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The plasma binding protein for vitamin D is a site of discrimination against vitamin D-2 compounds by the chick

Hector F. DeLuca, Masaki Nakada *, Yoko Tanaka *, Rafal Sicinski * and Mary Phelps

Department of Biochemistry, College of Agricultural and Life Sciences, University of Wisconsin-Madison, Madison, WI (U.S.A.)

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The binding of 25-hydroxy-[26,27-^{3}H]vitamin D-3 and 25-hydroxy-[26,27-^{3}H]vitamin D-2 to the vitamin D binding protein in the plasma of both rats and chicks has been studied. In the case of rats, sucrose density gradient analysis, competitive displacement, and Scatchard analysis demonstrate that 25-hydroxyvitamin D-3 and 25-hydroxyvitamin D-2 are bound equally well to the vitamin D binding protein. In contrast, 25-hydroxyvitamin D-2 is poorly bound, while 25-hydroxyvitamin D-3 is tightly bound to the vitamin D binding protein in chick plasma. On the other hand, the chick intestinal receptor binds 1,25-dihydroxyvitamin D-2 and 1,25-dihydroxyvitamin D-3 equally well with a K_D of $7 \cdot 10^{-11}$ M for both compounds. These results strongly suggest that the failure of the plasma transport protein in chicks to bind the vitamin D-2 compounds may be responsible for their relative ineffectiveness in these animals.

Introduction

Vitamin D and hydroxylated metabolites circulate in the blood bound to vitamin D binding protein (DBP). It is believed that DBP solubilizes relatively insoluble vitamin D compounds in the aqueous environment of plasma and protects them from oxidative inactivation [1,2]. A specific DBP has been purified from man [2–4], rats [5] and chicks [6]. The properties of the proteins are similar; they have a single binding site for the vitamin D compounds and preferentially bind 25-hydroxyvitamin D-3 (25-OH-D-3) above 1,25-dihydroxyvitamin D-3 (1,25-$(OH)_2$D-3).

Vitamin D-2 and vitamin D-3 have the same activity in preventing or curing rickets in most mammals; however, vitamin D-2 is approximately one-tenth as potent as vitamin D-3 in the chick [7]. Similarly, 25-OH-D-2 and 1,25-$(OH)_2$D-2 are one-tenth as potent as 25-OH-D-3 or 1,25-$(OH)_2$D-3, respectively, in the chick, in contrast to equal potency in the rat [8]. The reason has not been elucidated clearly, although it has been shown that D-2 compounds are more rapidly metabolized than D-3 compounds in the chick [9]. It has also been suggested but not proved that D-2 compounds are poorly bound by chick DBP [10]. We recently completed synthesis of 25-OH-[26,27-^{3}H]D-2 and 1,25-$(OH)_2$-[26,27-^{3}H]D-2 of high

* Present addresses: M. Nakada, Kobe University School of Medicine, Kobe, Japan; Y. Tanaka, VA Medical Center, Endocrinology and Metabolism Laboratory, Albany, NY, U.S.A.; R. Sicinski, University of Warsaw, Warsaw, Poland.

Abbreviations: DBP, vitamin D binding protein; 25-OH-D; 25-hydroxyvitamin D; 1,25-$(OH)_2$D, 1,25-dihydroxyvitamin D; 25-OH-[26,27-^{3}H]D, 25-hydroxy-[26,27-^{3}H]vitamin D.

Correspondence: H.F. DeLuca, Department of Biochemistry, University of Wisconsin-Madison, 420 Henry Mall, Madison, WI 53706, U.S.A.

specific activity [11], making it possible to measure directly the binding of 25-OH-D-2 to DBP. This report demonstrates that indeed chick DBP binds 25-OH-[26,27-^{3}H]D-3 as well as rat DBP but binds 25-OH-[26,27-^{3}H]D-2 very poorly in contrast to the rat protein. Furthermore, chick intestinal receptor for 1,25-$(OH)_2$D-3 binds 1,25-$(OH)_2$D-2 and 1,25-$(OH_2$D-3 similarly [11–13].

Materials and Methods

Chemicals. 25-OH-[26,27-^{3}H]D-3 (80 Ci/mmol) was prepared as previously described [12]. 25-OH-[26,27]^{3}H]D-2 (80 Ci/mmol) was recently synthesized [11]. It was purified by HPLC immediately before use and was over 97% pure by HPLC analysis [11]. Nonradioactive 25-OH-D-3 was the gift of the Organon Company (West Orange, NJ). Nonradioactive 25-OH-D-2 was synthesized in this laboratory [14].

Animals. 1-day-old white Leghorn cockerels (Northern Hatcheries, Beaver Dam, WI) were fed a vitamin D-deficient, soy protein diet for 4–6 weeks [15]. Weanling male rats were purchased from the Holtzman Co. (Madison, WI) and fed a vitamin D-deficient diet for 4–6 weeks [16]. The plasma from these animals was diluted with 0.05 M sodium phosphate buffer pH 7.4.

Sucrose-density gradient analysis. 0.5 ml diluted plasma from chicks and rats (dilution: chick, 50-fold; rat, 5000-fold) were incubated with 1 nM 25-OH-[26,27-^{3}H]D-3 or 25-OH-[26,27-^{3}H]D-2 in the absence or presence of 100-fold excess nonradioactive 25-OH-D-3 or 25-OH-D-2. After 2 h at 0–4°C, unbound ligand was removed with dextran-coated charcoal as described below and 0.2 ml aliquots were then analyzed on 4–20% sucrose gradients in 0.05 M sodium phosphate buffer by the method described previously [17]. Bovine serum albumin (BSA) and ovalbumin (OVA) were used for the estimation of sedimentation coefficients.

Equilibrium binding studies. Plasma from rats and chicks was diluted to 1 : 5000–10 000 and 1 : 1000–2000, respectively, with 0.05 M sodium phosphate buffer pH 7.4. Either 25-OH-[26,27-^{3}H]D-3 (0.01–1.0 nM) or 25-OH-[26,27-^{3}H]D-2 (0.01–1.0 nM)) was dissolved in 0.02 ml of absolute ethyl alcohol and mixed with each preparation (0.5 ml). The nonspecific binding was measured by parallel incubation of diluted plasma with 25-OH-[26,27-^{3}H]D-2 or 25-OH-[26,27-^{3}H]D-3 containing a 500-fold excess of nonradioactive 25-OH-D-2 or 25-OH-D-3, respectively. The mixtures were incubated for 16 h in an ice bath and then the unbound hormone was removed by adsorption to 200 µl of 1.0% activated charcoal and 0.1% dextran for 10 min at 0–4°C. This procedure removes only unbound ligand [18]. 0.4 ml aliquots were then withdrawn and added to 3.5 ml of scintillation fluid mixture consisting of 1.32 liters Triton X-100, 8.0 g of PPO (2,5-diphenyloxazole) and 0.2 g of POPOP (1,4-bis[2-(5-phenyloxazolyl)]benzene per 4 liters of toluene. Radioactivity determinations were carried out with a PRIAS liquid scintillation counter (Packard Instruments, Downers Grove, IL). Analysis of the data was performed by the method of Scatchard [19] and regression analysis was used for curve fitting.

Equilibrium binding studies with chick intestinal receptor for 1,25-$(OH)_2$D-3 was carried out with 1,25-$(OH)_2$-[26,27-^{3}H]D-3 or 1,25-$(OH)_2$-[26,27-^{3}H]D-2 as previously described [18].

Competitive displacement studies. Displacement of 25-OH-[26,27-^{3}H]D-3 or 25-OH-[26,27-^{3}H]D-2 from rat plasma or chick plasma by nonradioactive 25-OH-D-3 or 25-OH-D-2 was measured as described by Shepard and DeLuca [20]. 5 ml of diluted rat plasma (5000-fold dilution) or chick plasma (50-fold dilution) was incubated with 25-OH-[26,27-^{3}H]D-3 or 25-OH-[26,27-^{3}H]D-2 in the presence of graded amounts of nonradioactive 25-OH-D-3 or 25-OH-D-2 in an ice bath for 16 h. Dextran charcoal was used to remove the unbound free sterol as described above. Similarly, displacement of 1,25-$(OH)_2$-[26,27-^{3}H]D-3 from chick intestinal receptor protein was carried out as described by Shepard and DeLuca [20].

Protein concentrations in the plasma samples were determined by the method of Bradford [21].

Results

Diluted plasma from rats and chicks was incubated with identical concentrations of 25-OH-[26,27-^{3}H]D-2 and 25-OH-[26,27-^{3}H]D-3 and analyzed by sucrose density gradient sedimentation (Fig. 1). Fig. 1A and B show that 25-OH-

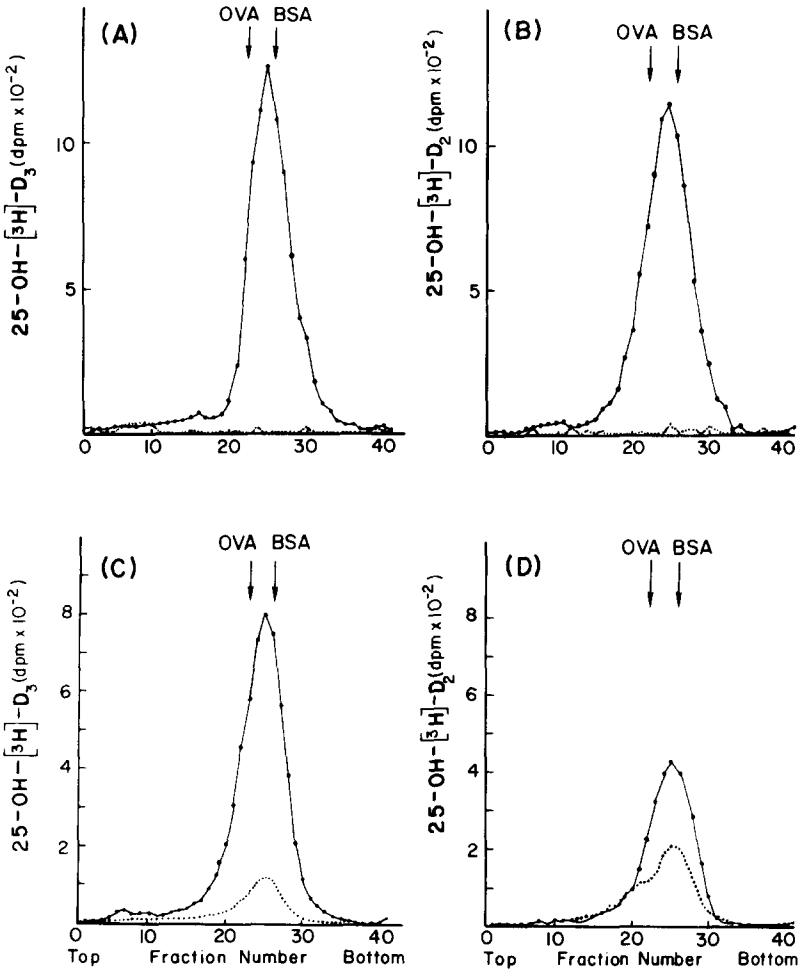

Fig. 1. Sucrose density gradient analysis of 25-OH-[26,27-^{3}H]D-3 and 25-OH-[26,27-^{3}H]D-2 binding components in rat or chick plasma. Diluted plasma from rats and chicks was incubated for 2 h at 0 ° C with 1 nM 25-OH-[26,27-^{3}H]D-3 or 25-OH-[26,27-^{3}H]D-2 in the absence or presence of 100-fold excess nonradioactive 25-OH-D-3 or 25-OH-D-2. After Dextran-charcoal treatment, 0.2 ml aliquots were analyzed on 4–20% sucrose gradients in 0.05 M sodium phosphate buffer. (A) Binding of 25-OH-[26,27-^{3}H]D-3 (———) and competition with nonradioactive 25-OH-D-3 (· · · · · ·) in rat plasma. (B) Binding of 25-OH-[26,27-^{3}H]D-2 (———) and competition with nonradioactive 25-OH-D-2 (· · · · · ·) to rat plasma. (C) Experiment as described for A except chick plasma was used. (D) Experiment as in B except chick plasma was used. OVA, ovalbumin; BSA, bovine serum ablumin.

[26,27-^{3}H]D-2 or 25-OH-[26,27-^{3}H]D-3 bound to rat plasma sedimented as a single component having the same sedimentation coefficient (approx. 4 S). Excess nonradioactive the 25-OH-D-2 and 25-OH-D-3 eliminated binding of the tritiated sterols in the 4 S region. With chick plasma, 25-OH-[26,27-^{3}H]D-3 sedimented as a single component with a 4 S sedimentation coefficeint (Fig. 1C). Excess nonradioactive 25-OH-D-3 displaced the radioactivity almost entirely from this component (Fig. 1C). In contrast, much less 25-OH-[26,27-^{3}H]D-2 bound to this component. Only a portion of the radioactivity was displaced by excess nonradioactive 25-OH-D-2 (Fig. 1D). Similar results were obtained with 1,25-$(OH)_2$-[26,27-^{3}H]D-2 and 1,25-$(OH)_2$D-2 (not shown). This reveals that much of the binding of 25-OH-D-2 to the chick plasma component is nonspecific.

To evaluate further the binding properties of the plasmas for 25-OH-D-2 and 25-OH-D-3, dis-

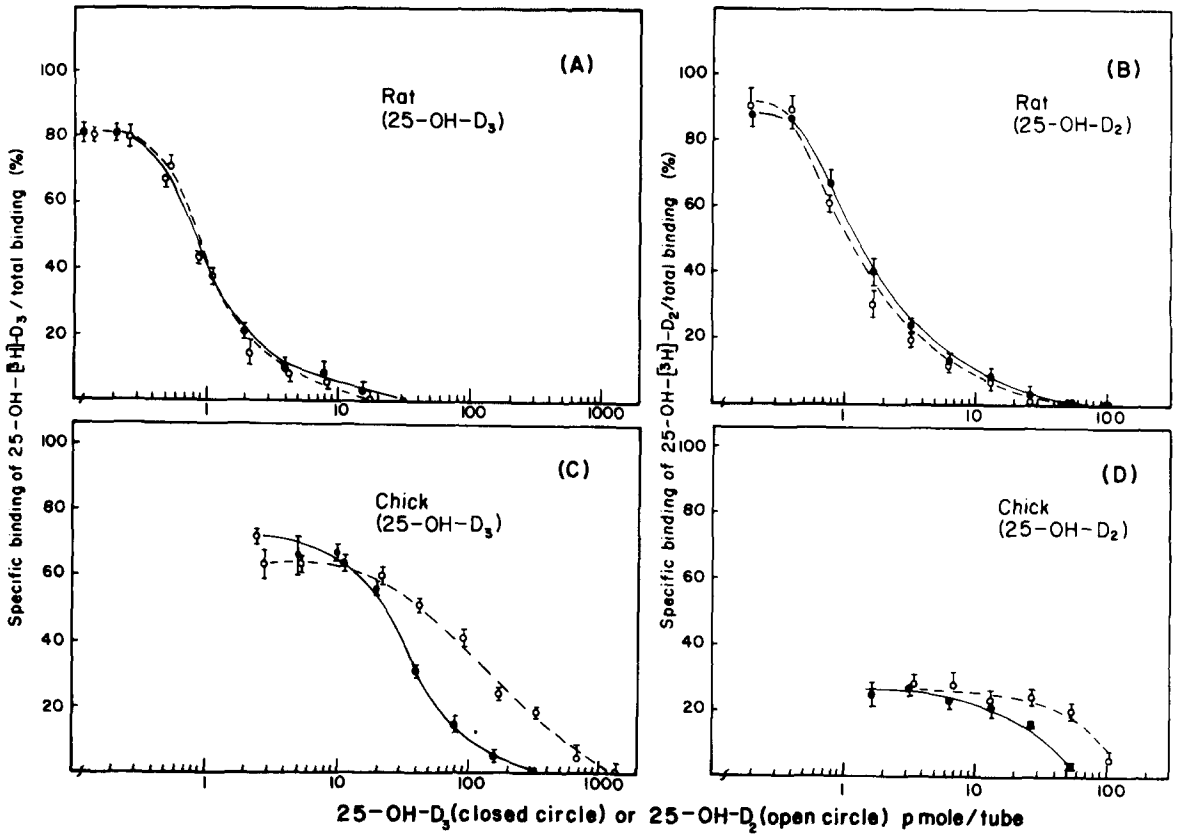

Fig. 2. Displacement of 25-OH-[26,27-^{3}H]D-3 or 25-OH-[26,27-^{3}H]D-2 from rat or chick plasma by 25-OH-D-3 (●) or 25-OH-D-2 (○). (A) Displacement of 25-OH-[26,27-^{3}H]D-3 from rat plasma protein. (B) Displacement of 25-OH-[26,27-^{3}H]D-2 from rat plasma protein. (C) Displacement of 25-OH-[26,27-^{3}H]D-3 from chick plasma. (D) Displacement of 25-OH-[26,27-^{3}H]D-2 from chick plasma. Total binding was obtained by incubation of plasma protein in the absence of competing nonradioactive compound. Specific binding was determined by subtraction of nonspecific binding from total binding. Each point represents the mean value from triplicate determinations. The vertical bars represent standard deviation from the mean.

placement of 25-OH-[26,27-^{3}H]D-2 or 25-OH-[26,27-^{3}H]D-3 with increasing amounts of nonradioactive 25-OH-D-2 or 25-OH-D-3 was determined (Fig. 2). With rat plasma, increasing amounts of 25-OH-D-2 or 25-OH-D-3 produced progessive and equivalent displacement of 25-OH-[26,27-^{3}H]D-3 binding (Fig. 2A). The displacement curve for 25-OH-[26,27-^{3}H]D-2 and rat plasma was identical to that produced with 25-OH-[26,27-^{3}H]D-3 (Fig. 2B). However, with chick plasma, increasing amounts of 25-OH-D-2 were much less effective than 25-OH-D-3 in displacing the 25-OH-[26,27-^{3}H]D-3 (Fig. 2C). 50% displacement of specifically bound 25-OH-[26,27-^{3}H]D-3 from chick plasma was achieved with $5 \cdot 10^{-7}$ M 25-OH-D-3, while $3 \cdot 10^{-5}$ M 25-OH-D-2 was required to achieve the same displacement. Thus, the affinity of chick DBP for 25-OH-D-2 is about 100-fold less than its affinity for 25-OH-D-3. Of considerable interest is the finding that little 25-OH-[26,27-^{3}H]D-2 binds to the chick protein and thus displacement could not be accurately studied (Fig. 2D). Similarly, no 1,25-$(OH)_2$-[26,27-^{3}H]D-2 binds to chick plasma, making competitive experiments impossible. This result was obtained even when more concentrated plasma samples were used (data not shown). To determine the affinity of the proteins for 25-OH-D-2 or 25-OH-D-3, Scatchard plot analysis was carried out [19]. K_D for 25-OH-D-3 and 25-OH-D-2 of rat plasma was $(4.1 \pm 1.0) \cdot 10^{-10}$) M and $(5.6 \pm 0.2) \cdot 10^{-10}$ M, respectively (Table I), with maximum binding capacities of

TABLE I

DISSOCIATION CONSTANTS AND MAXIMUM BINDING CAPACITY OF THE VITAMIN D BINDING PROTEIN IN RAT AND CHICK

Diluted plasma from rat and chick (0.5 ml) was equilibrated with 25-OH-[26,27-^{3}H]D-3 or 25-OH-[26,27-^{3}H]D-2 (0.01–1.0 nM each) for 16 h at 0–4°C. The nonspecific binding was measured in a parallel incubation with 25-OH-[26,27-^{3}H]D-3 or 25-OH-[26,27-^{3}H]D-2, and a 500-fold excess of nonradioactive 25-OH-D-3 or 25-OH-D-2. Bound fraction was measured through the use of dextran-coated charcoal (0.1%, 1%, w/v, respectively). Dissociation constants (K_D) for 25-OH-D-3 or 25-OH-D-2 were calculated from the slope of the regression line by Scatchard analysis. Regression analyses were performed to obtain the best fit. Maximum binding (n) was estimated from the abscissa intersect. Each value is the mean ± S.D. of three separate analyses.

	25-OH-D-3	25-OH-D-2
Rat		
K_D (M)	$(4.1 \pm 1.0) \cdot 10^{-10}$	$(5.6 \pm 0.2) \cdot 10^{-10}$
n (μmol/mg)	3.2 ± 0.4	2.6 ± 0.4
Chick		
K_D (M)	$(1.1 \pm 0.4) \cdot 10^{-9}$	– [a]
n (μmol/mg)	1.2 ± 0.3	–

[a] –, unmeasurable.

3.2 ± 0.4 μmol/mg protein and 2.6 ± 0.4 μmol/mg protein, respectively. The K_D of the chick DBP for 25-OH-D-3 was $(1.1 \pm 0.4) \cdot 10^{-9}$ M with a maximum binding capacity (n) of 1.2 ± 0.3 μmol/mg protein. The dissociation constant of the chick protein for 25-OH-D-2 could not be measured because of the small amount of specific binding observed. In contrast, the chick intestinal receptor for 1,25-$(OH)_2$D-3 provided virtually identical displacement curves for 1,25-$(OH)_2$D-2 and 1,25-$(OH)_2$D-3 (Fig. 3). Scatchard analysis revealed very similar K_D values for 1,25-$(OH)_2$-[26,27-^{3}H]D-2 and 1,25-$(OH)_2$-[26,27-^{3}H]D-3 when tested with the chick intestinal receptor using equilibrium binding methods (Table II).

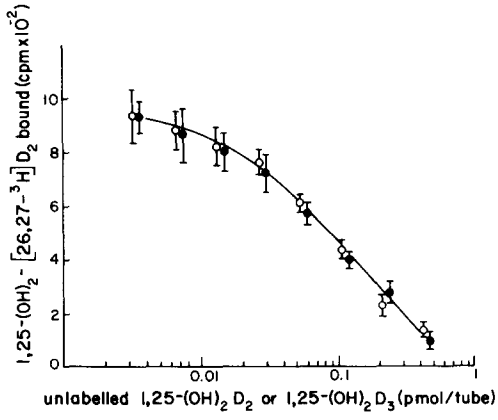

Fig. 3. Displacement of 1,25-$(OH)_2$-[26,27-^{3}H]D-3 from chick intestinal receptor by 1,25-$(OH)_2$D-2 (○) or 1,25-$(OH)_2$D-3 (●——●). The procedure is described in the text. Identical results are obtained if displacement of 1,25-$(OH)_2$-[26,27-^{3}H]D-2 is studied.

TABLE II

BINDING CONSTANTS FOR CHICK INTESTINAL RECEPTOR PROTEIN AND 1,25-$(OH)_2$D-3 AND 1,25-$(OH)_2$D-2

The constants were determined by Scatchard analysis as described in the text. The results are the most recent of three independent determinations.

Constant	1,25-$(OH)_2$D-3	1,25-$(OH)_2$D-3
K_D (M)	$7.1 \cdot 10^{-11}$	$10.4 \cdot 10^{-11}$
n (fmol/mg protein)	580	460

Discussion

The availability of 25-OH-[26,27-^{3}H]D-2 of high specific activity in this laboratory has made possible a quantitative examination of binding affinity and capacity of rat and chick plasma DBP for 25-OH-D-2. This was accomplished by three techniques: (1) Scatchard plot analysis, (2) sucrose denstiy gradient analysis, and (3) displacement analysis. By all three techniques, it is obvious that the chick DBP has little affinity for 25-OH-D-2 but is fully capable of binding 25-OH-D-3 with expected with high affinity ($K_D = 1.1 \cdot 10^{-9}$ M). Further, the rat DBP has approximately equal affinity for 25-OH-D-3 ($K_D = 4.1 \cdot 10^{-10}$ M) and 25-OH-D-2 ($K_D = 5.6 \cdot 10^{-10}$ M). This provides strong support for the previous study and suggestion [10]. Thus the chick protein is unable to bind and transport this compound. This observation also extends to the vitamin D-2 itself and its most active metabolite, 1,25-$(OH)_2$D-2 (data not shown). Unlike the plasma transport protein, the chick intestinal receptor protein does not appre-

ciably discriminate against the vitamin D-2 side chain (Table II, Fig. 3) [11–13].

It is well known that vitamin D-2 is one-tenth as active as vitamin D-3 in the bird [7]. This is true for 25-OH-D-2 versus 25-OH-D-3 and for 1,25-$(OH)_2$D-2 versus 1,25-$(OH)_2$D-3 [8]. It has been demonstrated that this discrimination results from increased metabolism and excretion [9]. It seems possible that the increased metabolism may result from rapid liver assimilation of the free or unbound vitamin D-2 compound. It is also possible that specific plasma transport protein delivery of vitamin D compounds to target organs is dependent upon DBP. In any case, the suggestion [10] that the discrimination against vitamin D_2 compounds by the chick is at the plasma binding protein level now appears very likely. However, it is unclear whether this is the only site of such discrimination and whether this discrimination alone can account for the reduced activity of vitamin D-2 compounds in birds. The fact that the chick intestinal receptor protein does not discriminate against the D-2 side chain provides strong support for the idea that the lack of plasma protein binding of D-2 compounds is responsible for their lack of biological activity in birds.

The affinity constants reported for rat and chick DBP for 25-OH-D-3 have been quite variable [22]. The K_D for rat plasma reported in this paper is $4.1 \cdot 10^{-10}$ M, in good agreement with that observed for pure rat DBP [5], while the chick DBP had a K_D of $1.1 \cdot 10^{-10}$ M, in agreement with data obtained with chick DBP [6]. A K_D of $5.6 \cdot 10^{-10}$ M was also found for 25-OH-D-2 and rat DBP. However, a K_D for 25-OH-D-2 for chick DBP could not be determined because of insignificant specific binding. The data are nevertheless sufficient to demonstrate that chick DBP binds 25-OH-D-2 poorly or not at all and that this might be responsible for the poor biological activity of vitamin D-2 compounds in birds.

The present study and those previously reported [10] support the idea that DBP has an important role in the metabolism of vitamin D. It is possible that DBP serves to store vitamin D and to prevent its rapid metabolism and elimination. Certainly, chick DBP binds vitamin D-2 compounds poorly, and in this species the vitamin D-2 compounds are rapidly metabolized and excreted [9].

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