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Quantitative Studies of the Interaction of Cholecalciferol (Vitamin D_3) and its Metabolites with Different Genetic Variants of the Serum Binding Protein for these Sterols

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Cholecalciferol (vitamin D_3) and its 25-hydroxy metabolite are transported in plasma bound to a specific protein, the binding protein for cholecalciferol and its metabolites (DBP). DBP is identical with the group-specific component (Gc) proteins, which are known to display genetic polymorphism. Studies were conducted to explore whether or not major differences in the transport of cholecalciferol and its biological metabolites might exist among persons with different Gc phenotypes. Detailed quantitative studies were first carried out on the interaction of 25(OH)D_3 with DBP in 21 different samples of serum, representing eight different Gc phenotypes. The studies used a filter disc assay method that provided highly reproducible quantitative results with cholecalciferol-related sterols. The Gc phenotypes studied included the three common types (Gc 1-1, 2-1, and 2-2) and several uncommon genetic variants (Gc Ab-Ab, Ab-1, Ab-2, Chip-1, and Chip-2). The binding affinities for 25(OH)D_3 observed with these different sera were all fairly similar to each other. More extensive studies were then conducted to compare the binding of four cholecalciferol-related sterols to each of three genetic variants of DBP, by using sera from homozygous persons with the Gc 1-1, Gc 2-2 and Gc Ab-Ab phenotypes. The ligands tested included cholecalciferol, 25(OH)D_3, 1,25$(OH)_2D_3$, and 24(R) 25$(OH)_2D_3$. The affinities of the three genetic types of DBP/Gc protein were found to be similar for each of the four cholecalciferol-related sterols. The apparent association constants for 25(OH)D_3 and 24,25$(OH)_2D_3$ were similar (approx. $1–2\times10^8\,M^{-1}$); lesser affinities were observed for 1,25$(OH)_2D_3$ (k_A approx. $1\times10^7\,M^{-1}$) and for cholecalciferol (k_A approx. $3–4\times10^5\,M^{-1}$). Thus the common genetic variants of DBP/Gc protein, and the uncommon genetic variants studied here, all appear to have similar binding properties for cholecalciferol and its several metabolites.

It is now well established that cholecalciferol and its hepatic metabolite 25-hydroxycholecalciferol are transported in human plasma bound to a specific protein, the binding protein for vitamin D and its metabolites (DBP). DBP has recently been isolated and partly characterized in three laboratories (Imawari *et al.*, 1976; Haddad & Walgate, 1976*a*; Bouillon *et al.*, 1976*a*). Purified DBP has a molecular weight that has been estimated to be from 52000 (Imawari *et al.*, 1976) to 59000 (Haddad & Walgate, 1976*a*), and there is one binding site per molecule for one molecule of 25(OH)D_3. A similar binding capacity, of approx. 1 mol/mol, was also observed with the isolated protein for cholecalciferol and for 1,25$(OH)_2D_3$ (Haddad & Walgate, 1976*a*). The normal concentration of DBP in plasma is of the order of 300–600 μg/ml (Haddad & Walgate, 1976*b*; Imawari & Goodman, 1977; Bouillon *et al.*, 1977).

Purified DBP was found to be immunologically identical with the group-specific component (Gc) protein of human plasma (Haddad & Walgate, 1976*a*; Bouillon *et al.*, 1976*a*; Imawari & Goodman, 1977). These findings confirmed and extended the work of Daiger *et al.* (1975), who demonstrated binding of cholecalciferol and 25(OH)D_3 by the Gc proteins of human serum. Before these reports, the possible physiological function of Gc protein was not known.

The Gc proteins have been investigated extensively as a marker for genetic studies during the past 15 years. Gc proteins display genetic polymorphism, with three common phenotypes (Gc 1-1, Gc 2-1, and Gc 2-2) being found in somewhat varying proportions throughout the world (Cleve, 1973;

Abbreviations used: DBP, binding protein for vitamin D and its metabolites; Gc protein, group-specific component protein; 25(OH)D_3, 25-hydroxycholecalciferol; 1,25$(OH)_2D_3$, 1,25-dihydroxycholecalciferol; 24,25-$(OH)_2D_3$, 24,25-dihydroxycholecalciferol.

Giblett, 1969; Reinskou, 1968). A moderately large number of uncommon and rare Gc genetic variants and phenotypes have also been observed (Cleve, 1973; Giblett, 1969).

The present studies were designed to explore whether or not major differences in the transport of vitamin D and its biological metabolites might exist among persons with different Gc phenotypes. In order to examine this question, quantitative studies of the interaction of 25(OH)D_3 with DBP/Gc protein were carried out with serum samples representing both the three common Gc phenotypes and also several uncommon genetic variants. In addition, detailed quantitative studies were conducted to compare the binding of four cholecalciferol-related sterols to each of three different genetic variants of DBP/Gc protein from persons homozygous as to Gc type.

Materials and Methods

Serum samples

Samples of venous blood were obtained from normal healthy Caucasian and Japanese adults. After clotting of the blood samples, sera were separated by centrifugation and stored at −20°C. The Gc phenotypes of the samples were determined by immunoelectrophoresis (Reinskou, 1968; Hirschfeld, 1962), by using the monospecific rabbit antiserum against human DBP (i.e. against Gc protein) described previously (Imawari & Goodman, 1977).

Serum samples from aborigines of Waisa Village, New Guinea, and from Chippewa indians, were generously provided by Dr. A. G. Bearn of Cornell University Medical College. The samples had been stored for more than 10 years at approx. −70°C, and had been characterized as to Gc phenotype as described by Cleve *et al.* (1963).

Fifteen sera representing the three common Gc phenotypes (Cleve, 1973; Giblett, 1969) were used for binding studies. These sera included six samples with the Gc 1-1 phenotype (obtained from three Caucasians, one Japanese, one aborigine, and one Chippewa indian); six samples with the Gc 2-1 phenotype (two Caucasians, two Japanese, one aborigine and one Chippewa indian); and three samples with the Gc 2-2 phenotype (one Caucasian, one aborigine and one Chippewa indian). In addition, six samples representing uncommon genetic variants were used for binding studies. Two samples with the Gc Ab-Ab phenotype and one each with Gc Ab-1 and Gc Ab-2 (Cleve, 1973; Cleve *et al.*, 1963) had been obtained from New Guinea aborigines. One sample each of Gc Chip-1 and Gc Chip-2 had been obtained from Chippewa indians (Cleve *et al.*, 1963).

Sterols

25-Hydroxy[26,27-^{3}H]cholecalciferol [^{3}H-labelled 25(OH)D_3] (sp. radioactivity 11.3 Ci/mmol; Amersham/Searle Corp., Arlington Heights, IL, U.S.A.) was purified by chromatography on a column (1 cm × 20 cm) of Sephadex LH-20 (Pharmacia Fine Chemicals, Piscataway, NJ, U.S.A.) (Holick & DeLuca, 1971), each time before use. Cholecalciferol (vitamin D_3) was purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Pure crystalline 25(OH)D_3 was kindly provided by Dr. J. Babcock of The Upjohn Co., Kalamazoo, MI, U.S.A. Pure 1,25-$(OH)_2D_3$ and 24(R),25$(OH)_2D_3$ were the generous gifts of Dr. M. Uskokovic and Dr. W. Scott of Hoffman–La Roche, Nutley, NJ, U.S.A. The pure unlabelled cholecalciferol-related sterols were dissolved in ethanol at concentrations of approx. 0.1 nmol/ml, and stored in small vials under N_2 in the dark at −20°C. Before use they were examined for purity by t.l.c. with hexane/acetone (1:1, v/v). The compounds remained stable in ethanol solution under the conditions used for at least 4 months.

DBP assay

The serum concentrations of DBP were determined by the single radial immunodiffusion assay as described previously (Imawari & Goodman, 1977). As a standard for this entire study, a preparation of normal human whole serum that had been standarized against purified DBP (Imawari *et al.*, 1976) was used. The molar concentration of DBP was calculated by using the value of 52000 as the mol.wt. of DBP (Imawari *et al.*, 1976).

Binding studies: general comments

Studies of the binding of vitamin D-related sterols to DBP in whole serum were carried out by a filter assay method similar to that developed for the assay of steroid receptors by Santi *et al.* (1973), and as used by Rosner *et al.* (1976) for the assay of plasma corticosteroid-binding globulin. The method involves the equilibration of increasing amounts of ligand (cholecalciferol-related sterol) with DBP in diluted whole serum, followed by the separation of the protein (DBP)-bound ligand from the unbound ligand by a DEAE-cellulose filter-disc method. Because of the lipid-soluble (rather than water-soluble) nature of the ligands studied here, the filter assay method of Santi *et al.* (1973) required modification in order to be applicable to the present studies. The conditions selected and used, and described in detail below, provided highly reproducible, quantitative results on the binding of the several cholecalciferol-related sterols to DBP. Because of the problems inherent in the use of lipid-soluble ligands, however, and the conditions utilized (see below) to effectively deal with these problems, the results should be considered

as quantitatively fully valid only for the conditions used. Despite these limitations, the results do provide valid comparisons of the relative affinity of DBP for the different ligands studied, and of the relative affinities of the different DBP/Gc protein genetic variants for the various ligands.

Equilibration of ligand with serum

In order to achieve complete and reproducible equilibration of added ligand with binding protein, methods were first sought that would be able to fully and stably disperse ('solubilize') the lipid-soluble ligands in the aqueous incubation medium. The methods examined included the addition of various amounts of ethanol, glycerol, Triton X-100 (Eastman-Kodak Co., Rochester, NY, U.S.A.), ovalbumin and human plasma lipoproteins of density (ρ) <1.063 g/ml. In experiments with ^{3}H-labelled 25(OH)D_3, excellent recovery of the added ^{3}H in the aqueous medium was achieved with Triton X-100 (at concentrations of greater than 0.05%, w/v), with ovalbumin (in concentrations above 1%, w/v), and with ρ<1.063 lipoproteins (in concentrations one-tenth or more of those present in whole plasma). Furthermore, reproducibility of recovery (>95%) was increased when the dried sterol was redissolved in 10μl of ethanol before the aqueous solution was added.

Subsequent preliminary binding studies with these latter additions showed that the apparent association constant obtained in studies with Triton X-100 in the incubation medium was lower than that obtained with the other systems. Ovalbumin was needed in higher concentrations, and gave higher non-specific binding of radioactivity to the filter discs than did the use of ρ<1.063 g/ml lipoproteins. Prior addition of 10μl of ethanol did not affect the binding-parameter estimates except for improvement in reproducibility. Accordingly, the final binding studies used human plasma lipoproteins (ρ<1.063 g/ml), diluted 1:10 (v/v) as compared with whole plasma (see below), in the incubation medium, together with the prior addition of 10μl of ethanol. In the absence of DBP, only negligible specific binding (see below) of 25(OH)D_3 to the filter disc was observed with medium containing this concentration of plasma lipoproteins, under the conditions used in the binding studies.

Preliminary experiments were conducted to determine the incubation time required to reach equilibrium between added ligand and serum DBP. These experiments used a final serum dilution of 1:350, at which approx. 50% of ^{3}H-labelled 25(OH)D_3 (5pmol added) was found specifically bound to DBP. At 24°C, all four ligands studied reached equilibrium after 3h of incubation. The time required for cholecalciferol to reach equilibrium was 3h at 24°C and 24h at 4°C, and was the longest among the cholecalciferol-related sterols studied.

In the experiments reported here, endogenous cholecalciferol-related sterols were not removed from the serum samples used for study because of the great molar excess of apo-DBP (probably more than 50 times the concentrations of cholecalciferol and its metabolites) known to be present in normal serum (Haddad & Walgate, 1976*b*; Imawari & Goodman, 1977; Bouillon *et al.*, 1977).

Separation of bound and free ligand

Unlike glucocorticoids and related steroids (Santi *et al.*, 1973), 'free' (i.e., not protein-bound) 25(OH)D_3 could not be removed effectively from the filter disc with 10mM-Tris/HCl buffer containing 1mM-EDTA alone, even when the disc was washed extensively. This finding presumably reflects the solubility characteristics of 25(OH)D_3. Experiments were then conducted to find an adequate buffer for washing the filter, namely one which effectively removed 'free' 25(OH)D_3, but did not disrupt the 25(OH)D_3–DBP complex bound to the filter. To this end the effects of ethanol or of Triton X-100 were examined. Ethanol did not improve washing effectiveness when added to the buffer in amounts up to 1% (v/v) final concentration. In contrast, buffer containing more than 0.2% (w/v) of Triton X-100 excellently removed ^{3}H-labelled 25(OH)D_3 from the filter discs. When ^{3}H-labelled 25(OH)D_3 dispersed in buffer alone was applied to the filter, washing with three 1ml portions of 10mM-Tris/HCl buffer containing 0.2% Triton X-100 allowed very little ^{3}H-labelled 25(OH)D_3 to remain on the filter disc, and five washes of 1ml each with this buffer removed virtually all the free ligand. On the other hand, when ^{3}H-labelled 25(OH)D_3 was applied after equilibration in the buffer containing diluted human whole serum or isolated DBP, substantial amounts of labelled ligand remained bound to the filters after five washes. Moreover, further washing (several times) with more than five washes did not decrease the value for 'bound' labelled ligand, indicating that the washing buffer was not progressively disrupting or dissolving the 25(OH)D_3–DBP complex bound to the filter. Increasing the concentration of Triton X-100 from 0.2 to 1% did not alter the values observed for 'total bound' ligand, but decreased slightly the values observed for 'non-specifically bound' ligand (see below).

Filter assay method for binding studies

The binding studies of DBP with 25(OH)D_3 were all carried out in exactly the same manner. In each study, serially increasing amounts of ^{3}H-labelled 25(OH)D_3 (from 1pmol to 50pmol) were put into glass tubes (12mm×75mm in size) and dried under N_2. The sterol was redissolved in 10μl of ethanol and incubated at room temperature (24°C) with 0.50ml of serum diluted 1:350 with 10mM-Tris/HCl buffer, pH7.4, containing 1mM-EDTA and <1.063 ρ plasma

lipoproteins diluted 1:10. During incubation the solution was mixed frequently on a vortex mixer. After exactly 180min, duplicate samples of 100μl each were assayed for 'bound' ^{3}H-labelled 25(OH)D_3 by the filter-disc method described below. Preliminary experiments on the capacity of the DEAE-cellulose filter disc to retain bound ^{3}H-labelled 25(OH)D_3 demonstrated that the capacity was in excess of 300μl of the diluted serum incubation medium used here.

In order to correct for 'non-specific' binding of labelled ligand to the filter disc, duplicate tubes containing 300-fold excess of unlabelled 25(OH)D_3 were incubated with each concentration of ^{3}H-labelled 25(OH)D_3 in parallel in the same experiment.

Binding assays were conducted with DEAE-cellulose filter-paper discs (DE81, 2.4cm diam.; Whatman, Clifton, NJ, U.S.A.) and a membrane-filter holder (model FH 244; Hoefer Scientific, San Francisco, CA, U.S.A.) The discs were first washed with three 1ml portions of 10mM-Tris/HCl buffer, pH7.4, containing 1mM-EDTA at 24°C under low vacuum (gauge 1); excess moisture was removed with high vacuum (gauge 5) for 30s. For each sample, 100μl of incubation mixture was applied to the washed filter and allowed to remain on the disc without vacuum for 3min. Preliminary experiments indicated that 3min was an adequate time interval to permit protein (i.e. DBP)-bound ^{3}H-labelled 25(OH)D_3 to become bound to the filter. The filters were then washed serially with five 1ml portions of ice-cold 10mM-Tris/HCl buffer, pH7.4, containing 1mM-EDTA and 1% Triton X-100, by using a low vacuum (gauge 1). These washes removed 'free' labelled ligand from the filter. After completion of the fifth wash, excess moisture was removed by application of high vacuum (gauge 5) for 1min. The filters were then transferred into scintillation-counting vials, each containing 2ml of ethanol, and allowed to soak in ethanol for a few minutes. To each vial was added 10ml of scintillation solvent (2,5-diphenyloxazole in toluene; 5g/litre), and the vials were assayed for ^{3}H in a Packard model 3003 liquid-scintillation spectrometer (Packard Instrument Co., Downers Grove, IL, U.S.A.), at an efficiency of approx. 30%.

In order to determine the total amount of ^{3}H-labelled 25(OH)D_3 present in each incubation mixture, duplicate 100μl portions of each incubation mixture were taken directly into counting vials, mixed with 10ml of ScintiVerse (Fisher Scientific Co., Pittsburgh, PA, U.S.A.) and 1ml of water, and assayed for ^{3}H. In both radioassay systems, the observed c.p.m. were corrected to d.p.m. by the use of an internal standard of [^{3}H]toluene.

For each serum sample studied, the apparent association constant (k_a) and maximum binding capacity were calculated by the method of Scatchard (1949). Linear regression lines for these calculations were determined by the method of least squares with a Wang model 700A electronic calculator (Wang Laboratories, Inc., Tewksbury, MA, U.S.A.) with programs prepared for that instrument.

Binding studies with 25(OH)D_3 alone

Studies of the interaction of 25(OH)D_3 with DBP were carried out by the filter assay method described above with the following sera: six samples with phenotype Gc 1-1, six sera with Gc 2-1, three sera with Gc 2-2, two sera with Gc Ab-Ab and one serum with each of Gc Ab-1, Gc Ab-2, Gc Chip-1 and Gc Chip-2. The binding parameters for the three common phenotypes (Gc 1-1, 2-1, and 2-2) were compared statistically by one-way analysis of variance (Snedecor & Cochran, 1967).

Binding studies with vitamin D_3, 1,25(OH)$_2D_3$ and 24(R),25(OH)$_2D_2$

The interaction of DBP with each of these three ligands was investigated indirectly, by studying the effects of competition between each unlabelled ligand and ^{3}H-labelled 25(OH)D_3 for the binding protein. Complete studies were carried out with three samples of serum, comprising one sample selected at random from the samples available for each of three homozygous genetic variants of Gc, namely Gc 1-1, Gc 2-2 and Gc Ab-Ab.

For each of these studies (for each ligand and each serum sample), a study of the binding of ^{3}H-labelled 25(OH)D_3 alone to DBP was carried out as described above. Replicate studies of the binding of ^{3}H-labelled 25(OH)D_3 were then conducted in the presence of a constant amount (in all tubes) of one of the other unlabelled sterols. For each unlabelled sterol (ligand), separate studies using each of two different amounts (the amount constant in any one study) were carried out. The amounts of unlabelled sterol used were determined from preliminary experiments that examined the displacement of 5pmol of ^{3}H-labelled 25(OH)D_3 from the binding protein by each sterol, in incubation media containing 1:350 diluted human serum. Concentrations of unlabelled sterols that displaced approx. 40% and 60–80% of the ^{3}H-labelled 25(OH)D_3 from the binding protein were selected for the competitive binding studies. These concentrations were: 1.05μM and 10.5μM for cholecalciferol; 137nM and 274nM for 1,25(OH)$_2D_3$; and 6nM and 12nM for 24(R),25-(OH)$_2D_3$.

For each sample in each study, the values for the 'bound' and 'total' amount of ^{3}H-labelled 25(OH)D_3 were determined by the filter assay method exactly as described above.

The association constants for the ligands other

than $25(OH)D_3$ were calculated by the equation (Edsall & Wyman, 1958).

$$k_B = \frac{1}{[B]}\left(\frac{k_A}{k'_A} - 1\right)$$

where k_A represents the apparent association constant of the binding protein for the labelled ligand [$25(OH)D_3$], and k_B the apparent association constant for the unlabelled ligand other than $25(OH)D_3$. k'_A is the observed association constant for $25(OH)D_3$ obtained in the presence of the unlabelled ligand in the total concentration [B].

It should be noted that, in this method, association constants for the ligands other than $25(OH)D_3$ (k_B values) were calculated from the values determined experimentally for k_A and k'_A. Thus it was desirable to try to determine the values for k_A and k'_A with a high degree of precision. Accordingly, for each of the three homozygous sera studied, k_A was determined repeatedly in four different binding studies. The values for k'_A were also obtained from data collected in replicate experiments. Thus for each unlabelled sterol, two or three binding experiments were carried out with each serum with each of the two concentrations of ligand used. The final estimates for k_A and k'_A were obtained from the combined pooled data from the replicate experiments carried out under each set of conditions. These final estimates were derived from linear-regression analysis of single Scatchard (1949) plots of the combined data under each set of conditions, as described above. In order to estimate the precision of these estimates of the association constants (k_A and k'_A values) the S.D. of the slope of the regression line for each Scatchard plot (from the combined data) was obtained as the sample S.D. of the regression coefficient (Snedecor & Cochran, 1967).

The S.D. values of the association constants for the ligands other than $25(OH)D_3$ (k_B values) were calculated from the S.D. values of k_A and k'_A by the equation (Mood *et al.*, 1974):

$$\text{S.D.}_{k_B} = \left(k_B + \frac{1}{[B]}\right)\sqrt{\frac{(\text{S.D.}_{k_A})^2}{(k_A)^2} + \frac{(\text{S.D.}_{k'_A})^2}{(k'_A)^2}}$$

Values for k_B obtained from the two different concentrations used for each unlabelled ligand were averaged to obtain the final estimates for k_B. The S.D. of each averaged k_B was calculated from the S.D. of each of the two k_B values for that ligand by the equation:

$$\text{S.D.}_{k_B} = \tfrac{1}{2}\sqrt{(\text{S.D.}_{k_{B1}})^2 + (\text{S.D.}_{k_{B2}})^2}$$

Other methods

Lipoproteins of $\rho < 1.063$ g/ml were obtained from fresh plasma collected from a fasting normolipidaemic investigator, by ultracentrifugal flotation at $\rho =$ 1.063 g/ml (Havel *et al.*, 1955). The separated lipoproteins were dialysed extensively against 10mM-Tris/HCl buffer, pH 7.4, containing 1mM-EDTA at 4°C. The dialysed preparation was then diluted with the same buffer to the same volume as that of the plasma from which the lipoproteins had been derived. The concentration of the final preparation was approx. 1.0mg of protein/ml, as determined by the method of Bradford (1976). The lipoprotein preparation was sterilized by passage through a sterile Millipore filter (0.22μm pore size; Millipore Corp., Bedford, MA, U.S.A.), and stored in sterilized Falcon culture tubes (12mm×75mm) at 4°C until use, within 2 weeks of preparation.

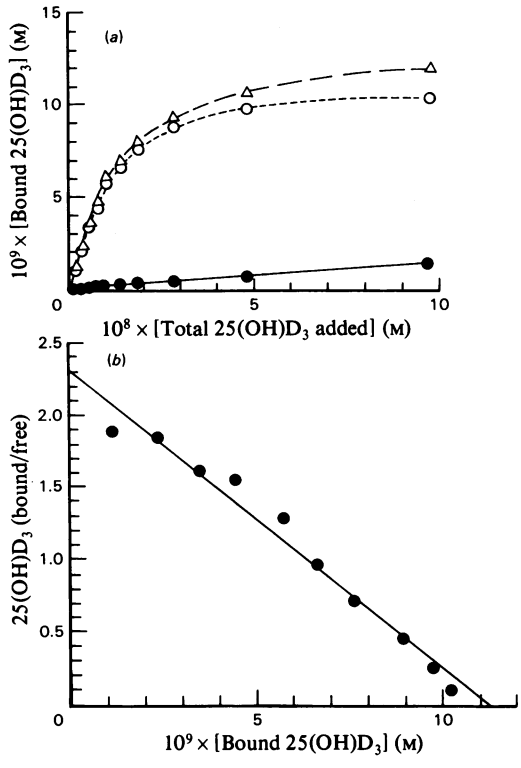
Results

Interaction of $25(OH)D_3$ alone with different genetic variants of DBP/Gc protein

Fig. 1 shows the results of a representative experiment, on the binding of $25(OH)D_3$ to DBP in one of the samples of Gc 1-1 serum. The data obtained are presented in two forms. In Fig. 1(*a*) the observed values for 'bound' $25(OH)D_3$ are plotted against the corresponding values for the total amount of $25(OH)D_3$ added to each incubation medium and equilibrated with DBP. The values for 'specifically bound' $25(OH)D_3$ were obtained by subtraction of the values for 'non-specifically bound' ligand (bottom curve) from the values for 'total bound' ligand (top curve). The values for 'free' $25(OH)D_3$ (not shown in Fig. 1*a*) were obtained as the difference between total added and total 'bound' $25(OH)D_3$. In Fig. 1(*b*) the same data are presented in the form of a Scatchard plot. The linearity of the plot indicates that serum contains a single class of high-affinity binding sites for $25(OH)D_3$ (presumably the single binding sites on the DBP molecules in the diluted serum). The apparent association constant was obtained from the slope of the line, and the maximum binding capacity of the serum from the intercept on the abscissa.

Table 1 shows the results of similar experiments carried out with 21 different samples of serum, representing eight different Gc phenotypes. The apparent association constants obtained for all the different sera were fairly similar to each other. The association constants of the three common phenotypes (Gc 1-1, 2-1, and 2-2) did not differ significantly from each other by one-way analysis of variance. The association constants of the other, uncommon, phenotypes were similar, but were not analysed statistically because of the limited number of samples available for each phenotype.

The maximum binding capacities and DBP concentrations of the different sera were also generally similar to each other (Table 1). The Gc 2-2 sera



showed slightly lower mean maximum binding capacities and DBP concentrations, with the Gc 2-2 mean maximum binding capacity being significantly different from that for the Gc 1-1 sera ($P<0.01$) or the Gc 2-1 sera ($P<0.05$). These very limited data are consistent with the report of Cleve & Dencker (1966) that individuals of type Gc 2-2 have significantly lower concentrations of Gc protein in serum than do individuals of types Gc 1-1 or 2-1. Similar values (range 0.39–0.52) were observed in all sera studied

Fig. 1. *Interaction of 25(OH)D_3 with Gc 1-1 serum* Gc 1-1 serum diluted 1:350 was incubated with serially increasing amounts of ^{3}H-labelled 25(OH)D_3 as described in the Materials and Methods section. In (*a*) the total amount of 25(OH)D_3 added to each tube is plotted on the abscissa, expressed as the final concentration in the incubation mixture. The ordinate shows the amounts of total bound (△) and of non-specifically bound (●) 25(OH)D_3 observed, expressed in terms of concentration in the incubation mixture. Specifically bound ^{3}H-labelled 25(OH)D_3 (○) was calculated by subtracting non-specifically from total bound radioactivity (d.p.m.). The specific binding sites in serum appeared to be virtually saturated at the highest concentration examined. In (*b*) the results are presented in the form of a Scatchard plot. The correlation coefficient (*r*) for the linear-regression analysis of these data was 0.99.

Table 1. *Interaction of 25(OH)D_3 with genetic variants of DBP/Gc protein*
Values in parentheses are means ± S.D. for the group above. Abbreviations used: k_A, apparent association constant; MBC, maximum binding capacity.

Gc type	$10^{-8} \times k_A$ (M^{-1})	$10^6 \times$MBC(M)	$10^6 \times$[DBP](M)	MBC/[DBP]
Gc 1-1	1.8	4.3	9.0	0.48
	2.1	3.9	8.3	0.47
	1.9	4.3	8.8	0.49
	2.1	4.0	8.3	0.48
	1.5	3.8	7.3	0.52
	2.1	4.8	10.0	0.48
	(1.9±0.2)	(4.2±0.4)	(8.6±0.9)	
Gc 2-1	2.2	3.8	8.1	0.47
	1.6	4.1	8.3	0.49
	1.9	3.8	7.9	0.48
	1.8	3.5	8.5	0.41
	1.6	4.2	8.8	0.48
	1.6	3.5	7.9	0.44
	(1.8±0.2)	(3.8±0.4)	(8.3±0.4)	
Gc 2-2	2.3	2.9	6.7	0.43
	1.7	2.6	6.7	0.39
	1.4	3.6	8.8	0.41
	(1.8±0.5)	(3.0±0.5)	(7.4±1.2)	
Gc Ab-Ab	2.1	3.8	8.5	0.45
	2.5	3.1	7.7	0.40
Gc Ab-1	1.9	4.3	9.0	0.48
Gc Ab-2	2.5	2.6	6.2	0.42
Gc Chip-1	2.3	4.4	10.6	0.42
Gc Chip-2	1.6	4.5	8.8	0.51

for the ratio between the maximum binding capacity and the DBP concentration. These values indicate that an average of 0.4–0.5 high-affinity binding sites for 25(OH)D_3 per molecule of DBP was apparent in the sera studied with the methods used.

Interaction of cholecalciferol (vitamin D_3), 25(OH)D_3, 1,25$(OH)_2D_3$ and 24(R),25$(OH)_2D_3$ with genetic varients of DBP/Gc protein

Detailed studies were conducted with samples of serum representing individuals homozygous as to Gc type. The three samples studied included types Gc 1-1, Gc 2-2 and Gc Ab-Ab.

Fig. 2 shows the Scatchard plots obtained for the binding of 25(OH)D_3 alone to each of these samples of serum. The plots represent combined data obtained in four different binding studies carried out at different times for each serum. For each of the three sera the combined data-points fit a straight line ($r > 0.80$), and the slopes of the lines could be estimated with good precision (coefficient of variation $< 13\%$).

Fig. 3 shows the data obtained for the binding of ^{3}H-labelled 25(OH)D_3 to the sample of Gc 2-2 serum, when studied both in the absence and in the presence of each of two concentrations of unlabelled cholecalciferol. The data points fit three straight lines (r values 0.87, 0.94 and 0.93 respectively) that meet at a common intercept on the abscissa. The association constants calculated from the studies conducted with each of the two concentrations of cholecalciferol were identical [see Fig. 3 (legend) and Table 2].

Fig. 4 shows the data obtained for the binding of ^{3}H-labelled 25(OH)D_3 to the sample of Gc Ab-Ab serum, when studied alone, and when studied in the presence of unlabelled cholecalciferol, of 1,25-$(OH)_2D_3$ or of 24(R),25$(OH)_2D_3$. The data points fit four straight lines (r values from 0.92 to 0.99) that meet at approximately the same intercept on the abscissa. Linear data with a similar x-intercept were also obtained with a second concentration of each of the three unlabelled ligands studied.

Table 2 presents the values for the association constants (k_B values) for the unlabelled ligands, as determined with each of two different concentrations for each ligand for each of the three homozygous sera. In every instance there was good agreement between the two k_B values determined at the two different concentrations of unlabelled ligand. This finding, together with the results shown in Fig. 4 (plus similar results obtained with the other sera), supports the assumption that all four cholecalciferol-related sterols studied compete for the same binding site on the protein.

Table 3 presents the final estimates for the association constants derived from these studies with the three different genetic variants of DBP/Gc protein. The values for 25(OH)D_3 were obtained from the plots shown in Fig. 2. The values for the other ligands represent the average values from the

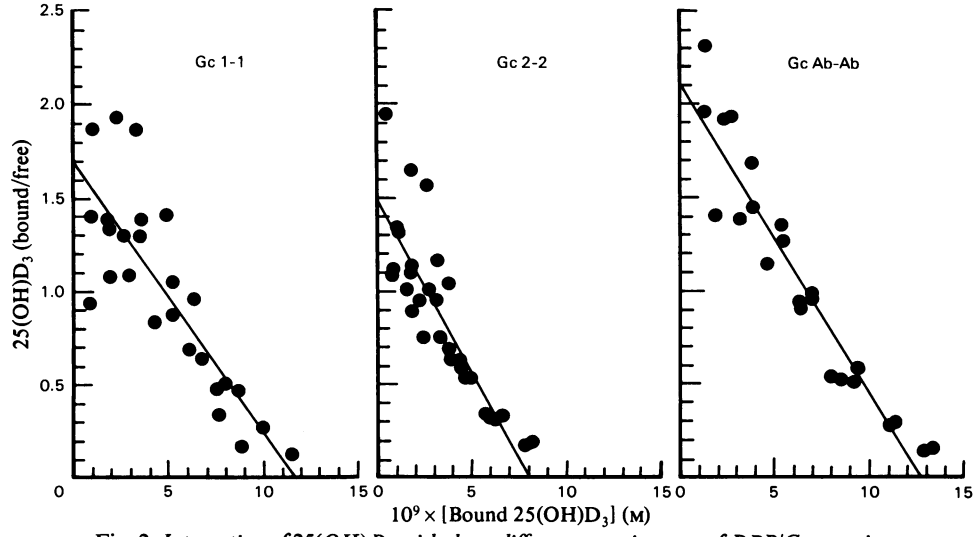


Fig. 2. *Interaction of 25(OH)D_3 with three different genetic types of DBP/Gc protein*
The sera were from individuals homozygous as to Gc type. Each of the three plots presents data obtained in four different experiments for each serum sample. Each individual binding experiment included five to eight tubes with serially increasing amounts of added ^{3}H-labelled 25(OH)D_3. The correlation coefficients (r) for the three plots were 0.84, 0.87 and 0.95 for Gc 1-1, Gc 2-2, and Gc Ab-Ab respectively. The association constants derived from these plots are presented in Table 3.

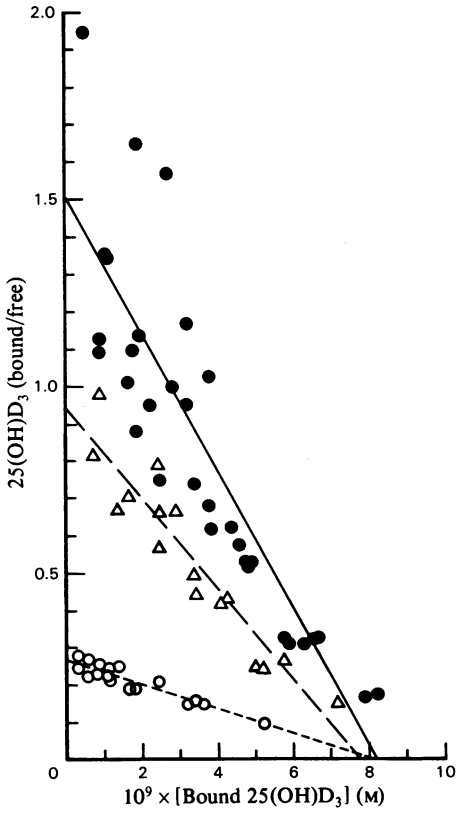


Fig. 3. *Effect of cholecalciferol on the interaction of 25(OH)D_3 with Gc 2-2 serum*

Combined data from several experiments are presented. Four experiments were carried out with ^{3}H-labelled 25(OH)D_3 alone (●, upper line); two experiments were carried out with 1.05 μM-cholecalciferol (△, middle line); and three experiments were carried out with 10.5 μM-cholecalciferol (○, lowest line). Analysis of the upper line and data points indicated that k_A (see the Materials and Methods section) = $1.8 \pm 0.2 \times 10^8$ M^{-1}. From the middle and lowest lines and data points the values of $k'_A = 1.2 \pm 0.12 \times 10^8$ M^{-1} and $k'_A = 0.33 \pm 0.034 \times 10^8$ M^{-1} were obtained. From these values, the association constant for cholecalciferol (k_B, see the Materials and Methods section) was calculated as $4.7 \pm 2.0 \times 10^5$ M^{-1} (for [cholecalciferol] = 1.05 μM) and as $4.2 \pm 0.76 \times 10^5$ M^{-1} (for [cholecalciferol] = 10.5 μM).

estimates shown in Table 2. With each of the different sera, markedly different affinities were observed for the four ligands studied. In each instance, the affinities of DBP (i.e., the association constants) were highest, and were similar to each other, for 25(OH)D_3 and for 24(R),25(OH)$_2$D$_3$. The affinities for 1,25-(OH)$_2$D$_3$ were an order of magnitude lower than those observed for 25(OH)D_3 (association constants of approx. 1×10^7 as compared with $1\text{–}2 \times 10^8$ M^{-1}). Cholecalciferol itself showed the lowest affinities, with association constants of the order of one-five-hundredth of those observed for 25(OH)D_3.

Similar results were obtained with each of the three different Gc-type sera. For each genetic variant the order of magnitude and the rank order of affinities for the four different ligands were the same. Moreover, the association constants estimated for each ligand were fairly similar among the three different sera. A statistical comparison of the association constants calculated for each ligand for the three different Gc-type sera was not carried out, because only one serum sample for each of these Gc types was studied in detail. The results (Table 3) do, however, clearly indicate that quantitatively similar binding affinities for cholecalciferol and its three major circulating metabolites were present in all three of the different genetic variants of DBP/Gc protein studied here.

Discussion

The present studies were designed to examine the

Table 2. *Apparent association constants estimated for the unlabelled ligands cholecalciferol, 1,25(OH)$_2$D$_3$ 24(R),25(OH)$_2$D$_3$*

The values listed are the calculated values (±S.D.) of k_B (see the Materials and Methods section) for each ligand, as determined at each of two concentrations of that ligand, with each sample of serum. The S.D. values listed for each k_B value were calculated as described in the Materials and Methods section.

		k_B		
	Ligand ...	Cholecalciferol	1,25(OH)$_2$D$_3$	24,25(OH)$_2$D$_3$
Gc type	Units ...	M^{-1} × 10^{-5}	M^{-1} × 10^{-7}	M^{-1} × 10^{-8}
Gc 1-1		1.8 ± 1.8	1.4 ± 0.4	1.1 ± 0.8
		2.6 ± 1.0	1.3 ± 0.2	2.1 ± 1.1
Gc 2-2		4.7 ± 2.0	0.9 ± 0.2	0.9 ± 0.4
		4.2 ± 0.8	1.2 ± 0.2	2.4 ± 0.3
Gc Ab-Ab		3.3 ± 1.0	0.9 ± 0.1	0.6 ± 0.4
		4.1 ± 0.4	0.9 ± 0.1	0.9 ± 0.3

Table 3. *Apparent association constants for cholecalciferol and its metabolites for sera representing three genetic variants of DBP/Gc protein*

The values listed are the final estimates (±S.D.) of the apparent association constants (see the text) for each of the three Gc-type sera studied. The S.D. values listed provide information about the precision of these estimates.

		k_A	k_B		
Gc type	Ligand ... / Units ...	25(OH)D_3 $M^{-1}\times10^{-8}$	Cholecalciferol $M^{-1}\times10^{-5}$	1,25$(OH)_2D_3$ $M^{-1}\times10^{-7}$	24,25$(OH)_2D_3$ $M^{-1}\times10^{-8}$
Gc 1-1		1.5±0.2	2.2±1.0	1.3±0.2	1.6±0.7
Gc 2-2		1.8±0.2	4.5±1.1	1.0±0.1	1.7±0.5
Gc Ab-Ab		1.7±0.1	3.7±0.6	0.9±0.1	0.7±0.2

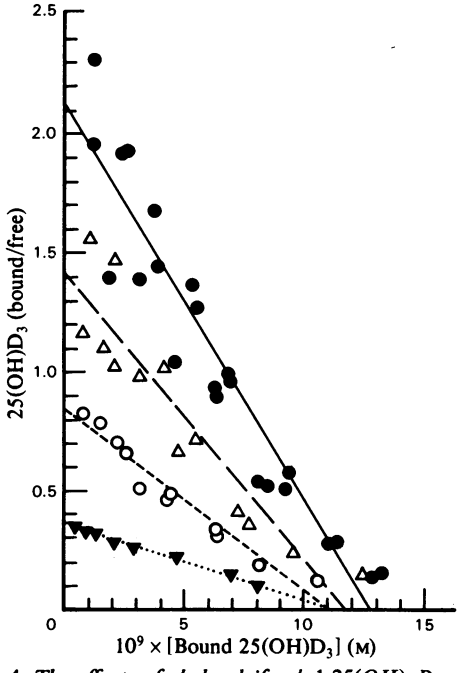


Fig. 4. *The effects of cholecalciferol, 1,25$(OH)_2D_3$ and of 24(R),25$(OH)_2D_3$ on the interaction of ^{3}H-labelled 25$(OH)D_3$ with Gc Ab-Ab serum*

Combined data from several experiments are presented. Four experiments were carried out with ^{3}H-labelled 25(OH)D_3 alone (●, uppermost line); two experiments were carried out with 6nM-24(R),25-$(OH)_2D_3$ (△, second line from top); two experiments were carried out with 137nM-1,25$(OH)_2D_3$ (○, third line from top); and one experiment was carried out with 10.5μM-cholecalciferol (▼, bottom line).

interaction of cholecalciferol and its major biological metabolites with different genetic variants of the plasma transport protein for these sterols. This transport protein has been called DBP, and has been shown to be identical with the Gc proteins of human plasma (Haddad & Walgate, 1976*a*; Bouillon *et al.*, 1976*a*; Imawari & Goodman, 1977; Daiger *et al.*, 1975). Binding studies reported here were carried out by a filter-disc assay method similar to the methods used by others for the study of the binding of steroid hormones to cytosol receptor proteins (Santi *et al.*, 1973) or to plasma corticosteroid-binding globulin (Rosner *et al.*, 1976). As discussed above (see the Materials and Methods section), because of the solubility characteristics of the ligands studied here, the filter assay method required modification in order to be applicable to the present studies. The present studies represent examples of the interaction in aqueous solution of a soluble protein with lipid-soluble ligands that possess some polarity. Because of the presence of polar (–OH) groups on these ligand molecules, they should possess some extremely low but finite water-solubility. Nevertheless, it is not known what form of unbound ('free') ligand was present in the incubation medium in equilibrium with the ligand–DBP complex. With the experimental system used here, it is probable that the equilibrated mixtures contained ligand tightly bound to DBP, ligand loosely associated with plasma lipoproteins, and some ligand molecules in aqueous solution. Only ligand bound to DBP was retained on the filter discs, and measured as 'bound' ligand, with the methods used. Because the exact physical state of the 'free' ligand (not bound to DBP) is not known, the affinities determined in these studies should be interpreted in terms of apparent association constants, quantitatively valid strictly for the experimental conditions used, rather than true association constants in thermodynamic terms. Despite this limitation in interpretation, however, the results do provide quantitative and valid comparisons of the relative affinity of DBP for the different ligands studied, and of the relative affinities of the different genetic variants of DBP for the several ligands.

Scatchard plots of the binding of ^{3}H-labelled 25(OH)D_3 to DBP in diluted whole serum showed excellent linearity for all plots obtained in the absence or in the presence of unlabelled cholecalciferol or of one of its metabolites. For each sample of serum, the several lines so obtained met together at approximately the same point on the abscissa. In addition, the apparent association constants for

each unlabelled ligand calculated from the analysis of the two lines corresponding to different concentrations of the ligand were found to be similar. Taken together, these findings strongly suggest that the four cholecalciferol-related sterols studied were all binding specifically to, and competing for, the same binding site on the same protein.

Previous studies by others have used a number of techniques to estimate the affinity of DBP for $25(OH)D_3$ and related compounds. Haddad & Walgate (1976*a*) used a sucrose-density-ultracentrifugation method to study the binding of labelled cholecalciferol, $25(OH)D_3$ and $1,25(OH)_2D_3$ to purified DBP. The apparent association constants of DBP for these three ligands were estimated as $2.3\times10^6M^{-1}$, $1.6\times10^7M^{-1}$ and $3\times10^6M^{-1}$ respectively. In addition, by using dextran-coated-charcoal-adsorbent removal of unbound sterol, $24(R),25(OH)_2D_3$ was found to be as potent as $25(OH)D_3$ in displacement of labelled $25(OH)D_3$ from DBP (Haddad & Walgate, 1976*a*). A higher estimate for the apparent association constant of DBP for $25(OH)D_3$ (namely $1.4\times10^9M^{-1}$) was reported from this laboratory (Haddad *et al.*, 1976), with diluted serum and a dextran-coated-charcoal method to separate 'free' and 'bound' sterol. Still higher estimates for the affinity of DBP for $25(OH)D_3$ were reported by Bouillon *et al.* (1976*b*) in studies using diluted whole human serum at 0°C (apparent k_A $1.9\times10^{10}M^{-1}$), or isolated DBP at 4°C (apparent k_A $1.2\times10^{10}M^{-1}$) (Bouillon *et al.*, 1976*a*). These latter binding studies (Bouillon *et al.*, 1976*a*; Bouillon *et al.*, 1976*b*) were described as having been carried out by competitive protein binding, but no details were provided as to the actual methods used, making the reported results somewhat difficult to evaluate.

The four ligands studied here represented both cholecalciferol itself and the three of its major known circulating metabolites. It is now well established that $25(OH)D_3$ normally represents the quantitatively major circulating cholecalciferol-related sterol, with plasma concentrations normally mainly in the range of approx. 10–30ng/ml (Haddad & Walgate, 1976*b*; Imawari & Goodman, 1977; Haddad & Stamp, 1974; Hillman & Haddad, 1974; McLaughlin *et al.*, 1974; Bouillin *et al.*, 1976*c*; Lambert *et al.*, 1977; Jones, 1978). The normal plasma concentrations of cholecalciferol itself are somewhat lower, and have been reported to be in the range of approx. 1–17ng/ml (Lambert *et al.*, 1977; Jones, 1978). Mean values of approx. 2ng/ml have been reported for the plasma concentration of $24,25(OH)_2D_3$ (Taylor *et al.*, 1976; Lambert *et al.*, 1977). The circulating concentrations of $1,25(OH)_2D_3$ (approx. 3–6ng/dl) (Brumbaugh *et al.*, 1974; Eisman *et al.*, 1976; Lambert *et al.*, 1977) are much lower than those of the other cholecalciferol metabolites.

Markedly different affinities were observed in the present studies for cholecalciferol and its metabolites. The apparent association constant for $25(OH)D_3$ was $1.5-2\times10^8M^{-1}$. The affinity of DBP for $24(R),25(OH)_2D_3$ was approximately similar to that for $25(OH)D_3$. In contrast, the affinity for $1,25(OH)_2D_3$ was an order of magnitude lower (approx. $1\times10^7M^{-1}$), and that for cholecalciferol was almost three orders of magnitude lower (approx. $3–4\times10^5M^{-1}$).

In the present studies, the ratio of the maximum binding capacity for $25(OH)D_3$ to the serum concentration of DBP was approx. 0.4–0.5 for all sera studied. Thus an average of 0.4–0.5 high-affinity binding sites per molecule of DBP was apparent in the sera studied. A similar value (approx. 0.4) was observed by Bouillon *et al.* (1977; 1976*b*), and a somewhat lower value (approx. 0.2) was observed by Haddad & Walgate (1976*b*) and Haddad *et al.* (1976) in previous studies on the maximum binding capacity of serum for $25(OH)D_3$. A higher value, of 1.0, might be anticipated if all of the DBP molecules in serum had binding sites available for the binding of ^3H-labelled and unlabelled added $25(OH)D_3$. The observed lower value (0.4–0.5) may partly reflect effects of the methods used for the binding studies, but other phenomena may also be involved that remain to be explored. In any event, the serum maximum binding capacity observed here (approx. $4\mu M$) is of the order of 25–50 times the probable usual concentrations of circulating cholecalciferol and its metabolites (see above). These data further emphasize the fact that serum normally contains a large molar excess of DBP, and of a binding capacity for cholecalciferol and its metabolites, as compared to the circulating concentrations of these sterols.

The discovery that Gc protein is identical with the plasma transport protein for cholecalciferol and its metabolites raised the interesting possibility that the different genetic forms of Gc protein might differ in their binding properties for cholecalciferol or its metabolites. Daiger *et al.* (1975) speculated that a difference in the binding affinity of the products of the common Gc alleles might be involved in the balancing selection that is maintaining the worldwide polymorphism at the human Gc locus. In pursuing this suggestion, Mourant *et al.* (1976) plotted on a world map all available data on the frequencies of the allele Gc^2, and compared the distribution with that of sunlight. With some exceptions, high frequencies of Gc^2 corresponded to low amounts of sunlight and vice versa. It was suggested that the main distribution of Gc^2-gene frequencies might be explained by natural selection if the Gc-2 protein were more efficient than Gc-1 in binding cholecalciferol.

The results reported here do not support this hypothesis. Quantitative binding studies with the Gc-1 and with the Gc-2 protein demonstrated comparable binding properties for all four cholecalciferol-

related sterols for both Gc proteins. In addition, quantitatively similar binding properties for these sterols were observed for the Gc-Ab protein, and with sera from heterozygous persons containing the Gc-Chip protein. Thus it does not appear that qualitative or quantitative differences in the binding and transport of cholecalciferol and its metabolites are involved in selection at the Gc locus or in maintaining the existing polymorphism of this system.

Two reports have recently appeared in which the binding of radioactive cholecalciferol and/or 25-hydroxycholecalciferol to a considerable number of rare variants of Gc protein was examined (Cleve & Patitschnick, 1977; Daiger & Cavalli-Sforza, 1977). Both studies used electrophoresis of serum, followed by radioautography to demonstrate the Gc-protein bands in the various phenotypes. No Gc variants clearly defective in their cholecalciferol-binding properties were observed in these qualitative studies. These radioautographic studies could not, however, explore whether physiologically significant quantitative differences in binding properties might exist between different genetic forms of Gc protein (Cleve & Patitschnick, 1977). The quantitative results reported here thus complement and extend these radioautographic studies, by demonstrating that quantitative differences in the affinities for cholecalciferol and its metabolites do not exist among the common and the major uncommon forms of Gc protein.

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