Vitamin D Plasma Binding Protein

TURNOVER AND FATE IN THE RABBIT

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A B S T R A C T The metabolic disposition of the plasma binding protein (DBP) for vitamin D and its metabolites was studied in adult rabbits. Apo-DBP was purified from rabbit plasma and enzymatically labeled with radioiodine. The radioiodine-labeled protein retained its ability to bind vitamin D sterols and its physicochemical properties. When 125I-labeled DBP and 131I-labeled rabbit albumin were simultaneously injected intravenously, the 125I was cleared from plasma at a faster rate (t1/2 = 1.7 d) than 131I (t1/2 = 5 d) and 131I was present in excess of 125I in kidney, liver, skeletal muscle, heart, lung, intestine, testis, and bone 1 h after injection. In contrast to DBP, 25(OH)D3 was cleared more slowly (t1/2 = 10.7 d). Compared to albumin, DBP radioactivity appeared earlier and in greater quantity in the urine of catheterized rabbits. Gel filtration analyses of plasma revealed most of the 125I to elute in the position of DBP, with only small amounts in the <1,000-dalton region. In contrast, almost all of the urine 131I eluted in this small molecular weight fraction. The molar ratio of DBP to 25(OH)D3 in normal rabbit plasma was 138/1. The extravascular pool of DBP was calculated to be 1.5–2.4 times larger than the intravascular DBP pool, and the molar replacement rate of DBP was 1,350-fold higher than that for 25(OH)D3. The plasma disappearance curves of holo-DBP, prepared either by saturating with 25(OH)D3 or by covalently linking 3β-bromoacetoxy-25(OH)D3, were very similar to that of apo-DBP. Neuraminidase treatment of DBP did not alter its plasma survival.

These studies indicate that DBP or DBP-25(OH)D3 complex is removed from plasma by a variety of tissues, that the DBP moiety is degraded during this process, and that a significant recirculation of 25(OH)D3 probably occurs. The molar excess of DBP to 25(OH)D3 in plasma, and the relatively rapid turnover of DBP indicate that a high capacity, high affinity, and dynamic transport mechanism for vitamin D sterols exists in rabbit plasma.

INTRODUCTION

Although recognized for 20 yr (1), the human plasma binding protein (DBP)1 for vitamin D and its metabolites has only recently been isolated and characterized (2–4). It is an alpha globulin of ~58,000 mol wt, containing one steroid binding site per molecule with the highest binding affinity for the vitamin D metabolites, 25-hydroxycholecalciferol [25(OH)D3] and 24,25-dihydroxycholecalciferol [24,25(OH)2D3]. This protein is now known to be physicochemically and immunologically identical to the group specific component (Gc) human plasma protein (2, 3, 5). There is some evidence to suggest that this protein is synthesized in the liver (6, 7) and it occurs in high concentrations (6–8 μM) in plasma of man (5, 7–9) and rat (10, 11), whether measured by immunoassay or by saturation analysis of plasma 25(OH)D binding capacity. When compared with the likely total content of the vitamin D sterols (0.1–0.2 μM) in plasma in vitamin D-sufficient mammals it is apparent that the apo-DBP is present in remarkable excess of its ligand.

The plasma concentration of DBP is unaffected by a wide variety of disorders of mineral homeostasis (5, 7, 9) and unlike the retinol-binding protein, which declines in concentration during deficiency of vitamin A (12), the plasma level of DBP does not decrease during vitamin D deficiency. Although patients with cirrhosis and nephrotic syndrome (7) have a reduced plasma concentration of DBP, it nevertheless remains far in

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1 Abbreviations used in this paper: 25(OH)D3, 25-hydroxy-vitamin D; 1,25(OH)2D3, 1,25-dihydroxyvitamin D; DBP, binding protein in plasma for 25(OH)D3.
excess of that required to bind the normal amounts of vitamin D sterols in the blood. In analyses of over 80,000 different samples of human plasma (13) none were found where DBP (Gc) was absent, thus suggesting a vital role for this plasma protein.

To date, there is no information on the plasma turnover rate or the metabolic fate of DBP. To help define these purified rabbit plasma DBP was labeled with radioiodine and its plasma clearance and tissue accumulation were studied in rabbits. These experiments reveal that DBP, either in the apo or the holot form, is cleared quickly from plasma by a number of tissues and during this process is degraded to small molecular weight fragments.

METHODS

Materials. Crystalline rabbit plasma albumin, bovine serum albumin, type V, lactoperoxidase (milk, 67 U/mg protein), neuraminidase (type VIII, Clostridium perfringens, dithiothreitol, iodoacetamide, and blue dextran were purchased from Sigma Chemical Co., Dorset, England. ¹²¹Iodine (carrier-free) and ¹³¹Iodine (free from reducing agents) were obtained from The Radiochemical Centre, Bucks., England. Bromoacetic acid (practical grade) and dicyclohexylcarbodiimide were obtained from Eastman Organic Chemicals Div., Eastman Kodak Co., Rochester, N. Y. Calibration proteins for determination of molecular weight were obtained as a kit from Boehringer Corp., East Sussex, England.

Sterol studies. 25-hydroxy [26,27-³H]cholecalciferol (25(OH)[26,27-³H]D₃) (9.6 Ci/mmol, The Radiochemical Centre) was 90% pure as determined by silica gel 19254 thin-layer chromatography (14). Unlabeled 25(OH) D₃ was a gift from Hoffmann-La Roche, Basel, Switzerland, and its purity and quantity were assessed by ultraviolet spectrometry. For studies in vivo, 1.5 ml rabbit plasma was mixed with 3 μCi of 25(OH)[26,27-³H]D₃ dissolved in 10 μl ethanol and kept at 4°C overnight. Polyacrylamide gel electrophoresis revealed >95% of the ³H to be associated with a protein in the alpha globulin region. After intravenous injection of the 25(OH)-[26,27-³H]D₃ complex into rabbits, blood samples were taken at intervals for plasma analysis. These samples (1 ml) were extracted with chloroform-methanol (15). Radioactivity was measured in the 25(OH)D₃ fraction obtained by silicic acid chromatography (16).

Purification of rabbit DBP. DBP, labeled with tracer quantities of 25(OH)[26,27-³H]D₃, was isolated in pure form from 100 ml of normal pooled rabbit plasma by dialysis, DEAE ion-exchange column chromatography, gel filtration, on an AcA 44 column, and preparative 7% polyacrylamide gel electrophoresis. The final product was purified 175-fold relative to plasma and was judged to be homogeneous by analytical polyacrylamide gel electrophoresis (Fig. 1). In addition, the final preparation induced a monospecific antiserum in sheep which recognized only DBP in rabbit serum on agar gel diffusion or electrophoresis. The sheep anti-rabbit DBP antiserum did not react with DBP in human, monkey, rat, or chicken plasma. The concentration of DBP in rabbit serum was measured using the sheep anti-rabbit preparation in radial immunodiffusion assay (17), on gels made with 1% agarose in 0.025 M sodium barbitone buffer, pH 8.6.

Labeling of proteins with ¹²¹I and ¹³¹I. Purified rabbit plasma DBP and rabbit plasma albumin were labeled with ¹²¹I and ¹³¹I, respectively by the lactoperoxidase method of Miyachi et al. (18). After labeling with radioiodine, 0.5 ml of 0.05 M sodium phosphate buffer, pH 7.5, containing 1.0 mg potassium iodide was added. The entire incubation volume was then layered onto a 0.5 × 17-cm column of Sephadex G-50 (Pharmacia Fine Chemicals Ltd., London, England) equilibrated in 0.02 M sodium phosphate containing 0.3 M sodium chloride, pH 7.4. The column had been precoated by running through it 1 ml 0.5% bovine serum albumin in the column buffer and the void volume had been determined with blue dextran. The latter phase of the void volume was collected. Radioiodine was removed by this step, and additional analyses by polyacrylamide gel electrophoresis or gel filtration on 1 × 40-cm columns of Ultragel AcA54 (LKB, Bromma, Sweden) were carried out. Specific activities of 25–50 μCi/μg protein were routinely obtained with this procedure. De-natured DBP was produced by treating ¹²¹I-labeled DBP in 1% sodium dodecyl sulphate and 2 mM dithiothreitol overnight at room temperature, followed by the addition of 0.02 M iodoacetamide for a further hour. More than 99% of the dialyzed product’s radioactivity precipitated in the presence of cold trichloroacetic acid.

Preparation of holo-25(OH)D₃-DBP. Three different approaches were taken in the studies with holo-25(OH)D₃-DBP.

(a) Radioiodinated apo-DBP was incubated in vitro with a twofold molar excess of unlabeled 25(OH)D₃. The radio-labeled DBP, in 1.5 ml cold 0.02 M sodium phosphate, 0.15 M sodium chloride, pH 7.5, was added to the 25(OH)D₃ in 10 μl ethanol and the preparation was mixed and incubated at 0°C for 2 h before injection into rabbits. Preliminary experiments with 25(OH)[26,27-³H]D₃ were conducted to assess the abilities of unlabeled and ¹²¹I-labeled apo-DBP to bind this sterol. After incubation the unbound sterol was removed with dextran-coated charcoal (16). The supernate was counted in a gamma spectrometer and extracted with chloroform-methanol (2:1, vol/vol). The chloroform phase was washed three times with water and assayed for ³H. Calculations based on the known specific activities of ¹²¹I-DBP and 25(OH)[26,27-³H]D₃ revealed that both preparations were capable of binding nearly equimolar amounts of the sterol.

(b) In one experiment an attempt was made to saturate plasma DBP in vivo by injecting an amount of unlabeled 25(OH)D₃ equivalent to 1.5 times the molar amount of DBP in the plasma pool. The sterol was injected intravenously in propylene glycol, 10 min before the injection of holo-DBP labeled with radioiodine.

(c) A covalent holo 25(OH)D₃-DBP preparation was made by a modification of an affinity labeling procedure previously described (19). Monobromoacetic acid was distilled and the fraction of boiling point 202–204°C was collected. Tetrahydrofuran was passed over a column of neutral alumina and dried over potassium hydroxide pellets. To a stirred solution of 1.2 mg (3 μmol) 25(OH)D₃ and 2 × 10⁶ dpm 25(OH)-[26,27-³H]D₃ in 1.1 ml dry tetrahydrofuran at 22°C were added 0.5 mg (3.6 μmol) bromoacetic acid in 0.5 ml of tetrahydrofuran. After 5 min 30 μl (0.36 μmol) dry pyridine was added and the mixture was stirred for 2 h at 22°C. The solution was filtered through a fritted funnel and dried under nitrogen. The material was dissolved in chloroform/acetonitrile (9:1, vol/vol) and applied to 0.4 mm silica gel thin-layer plates. Ascending chromatography was carried out in the same solvent mixture to a 15 cm height. The products, 3β-bromoacetoxy-25(OH)D₃ (retardation factor; Rf = 0.60) and 25-(OH)D₃ (Rf = 0.36) were located under ultraviolet (UV) light. The esterified sterol band was scraped from the plate and the silica gel was extracted with three 10-ml vol of diethyl ether. After removing the solvent under nitrogen the product had a UV spectrum in ethanol identical to that of 25(OH)D₃ and it transformed to 25(OH)D₃ during saponification in 0.1 N potassium hydroxide in ethanol. Specific activity and UV extinction
measurements indicated a 61% yield of ester. In equilibration competition experiments, the ester was 8-10 fold less potent than equimolar amounts of 25(OH)D₃ in displacing 25(OH)[26,27-3H]D₃ from DBP. However, when the ester was incubated with equimolar amounts of DBP for 4 h at 22°C, more than 85% of subsequently added 25(OH)[3H]D₃ was not bound by the DBP. The ester was covalently bound to the protein, as indicated by the presence of 3H in slices of sodium dodecyl sulfate-polyacrylamide gels loaded with the ester-protein mixture previously heated at 70°C in 1% sodium dodecyl sulphate for 1 h. On the basis of these observations, 30 μg ³¹I-DBP was incubated with 321 ng 3β-bromoacetoxy-25-25(OH)D₃ for 4 h at 22°C, and this preparation was used in vivo for assessment of the disposition of holo-DBP with covalently bound ligand.

Animal experiments. New Zealand White and Dutch Belted rabbits, 1.8-3.6 kg, were supplied with standard rabbit chow (Cranefeed Mills, Herts., England) ad lib., and given 0.005% potassium iodide drinking water starting at least 48 h before the experiment. Male rabbits were sedated with a single intravenous injection of pentobarbitol (25 mg/kg body wt) for the purpose of bladder catheterization with a number 8 paediatric bladder catheter (Warner Surgical Products Ltd., Hants., England). Radioactive DBP (1.9-5.5 μg; 106-275 μCi ³¹I) and/or radioactive albumin (1.5-4.9 μg; 75-243 μCi ³¹I) was injected in 0.5 to 1.5 ml 0.02 M sodium phosphate, 0.15 M sodium chloride, pH 7.5 into a marginal ear vein. Blood samples from the marginal vein of the opposite ear were collected in heparinized tubes. Plasma was obtained by centrifugation at 500 g for 15 min at 4°C. Plasma or cytosol protein, precipitated with cold trichloroacetic acid (7% wt/vol), was counted in a gamma spectrometer. Plasma and urine were also analyzed by gel filtration and gel electrophoresis. Urine was obtained by gravity flow, syringe aspirations of the bladder catheter and a bladder rinsing with 15 ml 0.9% (wt/vol) sodium chloride at intervals during each experiment. Rabbits were killed by the rapid intravenous injection of 240 mg pentobarbitol. Organs and tissues were removed within 20 min of death. They were rinsed in 0.9% sodium chloride and weighed before analyzing for radioactivity. Abdominal wall adipose tissue was excised and the entire right tibia was pulverized for a representative bone sample. Skeletal muscle was obtained from the right thigh and duodenum was used as the sample of small intestine. Gut lumenal contents were washed out before weighing and analysis of the intestinal tissue. Cytosols were prepared by homogenizing tissue (1:1, vol/wt) in cold 0.05 M Tris/HCl buffer pH 7.4 containing 0.15 M sodium chloride, 2 mM dithiothreitol and 2 mM EDTA. After centrifugation at 500 g for 10 min at 4°C the supernates were centrifuged at 100,000 g for 60 min at 4°C. The particle-free supernates were stored at -20°C before analysis.

In bred hooded rats (200 g) of the Dunn Nutritional Laboratory strain, given adequate diet and potassium iodide drinking water, were used in preliminary experiments to study the fate of labeled rabbit DBP.

Measurement of radioactivity. Tritium was assayed in a Packard Tri-Carb model 3375 scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.) using toluene or toluene-Triton X-100 scintillation solutions. Quenching was corrected by automatic external standardization.

Radioiodine in all the samples was analyzed in the same type of plastic counting vials and corrections were made for variation in counting efficiency, isotope decay and isotope spillover in a well-type, double-channel gamma spectrometer (ICN Instruments Division, Antwerp, Belgium). Variation in the counting efficiency for ³¹I was minimal in samples from different tissues. 30 min after intravenous injection of ³¹I-labeled and ³¹I-labeled rabbit serum albumin or ³¹I-labeled and ³¹I-labeled rabbit DBP into rabbits, the ratio of ³¹I to ³¹I in the plasma was within 1% of the original value, but the ratio was reduced by factors of 0.95 in liver and 0.91 in bone. Since in all other tissues the ratio was within 2% of the preinjection value, corrections for the presence of ³¹I in tissue were made only for liver and bone.

Other methods. Gel filtration analyses on columns of Ultragel AcA54 and AcA44 were performed under constant pump pressure at 4°C. Polyacrylamide gel-electrophoresis (20), sodium dodecyl sulphate-polyacrylamide-gel electrophoresis (21), serum creatinine (22) and 25(OH)D binding assays (16) were performed as previously described.

Statistical analysis. The turnover data was calculated by a noncompartmental, mathematical analysis of the plasma specific radioactivity curves (23). In this method, replacement rate was obtained by dividing the injected dose by the area under the plasma curve from zero time to infinity. Minimal transit time and minimal body pool are the transit time and pool size if it is assumed that catabolism is solely from the sampling pool. Minimal transit time and maximal body pool are the transit time and pool size if it is assumed that catabolism all occurred at a site removed as far as possible from the sampling pool. In the calculation of the maximal transit time, a sum of the reciprocals of the exponents was not employed, as the early exponential slopes were not comparable in magnitude to the terminal one; (a fit of a 2 exponential model to the data indicated the earlier exponentials to be 10 times the terminal one). Since the semilogarithmic plot of the plasma curve attained a terminal constant slope, maximal transit time was calculated from the reciprocal of the terminal exponential, and calculation of maximal body pool assumed that the fractional rate of catabolism was uniform throughout the system. Minimal transit time was calculated graphically. Synthesis was equated to the calculated replacement rate, assuming a steady state to have prevailed throughout the study. This was actually the case as judged by rabbit weights, hematocrit, and serum protein values.

The significance of the differences between the ratio of ³¹I/mg in the tissue and the ratio of the injected material (see Table II) was analyzed by the Student's t test.

RESULTS

Properties of rabbit plasma DBP. The final preparation of purified DBP was homogeneous when analyzed by polyacrylamide gel electrophoresis (Fig. 1). Gel filtration analyses revealed that the 25(OH)[26,27-³H]D₃-DBP complex had eluted in a single peak with an apparent mol wt of 58,000 (Fig. 2B). This was identical to the elution of radioactivity when the column was charged with normal rabbit plasma incubated with tracer quantities of 25(OH)[26,27-³H]D₃ (Fig. 2A). The purified DBP from rabbit plasma had alpha mobility on electrophoretic analyses in 7% polyacrylamide gels. The absorption spectrum of purified rabbit plasma DBP revealed maximum absorbance at 275 to 280 nm with a 260/280-nm absorption ratio of 1.20. As revealed by saturation analyses, rabbit DBP bound 25(OH)D₃ with higher affinity than cholecalciferol or 1,25-dihydroxycholecalciferol (1,25(OH)₂D₃). Radial immunodiffusion quantitation of DBP in rabbit plasma, utilizing purified DBP as reference standard, indicated the concentration to be 400 ng/ml (6.9 μM).
FIGURE 1 Sodium dodecyl sulphate-polyacrylamide (7%) gel electrophoresis of 40 μg purified rabbit plasma DBP. The wire indicates the position of migration of the anodal dye, bromophenol blue.

Rabbit plasma DBP, therefore, closely resembles human plasma DBP in its physicochemical characteristics, binding preference for the vitamin D metabolites, and plasma concentration. Under normal conditions, 25(OH)D₃ is the predominant form of vitamin D in mammalian plasma. Since only tracer amounts of ³H-labeled 25(OH)D₃ were used in purification of the DBP and since the normal rabbit plasma levels of 25(OH)D were 20 ng/ml (50 nM), ~99% of the purified rabbit DBP was in the apo form.

Properties of the radiolabeled proteins. When ¹²⁵I-labeled DBP from the void volume of Sephadex G-50 column was applied to a longer column of Ultragel AcA44, the elution profile presented in Fig. 2B was obtained. More than 95% of the ¹²⁵I-eluted in a symmetrical peak with an apparent mol wt of 58,000, corresponding to the elution patterns observed when plasma (Fig. 2A) or purified DBP (Fig. 2B) had been applied after incubation with 25(OH)[26,27-³H]D₃. Occasionally a small amount of radioactivity spread from the void volume to the DBP region. The nature of this dispersed ¹²⁵I-labeled material has not been determined although it may have resulted from protein alteration during the ¹²⁵I-labeling procedure. Radiolabeled material from the DBP region was also homogeneous when analyzed by 7% polyacrylamide gel electrophoresis. Since radioactivity in the void volume and small molecular weight regions increased slightly after protracted storage of ¹²⁵I-labeled DBP, experiments with this protein were done within 24 h of its preparation and purification by gel filtration.

The polyacrylamide gel electrophoretic analyses of gel-filtered, radiolabeled rabbit serum albumin revealed >95% of the radioiodine to filter or migrate as a homogenous band corresponding to that exhibited by reference albumin.

Preliminary studies in rats indicated a more rapid plasma clearance of radiolabeled rabbit DBP than albumin, and a preferential accumulation of DBP, compared to albumin, in most tissues examined. Unfortunately, the rat serum DBP has a high tendency to polymerize (11). This consideration, and our desire to examine a homologous system, prompted our carrying out further studies in rabbits.

Vitamin D Plasma Binding Protein 1553
Plasma survival of radiolabeled proteins and 25(OH)D₃. Fig. 3 presents the early disappearance of [¹²⁵I]-labeled DBP and [¹³¹I]-labeled rabbit albumin from the plasma of a normal rabbit after the simultaneous intravenous injection of these proteins. During the first minutes, both proteins disappeared from the plasma at similar rates but a more rapid egress of DBP was apparent by 1 h after injection. At this time the mean [¹²⁵I]/[¹³¹I] ratio found in four rabbits was 0.95, which was significantly less (P < 0.02) than the ratio of the injected material. The curves describing the disappearance of both proteins were multieponential within the first day. After a brief delay, both isotopes appeared in the urine and a greater rate of urinary excretion of [¹²⁵I] was observed. At 24 h, the plasma disappearance rate of DBP was clearly more rapid and only 16% of the dose remained in the vascular compartment. Over 24 h ~22% of the dose of [¹²⁵I] and 10% of the dose of [¹³¹I] appeared in urine. The divergent rates of plasma clearance of these proteins remained distinct after 48 h when only 9% of the injected DBP was present in plasma. In contrast to the behavior of these proteins, denatured DBP was cleared more rapidly with <10% of the dose remaining in plasma 8 h after injection (Fig. 3).

The later phases of the plasma disappearance of the two proteins and 25(OH)[26,27-³H]D₃ are depicted in Fig. 4. The apparent plasma removal rate of the sterol was similar to those of the proteins only during the first few hours after injection (Fig. 3). The later rate of sterol disappearance described a slope indicating a plasma t₁/₂ of ~11 days. The terminal slope of [¹²⁵I]-labeled DBP plasma disappearance indicated a t₁/₂ of 1.7 d and that for [¹³¹I]-labeled albumin was 5 d. A total of five rabbits was studied and these were found to exhibit plasma survival curves of DBP and albumin similar to those shown in Figs. 3 and 4. An additional rabbit, injected with 25(OH)[26,27-³H]D₃ bound to normal rabbit plasma, displayed a terminal plasma disappearance rate of this sterol closely similar (t₁/₂ = 10.5 d) to that indicated in Fig. 4. Base-line plasma concentrations of DBP or 25(OH)D were almost identical to those measured during and after the experiments. Table I summarizes the analyses of the plasma survival curves.

Plasma survival of neuraminidase-treated DBP and holo-25(OH)D₂-DBP. Factors which might affect the plasma clearance rate of DBP have been considered. The role of sialic acid residues in the uptake by liver of plasma proteins was shown by Ashwell and Morell (24) but we found that the presence of this residue on DBP is not essential for its normal turnover rate. Presumptive evidence for heterogeneity in purified rabbit DBP was revealed by neuraminidase treatment (3 U/0.1 ml in 0.1 M sodium acetate, pH 5.3, for 15 h at 37°C) of 3 μg [¹²⁵I]-labeled DBP. When this preparation and [¹³¹I]-labeled DBP incubated without enzyme were analyzed by polyacrylamide gel electrophoresis, a higher [¹²⁵I]/[¹³¹I] ratio was observed in cathodal slices of the DBP band. These findings suggest that sialic acid was present in anodal portions of the DBP preparation as had been reported for human DBP (25–27).
The plasma disappearance of trichloroacetic acid-precipitable $^{125}$I and $^{131}$I was studied in a rabbit injected simultaneously with $^{125}$I-labeled DBP which had been treated with neuraminidase and $^{131}$I-labeled DBP which had not been exposed to this enzyme in vitro. The plasma survival curves of these two preparations are shown in Fig. 5. The removal of sialic acid from the "fast" electrophoretic moiety of DBP did not cause any apparent alteration in its plasma survival.

It is also possible that the turnover rate of DBP is affected by the presence or absence of 25(OH)D$_3$. Saturation of $^{125}$I-labeled DBP with 25(OH)D$_3$ in vitro did not alter its rate of egress from plasma (data not presented). A similar preparation was injected 10 min after the slow intravenous injection of 480 $\mu$g (1.2 mmol) of 25(OH)D$_3$, which was a 1.5-molar excess of ligand for the calculated intravascular pool of DBP (~50 mg or 0.8 $\mu$mol for a 3-kg rabbit). As indicated in Fig. 6 the plasma disappearance of the $^{125}$I-labeled DBP was not substantially changed by these procedures. Plasma creatinine was not increased after this 25(OH)D$_3$ injection but plasma 25(OH)D$_3$ concentrations were only near DBP saturation levels (2.7 $\mu$g/ml) at 5 min after injection and they fell rapidly over 24 h to ~0.9 $\mu$g/ml, reaching a level of 0.2 $\mu$g/ml 7 d later.

The plasma survival of $^{125}$I-labeled DBP bound to the affinity labeling reagent, 3$\beta$-bromocetoxy-25(OH)D$_3$, is also shown in Fig. 6. With this preparation of ligand covalently bonded to its carrier protein, the plasma survival of $^{125}$I-labeled DBP was unchanged compared to apo-DBP. Total urinary excretion of $^{125}$I was similar to that found after injection of apo-DBP as was the presence of only low-molecular weight $^{125}$I in urine and the predominantly DBP-like $^{125}$I in gel-filtration analyses of plasma.

Properties of radiolabeled materials in plasma and urine. Analyses of plasma obtained 5 min after injection of $^{125}$I-labeled DBP revealed a gel filtration profile similar to the one shown in Fig. 2B. Samples taken at 9 d after injection revealed no less than 92% of the radioactivity to elute in a single peak with an apparent mol wt of 58,000 (Fig. 3C). The remainder of the radioactivity was found in the region of apparent mol wt < 1,000. Polyacrylamide gel electrophoresis revealed the large-molecular weight material to be DBP and the small-molecular weight material to have a net negative charge, running near the bromphenol blue front. The nature of this material has not been precisely determined but iodothyrosine, free iodine, or iodine attached

### Table I

<table>
<thead>
<tr>
<th>Parameter</th>
<th>DBP</th>
<th>25(OH)D$_3^*$</th>
<th>Molar ratio DBP/25(OH)D$_3$</th>
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<td>Plasma concentration</td>
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<tr>
<td>Replacement rate</td>
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<td>4.7 ng/h/kg</td>
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<tr>
<td>Minimal total body pool</td>
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<td>1708 ng/kg</td>
<td>162.1</td>
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<tr>
<td>Maximal mean transit time</td>
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<tr>
<td>Maximal total body pool</td>
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<tr>
<td>Fractional catabolic rate, fraction of intravascular pool per day</td>
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<td>Plasma t$_{1/2}$</td>
<td>40.6±0.46 h</td>
<td>257.8 h</td>
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</table>

Calculations for DBP represent the mean±SEM or range of means obtained in five separate experiments, and those for 25(OH)D$_3$ are the mean or range of values in two separate experiments.

* These calculations for 25(OH)D$_3$ assume, probably incorrectly, an irreversible removal of the 25(OH)D$_3$ from the sampling (plasma) pool.

![Figure 5](image-url)

**FIGURE 5** Plasma survival curves of neuraminidase-treated $^{125}$I-labeled DBP (○) and untreated, $^{131}$I-labeled DBP (●) after their simultaneous intravenous injection into a rabbit.
to small peptides are likely possibilities. This small-sized material comprises the major portion of the radioactivity in urine collected at any time within 48 h of injection of 125I-labeled DBP (Fig. 2C). Frequently a small amount of apparently intact 125I-labeled DBP appeared in urine samples but this never accounted for >1% of the urine radioactivity. Gel filtration profiles of urine from rabbits injected with 125I-labeled albumin revealed almost all of the 125I to be in a small mol wt form (data not presented). Tissue, rather than plasma alone, appeared to be required for the formation of low-molecular weight 125I-material since more than 99% of 125I-labeled DBP incubated with plasma at 37°C for 1 h remained intact. In a single experiment, the low-molecular weight 125I-material was found in plasma 2 h after the injection of 125I-labeled DBP into a bilaterally nephrectomized rabbit.

After injection of 25(OH)D3, the major distribution of 3H in plasma was observed to be associated with DBP. More than 90% of the radioactivity in plasma co-migrated with the binding protein on gel filtration or electrophoresis. Chromatography of lipid extracts of plasma indicated that >88% of plasma-3H ran with standard 25(OH)D3.

**Tissue distribution of radiolabeled proteins.** In preliminary experiments with rats, the simultaneous intravascular injection of 125I-labeled rabbit DBP and 131I-labeled rabbit albumin gave results suggesting the relatively greater accumulation of DBP in a variety of tissues at all times from 1 to 24 h after injection. Studies in one rabbit at each of several times after the simultaneous intravenous injection of 125I-labeled DBP and 131I-labeled rabbit albumin revealed an excess of 125I in many tissues shortly after injection. The significance of these early results was confirmed by statistical analysis of data obtained from four rabbits sacrificed 60 min after injection (Table II). When compared to the dose ratio and the values in the 5 min plasma, the plasma DBP/albumin ratio was significantly reduced in plasma obtained 60 min after injection. The previously demonstrated difference between the plasma survival of DBP and albumin, which are proteins of similar size, suggested the possibility of a tissue uptake of DBP that was not solely attributable to diffusion into the extracellular fluid. When tissue ratios were calculated on the basis of the total amount of each isotope administered, a significant excess of 125I was found in all tissues examined except adipose tissue. Highest ratios were found in kidney, intestine, and muscle.

When corrected for extracellular fluid contamination, the calculated organ distribution of 125I-labeled DBP still revealed highest accumulations in kidney, liver, and skeletal muscle (Table III). Four rabbits that had received the radiolabeled proteins 24 h earlier were also sacrificed. At this time the tissue data revealed 125I accumulation to be increased in bone and adipose tissue compared to the 1-h data. When considered as the amount of 125I accumulated per gram wet weight of tissue, kidney was the most efficient extractor and high concentrations were also noted in lung, liver, and heart.

**Properties of radiolabeled material in tissue cytosols.** Cytosols from tissues of one rabbit sacrificed

**TABLE II**

Relative Tissue Distribution of 125I and 131I at 1 h after the Simultaneous Intravenous Injection of 125I-labeled DBP and 131I-labeled Rabbit Serum Albumin

<table>
<thead>
<tr>
<th>Organ or tissue</th>
<th>125I/131I</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td>0.95±0.01*</td>
</tr>
<tr>
<td>Liver</td>
<td>1.20±0.02†</td>
</tr>
<tr>
<td>Kidney</td>
<td>1.83±0.09†</td>
</tr>
<tr>
<td>Skeletal muscle</td>
<td>1.49±0.04†</td>
</tr>
<tr>
<td>Heart</td>
<td>1.19±0.04*</td>
</tr>
<tr>
<td>Lung</td>
<td>1.24±0.05*</td>
</tr>
<tr>
<td>Small intestine</td>
<td>1.43±0.06†</td>
</tr>
<tr>
<td>Large intestine</td>
<td>1.62±0.06†</td>
</tr>
<tr>
<td>Testis</td>
<td>1.22±0.02†</td>
</tr>
<tr>
<td>Bone</td>
<td>1.12±0.02†</td>
</tr>
<tr>
<td>Adipose tissue</td>
<td>1.04±0.02</td>
</tr>
</tbody>
</table>

Four rabbits were injected. The ratios were calculated on the basis of the percentage of the total amount of each isotope administered. Values are mean±SEM.

* The value differs from 1.00 at P < 0.02.
† The value differs from 1.00 at P < 0.01.
60 min after the simultaneous injection of \(^{125}\text{I}\)-labeled DBP and \(^{131}\text{I}\)-labeled albumin, were analyzed on gel filtration columns (Fig. 7). Radioactivity in plasma was almost entirely in the elution volumes corresponding to these proteins but a small amount of isotope (1.7-2.2\% of total radioactivity eluted) formed a low-molecular weight (<1,000) peak (Fig. 7A). In tissue cytosols, the \(^{125}\text{I}\) in the small-molecular weight peak comprised a higher percentage of the total \(^{125}\text{I}\) eluted (5-25\%) than it did in plasma. Although diffusion of low-molecular weight material into extravascular fluid could explain its exaggerated presence compared with plasma, a comparison of the percentages of total column radioactivity in low-molecular weight form revealed a higher proportion of \(^{125}\text{I}\) to \(^{131}\text{I}\) in most instances. Analysis of muscle cytosol is shown in Fig. 7B and the \(^{125}\text{I}\) profile of kidney cytosol in Fig. 7C. The statistical significance of the relatively higher tissue accumulations of DBP (Table II) was not altered by subtraction of the low-molecular weight \(^{125}\text{I}\) components in cytosols.

In all cytosols examined, another feature consistently observed was the presence of high-molecular weight, \(^{125}\text{I}\)-labeled material eluting at or near the void volume and extending into the volume of DBP elution (Figs. 7B and 7C). The nature of this material was studied in several cytosols containing sufficient radioactivity for analysis by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (liver, heart, kidney, and lung). In each instance the high-molecular weight \(^{125}\text{I}\) was not found in slices of these gels and radioactivity was present only in positions characteristic of DBP and free \(^{131}\text{I}\). Most plasma samples from rabbits injected with radiolabeled DBP were free of the high-molecular weight material but small amounts were seen in samples from hemolyzed blood. The exact nature of the high-molecular weight radioactivity in such plasma was not determined.

**DISCUSSION**

The studies presented here show that rabbit plasma DBP can be labeled with radiiodine without causing significant alterations to its sterol-binding ability or physicochemical characteristics. Labeled DBP retained both its ability to bind \(^3\text{H}\)-labeled 25(OH)D\(_3\) in vitro as well as the electrophoretic and gel filtration behavior of the native protein. Since purposefully denatured labeled DBP was cleared much more rapidly from plasma after injection into rabbits, the more protracted plasma survival of native DBP apparently depends on the retention of its tertiary conformation. The large amount of endogenous plasma DBP prevented verification of the \(^{125}\text{I}\)-labeled DBP plasma survival data by immunoquantitation after its injection. However, the plasma survival of \(^{14}\text{C}\)-labeled DBP prepared by the method of Dottavio-Martin and Ravel (28)
disposition of this sterol and the carrier clearly diverge at some point. The experiments with 125I-labeled rabbit DBP indicate that this protein is cleared from plasma by a large number of tissues. The protein appears to be degraded during this process since shortly after the intravenous injection of 125I-labeled DBP, a form of 125I with a mol wt < 1,000 appears in the plasma and is rapidly excreted in the urine. From the assumption that the difference between the plasma survival of 125I-labeled DBP and 125I-labeled albumin represents the tissue uptake of 125I that is not solely attributable to diffusion into the extracellular fluid, an association of DBP with tissue elements must be considered. The preferential accumulation of 125I-labeled DBP compared to 125I-labeled albumin is supportive in this regard. However, the precise nature of the tissue accumulation and degradation of DBP is not revealed by our studies. After injection of 25(OH)-[26,27-3H]D₃ and 125I-labeled DBP, similar rates of disappearance from plasma were seen for only a very brief period (Fig. 3) with subsequent increases in the plasma ³H to 125I ratio. A dissociation of the sterol-protein complex must occur at some site yet to be identified.

25(OH)D₃ has been found distributed to many human tissues (30) and can exert a prolonged biological action after a single dose (31). Kinetic analyses of 25(OH)D₃ distribution suggest its tissue entry and subsequent egress into plasma (32). The apparently protracted plasma survival of 25(OH)D₃ (33, 34) may therefore reflect the difference between unmetabolized 25(OH)D₃ that recirculates between plasma and tissue and that which is metabolically transformed (Figs. 3 and 4). It is also possible that the dissociation of 25(OH)D₃ from DBP might occur in the extracellular compartment. The experiments with apo and holo-25(OH)D₃ DBP preparations (Figs. 4 and 6) indicated a very similar plasma clearance of these materials and therefore do not suggest a different clearing mechanism for these forms of DBP. Since we did not examine the relative tissue accumulations of apo-DBP and holo-DBP, a subtle difference in their tissue uptake remains a possibility. Our experiments with 125I-labeled DBP covalently bonded to 3β-bromoaceptoxy-25(OH)D₃ (Fig. 6) support the data obtained with in vivo and in vitro saturation of 125I-labeled DBP, but in vivo hydrolysis of the sterol ester may have occurred.

A substantial metabolism of plasma proteins is recognized to occur in liver, kidney, and intestine (35). Experiments with neuraminidase-treated DBP (Fig. 5) revealed that this plasma protein is not likely cleared by binding to hepatic receptors that recognize galactose residues after terminal sialic residues are removed (24). Since bilateral nephrectomy did not affect the initial plasma turnover rate of DBP, it seems that renal tissue is not the main site of DBP metabolism. Our failure to identify intermediate size fragments of DBP in the
plasma or urine, and the appearance of small molecular weight fragments in these fluids and tissue cytosols shortly after intravenous injection of DBP, suggest the action of multiple proteases in the degradation of this protein. This suggests the possibility of lysosomal activity which has been linked to receptor-mediated endocytosis of several proteins (36–38). Our experiments in vivo do not provide direct evidence for cellular association or uptake of DBP, and further studies are required to examine this possibility. It is of interest, however, that a cellular protein is recognized to bind DBP (39–41) and that DBP complexed to this protein has been observed in cytosols from well-washed, cultured fibroblasts (41), well-perfused kidney (42), in well-washed kidney subcellular fractions (42, 43) and in well-washed, cultured bone cells (44). Others, however, have not observed the complexing of DBP by extracts from well-washed cells cultured without serum (39). Recent evidence has been presented that the cellular DBP binding protein is actin. This provocative finding has yet to be explored, but does not guarantee an intracellular disposition of DBP because DBP might function to scavenge actin released into the circulation (45).

The metabolic pathway for vitamin D implicates many tissues in its formation, transformation, and biological action. Vitamin D itself and its potent metabolite, 1,25(OH)₂D₃, for example, are recognized to have plasma clearance rates and tissue distributions which are distinct from those of 25(OH)D₃ (30, 46–48). It is therefore difficult to present a unified hypothesis whereby DBP, the only specific binding protein for cholecalciferol sterols in mammalian plasma, plays a delivery function for all these sterols. Cholecalciferol and 1,25(OH)₂D₃ are bound by DBP with lower affinity than 25(OH)D₃ (2) and probably have additional mechanisms for their tissue delivery. The hepatic and adipose tissue accumulation of vitamin D may derive from the relatively greater association of cholecalciferol to plasma lipoproteins (49). The solubility of the more polar metabolite, 1,25(OH)₂D₃ may be such that at its usual plasma concentration (30 pg/ml) it is more available in the free form for uptake by target tissues for association with their high-affinity, soluble intracellular receptors (50–53).

Since the major sterol that is most avidly bound to mammalian plasma DBP is 25(OH)D₃, it is appropriate to consider the biological significance of it having a plasma transport protein of high capacity which turns over rapidly. For the transformation of 25(OH)D₃ to other metabolic forms, such a transport system would guarantee a continuing supply of substrate, even when circulating levels of 25(OH)D₃ are low (54), during which time DBP levels are unchanged (7). This interpretation, however, would only be attractive if carrier-mediated delivery of 25(OH)D₃ occurs. The rapid replacement of DBP with newly synthesized apo-DBP might also protect cells against excessive accumulation of 25(OH)D₃ by ensuring the cellular egress of unutilized 25(OH)D₃ away from 1,25(OH)₂D₃ receptor sites. A precise definition of the biological role of DBP, however, shall require further studies of the tissue disposition of this protein.

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