be rapidly induced by vitamin D treatment (*17*). The absence of specific nuclear vitamin D binding sites in central nervous system tissue in the present experiments is consistent with the unresponsiveness of brain tissue to vitamin D.

In preliminary experiments, we observed specific binding of 1α,25-$(OH)_2$-$[^3H]D_3$ to nuclear fractions from intact pituitaries of lactating rats and from cultures of rat pituitary adenoma GH_3 cells. Sites in these tissues show the same high-affinity kinetic characteristics as those found with the bovine pituitary receptors. Our observations are particularly interesting in view of the autoradiographic evidence (*18*) of 1α,25-$(OH)_2D_3$ binding to nuclei of cells of the rat pars distalis. Association of 1α,25-$(OH)_2$-$[^3H]D_3$ with a pituitary cytoplasmic site has been demonstrated in vivo in chickens (*19*). It has also been reported that 1α,25-$(OH)_2D_3$ may regulate protein phosphorylation and prolactin production in pituitary GH_4 cells (*20*).

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Cultivation in vitro of the Quartan Malaria Parasite *Plasmodium inui*

Abstract. *The simian quartan malaria parasite* Plasmodium inui *(OS strain) was cultured in a continuous flow system with rhesus monkey erythrocytes and RPMI 1640 medium supplemented with Hepes buffer and rhesus serum. Over a 10-week period, the growth of the parasite permitted a 61,000-fold cumulative dilution of the original inoculum. After 5 weeks in culture, the parasites were still infective to the monkey* Saimiri sciureus *and to* Anopheles freeborni *mosquitoes.*

The cultivation of malaria parasites offers a valuable research tool against a disease that still claims a heavy toll in many developing nations (*1*). Successful long-term cultivation has been achieved with the human malaria parasite *Plasmodium falciparum* (*2*) and the simian parasites *P. knowlesi* (*3*) and *P. fragile* (*4*). These species share common characteristics: they cause a severe disease in the infected host, and their erythrocytic asexual cycles have a short periodicity, with quotidian (24-hour) cycles for *P. knowlesi* and tertian (48-hour) cycles for *P. falciparum* and *P. fragile*. We now report the successful cultivation of *P. inui*, a parasite with fundamentally different characteristics.

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