Isolation and Partial Characterization of a Human Vitamin D-binding Plasma Protein*

PER A. PETERSON

From the Department of Nutrition, Institute of Medical Chemistry, University of Uppsala, Uppsala, Sweden

SUMMARY

Vitamin D circulates in human plasma bound to a specific transport protein. This protein differs from the lipoproteins and has a hydrated density greater than 1.21. The purification of the human vitamin D-binding protein was accomplished by use of ammonium sulfate fractionation, DEAE-Sephadex chromatography, sulfoethyl-Sephadex chromatography, and gel chromatography. These procedures resulted in a highly purified preparation of the vitamin D-binding protein which had been purified approximately 15,000-fold. The purified protein appeared homogeneous by Ouchterlony immunodiffusion analyses, immunoelectrophoresis, and by analytical ultracentrifugation. The vitamin D-binding protein separated into two components on electrophoresis, both with $\alpha_1$ mobility. The most anodal component carried vitamin D$_3$, whereas the cathodal form of the vitamin D-binding protein was devoid of this form of the vitamin. The molecular weight of the vitamin D-binding protein determined by equilibrium ultracentrifugation and estimated from the sedimentation coefficient and gel chromatography was approximately 53,000. Determinations of the molecular weight of reduced and alkylated vitamin D-binding protein in 6M guanidine hydrochloride gave the same value as found under physiological conditions, suggesting that this protein is not composed of subunits. The frictional ratio ($f/f_0$) was low for the vitamin D-binding protein, indicating a close to spherical appearance for this protein.

The occurrence of the vitamin D-binding protein in normal serum, normal urine, and normal cerebrospinal fluid was established by Ouchterlony immunodiffusion analyses with use of a specific antiserum against the vitamin D-binding protein. Indirect estimates indicated that the normal concentration of this protein in serum is approximately 5 $\mu$g per ml.

EXPERIMENTAL PROCEDURE

Materials

Plasma—Outdated blood was obtained from the Blood Center, University Hospital, Uppsala. After centrifugation of the blood the plasma was sucked off and used immediately or after storage at -23°.

Urine—Normal urine was obtained from healthy individuals. Urine collection and sample processing were carried out as described elsewhere (6).

Cerebrospinal Fluid—Samples of cerebrospinal fluid were taken from five individuals in connection with diagnostic myelographies. The cerebrospinal fluids were considered normal, and the subjects were judged to be free from neurological disease. The samples were concentrated as described in a previous publication (7).

Antisera—A polyvalent antiserum against urinary proteins from patients with tubular proteinuria was kindly provided by Dr. I. Bergqvist. Anti-human serum protein serum and anti-Gc-globulin serum were purchased from Behringwerke AG (Marburg/Lahn, Germany). An antiserum against a highly purified preparation of the vitamin D-binding protein was raised in a rabbit with a previously described technique (8).

Other Materials—Sephadex G-100 and G-200, DEAE-Sephadex A-50, and sulfoethyl-Sephadex C-50, products of Pharmacia Fine Chemicals AB (Uppsala), were treated according to the instructions supplied by the manufacturer. $^{14}$C-Labeled vitamin

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D₄ (specific activity 23.6 mCi per mmole) was obtained from Phillips-Duphar (Petten, Netherlands).

All other chemicals were of the highest grade available and were used without further purification.

Methods

Large Scale Gel Chromatography—Gel chromatography on Sephadex G-200 was performed on a column consisting of six cylindrical segments (15 × 48 cm) comprising a total volume of 144 liters. Details of the procedure will be described elsewhere.

Procedure for Concentration of Proteins—Protein isolated during the purification procedure was concentrated by ultrafiltration (9) with Visking ?这儿-inch dialysis tubing (Union Carbide Corp., Chicago, Ill.) as the ultrafiltration membrane. The losses of material during the ultrafiltrations were small and always less than 10%.

After the final purification step fractions containing the vitamin D-binding protein were concentrated either by ultrafiltration or by lyophilization. Prior to lyophilization the protein was extensively dialyzed against distilled, deionized water.

Polyacrylamide Gel Electrophoresis—Vertical polyacrylamide gel electrophoresis was carried out in the apparatus of E-C Apparatus Co. (Philadelphia, Pa.). The electrophoreses were performed in 0.4 M Tris-glycine buffer, pH 8.9, with 4% Cyano- gum-41 (E-C Apparatus Co., Philadelphia, Pa.) in the spacer gel and 8% in the running gel.

Some experiments were performed with 10% glycerol in the gel under conditions otherwise identical with those described above. The glycerol permitted freezing and slicing of the gel. The sliced gels were handled as described previously (7).

Molecular Weight Determination by Gel Chromatography—Highly purified vitamin D-binding protein was labeled with carrier-free ³²P (Radiochemical Centre, Amersham) according to the chloramin T procedure of Greenwood and Hunter (10). The labeled protein was dialyzed against 6 M guanidine hydrochloride, reduced with 0.1 M dithiothreitol for 1 hour, and alkylated with 0.24 M iodoacetic acid. Reduced and alkylated protein was subsequently chromatographed on a Sepharose 6B (Pharmacia Fine Chemicals AB, Uppsala) column (120 × 1.5 cm) equilibrated with 6 M guanidine hydrochloride. Most proteins behave as random coils under these conditions and thus accord to molecular weight (11). The Sepharose 6B column used was calibrated with heavy and light chains of human IgG (12), retinol-binding protein (13), prealbumin (14), albumin, thyroxine-binding globulin, 2 ribonuclease and carboxypeptidase B (Worthington), and β₂-microglobulin (15). All proteins were reduced and alkylated in 1 M Tris-HCl buffer, pH 8.0, containing 6 M guanidine hydrochloride as described above for the vitamin D-binding protein.

Analytical Ultracentrifugation—Analytical ultracentrifugations were performed in a Spinco model E analytical ultracentrifuge equipped with an RTIC temperature control unit and an electronic speed control device. Determinations were carried out in standard 12-mm double sector cells with sapphire windows. All experiments were performed in 0.02 M Tris-HCl buffer, pH 8.0, containing 0.2 M NaCl. The centrifuge was operated at 60,000 rpm for sedimentation analyses, and recordings were accomplished with the photoelectric scanning system set at 280 nm. Calculations were carried out according to the method of Schachman (16). All values given are corrected to 20° and water.

Sedimentation equilibrium analyses were performed by the meniscus-depletion technique of Yphantis (17) as devised by Teller et al. (18). Recordings were made with the photoelectric scanning system set at 280 nm. The speed and time required for establishing equilibrium were estimated as described by van Holde (19). A final check of the equilibrium conditions was obtained by taking recordings over a time period of several hours. It was assumed that when no redistribution of material could be detected in recordings taken with a 5-hour interval, equilibrium had been established.

Calculations of apparent weight average molecular weights were computed from the following equation (20)

$$M_w = 2RT(dln C/dx^2)/(1 - \bar{b}ρ)u$$

where the symbols have their usual meaning. The partial specific volume was calculated from parallel sedimentation equilibrium runs in solvents with H₂O and D₂O, respectively, according to the equation given by Edelstein and Schachman (21)

$$kM_w = 2RT(dln C/dx^2)/(1 - V_{w20}/k)_o$$

where a value of 1.0155 for k was assumed (21).

Determination of Stokes Radius—Analytical gel chromatography was performed on a Sephadex G-200 column (100 × 1.0 cm) equilibrated with 0.02 M Tris-HCl buffer, pH 8.0, containing 0.15 M NaCl. The details of this procedure have been outlined elsewhere (9). All experiments were carried out at +4°C, fractions of 1.0 ml were collected, and analyses were performed in duplicate.

Stokes molecular radius, rs, was calculated from the equation of Laurent and Killander (22)

$$K_w = e^{-rL(r_f + r_s)^2}$$

where K_w is the volume available for the analyzed protein in the gel phase, and L and r_s are constants characteristic of each gel type. L was determined from the K_w of human albumin (r_s = 35.5 Å) and r_s was assumed to be 6.5 Å (23). The Stokes radius of the vitamin D-binding protein was used for the calculation of the molecular weight according to the method of Siegel and Monty (24).

Other Methods—Ouchterlony immunodiffusion analyses (25) were carried out with a previously described microtechnique (26). Immunoelectrophoresis was performed according to Scheidegger (27).

Plasma lipoproteins were separated from non-lipoproteins by ultracentrifugation in a Spinco L-2 65 preparative ultracentrifuge with use of rotor 65. Samples of plasma were adjusted to a density of 1.21 and centrifuged as described by Havel, Eder, and Bragdon (28).

Protein concentrations in the unpurified fractions containing the vitamin D-binding protein were estimated by the modified Folin procedure of Lowry et al. (29) with immunoglobulin G as the standard. More highly purified fractions were quantitatively assayed for protein by measuring the absorbance at 280 nm assuming an E₂₈₀ of 15.0 based on the relation between the absorbance at 280 nm and the measured quantity determined by the Folin method.

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All operations during the isolation procedure were carried out at +4°.

RESULTS

Distribution of Vitamin D₃ among Human Plasma Proteins

To achieve some information about the number and the size of vitamin D₃-binding proteins in human plasma the following experiment was undertaken.

Plasma, to which small amounts of ³⁵Cl-labeled vitamin D₃ had been added, was chromatographed on a column (137 × 2.4 cm) of Sephadex G-200 equilibrated with 0.02 M Tris-Cl buffer, pH 8.0, containing 0.15 M NaCl. It was evident from the chromatogram that ³⁵Cl-labeled vitamin D₃ was eluted at two positions. The material emerging at the void volume of the column contained about half of the added radioactivity whereas the residual radioactivity was eluted at a position somewhat later than that of albumin. Fractions in the two positions containing radioactivity were separately pooled, concentrated, and subjected to ultracentrifugation after the density of each sample had been raised to 1.21. All radioactivity of the material recovered in the void fraction was associated with material of density less than 1.21, whereas the ³⁵Cl-labeled vitamin D₃ eluted later than albumin had sedimented and thus was of a density greater than 1.21. A similar distribution of vitamin D₃ has been described for dog plasma after addition of the compound in vivo (3). This pattern is also obtained in plasma from monkeys. In a study on the time course of the distribution of ³⁵Cl-labeled vitamin D₃ after in vivo administration to monkeys, it has been shown that the radioactivity first associates with the lipoproteins and later appears in a protein which has similar characteristics as the human counterpart.

Purification of Human Vitamin D₃-Binding Protein

The human vitamin D₃-binding protein was isolated from altogether 30 liters of outdated plasma. During the isolation procedure ³⁵Cl-labeled vitamin D₃ was added in order to trace the vitamin D-binding protein. In later stages of the purification procedure the vitamin D binding protein was occasionally followed by polyacrylamide gel electrophoresis.

During the first preparations, the starting material consisted of 0.5 to 4 liters of plasma but it was noted that the vitamin D-binding protein was present in amounts too low to obtain quantities of the purified protein sufficient for characterization. The isolation procedure adopted was very reproducible and a large scale purification was achieved. The radioactivity first associates with the lipoproteins and later appears in a protein which has similar characteristics as the human counterpart.

Ammonium Sulfate Fractionation—The vitamin D-binding protein is obviously a minor constituent of the plasma proteins. It was therefore necessary to develop a simple fractionation step in order to separate the vitamin D-binding protein from some of the contaminating plasma proteins, thereby accomplishing a concentration to obtain suitable volumes for the further purification steps. Such a separation could be accomplished by means of ammonium sulfate fractionation. Small volumes of plasma, containing trace amounts of [³⁵Cl]D₃, were adjusted to different concentrations of ammonium sulfate and left at +4° overnight with continuous stirring. The precipitates were centrifuged off and the radioactivity in the supernatants was determined. The content of [³⁵Cl]D₃ in the supernatants decreased considerably at an ammonium sulfate concentration of more than 30%. Accordingly, two-liter portions of plasma were adjusted with ammonium sulfate to a final concentration of 40%. The precipitate (406,000 mg of total protein) from 16 liters of plasma was dissolved in 5,000 ml of 0.02 M Tris-Cl buffer, pH 8.0, containing 0.5 M NaCl. The resulting solution, which was slightly turbid, was recentrifuged and the clear supernatant was subjected to Sephadex G-200 chromatography.

Sephadex G-200 Chromatography—The experiment on the size distribution of protein-bound [³⁵Cl]D₃ (see above) showed that the carrier protein was eluted somewhat later than albumin on Sephadex G-200 chromatography. The dissolved precipitate, 306,000 mg of total protein, obtained from the ammonium sulfate fractionation step, was subjected to gel chromatography on a Sephadex G-200 column, consisting of six stacked segments (15 × 48 cm each), equilibrated with 0.02 M Tris-Cl buffer, pH 8.0, containing 0.15 M NaCl. Fractions of 2,000 ml were collected at a flow rate of 6,000 ml per hour. The chromatogram showed that only minor amounts of albumin was present. As most plasma proteins were eluted ahead of albumin, a considerable purification of the vitamin D binding protein was achieved. The fractions eluted at the position corresponding to the Kᵥ of [³⁵Cl]D₃ on analytical gel chromatography, elution volume 84 to 100 liters, were pooled and concentrated by ultrafiltration. Adjacent fractions contained at most negligible quantities of the vitamin D-binding protein. This was shown by addition of [³⁵Cl]D₃ to these concentrated fractions followed by analytical gel chromatography. For the further purification of the vitamin D-binding protein the concentrated material, 22,700 mg of total protein, was divided into four aliquots which were separately purified according to the procedure described below.

First DEAE-Sephadex Chromatography—The fraction of concentrated vitamin D-binding protein from the gel chromatography step was exhaustively dialyzed against 0.05 M Tris-Cl buffer, pH 7.4, and thereafter subjected to ion exchange chromatography on a column (42 × 6.5 cm) of DEAE-Sephadex, equilibrated with the same buffer. Elution was performed at pH 7.4 with a 5000-ml linear gradient of NaCl from 0 to 0.3 M. Prior to application, ³⁵Cl-labeled vitamin D₃ was added to the dialyzed sample. To enhance equilibration between unlabeled endogenous and labeled exogenous vitamin D the sample was incubated at 37° for 2 hours. A good separation was achieved between the [³⁵Cl]D₃-labeled vitamin D-binding protein and the bulk of the contaminating proteins. The fractions containing radioactivity were combined and concentrated by ultrafiltration.

Chromatography on Sulfophethyl Sephadex—Various conditions for further purification of the vitamin D-binding protein were tested. The following procedure was finally adopted.

A column (31 × 1.9 cm) of sulfoethyl-Sephadex was equilibrated with 0.02 M sodium acetate buffer, pH 5.0, containing 0.03 M NaCl. The fraction containing the vitamin D-binding protein from the DEAE-Sephadex chromatography step was dialyzed against three changes of the same buffer and then applied to the column. The starting pH is critical since protein in this fraction tends to precipitate at pH 5.0 at the low ionic strength employed. The column was eluted with a linear salt gradient of 600 ml from 0.03 to 0.15 M NaCl in the sodium acetate buffer. Multiple protein peaks were obtained. The [³⁵Cl]D₃
radioactivity was, however, eluted in a position which did not coincide with any of the protein peaks. Thus a considerable purification of the vitamin D-binding protein was achieved. The radioactive vitamin D$_3$ fractions were pooled and concentrated.

**Gel Chromatography on Sephadex G-100**—The concentrated fraction containing the vitamin D-binding protein obtained from the sulfoethyl-Sephadex chromatography step was subjected to gel chromatography on a Sephadex G-100 column (130 x 2 cm) equilibrated with 0.02 M Tris-HCl buffer, pH 8.0, containing 0.15 M NaCl. The applied protein consisted of at least three components as indicated by the partly resolved elution peaks. The last eluted material coincided with the distribution of the radioactivity. Eluates corresponding to this position were combined and concentrated by ultrafiltration.

**Second DEAE-Sephadex Chromatography**—The fraction containing the vitamin D-binding protein obtained from the previous purification step was chromatographed on a column of DEAE-Sephadex, equilibrated with 0.05 M Tris-HCl buffer, pH 9.0, containing 0.10 M NaCl. The sample was applied after extensive dialysis against the same buffer and the column was run as described in the legend of Fig. 1. The protein was eluted at three positions. $^{14}$C-Labeled vitamin D$_3$ was present only in the last protein peak. It can be seen from Fig. 1 that the specific activity of $^{[14C]}$D$_3$ was constant over the frontal part of this peak. Fractions with the same specific activity were pooled as indicated in the figure, dialyzed exhaustively against distilled, deionized water, and lyophilized. This material constituted purified vitamin D-binding protein.

The tail of the last protein peak which contained the residual amount of the vitamin D-binding protein was rechromatographed under identical conditions. The elution profile was similar to that depicted in Fig. 1, except that the first protein peak of the elution diagram was missing. The protein peak immediately preceding the position of the vitamin D-binding protein was always present on rechromatographs. This material was pooled and concentrated. Evidence will be given below to certify that it represents vitamin D-binding protein. Part of the last eluted protein peak had on rechromatography a constant specific radioactivity. This material was accordingly treated as highly purified vitamin D-binding protein. The total amount of purified vitamin D-binding protein obtained after this purification step was 7.2 mg.

**Homogeneity, Purity, and Immunological Properties of Vitamin D-binding Protein**—The homogeneity of the isolated preparation of the vitamin D-binding protein was tested by gel chromatography on a column of Sephadex G-200. The applied sample emerged as a symmetrical protein peak from the column as did the $^{[14C]}$D$_3$ radioactivity. Thus, the specific radioactivity was constant over the entire protein distribution indicating a high degree of purity and size homogeneity for the tested preparation.

Although the vitamin D-binding protein appeared homogeneous on gel chromatography, polyacrylamide gel electrophoresis revealed charge heterogeneity. Fig. 2 shows that highly purified vitamin D-binding protein gave two protein zones (Fig. 2A), both with $a_2$ mobility, whereas the tail part of the peak containing the vitamin D-binding protein obtained from the second DEAE Sephadex chromatography step contained several additional components (Fig. 2B). The two zones of the electrophoretically separated vitamin D-binding protein was eluted from the gel. Analyses of the distribution of $^{14}$C-labeled vitamin D$_3$ revealed that only the zone with highest anodal mobility contained radioactivity. To exclude that the electrophoretically slower component was unrelated to the vitamin D-binding protein, Ouchterlony immunodiffusion analysis was performed. It is evident from Fig. 2 that both components reacted with the specific anti-vitamin D-binding protein serum and showed complete immunological identity. This indicated that the vitamin D-binding protein, on complexing with vitamin D$_3$, had a higher electrophoretic mobility than when free from this form of the vitamin. This was corroborated by the finding that the slower component, after addition of vitamin D$_3$ changed its electrophoretic mobility to that of the faster $^{[14C]}$D$_3$-containing component. In the same experiment, it was shown that the protein peak always emerging somewhat earlier than $^{[14C]}$D$_3$ on the second DEAE-Sephadex chromatography step (cf. Fig. 1), reacted with highly purified vitamin D-binding protein whereas both antisera reacted with the contaminating protein present in the tail part of the $^{[14C]}$D$_3$-containing peak obtained by the second DEAE-Sephadex chromatography step (cf. Fig. 1 and Fig. 2B). This contaminant was identified as Gc-globulin by use of a specific anti-serum against this protein. The antisera raised against the preparation of highly purified vitamin D-binding protein gave a single arc of precipitation with the vitamin D-binding protein when examined on immunoelectrophoresis, giving further evidence for the purity of the vitamin D-binding protein.
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FIG. 2 (left). Polyacrylamide gel electrophoresis in Tris-glycine buffer, pH 8.9, of highly purified vitamin D-binding protein (A) and of the tail part of the peak containing the vitamin D-binding protein obtained from the second DEAE-Sephadex chromatography (B) (cf. Fig. 1).

Fig. 3 (center). Results of Ouchterlony immunodiffusion analysis of different vitamin D-binding protein components. Comparison of the anodal [14C]D-containing component (1) and the cathodic component (2) eluted from a polyacrylamide gel after electrophoretic separation (cf. Fig. 2A), and of the protein peak immediately preceding that containing [14C]D obtained on the second DEAE-Sephadex chromatography (3) (cf. Fig. 1). The center well contained an antiserum against the vitamin D-binding protein.

FIG. 4 (right). Demonstration of the vitamin D-binding protein in normal serum (1), normal urine (2), and normal cerebrospinal fluid (3) by Ouchterlony immunodiffusion analysis. The center well of the plate contained an antiserum against the vitamin D-binding protein.

TABLE I

<table>
<thead>
<tr>
<th>Physical properties of human vitamin D-binding protein</th>
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<tr>
<td>Sedimentation constant, $s_{20,w}$ (S)</td>
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<tr>
<td>Partial specific volume, $v_p$ (ml/g)</td>
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<tr>
<td>Stokes molecular radius, $A$ (Å)</td>
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<td>Diffusion constant, $D_{20,1}$</td>
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<td>Frictional ratio, $f/f_s$</td>
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<td>Sedimentation Stokes radius</td>
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$^a$ Determined by parallel sedimentation equilibrium ultracentrifugations in H₂O and D₂O.

$^b$ Estimated by analytical gel chromatography. The diffusion constant is given as $10^{-11}$ cm² sec⁻¹.

$^c$ The speed used for the ultracentrifugations was 30,000 rpm.

$^d$ Determined in 6 M guanidine hydrochloride on reduced and alkylated vitamin D-binding protein.

normal serum, normal cerebrospinal fluid, and normal urine was established by Ouchterlony immunodiffusion analyses. As can be seen in Fig. 4, material reacting like the vitamin D-binding protein was present in these three biological fluids. The vitamin D-binding protein from the different sources gave reactions of complete immunological identity.

Physical Properties of Vitamin D-binding Protein—Some physical properties of the vitamin D-binding protein are summarized in Table I.

Sedimentation velocity analyses were carried out at protein concentrations of 0.02 to 0.1%. The vitamin D-binding protein behaved as a single homogeneous component within this con-
From these experiments a molecular weight of 53,000 was estimated for the vitamin D-binding protein, a value in good agreement with the ultracentrifugation data. Furthermore, this result indicates that the vitamin D-binding protein probably consists of a single polypeptide chain.

**DISCUSSION**

The experiments reported here demonstrate that vitamin D circulates in human plasma bound to a specific plasma protein. This protein has $\alpha_1$ mobility, a molecular weight of 53,000, and apparently differs from all previously identified plasma proteins. The vitamin D-binding protein has a sedimentation constant ($s_{20,w}$) of 3.8 S, a frictional ratio ($f/f_0$) of 1.22, indicating a close to spherical appearance assuming "normal" hydration, and consists probably of a single polypeptide chain.

From the level of vitamin D normally circulating in plasma (30), one can estimate the usual level of the vitamin D-binding protein to be approximately 5 $\mu$g per ml. This figure, however, assumes that the vitamin D-binding protein carries a single molecule of vitamin D and is saturated with the vitamin. Preliminary studies with use of an immunological method for quantitation of the vitamin D-binding protein have given results in accord with this estimate. The concentration of the vitamin D-binding protein in serum indicates that the final preparation of the vitamin D-binding protein must have been purified about 15,000-fold. The adopted isolation procedure gave a recovery of highly purified vitamin D-binding protein of about 0.5 $\mu$g per ml of starting material, indicating a yield of about 10%.

The vitamin D-binding protein isolated from plasma consisted of a mixture of two components, both with $\alpha_1$ mobility, but which separated from each other on polyacrylamide gel electrophoresis. The two components had apparently the same molecular size and weight and exhibited complete immunological identity on Ouchterlony immunodiffusion analysis. The major of these two components contained bound vitamin D$_2$, whereas the other component did not. Since small amounts of protein-bound vitamin D$_2$ were lost during the purification procedure, it is likely that much of the vitamin D-binding protein devoid of vitamin D$_2$ found in the purified preparation arose during the course of the purification. It is, however, also possible that a small amount of vitamin D-free vitamin D-binding protein is normally present circulating in plasma, in analogy to the vitamin A-transporting protein (31). Further work, now in progress, is required for elucidating this question.

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Per A. Peterson


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