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Purification of the Receptor for 1α,25-Dihydroxyvitamin D_3 from Chicken Intestine†

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ABSTRACT: Methods were investigated for use in the purification of the chicken intestinal receptor for 1α,25-dihydroxyvitamin D_3. The techniques investigated include column isoelectric focusing, gel exclusion, polyacrylamide gel electrophoresis, and DNA–cellulose, DEAE-cellulose, and hydroxylapatite chromatography. For the starting receptor preparation, a nuclear extract of chicken intestinal mucosa was found to be enriched above cytosol preparations and a plentiful source of receptor. A five-step purification scheme that resulted in the purification of the receptor protein by 5800-fold with 8% yield has been described. Analysis of the purified proteins on polyacrylamide gel electrophoresis containing sodium dodecyl sulfate suggests homogeneity. Analysis using two-dimensional polyacrylamide electrophoresis characterized the purified protein as having a molecular weight of approximately 63 000 and a p*I* of 6.0–6.2. Furthermore, assessment of protein purity by ^{125}I iodination followed by sucrose gradient analysis revealed that approximately 90% of the iodinated macromolecules have the same sedimentation coefficient as the titrated 1α,25-dihydroxyvitamin D_3 receptor complex. The final purified receptor that bound tritiated 1α,25-dihydroxyvitamin D_3 retained affinity for DNA–cellulose and possesses a 3.7S sedimentation coefficient. The receptor has an estimated Stokes radius of 37 Å.

Vitamin D_3 has been shown to exert its physiological actions involved in the control of serum calcium and phosphate homeostasis via metabolic activation. It is now accepted that 1α,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$][1] is the hormonally active vitamin D_3 metabolite responsible for the actions of the vitamin (DeLuca & Schnoes, 1976).

Studies have shown that the intracellular mechanism of action of 1,25-$(OH)_2D_3$ for control of intestinal calcium absorption involves the modulation of gene transcription (Eisenstein & Passavoy, 1964; Zull et al., 1965; Norman, 1965). Autoradiographic and subcellular fractionation studies have further shown specific nuclear localization of 1,25-$(OH)_2$-[^3H]D_3 (Haussler et al., 1968; Chen & DeLuca, 1973) and revealed specific high-affinity 1,25-$(OH)_2D_3$ receptor-like protein in intestinal cytosol and nuclei (Brumbaugh & Haussler, 1974; Kream et al., 1976). Current findings suggest that 1,25-$(OH)_2D_3$ acts on the intestine in a manner similar to that proposed for other steroid hormones. Central to the intraceullular mechanism of action of 1,25-$(OH)_2D_3$ is the interaction of the hormone with specific receptor proteins.

Our laboratory along with others have characterized 1,25-$(OH)_2D_3$ receptor-like proteins. They exist in over 20 target organs [for review, see Norman et al. (1982)] including intestine (Brumbaugh & Haussler, 1974; Kream et al., 1976), bone (Kream et al., 1977; Mellon & DeLuca, 1980), kidney (Simpson et al., 1980; Chandler et al., 1979), pancreas (Christakos & Norman, 1979), skin (Simpson & DeLuca, 1980; Colston et al., 1980), and mammary tissue (Reinhardt

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[1] Abbreviations: 1,25-$(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol.

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& Conrad, 1980). The receptor has a molecular weight of 60 000–72 000, as estimated by gel-exclusion chromatography (Franceschi & DeLuca, 1979; Wecksler et al., 1980). Our laboratory recently demonstrated that purification of the 1,25-$(OH)_2D_3$ receptor from chicken intestines yielded a protein of a molecular weight approximate to those of bovine serum albumin (67 000) and catalase (60 000) as determined by $NaDodSO_4$ gel electrophoresis (Simpson & DeLuca, 1982). Others, using similar techniques, have independently supported this estimate (~64 000) of the purified 1,25-$(OH)_2D_3$ receptor (Pike et al., 1982a,b). A higher molecular weight form of the receptor (<100 000) has also been measured by gel-exclusion chromatography when phenylmethanesulfonyl fluoride (PMSF) is added to receptor preparations before or after tissue homogenization or receptor labeling (Bishop et al., 1982). The importance of this form remains to be established. We recently showed the receptor to be an acidic macromolecule (~p*I* = 6.0) (Simpson & DeLuca, 1982). It has been found to have affinity for DNA, to be stabilized by sodium molybdate, and to aggregate under low-salt conditions (Franceschi et al., 1981). The physiological importance of these findings is unknown.

We have previously reported the isolation of microgram quantities of this receptor protein (Simpson & DeLuca, 1982). Critical to this purification was the use of a crude nuclear extract obtained from mature chicken intestines. This provided an enriched and plentiful receptor preparation. We purified the preparation by selective precipitation, gel filtration, ion exchange, and chromatofocusing (Simpson & DeLuca, 1982).

Here we report additional techniques for purification of this protein, including nondenaturing gel electrophoresis, preparative isoelectric focusing, and batch absorption to and specific elution from DNA–cellulose. We also report a purification scheme utilizing DNA–cellulose followed by gel-exclusion and ion-exchange chromatography that results in an apparently pure protein with a similar molecular weight (63 000) to that previously reported (Simpson & DeLuca, 1982; Pike et al., 1982a,b). This paper demonstrates the purity and character of this protein.

Materials and Methods

Chemicals. Nonradioactive 1,25-$(OH)_2D_3$ was provided by the Hoffmann-La Roche Co. of Nutley, NJ. 1,25-$(OH)_2$-[26,27-$^3H_2$]D_3 (160 Ci/mmol, 1 Ci = 3.7 × 10^{10} Bq) was prepared as previously described (Napoli et al., 1980). The Bolton–Hunter reagent [iodinated (*p*-hydroxyphenyl)propionic acid *N*-hydroxysuccinimide ester] (2000 Ci/mmol) for protein iodination was obtained from New England Nuclear, Inc. (Boston, MA). All additional chemicals were at least reagent grade.

Intestinal Tissue Preparation. Mature chicken duodena were obtained from Arcadia Fryers, Inc. (Arcadia, WI). For receptor preparation, intestines were used fresh or after quick freezing on dry ice followed by thawing. They were rinsed with TED buffer (50 mM Tris-HCl–1.5 mM EDTA–5 mM DTT, pH 7.4), and the mucosa was scraped from the serosa. The mucosa was rinsed with 3 volumes of TED buffer. A 30% tissue homogenate was prepared in this buffer with 10-s bursts from a Polytron homogenizer (Brinkmann Instruments, Westbury, NJ). The homogenate was centrifuged at 4000*g* for 10 min to prepare a low-speed "crude nuclear pellet". The pelleted material was rinsed 3 times by resuspension and centrifugation with 4 volumes of TED buffer. The receptor was extracted from the crude nuclear pellet with TED buffer containing 300 mM KCl and 10 mM $MgCl_2$. The extract was then centrifuged for 60 min at 22000*g*, and the supernatant fraction (the crude nuclear extract) was used immediately. Before isolation procedures were initiated, the crude nuclear extract was labeled with 4 nM 1,25-$(OH)_2[^3H]D_3$ (5–16 Ci/mmol) added in ethanol (final concentration 1.0%) and incubated on ice for 12–16 h.

Receptor Binding Assays. The hydroxylapatite technique (Wecksler & Norman, 1979), the dextran-coated charcoal technique (Mellon et al., 1980), or both assays were used for assessment of receptor-bound hormone.

Ammonium Sulfate Precipitation. The crude receptor preparation was precipitated by addition of ice-cold $(NH_4)_2SO_4$ (100% saturated) to yield a final concentration of 38% (v/v). After 10–30 min, the suspension was centrifuged at 9000*g* for 10 min. The surface of the pellet was then rinsed with ice-cold distilled water and resuspended in TED buffer.

Chromatographic Procedures. All purification procedures were performed on ice or at 0–4 °C.

DNA–Cellulose. Aliquots of $(NH_4)_2SO_4$-precipitated receptor were diluted with TED buffer to achieve an ionic strength equal to 50 mM KCl. DNA–cellulose was prepared by the procedure of Alberts & Herrick (1971) with calf thymus DNA (Sigma) and cellulose (Bio-Rad, Cellex 410). Routinely, a 10% (v/v) suspension of DNA–cellulose was used for batch adsorption of receptor. The suspension was gently swirled on ice for 30 min. After this period, the DNA–cellulose–receptor complex was collected by gentle centrifugation at 100*g* for 5 min and rinsed once with TED buffer containing 50 mM KCl (TED K_{50}). The DNA–cellulose was then packed into an appropriate column (2.6 × 45 cm) and rinsed with 2 column volumes of TED K_{50}. The receptor was eluted from the column with a linear 0.1–0.4 M KCl gradient in TED buffer.

Hydroxylapatite. The eluted receptor was purified and concentrated on a small (1 × 5 cm) column packed with 4 mL of hydroxylapatite (Bio-Gel HTP, Bio-Rad, Richmond CA). The column was equilibrated with TED buffer, and the receptor–1,25-$(OH)_2[^3H]D_3$ complex was applied with a slow flow rate (~0.5 mL/min). The ionic strength of the sample was adjusted to equal that of 0.1–0.2 M KCl. The column was rinsed with 2 volumes of TED buffer and 2 volumes of TED buffer containing 0.5% Triton X-100. A linear 50–500 mM gradient of KH_2PO_4 in TED buffer was used to elute the receptor.

Sephacryl S-200. A column (2 × 90 cm) of Sephacryl S-200 (Pharmacia, Piscataway, NJ) was packed to a bed volume of 240 mL. The column was equilibrated in TED K_{50} containing 0.5% Triton X-100 and eluted at a flow rate of 30 mL/h with a peristaltic pump. Fractions were collected in an LKB RediRac (Stockholm, Switzerland) fraction collector. The column was calibrated with blue dextran (2 × 10^6 daltons) and 20 mg each of bovine γ-globulin (160 000), bovine serum albumin (67 000), ovalbumin (43 000), myoglobin (18 000), and cytochrome *c* (12 000).

DEAE-cellulose. A 1.0 × 10 cm column was packed with 10 mL of DEAE-Sepharose (Pharmacia) and eqilibrated with TED K_{50} containing 0.5% Triton X-100. After sample application, the column was rinsed with 2 column volumes of this buffer. A linear gradient of 50–500 mM KCl routinely eluted the receptor at 0.15–0.20 M KCl.

Preparative Isoelectric Focusing. Preparative isoelectric focusing of the receptor was carried out in a 110-mL LKB preparative column apparatus by a modification of the procedures of Puca et al. (1975). A 5–50% sucrose gradient containing 1% ampholine, pH 4–9, was used. The temperature of the water running through the double-jacketed column was

kept at 0–4 °C. A partially purified, desalted sample (3 mL) of 1,25-$(OH)_2[^3H]D_3$–receptor complex was positioned in a prefocused column at pH 7.2. The sample was focused with 0.8 mA at 800 V. After approximately 5 h, the gradient was collected with a peristaltic pump. The pH of each fraction was measured on an Orion pH meter.

Nondenaturing Polyacrylamide Gel Electrophoresis. The technique of Tindall et al. (1975) was used for electrophoresis of 1,25-$(OH)_2D_3$–receptor complex. Gels were prepared containing 6% total acrylamide with 10% glycerol and 10 mM mercaptoethanol in Tris–glycine, pH 8.3. Buffer (upper and lower) contained 0.6% Tris and 2.9% glycine in water, pH 8.6 at 4 °C. Electrophoresis was peeformed at 2 mA/tube in a 4 °C cold room with the lower buffer chamber immersed in ice water.

A receptor (40-μg) preparation labeled with 1,25-$(OH)_2$-$[^3H]D_3$ (5 Ci/mmol) was purified 2600-fold by $(NH_4)_2SO_4$ precipitation, DNA–cellulose, hydroxylapatite, and DEAE-cellulose. The sample was dialyzed against distilled water containing 5 mM Tris-HCl, pH 7.4 at 0–4 °C, and lyophilized. A similar receptor preparation was labeled with 160 Ci/mM 1,25-$(OH)_2[^3H]D_3$ and purified by $(NH_4)_2SO_4$ precipitation, DNA–cellulose, and DEAE-cellulose. This receptor sample was utilized for assessment of 1,25-$(OH)_2[^3H]D_3$-bound protein. Both receptor preparations were in TED buffer containing 20% sucrose and 150 mM KCl and were layered directly over running gels. The former gel was stained with Coomassie brilliant blue for 2 h and then destained as described by Weber & Osborne (1969). The gel with the (160 Ci/mM) 1,25-$(OH)_2[^3H]D_3$-labeled receptor sample was sliced in 2.0-mm slices and radioactivity assessed as described by Tindall et al. (1975).

Iodination of Purified Receptor Protein. The receptor protein (~8 μg) was iodinated by the Bolton–Hunter technique (Bolton & Hunter, 1973). The lyophilized purified receptor preparation, suspended in 0.1 M borate buffer, pH 8.5, was added to the dried ^{125}I iodination ester reagent (New England Nuclear, Inc., Boston, MA), and the reaction mixture was agitated for 15 min at 0 °C. The esterification reaction was quenched with 0.5 mL of 0.2 M glycine in 0.1 M borate buffer, pH 8.5. After 30 min at 0 °C, the ^{125}I-labeled protein was taken up in Tris buffer, pH 7.4, containing 0.1% gelatin as carrier protein. The ^{125}I-labeled macromolecules were separated from free ^{125}I-labeled ester on a Sephadex G-25 column (2.5 × 60 cm) equilibrated with Tris-HCl, pH 7.4, containing 0.1% gelatin. The specific activity of labeled proteins was 6×10^5 cpm/μg. The ^{125}I-labeled proteins were analyzed by sucrose density gradient (4–20%) as previously described (Simpson et al., 1980).

Electrophoresis in Polyacrylamide Gels Containing Sodium Dodecyl Sulfate. The electrophoresis and protein staining procedures of Weber & Osborne (1969) were used with 8% total acrylamide gels. Protein samples were dialyzed overnight in 0.01 M sodium phosphate and for 2 h in distilled water at 4 °C. Samples were then lyophilized and dissolved in the denaturing buffer as described by Weber & Osborne (1969).

Two-Dimensional Polyacrylamide Gel Electrophoresis. The purified receptor protein was analyzed by two-dimensional gel electrophoresis as initially described by O'Farrell (1975) and modified by Kendrick et al. (1981). The gels were run by Kendrick Laboratory (Madison, WI). Briefly, the methods are as follows: Ampholine, pH 5–7, was used in 130-mm gels, and isoelectric focusing was performed at 400 V for 12 h and 800 V for 1 h. The second dimension was a uniform 95 × 140 mm slab gel containing 10% acrylamide. Protein standards including phosphorylase *a* (94 000 daltons), catalase (60 000 daltons), and actin (43 000 daltons) were layered directly on the second dimension prior to electrophoresis. A parallel gel containing no receptor protein was also run, and only standard proteins were present.

Miscellaneous Measurements and Methods. Radioactivity was determined by liquid scintillation counting in a Prias PLD TriCarb minivial counter (Packard, Elk Grove, IL); quench correction was monitored with automatic external standards. The scintillation fluid mixture used in these experiments consisted of 1.32 L of Triton X-100, 8.0 g of 2,5-diphenyloxazole (PPO), and 0.2 g of 1,4-bis(4-methyl-5-phenyloxazol-2-yl)benzene (Me_2POPOP) in 4 L of toluene.

Sucrose gradient (4–20%) analysis was performed as previously described (Simpson et al., 1980). Protein concentration was determined by the method of Bradford (1976) with crystalline bovine serum albumin as the standard.

Results

We have found that duodenal mucosa from mature chickens provided an abundant and adequate source of the 1,25-$(OH)_2D_3$ receptor protein (Simpson & DeLuca, 1982). The use of frozen–thawed intestines stored at −20 °C for up to 2 weeks yields receptor preparations with greater than 70% of the binding sites found in fresh tissue (data not shown). Furthermore, we have found that the receptor protein localized in a low-speed (crude-nuclear) pellet of a mucosal homogenate when the hypotonic TED buffer was used. This low-speed pellet could be rinsed of soluble proteins with TED buffer and the receptor then extracted with high salt buffer (TED containing 0.3 M KCl). We previously reported that the crude nuclear preparation had approximately 75% of the total 1,25-$(OH)_2D_3$ binding sites found in the high-salt cytosol preparation (Simpson & DeLuca, 1982). Others have reported that greater than 90% of the 1,25-$(OH)_2D_3$ receptors are in the nuclear fraction (Walters et al., 1980). The data suggest that mature chicken intestines provide a satisfactory source of the 1,25-$(OH)_2D_3$ receptor. Furthermore, the crude low-speed pellet extract provides a significantly enriched receptor preparation. We included 5 mM $MgCl_2$ in our final receptor preparation before 1,25-$(OH)_2[^3H]D_3$ labeling. Magnesium chloride has been found to increase the stability of the DNA binding site (R. T. Franceschi, personal communication).

Selective Precipitation. Ammonium sulfate precipitation was found to be the most reproducible and effective means of precipitating the 1,25-$(OH)_2D_3$ receptor from the low-speed pellet extract. We found that centrifuging the $(NH_4)_2SO_4$ suspension at 9000*g* for 10 min provided a sufficiently hard pellet that could be gently rinsed with cold distilled water to decrease the salt contamination of the resuspended receptor. Although we previously used Polymin P (polyethylenimine–BRL) for precipitation of the receptor, we have routinely found that this technique did not afford significantly greater purification of the receptor preparation than does ammonium sulfate.

Batch Adsorption to DNA–Cellulose and Column Elution. We investigated methods for efficient adsorption of the 1,25-$(OH)_2D_3$–receptor complex to DNA–cellulose. Batch adsorption of the resuspended receptor in TED buffer possessing an ionic strength equivalent to 0.05 M KCl was found to be optimal. The optimal time for batch incubation of DNA–cellulose with receptor solution was found to be 30 min at 0–4 °C (data not shown). The optimal ratio of DNA–cellulose to receptor solution was also investigated. We analyzed 50%, 10%, and 3% suspensions of DNA–cellulose in TED buffer for adsorption of 1,25-$(OH)_2D_3$–receptor complex.

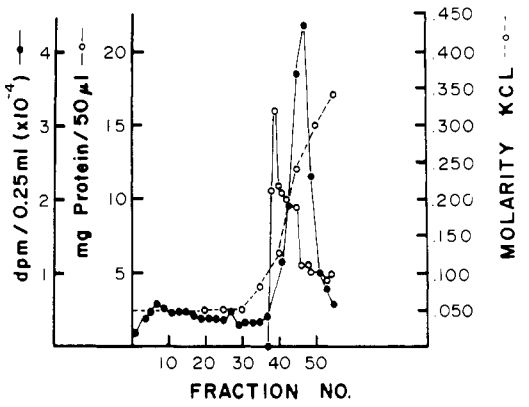

FIGURE 1: Elution of 1,25-$(OH)_2[26,27\text{-}^3H_2]D_3$–receptor complex from DNA–cellulose with a 0.1–0.4 M KCl gradient. The receptor–hormone complex was batch adsorbed onto DNA–cellulose. The receptor solution had been precipitated with ammonium sulfate and resuspended as described under Materials and Methods. Radioactivity (●), protein (O—O), and salt (KCl) (O- - -O) gradient are shown.

We found after 30 min of incubation at 0–4 °C that a 50% suspension had 3124 dpm bound, a 10% suspension had 3204 dpm bound, and a 3% suspension had 2008 dpm bound. The data suggested a 10% suspension of DNA–cellulose in buffered receptor solution was optimal.

Figure 1 demonstrates the chromatography achieved with KCl gradient elution of the labeled receptor protein from DNA–cellulose after batch adsorption. The low-speed, salt-extraction receptor preparation labeled with 1,25-$(OH)_2[^3H]D_3$ (5 Ci/mmol) was $(NH_4)_2SO_4$ (38%) precipitated and batch adsorbed as described. The DNA–cellulose was rinsed 2 times with TED K_{50} by gentle centrifugation (50*g* for 5 min) and resuspended. The total column volume was 150 mL of DNA–cellulose, and this was rinsed with sufficient TED K_{50} to decrease eluted protein to below detectability by protein assay. The 0.1–0.4 M KCl gradient was then initiated. The protein elution profile demonstrates that a major protein fraction elutes prior to the 1,25-$(OH)_2[^3H]D_3$–receptor complex. The overall purification from this procedure was estimated to be 62-fold with approximately 65% recovery as assayed by the hydroxylapatite method.

Preparative Isoelectric Focusing. Using the method of Puca et al. (1975), we investigated the isoelectric point of the 1,25-$(OH)_2D_3$ receptor (Figure 2). The 1,25-$(OH)_2D_3$ receptor protein was purified 110-fold by $(NH_4)_2SO_4$ (39% v/v) precipitation and DNA–cellulose chromatography and then initially introduced at pH 7.2 in the prefocused column. The labeled receptor focused at a pH of 6.0–6.2. The overall purification with these procedures was 3.6-fold, and there was a 65% recovery. The sample size of 3 mL was somewhat limiting for this particular technique. However, knowledge of the receptor protein's isoelectric point supports our earlier investigation of chromatofocusing (Simpson & DeLuca, 1982). The elution position of this receptor is similar for the chromatofocusing and isoelectric focusing techniques (Simpson & DeLuca, 1982).

Hydroxylapatite Chromatography. Figure 3 illustrates the chromatographic behavior of the 1,25-$(OH)_2D_3$ receptor on a small (3-mL packed volume) hydroxylapatite column. The 1,25-$(OH)_2D_3$ receptor was purified 110-fold with DNA–cellulose and $(NH_4)_2SO_4$ before application to hydroxylapatite. The preparation contained 6.8 mg of protein in 67 mL. The use of this step affter DNA–cellulose, which results in a large sample volume, and before the gel exclusion step has obvious benefits. The overall purification by this procedure was approximately 4-fold with a 64% recovery. Importantly, the

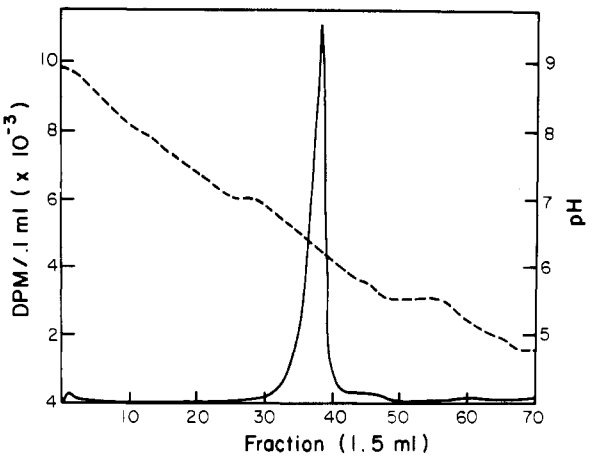

FIGURE 2: Electrofocusing profile of 1,25-$(OH)_2[26,27\text{-}^3H_2]D_3$–receptor complex. Electrofocusing was performed in a 110-mL preparative column with a 5–50% sucrose gradient containing 1% ampholine, pH 4–9. The sample was positioned at pH 7.2. The receptor complex focused at a pH of 6.0–6.2. The pH (– –) and radioactivity (—) profiles are shown. Approximately 6.2×10^5 dpm and 9 mg of protein were introduced to the column and 4.0×10^5 dpm and 1.8 mg protein focused at pH 6.0–6.2.

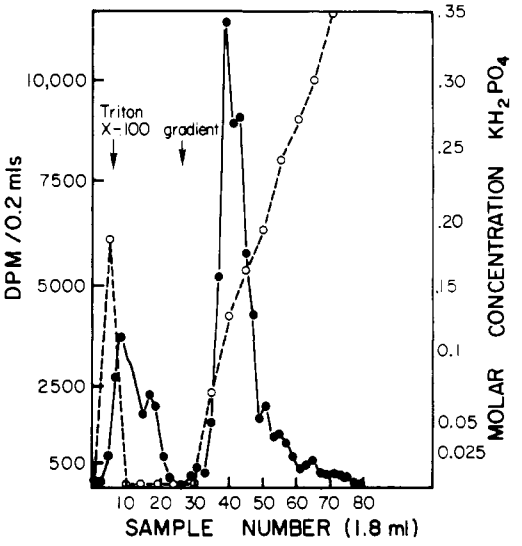

FIGURE 3: Chromatography of partially purified 1,25-$(OH)_2D_3$ receptor on hydroxylapatite (3.5-mL column). Initial receptor samples (6.8 mg in 67 mL containing 3×10^6 dpm) were applied and then rinsed with 10 mL of TED buffer and 30 mL of TED buffer containing 0.25% Triton X-100 at which time the 0.025–0.4 M KH_2PO_4 gradient was initiated. The flow rate was 0.5 mL/min. The radioactivity (—) and KH_2PO_4 gradient (– –) are shown.

volume of pooled receptor is one-fourth that of the initial applied receptor preparation. Furthermore, Triton X-100 (0.5% in TED K_{50} buffer) was found to be effective in removing free 1,25-$(OH)_2[26,27\text{-}^3H_2]D_3$ from the column prior to receptor elution with a 0.025–0.5 M KH_2PO_4 gradient in TED buffer. A slow initial sample application flow rate (~0.5–1.0 mL/min) was required for optimal adsorption of the labeled protein.

Sephacryl S-200 Gel-Exclusion Chromatography. The use of this gel-exclusion medium for purification of the chicken intestinal 1,25-$(OH)_2D_3$ receptor has previously been reported by us (Simpson & DeLuca, 1982) and others (Pike & Haussler, 1979). The stability of the Sephacryl column affords high flow rates with good resolution. We noted in our investigation significant decreases in recovery when the gel-exclusion chromatography step was used with a highly purified receptor preparation. Our laboratory previously showed that

Table I: Receptor Purification Scheme

step	protein (mg)	receptor (dpm × 10^{-6})	sp act. (dpm × 10^{-3}/mg)	purificn (x-fold)	yield (%)
nuclear extract	2100	9.00	4.3	1	100
$(NH_4)_2SO_4$	640				
DNA–cellulose	6.8	3.00	441.2	103	33
hydroxylapatite	2.0	1.80	900.1	214	20
Sephacryl S-200	0.34	1.22	3614.0	861	14
DEAE–cellulose	0.029	0.72	24828.4	5774	8

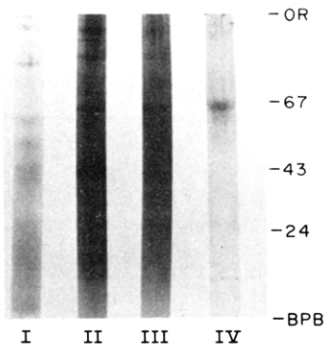

FIGURE 4: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of fractions from purification scheme. Samples are (I) $(NH_4)_2SO_4$ precipitate (40 μg), (II) hydroxylapatite (20 μg), (III) Sephacryl S-200 (10 μg), and (IV) DEAE-cellulose (4 μg). Bovine serum albumin (67000), ovalbumin (43000), trypsinogen (24000), and bromophenol blue (tracking dye) were run as molecular weight standards.

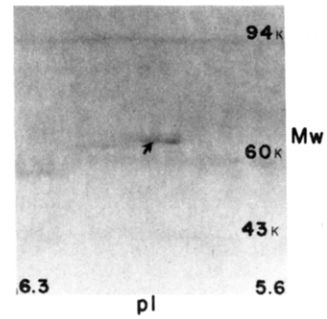

FIGURE 5: Purity, molecular weight, and isoelectric point of purified polypeptide was determined by the two-dimensional electrophoresis procedure of O'Farrell (1975). The protein was isoelectric focused horizontally and electrophoresed in sodium dodecyl sulfate along the vertical axis. The major polypeptide evident has a molecular weight of 64000 and a p*I* of approximately 6.0. The purified polypeptide appears as a continuous stained spot that is elongated along the isoelectric focus axis. It is important to note that phosphorylase *a* (94000), catalase (60000), and actin (43000) were run as internal standards in the second dimension of this gel as described under Materials and Methods.

TED buffer was as effective as TED K_{300} for elution of the partially purified receptor on Sephacryl S-200 (Franceschi & DeLuca, 1979). We have found, however, that buffer containing 0.5% Triton X-100 in TED K_{50} was effective in increasing recovery of the purified receptor. Furthermore, we found that a flow rate of 0.5 mL/min was optimal for peak resolution.

DEAE-Sepharose. The adsorption of 1,25-$(OH)_2D_3$ to DEAE-Sepharose was as previously described (Simpson & DeLuca, 1982). We found that addition of Triton X-100 was helpful for recovery of the 1,25-$(OH)_2[^3H]D_3$–receptor complex. The purification using this column was usually 10-fold with greater than 75% recovery.

Purification Procedure. The purification scheme is shown in Table I. Starting with 100 mature chicken intestines, we obtained approximately 600 g of mucosa. The resulting nuclear extract receptor preparation possessed 2.100 g of protein and 9×10^6 dpm of 1,25-$(OH)_2[^3H]D_3$ (5 Ci/mmol) binding. The receptor was precipitated in a 38% saturated solution of $(NH_4)_2SO_4$. The total protein content of the receptor preparation was decreased by 3.3-fold. DNA–cellulose adsorption and specific elution yielded a preparation 103-fold purified with 33% recovery of binding activity. Hydroxylapatite gave a 2-fold purification, and gel exclusion on Sephacryl S-200 gave a 6-fold purification. The final column of DEAE-Sepharose utilizing ion exchange resulted in the protein being purified approximately 5800-fold with an 8% yield, and the final total protein content was $^1/_{72000}$ of the starting extracted material from nuclei.

Electrophoresis in Polyacrylamide Gels. Polyacrylamide gels containing sodium dodecyl sulfate were utilized. The electrophoretic pattern obtained is shown in Figure 4. The initial gel (I) is shown from the post-$(NH_4)_2SO_4$ fraction. The subsequent gels are from post-hydroxylapatite (II) and post-Sephacryl S-200 (III), and the final gel is from the DEAE-Sepharose (IV). The gels display stepwise purification of a protein with a molecular weight of approximately 62000–65000. The final gel shows the protein as a single band.

To more accurately estimate the molecular weight, the isoelectric point, and the purity of the protein fraction from DEAE-cellulose, we used two-dimensional electrophoresis as described by O'Farrell (1975). The gel is shown in Figure 5. This showed a single major spot. The molecular weight from four gels was found to be 63000 ± 3900 with a p*I* of 6.0–6.3. The spot is shown to be elongated along the isoelectric separation axis and to resemble single-charge isomers as were described by Anderson & Hickman (1979) for carbamylated isomers of hemoglobin. The molecular weight and isoelectric point of the receptor as measured supports the fact that the protein purified is the receptor for 1,25-$(OH)_2D_3$.

Iodination of Receptor Protein. To further assess the purity of the receptor, we iodinated polypeptides present in the final preparation from DEAE-cellulose. The iodination macromolecules were separated from free ^{125}I-labeled ester by G-25 chromatography as described under Materials and Methods. Approximately 80000 cpm of labeled proteins in 200 μL of TED buffer containing 0.1% gelatin were layered on a 4–20% linear sucrose gradient. The gradient was fractionated and the resulting profile of radioactivity is shown in Figure 6. A major peak of ^{125}I cosedimented with ovalbumin and the 1,25-$(OH)_2[^3H]D_3$-labeled chicken intestinal receptor. A minor peak of radioactivity is shown to exist that sediments at approximately 4.6 S. The higher sedimentation peak of ^{125}I-labeled macromolecule represents less than 10% of the total radioactivity present in the gradient profile. The data suggest that, in the final preparation, approximately 90% of the macromolecules present have the same sedimentation coefficient as the intact 1,25-$(OH)_2D_3$–receptor complex. It may be concluded that the higher sedimenting species is a contaminant of this final preparation. However, this faster sedimenting species may be an aggregate or denatured receptor protein that has lost its assymmetric form during the purification procedures.

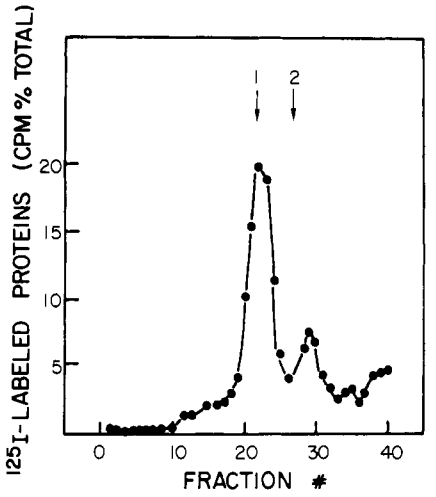

FIGURE 6: A sucrose (4–20%) density gradient analysis of ^{125}I-labeled proteins from the final fraction of scheme II. The polypeptides from DEAE-cellulose were iodinated by the Bolton–Hunter method. The iodinated species with an approximate 3.7S sedimentation coefficient represents approximately 90% of the iodinated materials. Free ^{125}I, determined by cosedimentation, was subtracted from the gradient profile. (1) The sedimentation of the purified 1,25-$(OH)_2[^3H]D_3$–receptor complex and ovalbumin (3.7 S). (2) The sedimentation of bovine serum albumin (4.4 S).

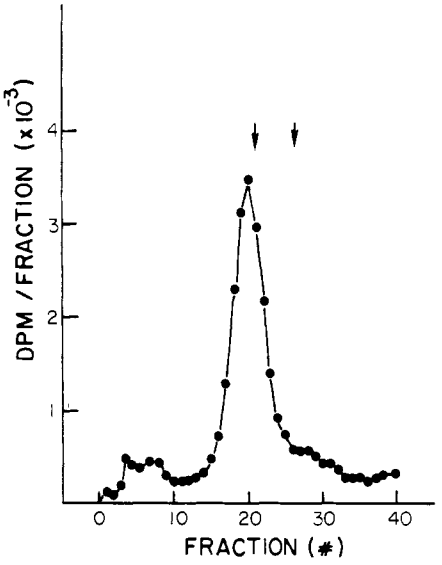

FIGURE 7: Sucrose gradient (4–20%) analysis of purified 1,25-$(OH)_2D_3$ receptor. The receptor was purified as outlined in Table I and concentrated by hydroxylapatite as described under Materials and Methods. The arrows reveal the sedimentation position of purified ovalbumin (3.7 S) and bovine serum albumin (4.4 S).

To analyze the viability and integrity of the purified receptor protein after purification, we concentrated the final fraction in a hydroxylapatite column (1 mL) at a slow flow rate and eluted the 1,25-$(OH)_2D_3$ receptor with 0.3 M KH_2PO_4 in TED buffer. This concentrated receptor solution (200 μL) was then applied to a 4–20% linear sucrose gradient and centrifuged for 18 h as described under Materials and Methods. Figure 7 shows the sucrose gradient profile of the purified 1,25-$(OH)_2[^3H]D_3$–receptor complex. We applied 32 270 dpm of labeled receptor fraction ($\sim$2 μg) to the sucrose gradient. A single 3.7S peak of radioactivity is evident. This gives further evidence that the protein isolated is the receptor for 1,25-$(OH)_2D_3$ from chicken intestines.

A further characteristic of the receptor for 1,25-$(OH)_2D_3$ is the binding to DNA–cellulose. Figure 8 shows that the purified protein that specifically binds 1,25-$(OH)_2[^3H]D_3$ has

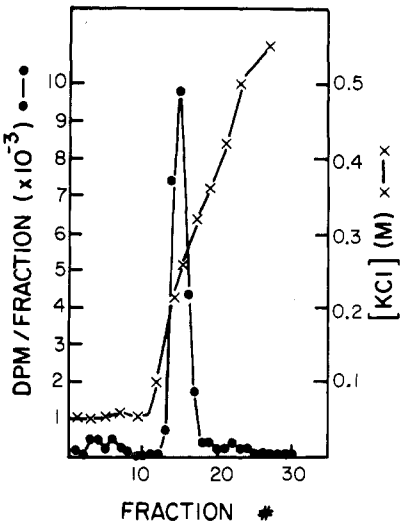

FIGURE 8: Purified 1,25-$(OH)_2[26,27-^3H_2]D_3$ occupied receptor (34 000 dpm) from purification scheme shown in Table I was assessed for binding to and elution from DNA–cellulose. The radioactivity (●) and KCl gradient (×) are shown. A 3-mL column of DNA–cellulose was used, and 1-mL fractions were taken.

high affinity for DNA–cellulose. The KCl gradient elution is similar to that found in the crude preparation. The protein elutes at 0.20–0.22 M KCl.

Nondenaturing Gel Electrophoresis. A chicken intestinal nuclear extract of 1,25-$(OH)_2D_3$ receptor was purified 2600-fold by $(NH_4)_2SO_4$ precipitation followed by DNA–cellulose, hydroxylapatite, and DEAE-cellulose chromatography. The sample was then dialyzed, lyophilized, and resuspended as described under Materials and Methods. A second gel was run in parallel with a partially purified receptor preparation labeled with 160 Ci/mmol of 1,25-$(OH)_2[^3H]D_3$. A tube gel was stained with Coomassie brilliant blue and scanned at 595 nm in a Gilford spectrophotometer. The radioactivity of 2-mm slices of the second gel was measured (Figure 9). The major protein band was found to comigrate with the 1,25-$(OH)_2[^3H]D_3$ peak. The receptor band could be easily sectioned out and represented approximately 30% of the total stained protein in the gel.

Discussion

We describe in this paper procedures useful for the purification of the chicken intestinal receptor for 1,25-$(OH)_2D_3$. Techniques investigated and optimized include (1) DNA–cellulose batch adsorption and column elution, (2) hydroxylapatite chromatography, (3) gel exclusion, (4) DEAE-cellulose, (5) preparative isoelectric focusing, and (6) polyacrylamide gel electrophoresis under nondenaturing conditions. Furthermore, we present a series of methods that were found to yield a small amount of purified receptor protein. Purity was determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, two-dimensional polyacrylamide gel electrophoresis as described by O'Farrell (1975), and ^{125}I iodination of the purified polypeptide followed by sucrose gradient analysis. The overall purification was 5800-fold on the basis of specific activity with a yield of 8%. The final receptor fraction possessed $^1/_{72\,000}$ of the protein in the initial receptor preparation.

A major problem involved in isolating the receptor from chicken intestines was to obtain a sufficiently plentiful and enriched source. We recently reported that the use of slaughterhouse chicken intestines is an adequate source (Simpson & DeLuca, 1982). We further found that prepa-

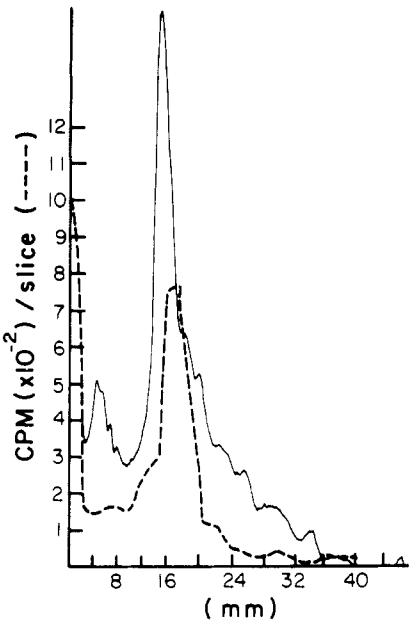

FIGURE 9: Purification of receptor by nondenaturing polyacrylamide gel electrophoresis. The methods for sample preparation and electrophoresis were those of Tindall et al. (1975). A receptor preparation was purified 2600-fold, and electrophoresis was performed. The tube gel was stained with Coomassie blue and scanned at 595 nm in a Gilford spectrophotometer (—). Radioactivity of gel slices (2 mm) of parallel run gels containing partially purified 1.25-$(OH)_2$-[^{3}H]D_3-receptor complex was measured (--). The major protein band was found to comigrate with the 1,25-$(OH)_2$[^{3}H]D_3-containing peak.

ration of a crude nuclear–high salt extract yielded a receptor preparation that is 4–5-fold enriched with receptor as compared to a corresponding high-salt cytosol preparation. This enriched receptor preparation allowed us to then purify a single polypeptide species that migrated on electrophoresis with a molecular weight similar to that of bovine serum albumin (67 000) and catalase (60 000) as determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Simpson & DeLuca, 1982). The techniques employed were selective precipitation, ion exchange, gel exclusion, and chromatofocusing.

It can be estimated that purification of the labeled 1,25-$(OH)_2D_3$–receptor is 14% of that required as determined by specific activity. We found a similar result in a previous purification procedure (Simpson & DeLuca, 1982). The noted approximate 6000-fold purification from these purification schemes does not include the 1,25-$(OH)_2D_3$–receptor in the initial extract that is occupied with endogenous unlabeled 1,25-$(OH)_2D_3$ or that which has become unoccupied during the purification procedure. Purification of other steroid receptors have shown similar discrepancies between theoretical and expected requirements for total purification (Schrader et al., 1977; Coty et al., 1979; Greene et al., 1977). The B subunit of the progesterone receptor was purified to an estimate of only 2% as measured by specific activities of the final product. On gel electrophoresis, the final protein was shown to be homogeneous, leading the authors to conclude that 98% of the progesterone receptors were endogenously occupied (Schrader et al., 1977). Similarly, the A subunit of the progesterone receptor was purified to a maximum of 18% of theoretical (Coty et al., 1979), and the estrogen receptor was purified to 10–30% of theoretical as estimated by specific activity (Greene et al., 1977).

The possibility exists that the pool of steroid receptors that bind radiolabeled ligands may be only a portion of the total receptor protein present in cells. The recent findings by Pratt and Toft and their co-workers (Leach et al., 1982; Nishigur & Toft, 1980) suggest that endogenous factors are required for stability and activity of receptor steroid binding sites. Thus, receptor estimation by radiolabeled ligand may fail to estimate total receptor proteins present. Therefore, our original estimation of receptor concentration by specific activity of radiolabeled sites would be an underestimation. Further work in this area for the 1,25-$(OH)_2D_3$ receptor will surely yield information important in our understanding of steroid receptor regulation.

In our efforts to purify the 1,25-$(OH)_2D_3$ receptor, we have investigated a number of chromatographic procedures that have been successfully used to purify other steroid receptor proteins. A partial purification of the intestinal 1,25-$(OH)_2D_3$ receptor has been reported (Pike & Haussler, 1979). In the previous purification attempt, the labeled receptor was directly applied to a small DNA–cellulose column (Pike & Haussler, 1979). The large volumes of the receptor preparation and high total protein content at this stage of purification make this a slow procedure that results in column clogging (R. U. Simpson, A. Hamstra, and H. F. DeLuca, unpublished results). We found that adsorption of the receptor in batch to DNA–cellulose, sedimentation, and rinsing, followed by packing of the DNA–cellulose into a column and a KCl gradient elution, results in a rapid and efficient procedure. The optimal concentration of DNA–cellulose for adsorption to the receptor was found to be 10%. Although this concentration of DNA–cellulose is greater than would be expected on the basis of the binding capacity of DNA, we believe that the large volume of DNA–cellulose is required for the optimal physical interaction of receptor and DNA.

To further optimize receptor purification, we found that broadening of the KCl gradient results in a greater separation of a contaminating protein peak from the receptor protein. The use of a 0.1–0.4 M KCl gradient after rinsing of the packed column with 0.05 M KCl in TED buffer routinely eluted the receptor protein at approximately 0.2 M KCl with a purification of 50–100-fold and approximately 50–75% yield.

An important property of a polypeptide macromolecule is its isoelectric point. We have previously utilized chromatofocusing, a recently developed technique of isoelectric chromatography using pH elution of proteins from an ionically charged column matrix to purify the receptor protein. The chicken intestinal receptor for 1,25-$(OH)_2D_3$ was found to specifically elute from this column at a pH of approximately 6.0. In the present experiments using an LKB 110-mL preparative column apparatus with a 5–50% sucrose gradient supporting a pH 9–4 gradient, we subjected 3 mL of a semipure preparation of the 1,25-$(OH)_2D_3$–receptor complex to isoelectric focusing. The receptor selectively focuses at a pH of 6.1 on this column (Simpson & DeLuca, 1982). This further supports our finding of the acidic pI of the receptor protein. The overall purification was 3.6-fold with a 65% recovery. The time required and the small sample volume that can be used for the selective positioning of the receptor make this technique not suited for initial steps in the purification scheme. However, this procedure may be useful for a final purification step as we previously described. A direct comparison of chromatofocusing vs. column isoelectric focusing was not performed, but the prior procedure is much easier and more rapid, while providing the same result.

We previously utilized hydroxylapatite in batch to purify the chicken intestinal receptor (Simpson & DeLuca, 1982). A major difficulty in the purification of reeceptor is the large

volume in the initial preparation. We, therefore, investigated methods for concentrating the receptor protein. We found that a hydroxylapatite column (3–4 mL) had the capacity to adsorb up to 20 μg of partially purified receptor. Other techniques such as ultrafiltration or specific precipitation were found less reproducible and effective than hydroxylapatite for concentrating the protein. We also found that gradient elution with 0.025–0.50 M KH_2PO_4 in TED buffer, pH 7.4, eluted the receptor with 5–15-fold purification and concentrated the receptor 3–7-fold. The utility of this column also allows separation of free 1,25-$(OH)_2[^3H]D_3$ from bound by rinsing the column after sample adsorption with 0.5% Triton X-100 as described by Wecksler & Norman (1979) for a batch hydroxylapatite technique for separation of bound 1,25-$(OH)_2D_3$ from free.

Other chromatographic techniques used are similar to those described previously (Simpson & DeLuca, 1982; Pike & Haussler, 1979). We have routinely found that a major protein peak elutes prior to the 1,25-$(OH)_2[^3H]D_3$ receptor peak on both Sephracryl S-200 gel exclusion and DEAE-cellulose as described under Materials and Methods. We, therefore, fractionate the receptor peak so as to avoid the contaminating protein.

We have shown that the partially purified 1,25-$(OH)_2D_3$ receptor can be purified by nondenaturing polyacrylamide gel electrophoresis (Figure 8). The methods utilized were a modification of those employed for the androgen receptor as described by Tindall et al. (1975). The purification by electrophoresis is a further technique that permits ultimate isolation of the 1,25-$(OH)_2D_3$ receptor. The partially purified 1,25-$(OH)_2[^3H]D_3$-labeled receptor comigrates with a protein band that represents approximately 35% of the total stained protein bands. The applied receptor preparation was purified 2600-fold with approximately 43% of that required for purity. This supports our identification of the radiolabeled protein as the 1,25-$(OH)_2D_3$ receptor on electrophoresis.

The procedure described under Materials and Methods and presented in Table I illustrates a purification scheme that yields a highly purified receptor from chicken intestines. As judged by sodium dodecyl sulfate–polyacrylamide gel electrophoresis it is apparently pure (Figure 4). The overall yield is routinely found to be 5–9% of the initial labeled receptor and for purification is approximately 6000-fold. The molecular weight of the polypeptide can be estimated to be approximately 62 000–66 000 by using the methods of Weber and Osborne for sodium dodecyl sulfate–polyacrylamide gel electrophoresis. These authors estimate the accuracy of molecular weight determinants to be ±10%. To more accurately determine the purity, molecular weight, and isoelectric point of the purified protein, we analyzed the final receptor fraction using the O'Farrell technique of two-dimensional gel electrophoresis. The approximate molecular weight by this procedure is 63 000 ± 3900, and the p*I* is 6.0–6.2. The protein is the major polypeptide spot seen on the two-dimensional slab gel electrophoresis. Interestingly, the purified protein spot is shown to streak along the isoelectric focusing axis. This finding is very similar to the streaking shown to occur with carbamylated single-charge isomers of hemoglobin (Anderson & Hickman, 1979). Recently, the purified progesterone receptor has been shown to exist as single-charge isomers as shown in similar two-dimensional gels. The authors presented evidence that phosphorylation of the receptor is responsible for the charge variances (Schrader et al., 1977). The gels presented suggest that the 1,25-$(OH)_2D_3$ receptor may exist as charged isomers. However, the streaking of the spot on two-dimensional gels could be due to other technical factors, and the possible posttranslation modification of the 1,25-$(OH)_2D_3$ receptor remains to be established.

To further assess purity of the proteins, we iodinated them using the Bolton–Hunter technique (Bolton & Hunter, 1973) and analyzed the proteins by sucrose density gradient. The final isolated protein that has a molecular weight of 63 000 on gel electrophoresis is shown to have a major radioactive peak that retains a 3.7S sedimentation coefficient and a minor radioactive peak that sediments at 4.5–5.0 S. The 3.7S peak is shown to represent 90% of the total ^{125}I of macromolecules iodinated. It must be cautioned that the Bolton-Hunter iodination reagent is specific for accessible lysine residues and the estimation of the amount of polypeptide is relative to the number of lysine residues per molecule.

The information presented here is useful for the isolation and purification of the chick intestinal 1,25-$(OH)_2D_3$ receptor. We have described a purified protein with a molecular weight of 63 000 and a p*I* of 6.2. The protein may exist as single-charge isomers and retains its 3.5–3.7S sedimentation coefficient and affinity for DNA–cellulose. These procedures should prove to be important in the routine purification of the receptor protein, which will further assist in our design of experiments to understand the intercellular mechanism of action of the protein and our understanding of the events subsequent to hormone–receptor interaction.

Registry No. 1,25-$(OH)_2D_3$, 32222-06-3.

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