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Group-Specific Component (Gc) Proteins Bind Vitamin D and 25-Hydroxyvitamin D

[vitamin-D-binding α-globulin/human genetic polymorphism/plasma transport proteins/gel and immuno-autoradiography/group-specific component (Gc) in other mammals]

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ABSTRACT **Group-specific component (Gc) proteins are human plasma proteins for which a worldwide polymorphism exists. As yet no functional role has been assigned this protein. We show that the products of both Gc alleles, proteins Gc 1 and Gc 2 (distinguished electrophoretically), bind substantial quantities of vitamin D and 25-hydroxyvitamin D. Three lines of evidence are reported: (*1*) Polyacrylamide gel electrophoresis and autoradiography of serum labeled with [^{14}C]vitamin D_3 revealed patterns of radioactive bands identical to those expected of the two Gc alleles. Population gene frequencies for these proteins binding vitamin D were in the range of those reported for Gc, and individuals of known Gc phenotype were found to have the corresponding vitamin-D-binding phenotype. (*2*) Immunoelectrophoresis and autoradiography of labeled serum reacted against antiserum to human Gc revealed labeling by [^{14}C]vitamin D_3 of Gc–antibody precipitation arcs. (*3*) [^{14}C]vitamin D_3 or 25-hydroxy[^{3}H]vitamin D_3 was found to coprecipitate specifically with Gc in serum incubated with Gc antiserum. Use of these techniques demonstrated further that plasma proteins that bind vitamin D and that are immunologically similar to human Gc are found in mouse, rat, cow, horse, dog, rhesus monkey, and chimpanzee. We propose that Gc and "vitamin-D-binding α-globulin" are in fact the same protein, and that the ability of Gc to bind vitamin D may be directly related to the action of selection on this locus. These techniques may also find application in the study of other plasma transport proteins.**

Group-specific component (Gc) proteins are human plasma proteins first detected immunologically more than 15 years ago (1). There is a worldwide polymorphism at the *Gc* locus; two alleles, Gc^1 and Gc^2, are found in *all* human populations (2). The products of the two alleles can be separated electrophoretically in polyacrylamide, starch, and agar gels, the Gc 1 protein being faster than Gc 2 (reviewed in refs. 3 and 4). Gc proteins are synthesized in the liver, appearing early in fetal life (5, 6). The absence of Gc has never been reported, although more than 75,000 individuals have been studied (2). Immunologically similar proteins also occur in several other mammals (4, 7). It is remarkable, however, that no physiologic role has been assigned this protein.

In the same year that Gc proteins were reported another human plasma protein, vitamin-D-binding α-globulin (VDBG), was described (8). This protein is found in neonatal blood and in all adults, and has an electrophoretic mobility similar to Gc (reviewed in ref. 9). VDBG binds not only the natural vitamin D's (D_2, D_3) but also the metabolically more active forms, 25-hydroxyvitamin D and 1α,25-dihydroxyvitamin D (9). A similar plasma protein occurs in other mammals and in chickens (10).

We became interested in VDBG while engaged in a general screening program for genetic variants of transport proteins in human plasma. The first genetic polymorphism we detected was for a plasma protein that binds vitamin D_3. For reasons which will become clear, we then investigated the properties of Gc. These investigations demonstrated that Gc proteins in man are capable of binding substantial quantities of vitamin D_3 and 25-hydroxyvitamin D_3 *in vitro* under physiologically normal conditions (11). Further, a Gc-like protein in serum from other mammalian species also binds vitamin D_3. We, therefore, propose that "vitamin-D-binding α-globulin" and Gc are the same protein and that the binding of vitamin D by Gc is physiologically and selectively significant.

The methods we developed provide a simple, high-resolution means of screening for Gc variants, and are also applicable to the study of other transport proteins. The methods utilize the capacity of small radioactive molecules, such as vitamins and hormones, to bind to and thus "label" proteins with an affinity for these ligands.

MATERIALS

Blood Samples. Serum samples were from (*1*) 80 Caucasians (65 females, 1–2 days post partum; 7 male and 8 female volunteers), and (*2*) a variety of animal species. Twenty-five human serum samples of known Gc phenotype were supplied by the Department of International Health, University of California, San Francisco.

Reagents. [^{14}C]Vitamin D_3 (32.3 Ci/mol) and 25-hydroxy-[^{3}H]vitamin D_3 (1.1 Ci/mmol) were purchased from Amersham-Searle, Inc. Before use the labeled compounds, supplied in benzene, were dried at room temperature in a nitrogen atmosphere and resuspended in ethanol.

Antiserum to human Gc and antiserum to human α_1-lipoprotein were purchased from the Behring Diagnostics Division of Hoechst Pharmaceuticals, Inc. The antigenic specificity of the Gc antiserum was tested by immunoelectrophoresis; the serum gave a single arc in the expected position for each Gc phenotype.

METHODS

Radiolabeling of Serum Proteins. Blood samples were labeled by adding [^{14}C]vitamin D_3 or 25-hydroxy[^{3}H]-vitamin D_3, in ethanol, to 100 μl aliquots of serum. Labeled

Abbreviations: Gc, group-specific component; VDBG, vitamin-D-binding α-globulin.

samples were incubated at 37° for 1 hr and then stored at 3° for up to 24 hr before use. Final vitamin concentrations ranged from 0.11 μM (0.0035 μCi/ml of ^{14}C, 0.121 μCi/ml ^{3}H) to 1.1 μM with 1–4% ethanol. [Normal vitamin D concentrations in serum of human adults, as assayed by biologic activity, range from 0.065 μM to 0.20 μM (8).]

Polyacrylamide Gel Electrophoresis and Autoradiography ("Gel Autoradiography"). Vertical polyacrylamide gel electrophoresis was performed on radiolabeled blood samples using the standard Ornstein-Davis system with minor modifications (12, 13). A Tris–glycine upper buffer and Tris·HCl gel buffer were used. At 3° the pH values of this system are 9.6 upper buffer, 7.6 stacking gel, and 10.3 separating gel. The concentration of acrylamide in the separating gel was 10%.

Initial studies were carried out with column (disc) gels. Samples of 30 μl each were loaded and electrophoresed at 3° for 4 hr with a constant current of 2.5 mA per tube. (The binding affinity of transport proteins is often enhanced at low temperatures.) After electrophoresis the acrylamide columns were sliced into four longitudinal sections. One of the two flat inner ribbons (representing 30% of the total gel) was placed on damp filter paper and dried under an infrared lamp (14). The remaining gel ribbons were fixed in 10% trichloroacetic acid and stained with 0.05% Coomassie blue.

More recent electrophoretic work was accomplished using a slab gel apparatus purchased from Bio-Rad, Inc. Ten microliter samples were loaded and a constant voltage of 13.5 V/cm per slab was maintained for 4 hr. Electrophoresed gels were dried on filter paper as noted above.

Autoradiographs were made by exposing the dried gels, either column or slab, to Kodak Rapid Processing Film, RP54, for from 2 to 30 days. The films were developed in a Kodak Rapid Processor.

Immunoelectrophoresis and Autoradiography ("Immuno-Autoradiography"). Immunoelectrophoresis was performed in 1% agar (0.1% sodium azide added) on 2 × 3 inch slides with a 0.05 M sodium barbital buffer system, pH 8.3, at 3°. Radiolabeled blood samples of 5 μl were loaded in each well; electrophoresis proceeded at 3° for 2 hr with a constant voltage of 4 V/cm per slide; and, subsequently, parallel troughs were filled with 100 μl of antiserum. Immunodiffusion was allowed to continue until distinct precipitation bands appeared, usually within 14 hr at 3°. The completed immunoelectrophoretic slides were dried in a stream of warm air and autoradiographs were then made as above. After autoradiography the dried slides were rinsed several hours in buffer and stained with amido black.

Radio-Immunoprecipitation. Radiolabeled blood samples were placed in polyethylene Microfuge tubes and equal volumes of specific antiserum were added. Following a 1 hr incubation at 37° the tubes were centrifuged at 11,000 rpm in a Sorvall SS-34 rotor for 30 min at 3°. One hundred microliters of supernatant were removed. The material remaining in the tube was frozen in an acetone/dry ice bath and the lower tip, containing precipitate and approximately 10 μl of supernatant, was cut off with a razor blade. The β activity in the supernatant sample and the precipitate was determined by incubating each in 0.5 ml of NCS Tissue Solublizer for 1 hr at 37°, adding 5 ml of Liquifluor–toluene, 1:25, and measuring radioactivity in a Packard Tri-Carb Liquid Scintillation Spectrometer.

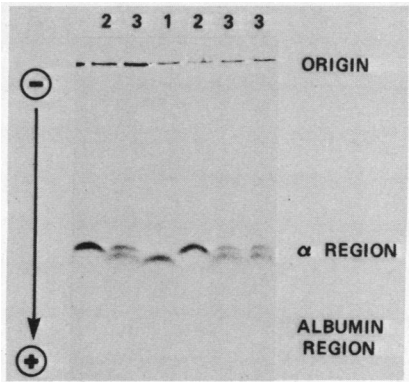

FIG. 1. An autoradiograph of a dried slab gel with human serum samples labeled by [^{14}C]vitamin D_3. Vitamin concentration was ten times physiologic normal, electrophoresis was conducted at 3°, and the gel was exposed to x-ray film for 10 days. Individuals having labeled proteins with (*1*) fast mobility (cathodal), (*2*) slow mobility (anodal), and (*3*) both mobilities can be observed. Under these conditions no binding to albumin is detectable, but some radioactivity does not enter the gel. This may represent unbound [^{14}C]vitamin D_3 or radioactivity associated with other, lipophilic serum components.

Gc Phenotyping. Electrophoretic separation of human serum samples was conducted on polyacrylamide gels with a continuous buffer system, Tris/EDTA/boric acid, pH 8.4, at room temperature. Electrophoresis was continued until maximum separation of α-globulins was achieved. Protein bands were visualized by two-step serial staining in Coomassie blue (15). The samples used in this work had their Gc type determined as a part of a large series; the validity of this typing procedure was periodically confirmed by immunoelectrophoresis.

RESULTS

Gel Autoradiography. Our initial observation was that individual differences exist in the electrophoretic patterns of a protein that binds vitamin D_3. The first data that were obtained suggested that this polymorphism might be related to Gc.

Gel autoradiography of serum samples labeled with 1.1 μM [^{14}C]vitamin D_3 revealed three distinct phenotypes for what we believed to be VDBG (Fig. 1). With [^{14}C]vitamin D_3 concentrations in the physiologic range these were the only labeled proteins found to have entered the gel even after relatively long exposures of gel to x-ray film.

On the assumption that these patterns represented the two homozygotes and single heterozygote for a two-allele autosomal locus, the "vitamin-D_3-binding genotype" of 80 Caucasians was determined. From these genotypes gene frequencies of 0.73 and 0.27 were calculated, the allele coding for the fast protein being the more common.

Gene frequencies of Gc in Caucasians are on the average 0.75 (Gc^1) and 0.25 (Gc^2). From this apparent coincidence and in view of the remarkable similarity in the general electrophoretic patterns of Gc proteins and VDBG, we hypothesized that these might actually be the same molecules. As a direct test of this hypothesis 25 serum samples of known Gc phenotype (8 Gc 1–1, 13 Gc 2–1, and 4 Gc 2–2) were tested, in a single blind study, for the phenotype of their vitamin-D_3-binding protein. The correspondence between the two pheno-

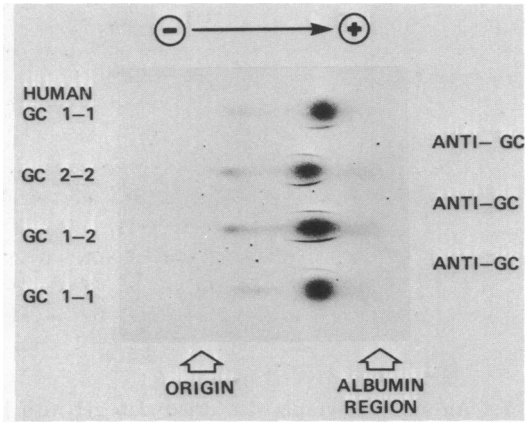

FIG. 2. Immuno-autoradiograph of individual human serum samples labeled with [^{14}C]vitamin D_3 and tested against antiserum to human Gc. Vitamin D_3 concentration was five times physiologic normal, the experiment was conducted at 3°, and the slide was exposed for 28 days. The position of the darkly labeled oval spots and the configuration of their corresponding precipitation arcs are consistent with that expected of each Gc phenotype. (Much of the radioactivity is associated with protein remaining near the point to which it was electrophoresed and which thus has not encountered Gc antiserum.) The arcs revealed by staining for protein the slide that produced this autoradiograph were directly superimposable on the radioactive arcs. A small amount of label is found in association with albumin and with a protein near the origin.

types was complete. The probability that this result would occur by chance is extremely small.

To establish that the binding was not merely artifactitious, we conducted gel autoradiography (*1*) with fresh heparinized plasma, (*2*) with fresh (not frozen) serum, (*3*) immediately after labeling and incubation, and (*4*) at room temperature. None of these experimental modifications altered the autoradiographic patterns.

Immuno-Autoradiography. Since the implicit definition of the Gc system is based on immunologic characterization, we proceeded to further establish the identity of Gc and VDBG by immuno-autoradiography.

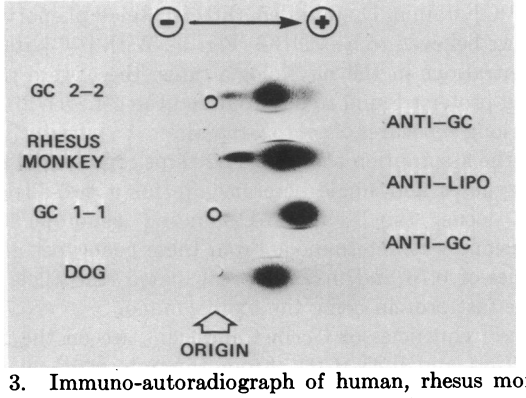

FIG. 3. Immuno-autoradiograph of human, rhesus monkey, and dog serum labeled with [^{14}C]vitamin D_3 (20 times normal concentration, 10 day exposure). Serum samples were tested against antiserum to human Gc and anti-α_1-lipoprotein. Although distinct precipitation arcs were seen in each case, only arcs formed against anti-Gc had radioactivity associated with them. In the human samples, a substantial fraction of the vitamin did not enter the gel.

TABLE 1. *Results of radio-immunoprecipitation experiments*

Experiment	Vitamin concentration (in μM) (1% ethanol)	% Total activity in precipitate ± SEM
1. Serum + [^{14}C]vitamin D_3 + anti-Gc	0.10	19.5 ± 2.5
2. Serum + [^{14}C]vitamin D_3 + anti-α_1-lipoprotein	0.10	<1
3. Serum + [^{14}C]vitamin D_3 + normal saline	0.10	<1
4. Serum + 25-hydroxy[^{3}H]-vitamin D_3 + anti-Gc	0.01	44.3 ± 8.5

Human serum samples, labeled with [^{14}C]vitamin D_3, were electrophoresed and allowed to diffuse against antiserum to human Gc. Once distinct precipitation arcs had formed, the agar slide was dried and autoradiographs were made (Fig. 2). The radioactive arcs seen in the x-ray film were exactly superimposable on the arcs revealed by staining the original slide for protein. Only a single labeled arc, of appropriate electrophoretic mobility, was produced from the serum of each known Gc phenotype. Under these conditions sera of Gc type 1–2 produce long, continuous bands embracing the entire Gc region.

As controls [^{14}C]vitamin-D_3-labeled serum was tested against anti-human α_1-lipoprotein and serum labeled with other radioactive lipids was tested against anti-Gc. Distinct precipitation arcs formed but in no instance were these arcs found to have bound the available ^{14}C-labeled ligand (center trough, Fig. 3).

Radio-Immunoprecipitation. Our next consideration was whether Gc binds the other forms of vitamin D normally present in human serum. Of these, 25-hydroxyvitamin D_3 is commercially available in radioactive form but labeled with ^{3}H, which did not prove satisfactory for gel autoradiography. A method using immunoprecipitation was, therefore, developed.

Human serum labeled with [^{14}C]vitamin D_3 or 25-hydroxy-[^{3}H]vitamin D_3 was precipitated with Gc antiserum or with antiserum to α_1-lipoprotein. An additional serum sample, with [^{14}C]vitamin D_3, was further treated with normal saline rather than antiserum. Table 1 summarizes the results of these experiments.

Antiserum to human Gc precipitated a major fraction of the available vitamin D; the α_1-lipoprotein antiserum and saline did not. The greater efficiency with which 25-hydroxyvitamin D_3 was precipitated with respect to vitamin D_3 probably reflects the difference in concentration of the two ligands and/or the substantial difference in the binding half-time of each (10). We believe that this may be an effective technique for detecting inherent differences in the binding capacities or affinities of the two Gc alleles.

Vitamin D Binding in Other Mammalian Species. Further experiments were directed toward determining if the Gc-like proteins from other mammals were also capable of binding vitamin D_3.

Serum samples from several animals were labeled with

[^{14}C]vitamin D_3 in amounts 10 times physiologic normal for humans. Gel autoradiography revealed one or more labeled proteins, distinct from albumin, in each animal tested (mouse, rat, rabbit, guinea pig, horse, cow, dog, rhesus monkey, and chimpanzee).

Next, immuno-autoradiography was performed on these labeled samples using antiserum (raised in rabbits) specific to human Gc. In the case of mouse, rat, horse, cow, dog, rhesus monkey, and chimpanzee, at least one batch of antiserum produced distinct precipitation arcs. Precipitation did not occur in rabbit or guinea pig serum. In each case where arcs formed autoradiography revealed that these bands had bound substantial amounts of [^{14}C]vitamin D_3 (Fig. 3). The electrophoretic mobility of the stained and labeled arcs was consistent with that determined by gel autoradiography.

An elaboration of this work on animal sera will be published at a later date.

DISCUSSION

Does Gc Bind Vitamin D In Vivo? Immunoprecipitation studies demonstrate that Gc binds vitamin D_3 and 25-hydroxyvitamin D_3 in physiologically normal concentrations and at normal serum pH and temperature. Displacement studies with other steroids indicate this binding is specific for vitamin D and its metabolites (details to be reported at a later date). It is highly probable that Gc retains these properties *in vivo*. The apparently long evolutionary history of this specificity, attested to by the fact that Gc-like proteins in other mammalian species have an affinity for vitamin D, supports the argument that this property of human Gc proteins is certainly not just coincidental.

One interesting point is that the normal concentration of human Gc in adults (8–14 μM) (cited in ref. 4) is 30 to 50 times the combined molar concentration of vitamin D and its congeners. Gc proteins in fresh serum labeled with ten times normal concentrations of [^{14}C]vitamin D_3 retain more than 50% of the available radioactivity after gel electrophoresis, as revealed by microdensitometry tracings of resultant autoradiographs (e.g., Fig. 1). This magnitude of excess binding capacity has also been noted in the rat (16). One can speculate that since these vitamins are lipophilic and essentially insoluble in an aqueous medium, large excesses of Gc are necessary to assure retention of every vitamin molecule and prevent their loss into noncirculating deposits. Gc may also act as a buffer to prevent toxic vitamin D effects at a local tissue level.

Are Gc and Vitamin-D-Binding α-Globulin the Same Protein? The remarkable number of similarities in the reported properties of these molecules, including electrophoretic mobility, binding specificities, excess binding capacity, and inter-species relatedness, makes it very probable that they are the same protein known by different names. One report, by Peterson in 1971, appears to cast doubt on this conclusion (17). A second related paper was published in 1972 (18). Peterson reported the purification of a putative "vitamin-D-binding plasma protein" with α_1 mobility and claimed that this was *not* a known plasma protein. In fact, Gc was stated to be a "contaminant." The purification, conducted on pooled, outdated human plasma, included precipitation in a 40% (by weight) ammonium sulfate solution, five stages of column chromatography, and repeated dialyzation. Trace amounts of [^{14}C]vitamin D_3 were used to mark the semi-purified protein at each step. From 16 liters of plasma 7.2 mg of pure protein were produced. This quantity represents less than one thousandth of the total available Gc.

Peterson reported that (*1*) antiserum to whole human serum did not cross-react with their purified protein (presumably the concentration of protein in the serum originally used to raise antiserum was too low to produce detectable titers of antibody); (*2*) the protein had electrophoretic mobility and molecular weight similar to but different from Gc; and (*3*) Gc was present as a contaminant at most stages of the purification but was eliminated at the final step. The nearly total exclusion of Gc was, however, to be expected, since most would remain in suspension until ammonium sulfate concentrations approached 60% (19).

Among possible explanations of the contrast between Peterson's results and ours are the following. Of 105 individuals screened by gel autoradiography one was found to have serum proteins in addition to Gc that bound vitamin D_3, thus suggesting that rare variants may be present in pooled plasma. Alternatively, Peterson may have extracted an antigenically distinct subfraction of Gc-related molecules. This may be a normal structural component or a breakdown product of Gc, the result of cellular and bacterial contamination, which is said to run in the α region (20, 21). Further work will be needed to completely resolve this question.

Proteins other than Gc may play a limited role in the transport of vitamin D in plasma. Albumin can bind vitamin D but at physiologic vitamin concentrations, and in the presence of Gc, only a small amount is associated with this protein (ref. 9, and Fig. 1). DeLuca and colleagues have shown that radiolabeled vitamin D, after *in vivo* administration, is first found predominantly in association with serum lipoproteins but in a matter of hours has shifted almost exclusively to VDBG. Immunoelectrophoretic separation, showing some radioactivity close to the origin, appears to support this observation (Fig. 3). Some vitamin D may also be associated with the cellular and chylomicron fractions of whole blood. Another protein, in addition to Gc-VDBG, may be involved in the transport of 1α,25-dihydroxy-vitamin D_3, the hormonal form of the vitamin which occurs in very low concentrations (9, 22). These observations notwithstanding, the evidence strongly supports the contention that Gc-VDBG plays a primary role in vitamin D transport *in vivo*.

Physiologic Significance of Gc–Vitamin D Binding. The physiologic significance of VDBG was reviewed quite extensively in a recent article (9). One difficulty is the ubiquitous occurrence of VDBG with seemingly unvarying properties. Perhaps the identification of VDBG with Gc, which is polymorphic, will contribute to a resolution of this dilemma.

Is a diminution in the concentration of Gc or the presence of a variant form of the protein associated with a vitamin D dysfunction such as rickets? Two instances of a silent allele at the Gc locus have been reported, but only as heterozygotes (cited in ref. 2). A related observation is that a vitamin-D-deficient diet appears to induce increased synthesis of VDBG (presumably Gc) in 7-week-old rats (10). From this point of view, certain cases of inherited vitamin-D-resistant or vitamin-D-dependent rickets may result from an abnormal Gc.

The wide distribution of the three Gc phenotypes will make it possible to test for differences at the molecular level in the properties, such as binding specificities, of the two alleles. We believe that some of the techniques suggested in

this report may be applicable to these investigations. Unfortunately many such studies in the past have been done on pooled plasma samples so that any individual differences would be confounded in the results.

Selective Significance. Based on an excess of heterozygotes in the progeny of Gc 2-2 × Gc 1-2 matings, it has been proposed that balancing selection is maintaining the worldwide polymorphism at the human Gc locus (4). Is vitamin D the focus of these selective forces? On the simplest level one can speculate that a difference in the binding affinity of the products of the two alleles for the various vitamin D's confers a selective advantage on the heterozygote. As D_2 is considered to be the dietary form of the vitamin and D_3 the form synthesized in the skin by ultraviolet light (though also available exogenously), it would be reasonable to seek associations between Gc gene frequencies and diet, incident ultraviolet—a function of cloud cover, latitude, and altitude—or, possibly, skin color. Naturally, in the absence of specific, supportive evidence of a biochemical or physiologic nature, any environmental correlation with Gc phenotype can at best be only suggestive.

An unusual feature of the electrophoretic properties of Gc is a "splitting" of the protein band associated with the Gc^1 allele (4). This phenomenon is seen in both Gc 1-1 individuals and heterozygotes but only under conditions permitting maximum, high-resolution separation. Our best preparations, using autoradiography of column gels, consistently reveal splitting of the Gc 1 protein, indicating that this presumed subfraction of Gc molecules binds [^{14}C]vitamin D_3. The apparent heterogeneity of the Gc 1 protein may thus bear on the question of the selective forces at work at this locus.

Finally, studies at both a molecular and population level should eventually include the other Gc alleles, Gc Aborigine and Gc Chippewa, which are found at polymorphic levels in some populations.

Application. Gel autoradiography of radio-labeled proteins is a rapid, high-resolution technique for the study of serum transport proteins. By labeling them with radioactive ligands, these proteins can be detected by virtue of a functionally significant property, binding specificity, in a manner analogous to stains dependent upon enzyme activity. One could apply this method to screen for genetic variants of several other transport proteins.

Conclusion. Our proof of the identity of vitamin-D-binding α-globulin and Gc relies on two lines of evidence. (*1*) Antiserum against Gc coprecipitates Gc and vitamin D. This still leaves the doubt that antigen used to prepare anti-Gc serum was not sufficiently purified and contained in addition to Gc, some VDBG. This is, of course, a very general problem in the purification and characterization by antibodies of any particular substance. But in this instance the doubt is resolved by the second line of evidence, namely (*2*) that Gc and VDBG *electrophoretic* variants are superimposable. This is an interesting example of how genetic differences can resolve a problem that would otherwise demand very elaborate biochemical procedures.

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