

Radioimmunoassay of the Binding Protein for Vitamin D and its Metabolites in Human Serum

CONCENTRATIONS IN NORMAL SUBJECTS AND PATIENTS WITH DISORDERS OF MINERAL HOMEOSTASIS

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ABSTRACT A radioimmunoassay for the binding protein for vitamin D and its metabolites (DBP) has been developed. Suitable rabbit anti-DBP antiserum was elicited after primary and one booster injection. Anti-DBP antisera, as well as antigroup-specific component antisera, produced a single, monospecific line of precipitation when reacted against purified DBP and human serum. DBP was iodinated with ^{125}I and ^{125}I -DBP was purified by gel filtration on Sephadex G-200. Binding of ^{125}I -DBP by 20 nl of rabbit anti-DBP antisera was approximately 50% and was sharply competed for by 0.4–4.0 ng of DBP standard. Displacement of ^{125}I -DBP by human serum dilutions or standard DBP gave identical curves, and only weak competition was observed with old and new world primate sera. Apo- and holo-DBP possessed indistinguishable immunoreactivity. The assay detects DBP in 1–10 nl of human serum with reasonable accuracy and with reasonable intra- and interassay precision. The mean serum concentration (\pm SEM) for a group of 40 normal adults was 525 ± 24 $\mu\text{g}/\text{ml}$ and no sex difference was observed. Higher levels were found in sera from pregnant women and women receiving oral contraceptives, and decreased concentrations were observed in premature cord and hypoproteinemic sera. No significant correlation between serum DBP levels and serum 25-hydroxycalciferol levels was found, and the DBP content of sera from vitamin D-deprived and vitamin D-treated subjects was indistinguishable from that of normal adults. DBP accounts for 6% of the alpha globulin in normal human serum. Considering the normal serum content of the parent vitamin and its metabolites to be ap-

proximately 0.1–0.2 μm , these immunoassay data confirm previous saturation analyses of human serum antiricketic sterol-binding capacity and suggest that >95% of DBP circulates as the apoprotein under normal conditions.

INTRODUCTION

Antiricketic sterols are transported in the human circulation by an inter-alpha globulin which has recently been characterized (1–4). This transport protein sediments at 3.46 S in the analytical centrifuge (4) and possesses stringent ligand recognition properties which have permitted its use in the radiocompetitive assay of the calcifidiols or 25-hydroxycalciferols (25-hydroxycholecalciferol [25-OHD_3] and 25-hydroxyergocalciferol [25-OHD_2]) (5).¹ Quantitation of this protein has previously been estimated by analyses of the saturation of specific binding sites in serum by 25-OHD_3 in vitro and indicated a dominance of the apoprotein in normal sera (5, 6). We report the development of a radioimmunoassay for the human serum binding protein for vitamin D and its metabolites (DBP). The serum level of DBP has been determined in normal subjects and patients, and the relation between serum DBP and 25-hydroxycalciferol has been examined.

METHODS

Purification of DBP. DBP was isolated from Cohn IV fractions of human plasma by DEAE-cellulose and DEAE-

¹ *Abbreviations used in this paper:* DBP, binding protein for vitamin D and its metabolites in human serum; Gc, group-specific component; 25-OHD, 25-hydroxycalciferol (both 25-hydroxycholecalciferol [25-OHD_3] and 25-hydroxyergocalciferol [25-OHD_2]).

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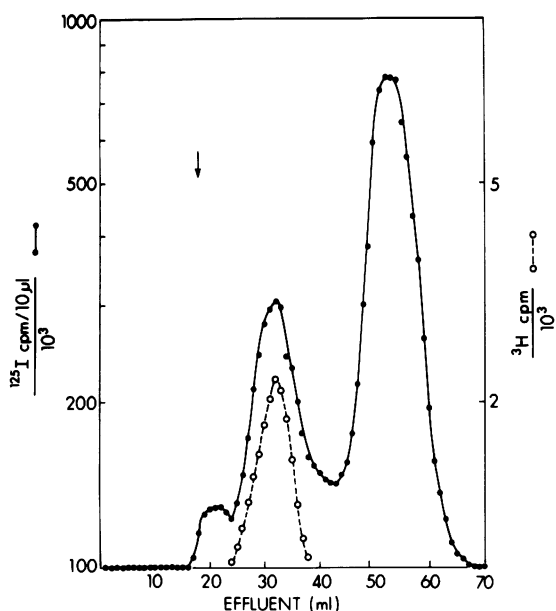


FIGURE 1 Gel filtration of the iodination mixture. 1 ml of the iodination solution was applied to a 1×45 -cm column of Sephadex G-200 equilibrated in 0.01 M Tris buffer, pH 7.4, containing 0.025% egg albumin and 0.025% sodium azide. Ascending flow with the same buffer was carried out at 15 ml/h at 4°C . The arrow depicts the elution volume (V_0) of blue dextran. The middle ^{125}I peak is ^{125}I -DBP, shown to share the V_e of the DBP-[^3H]25-OHD₃ complex, which was observed during calibration of the column. The initial ^{125}I peak presumably consists of polymerized ^{125}I -DBP, and the last ^{125}I peak is ^{125}I .

Sephadex chromatography (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) gel-filtration on Sephadex G-200, and preparative polyacrylamide gel electrophoresis. The purified preparation was homogeneous by analytical polyacrylamide gel electrophoresis, and by double immunodiffusion studies. These procedures have been described in detail in a previous publication (4).

Preparation of antisera. DBP was solubilized in 0.05 M sodium phosphate buffer, pH 7.4, and the solution was emulsified with an equal volume of complete Freund's adjuvant (Difco Laboratories, Detroit, Mich.) 1 mg of DBP was distributed by multiple subcutaneous injections into each of two 4-mo-old New Zealand rabbits. 4 wk later each rabbit received 0.5 mg of DBP emulsified in the same buffer and incomplete Freund's adjuvant (Difco Laboratories) by the same route. Ear artery bleedings 2 and 3 wk after the booster injection revealed suitable titers, and the serum from one of these animals was used in all of the immunassay studies.

Iodination of DBP. DBP was iodinated by utilizing chloramine-T. Lyophilized DBP (12 μg) (7) was dissolved in 0.5 M sodium phosphate buffer, pH 7.4, and 3–6 μg in 25 μl were transferred to a small glass tube containing 1–2 mCi of carrier-free [^{125}I]Na (Industrial Nuclear Co., St. Louis, Mo.) in 25 μl of 0.5 M sodium phosphate buffer, pH 7.4. Chloramine-T (Eastman Organic Chemicals Div., Rochester, N.Y.), 50 μg in 50 μl of buffer, was added, and the tube was tapped lightly for 20 s. Sodium metabisulfite, 100 μg in 50 μl of buffer was added, followed by 20 μg of KI

in 50 μl of buffer. Egg albumin (Egg white solids, Teklad Test Diets, Life Sci. Div., The Mogul Corp., Madison, Wisc.), 0.025% in 0.01 M Tris-HCl buffer, pH 7.4, containing 0.025% sodium azide, was transferred to the iodination tube.

Purification of ^{125}I -DBP. Immediately after the iodination procedure, 1 ml of the iodination solution was applied to a 1×45 -cm column of Sephadex G-200 that had been equilibrated in the Tris-albumin buffer. The filtration was carried out by ascending flow (15 ml/hr) at 4°C , and 1–2-ml fractions were collected. ^{125}I -DBP (mol wt $\sim 58,000$) eluted after the void volume and before ^{125}I (Fig. 1). Estimates of specific activity achieved were usually 50 $\mu\text{Ci}/\mu\text{g}$, and $>95\%$ of the ^{125}I -DBP purified on the G-200 column was bound by an excess of the rabbit anti-DBP antiserum.

Immunoassay procedure. Pilot studies indicated the merit of a double-antibody precipitation technique, and the relatively low nonspecific precipitation of ^{125}I -DBP in the Tris-albumin buffer. Suitable binding curves were reproducibly achieved by permitting a 2-day incubation of first antibody, followed by an overnight incubation with goat-antirabbit gamma globulin.

Tris-albumin buffer, DBP reference standard diluted in Tris-albumin buffer, or human serum diluted in Tris-albumin buffer were added to 10×75 -mm disposable glass tubes. ^{125}I -DBP (approximately 15,000 cpm or 300–400 μg), diluted in the same buffer containing carrier rabbit (non-immune) sera, was added, followed by 20 μl of rabbit anti-DBP antisera in 0.1 ml of the Tris-albumin buffer (final dilution 1:25,000). The tubes were kept at 0°C during the procedure, and the order of additions and final incubation volume (0.5 ml) were always the same. After mixing on a Vortex apparatus, the tubes were covered and placed at 4°C for 48 h. Goat antirabbit gamma globulin antiserum (Gateway Immunosera Co., Cahokia, Ill.) (50 μl) was added and the tubes were kept at 4°C for an additional 16 h. After the second incubation, the tubes were centrifuged at 2,000 rpm for 20 min at 4°C . Supernates were removed, and the precipitates were washed with 0.5 ml of the Tris-albumin buffer. After a second centrifugation and removal of supernates, the precipitates were assayed in a gamma spectrometer (Searle Analytic Inc., Des Plaines, Ill.) with a counting efficiency of 40% of ^{125}I .

Each assay contained replicate tubes without antibody, replicate tubes with antibody and ^{125}I -DBP, reference DBP dilutions in triplicate, and dilutions of reference pooled sera. Typically, the antibody control tubes revealed 40–50% of the ^{125}I -DBP to be antibody-bound in the absence of unlabeled DBP. In each assay, tubes containing excess anti-DBP (0.1 ml of 1:100 dilution) and ^{125}I -DBP revealed $>95\%$ of the ^{125}I -DBP to be bound by the rabbit anti-DBP. Nonspecific ^{125}I -DBP precipitation (in absence of anti-DBP) was 8% or less, and was subtracted from the counts per minute observed in the precipitates of tubes containing anti-DBP. Radioactivity precipitated in the presence of standard or serum was divided by that precipitated in the absence of unlabeled DBP to calculate B/Bo.

25-OHD assay. The serum content of 25-OHD (25-OHD₃ and 25-OHD₂ equally recognized) was determined by ether extraction, silicic acid chromatography, and competitive protein binding radioassay as previously described (8).

Serum samples. Venous blood was obtained from healthy men and women and from patients with a variety of disorders (Table II). Samples were also obtained from healthy women receiving chronic oral contraceptive therapy (estrogen and progestins), pregnant (last trimester) women, and from cord at the delivery of term and premature infants. Sera from patients previously identified to have osteomalacia

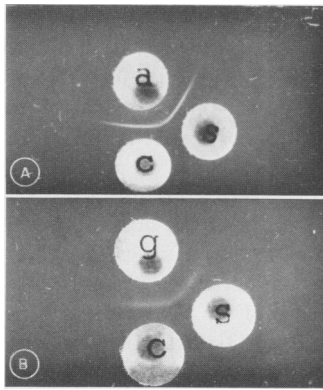


FIGURE 2 Agar gel immunodiffusion studies. (A) 40 μ l of rabbit antihuman DBP antiserum (a) was reacted with 2 μ l of whole human serum (s) and 1.5 μ g of pure human serum DBP (c). The anti-DBP antiserum IgG was concentrated fourfold by lyophilization. The well contents were delivered in 10 μ l of the agar gel buffer. (B) 40 μ l of rabbit antigroup-specific component antiserum (g) was reacted with 2 μ l of whole human serum (s) and 1.5 μ g of pure human serum DBP (c). The antigroup specific-component antiserum IgG was concentrated fourfold by lyophilization. The well contents were delivered in 10 μ l of the agar gel buffer.

or rickets and very low serum 25-OHD levels were studied. Sera from hypoparathyroid patients and patients diagnosed to have vitamin D-resistant rickets receiving high dose vitamin D₂ therapy (serum 25-OHD > 200 ng/ml) were analyzed. Bovine and swine sera were purchased from Colorado Serum Co., Denver, Colo. and serum from capuchin monkey, *cebus apella*, was purchased from Pel-Freez Bio-Animals, Inc., Rogers, Ark. Rhesus monkey sera were kindly provided by I. Mariz of the Metabolism Division at Washington University.

Immunodiffusion studies. Agar (0.6% in 0.01 M Tris-HCl containing 0.1 M NaCl, pH 7.5) slides were prepared. IgG fractions of rabbit anti-DBP and antigroup-specific component (Gc) antisera (Behring Diagnostics, American Hoechst Corp., Somerville, N. J.) were prepared by 33% (NH₄)₂ SO₄ precipitation, dialysis, and chromatography on 1 \times 17-cm columns of DEAE-cellulose equilibrated in the dialyzing buffer, 0.02 M potassium phosphate, pH 8.0. These IgG fractions were lyophilized, reconstituted, and allowed to diffuse against various sera and the purified DBP preparation.

RESULTS

Anti-DBP antiserum. The antiserum obtained after injections of DBP gave a single precipitin line when allowed to diffuse against DBP (Fig. 2A). Similarly, when tested against normal human serum, the anti-DBP caused a single line of precipitation. The reactions between anti-DBP and DBP and anti-DBP and human serum described a single, monospecific immunoprecipitin line (Fig. 2A). Fig. 2B indicates that indistinguishable results were observed when anti-Gc antiserum was substituted for the anti-DBP. Identical results were obtained over a range (1–10

μ l) of human serum used, and provided reasonable support for the monospecificity of the anti-DBP antiserum and the strong similarity, if not identity, of DBP and Gc.

Standard curve. Fig. 3 indicates that the displacement of ¹²⁵I-DBP by unlabeled DBP describes a sigmoid curve on a semilogarithmic plot. The steep portion of the curve was effected by 0.3–3.0 ng of unlabeled DBP, and virtually all of the ¹²⁵I-DBP was displaced in the presence of 50 ng of unlabeled DBP.

When normal human serum was diluted in the albumin buffer and assayed concurrently with standard DBP, the assay curve obtained with the serum dilutions was essentially identical to displacement observed with reference DBP (Fig. 3).

Accuracy. Known amounts of standard DBP were added to replicate dilutions of normal human serum and analyzed in the immunoassay. As indicated in Table I, the mean recovery of added DBP was 102% in both instances.

Intra-Assay variation. Two sera were analyzed in 10 replicate tubes within the same assay, and the coefficients of variation were 3.2 and 5.7% (Table I). Serum samples were routinely assayed in duplicate and at two separate dilutions (2 and 10 nl/tube). When duplicate assays differed from each other by more than 20%, the assay of the sample was repeated.

In each of eight separate assays over a 4-mo period, dilutions from the same serum pool were analyzed in duplicate at two different dilutions. As indicated in Table I, the mean \pm SD observed was 633 \pm 58 μ g/ml.

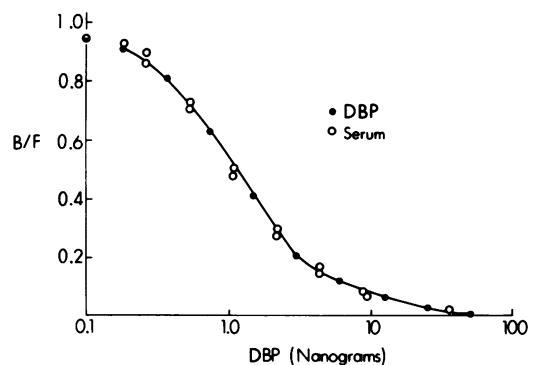


FIGURE 3 DBP radioimmunoassay standard curve. The figure demonstrates the displacement of ¹²⁵I-DBP from antibody by purified DBP (●) and by dilutions of normal human serum (○). The mean value for the level of DBP in the serum sample was determined by assaying the sample in four separate assays. The expected amount of DBP added per assay tube was calculated from the amount of diluted serum added, and plotted against the observed (B/F) for the corresponding samples.

TABLE I
Precision and Accuracy of Human Serum
DBP Radioimmunoassay

	Number	DBP		Coefficient of variation
		$\mu\text{g/ml} \pm \text{SD}$		
Intra-assay variation	10	407 \pm 23		5.7%
	10	1,189 \pm 38		3.2%
Inter-assay variation	8	633 \pm 58		9.2%
		Measured		
	Num-ber	Added ng/tube	ng/tube \pm SEM	% Recovery mean (range)
Accuracy	6	2.53	2.58 \pm 0.08	102 (95-117)
	6	4.03	4.10 \pm 0.15	102 (87-112)

Specificity of the assay. Whereas total displacement of ^{125}I -DBP from the antibody was achieved with 50–100 nl of human serum or 50 ng of DBP, human serum albumin and gamma globulin were noncompetitive in microgram amounts. Preincubation of DBP or dilutions of serum with microgram amounts of calciferol or calcifidiol did not affect their displacement potency in the DBP radioimmunoassay, indicating the equal potency of the apoprotein and holoprotein in the assay. As indicated in Fig. 4, the displacement of ^{125}I -DBP by human sera was most potent, with old and new world primate sera much less so. Dog and chicken sera were also noncompetitive in this system. When serum was fractionated by polyacrylamide gel electrophoresis or sucrose gradient ultracentrifugation, a homogeneous peak of DBP was observed by immunoassay of gel slice eluates and gradient fractions.

Serum DBP concentrations. The mean serum DBP concentration (\pm SEM) for normal adults was 525 \pm 24 $\mu\text{g/ml}$ (Table II). No difference in DBP levels between men and women were observed. Similarly, serum DBP levels were not altered in vitamin D-deprived nor vitamin D-treated subjects. Significantly lower values were observed in cord sera from prematurely delivered infants and patients (cirrhosis, nephrotic syndrome, and intestinal telangiectasia) with hypoproteinemia (serum albumin <2.8). Increased levels were observed in sera from pregnant females (1,254 \pm 89 $\mu\text{g/ml}$, mean \pm SEM) and females taking oral contraceptives (824 \pm 51 $\mu\text{g/ml}$, mean \pm SEM). Subjects with X-linked vitamin D-resistant rickets, sarcoidosis, as well as those receiving chronic adrenocorticosteroid or anticonvulsant therapy, had normal serum DBP levels. The sera of normal children (aged 7–13 yr) and term cord sera contained levels of DBP that were indistinguish-

able from those of normal adults. A normal DBP concentration was also found in the serum from one patient with vitamin D-dependent rickets.

Serum DBP and serum 25-OHD. Although no differences in serum DBP content were observed among vitamin D-deprived, normal, and vitamin D-treated subjects, we analyzed sera from subjects before and after the oral or parenteral administration of vitamin D or 25-OHD $_3$. At hours, days, and weeks after the doses, no significant changes in serum DBP concentrations were found. Fig. 5 displays the paired serum DBP and serum 25-OHD levels observed in 31 adult sera. No significant correlation was found in this group of sera. The mean DBP concentration is 572 $\mu\text{g/ml}$ (approximately 10 μM) and the mean 25-OHD concentration is 19.7 ng/ml (50 nM). The data clearly indicate the striking molar excess of binding protein to this ligand in normal sera.

DISCUSSION

An accurate and convenient radioimmunoassay for the binding protein for vitamin D and its metabolites in human serum (DBP) has been developed. The immunoassay has a reasonable degree of intra-assay and inter-assay precision. The assay is sensitive to nanogram and sub-nanogram amounts of DBP, thus requiring dilution of serum. This degree of sensitivity permits the reliable quantitation of DBP in as little as 2–10 nl of serum per assay tube. Serum DBP is apparently stable for immunoassay purposes, since aliquots from the same frozen serum pool were repetitively measured to be very similar over a 6-mo period.

The immunoassay is specific for human DBP, and

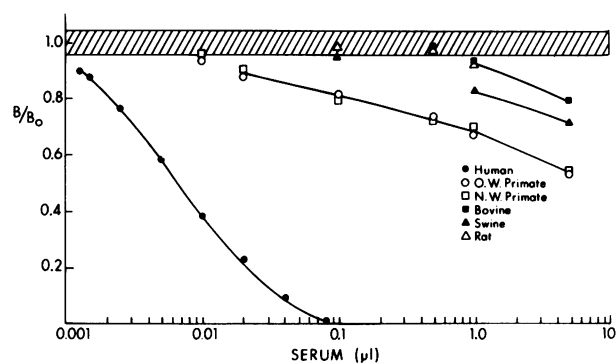


FIGURE 4 Specificity of the DBP radioimmunoassay for human serum. The figure illustrates the sensitive recognition of human DBP (●) in contrast to sera from other species: (□) new world monkey; (○) old world monkey; (▲) swine; (■) bovine; (△) rat. Also noncompetitive in displacing ^{125}I -DBP from the antibody were up to 10 $\mu\text{g/tube}$ of human albumin and gamma globulin and bovine serum albumin.

TABLE II
DBP Concentrations in Human Serum
Determined by Radioimmunoassay

Group	Number	DBP,
		Mean±SEM
		μg/ml
Men and women	40	525±24
Men	19	555±32
Women	21	499±36
Normal children	12	524±19
Cord—(Term)	10	558±78
Cord—(Premature)	9	359±38*
Pregnant women (third trimester)	11	1,254±89*
Oral contraceptive therapy	10	824±51*
Vitamin D deficiency	11	576±33
Vitamin D therapy	12	496±72
Sex-linked hypophosphatemic rickets	14	551±66
Sarcoidosis	6	492±78
Hypoproteinemia	10	234±28*
Anti-convulsant therapy	5	485±34
Adrenocorticosteroid therapy	6	660±75

* $P < 0.001$ Compared to adult levels.

identical displacement of ^{125}I -DBP from anti-DBP antibody was observed with pure DBP and human serum. DBP and human sera provide identical lines of immunoprecipitation when tested against anti-DBP in agar immunodiffusion experiments. Further, protein eluates from serum fractions in polyacrylamide electrophoresis gel slices and sucrose density gradient fractions can be identified by radioimmunoassay and indicate an identical migration and sedimentation of DBP and radioactive D_3 or 25-OHD_3 . Other serum proteins, and sera from other species were weakly competitive or noncompetitive in the radioimmunoassay. Apo- and holo- human DBP are recognized identically in the immunoassay.

Although underestimated by previous saturation analyses with ligand (5), the present findings confirm the large number of antiricketic sterol binding sites in human serum (Table II). One can estimate the normal serum combined content of $\text{D}(9)$, 25-OHD (10), and $24,25\text{-dihydroxycalciferol}$ ² to be approximately $0.1\text{--}0.2\ \mu\text{M}$. It has been shown that 1 mol of 25-OHD_3 is bound by 1 mol of human DBP (4). Further, our present observations (Table II) indicate the concentration of DBP in human sera to be about $9\ \mu\text{M}$. Assuming a mole per mole relationship between DBP and each of the vitamin D sterols (4) only 2–3% of the binding sites are occupied in normal man. Since no apparent relationship between serum DBP concentration and serum 25-OHD concentration was

² Minh, C., and J. G. Haddad, Jr. Unpublished observations.

observed (Fig. 5) and since DBP levels were similar in vitamin D-deprived and vitamin D-treated subjects (Table II), it seems probable that vitamin D availability and metabolism does not significantly affect the synthesis or catabolism of DBP. If this proves to be the case, the serum binding protein for vitamin D sterols differs from retinol-binding protein, since the hepatic egress of retinol-binding protein is made possible by the availability of its ligand (11).

Our studies do not identify the organ(s) of genesis of DBP but indicate that DBP levels are increased in pregnant women and women taking oral contraceptives (Table II). It seems likely, therefore, that DBP might originate in the liver and that its synthesis, as with certain other serum proteins during estrogen therapy (12), is augmented by estrogen administration. The lower DBP levels in premature cord sera might also reflect hepatic immaturity, since term cord levels were found to be like those of adults. Since even the premature cord levels of DBP most likely represent an excess of carrier protein to ligand, the demonstrated close relationship of maternal serum and premature cord 25-OHD concentrations (13) is not surprising. The recent demonstration of the vitamin D and 25-OHD binding properties of Gc is of considerable interest (14). Earlier estimates of serum Gc concentration were slightly higher than those presently observed and indicated the likelihood that Gc or DBP was of hepatic origin (15). Available evidence suggests the identity of Gc and DBP and provides the basis for asking whether or not the recognized Gc variants (16) have physiological roles in man's adaptation to differences in ultraviolet exposure or vitamin D metabolism. Studies of the relationships among vitamin D metabolites and these genetic variants of the protein are required to explore this possibility.

Tracer studies indicate a widespread distribution

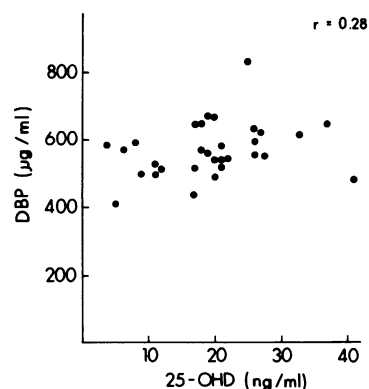


FIGURE 5 DBP concentration plotted as a function of the corresponding $25\text{-hydroxycalciferol}$ concentration in the sera of 31 normal adults.

of vitamin D and 25-OHD in human tissues (17). Recent attention has focused on the metabolite 1,25-dihydroxycalciferol, which is most potent in influencing skeletal and intestinal calcium transport (18), but a direct influence of 25-OHD on muscle metabolism has been observed (19). Although a separate carrier protein for 1,25-dihydroxycalciferol may exist, available evidence indicates its transport on DBP (4). Human serum DBP binds 1,25-dihydroxycalciferol less avidly than 25-OHD (4) and might suggest a relationship akin to that observed with corticosteroid binding globulin, aldosterone, and desoxycorticosterone (20). 24,25-Dihydroxycalciferol has been recently shown to compete strongly with 25-OHD₃ or 25-OHD₂ for binding to human DBP² (4). The recent observation of specific interaction between human serum retinol binding protein and membranes of retinol target tissues is provocative in its suggestion of new functions for carrier proteins (21). Although complex roles can be envisioned for DBP in modulating the storage, metabolism, and tissue availability of the parent vitamin and its metabolites, it presently seems most likely that the protein serves as a carrier-solubilizer of these sterols. Possibly, it provides a large (9 μ M) circulating reservoir which both conserves antirickettic sterols and protects against excessive delivery to the tissues, thereby accounting for the wide gap between therapeutic and toxic amounts of vitamin D. The concentrations of DBP in normal sera (Table II) identify it to be a major serum constituent since it represents approximately 6% of the serum alpha globulins.

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