Specific Binding of $1\alpha,25$-Dihydroxycholecalciferol to Nuclear Components of Chick Intestine*

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Specific binding of $1\alpha,25$-dihydroxycholecalciferol to macromolecular components of small intestinal mucosa nuclei is demonstrated in vitamin D-deficient chicks. The nuclear $1\alpha,25$-dihydroxycholecalciferol-macromolecule complex was isolated on sucrose density gradients and sediments at 3.7 S in the presence of 0.3 M KCl. Agarose gel filtration of the nuclear component indicated an apparent molecular weight of 47,000. The nuclear receptor complexes could not be distinguished from previously described cytoplasmic $1\alpha,25$-dihydroxycholecalciferol-binding components by the ultracentrifugation and chromatographic procedures employed. The association of the $^3$H-sterol with the nuclear component is thermolabile and is destroyed by treatment with pronase, but not by nucleases; the receptor component is therefore presumed to be a protein.

The macromolecular-$1\alpha,25$-dihydroxycholecalciferol complex formed in vivo or in vitro at 25° can be extracted from intestinal nuclei by 0.3 M KCl, but not by low salt buffers. Smaller amounts of the 3.7 S binding component can be detected in isolated purified chromatin or after incubation of $1\alpha,25$-dihydroxy$[^3]$Hcholecalciferol with reconstituted cytosol-chromatin at 0°. Following incubation of the labeled hormone with reconstituted cytosol-chromatin at 0°, $1\alpha,25$-dihydroxy$[^3]$Hcholecalciferol is primarily associated with the cytoplasmic receptor. After shifting the incubation temperature to 25°, a progressive increase in the concentration of the nuclear receptor complex and a concomitant decrease in the concentration of the cytoplasmic binding component occur. Thus the $1\alpha,25$-dihydroxycholecalciferol binding molecules appear to exist primarily in the cytoplasm, where they presumably function to transport the hormone into the nucleus.

Experiments employing incubation of $1\alpha,25$-dihydroxy$[^3]$Hcholecalciferol with reconstituted cytosol-chromatin from nontarget tissues indicate a requirement for both intestinal cytosol and chromatin for maximal formation of the nuclear hormone-receptor complex. These results suggest that the nuclear-binding component arises from hormone-dependent transfer of the cytoplasmic $1\alpha,25$-dihydroxycholecalciferol receptor to intestinal chromatin acceptor sites.

Recent investigations have suggested that the stimulation of calcium transport across the intestine in response to $1\alpha,25$-dihydroxycholecalciferol involves a cytoplasmic to nuclear migration of the sterol as a key event in the hormone’s action (1-3). Although the translocation of the sterol to the nucleus is not clearly understood, the hormone forms a complex with a cytoplasmic protein (4). The association of $1\alpha,25$-dihydroxycholecalciferol with this cytoplasmic receptor is accompanied by a temperature-dependent redistribution of the sterol from the cytoplasm to the nucleus, where it associates with the chromatin fraction in vitro (1, 4). Subsequent to the localization of $1\alpha,25$-dihydroxycholecalciferol in the chromatin, nuclear DNA-dependent RNA polymerase II activity is enhanced (5). In addition, increased messenger RNA activity specific for intestinal calcium-binding protein is observed in response to administration of cholecalciferol (vitamin D₃)¹ to vitamin D-deficient chicks (7), and the stimulation of calcium transport by $1\alpha,25$-dihydroxyvitamin D₃ is inhibited by actinomycin D in intestinal organ culture and in intact animals (8, 9). These findings provide the basis of the proposal that $1\alpha,25$-dihydroxycholecalciferol acts at the level of gene transcription.

The proposed initial events in steroid hormone action in target organs are generally similar. The steroid enters the target cell and interacts reversibly with an intracellular receptor protein. The steroid-receptor complexes are subsequently transported to the nucleus where they interact with the chromatin and thereby alter nuclear RNA metabolism. These specific protein receptors have been identified for estrogens.

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¹ According to the Revised Tentative Rules for the Nomenclature of Steroids, of IUPAC and IUB (6), cholecalciferol (vitamin D₃) may be defined as a steroid and termed 9,10-Sec0-5,7,10(19)-cholestatrien-3β-ol.

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The cytoplasm of the target tissue (4). These receptor macromolecules are tissue specific, heat-labile proteins with a violet absorbance of each fraction was measured by means of a Gilford spectrophotometer. The fractions were collected in counting vials and were counted in 5 ml of liquid scintillation mixture A (5% Liquifluor in xylene-Triton X-114, 3:1) in a Beckman LS-233 scintillation counter (25% efficiency). Quenching of radioactivity did not vary significantly with the concentration of sucrose in the fractions. Sedimentation coefficients were estimated by comparison with protein markers (chymotrypsinogen, 2.5 S; ovalbumin, 3.7 S; and bovine serum albumin, 4.4 S).

**Materials and Methods**

Materials—Animals used in experiments were White Leghorn cockerels (kindly donated by Demler Farms, Anaheim, Calif.) that were raised on a vitamin D-deficient diet (20). 25-Hydroxy-26(27)-methyl-3H-cholecalciferol (6.0 Ci per mmole) was obtained from Amersham-Searle. Crystalline 25-hydroxycholecalciferol was a gift from Dr. John C. Babcock of the Upjohn Co. RNase-CB (ribonuclease T1 with some v1 - v2 - v3 enzyme activities) was kindly donated by Demler Farms, Anaheim, Calif. 1,25-Dihydroxycholecalciferol receptor complexes further support a role for 1,25-dihydroxycholecalciferol in the regulation of gene expression.

**Preparation of 1a,25-Dihydroxy[3H]cholecalciferol, In Vitro—1a,25-Dihydroxy-26(27)-methyl-3H-cholecalciferol was prepared by a modification of the method of Lawson et al. (21) as previously described (9). Radiochemical purity of generated 1a,25-dihydroxy-3H-cholecalciferol was 98%. 25-Hydroxy-26(27)-methyl-3H-cholecalciferol substrate for the reaction was purified by Celite liquid-liquid partition chromatography (22). The radiochemical purity of the 25-hydroxy-3H-cholecalciferol was 95%, and its specific activity was determined by ultraviolet absorbance spectrophotometry at 265 nm.

In vitro and in vivo Exposure of Chicks to 1a,25-Dihydroxycholecalciferol—The small intestine, liver, and kidneys of 3- to 4-week-old vitamin D-deficient chicks were removed and minced in ice-cold buffer. Intestinal mucosa was scraped free from serosa with a microscepe slide, and liver was minced prior to homogenization. Twenty percent homogenates in 0.25 M sucrose, 0.05 M Tris-HCl, pH 7.4, 0.025 M KCl, and 0.5 mg MgCl2 (0.25 M sucrose-Buffer A) or 0.3 M KCl, 0.3 M Tris-HCl, pH 7.4, 1.5 M EDTA (0.3 M sucrose-Buffer B) were prepared with a Potter-Elvehjem homogenizer equipped with a Teflon pestle at 0°C by four passes, with 2-min cooling periods between passes. Homogenates were centrifuged at 1200 x g for 10 min, or incubated with sterol for 20 min at 0°C or 25°C (40 μl of ethanol) prior to centrifugation (Fig. 1B). Nuclear pellets were removed, and the resulting supernatants were centrifuged at 100,000 g for 1 hour at 0°C to yield a final supernatant fraction (cytosol).

Purified nuclear extracts (chromatin) were prepared from nuclear pellets by the method of Haussler et al. (23) and were resuspended and centrifuged in 0.01 M Tris-HCl, pH 7.5. The pellet was then extracted with 0.3 M KCl-Buffer B (Fig. 1, A and B) or reconstituted with cytosol and incubated at 25°C or 0°C with sterol (in 40 μl of ethanol) prior to extraction. Extracts were centrifuged at 30,000 x g for 20 min, and the resulting supernatants analyzed for 1a,25-dihydroxycholecalciferol-binding activity. In experiments where 1a,25-dihydroxycholecalciferol binding was analyzed directly in the cytosol fraction (Figs. 5 and 6), intestinal mucosa was homogenized in 0.3 M KCl-Buffer B and centrifuged at 100,000 x g for 1 hour at 0°C. The supernatant was incubated with sterol for 30 min at 0°C and analyzed for 1a,25-dihydroxycholecalciferol receptor complexes.

For studies in vivo, 1a,25-dihydroxycholecalciferol and/or vitamin D-deficient chicks were dosed orally with 1a,25-dihydroxy-3H-cholecalciferol dissolved in 0.2 ml of 1.2-propanediol and sacrificed at the prescribed time. 1a,25-Dihydroxycholecalciferol-binding activity was analyzed in 0.3 M KCl-Buffer B-chromatin extracts (prepared from intestinal mucosa homogenates as described above).

**Sucrose Gradient Centrifugation—Linear gradients (5.0 ml) of 5 to 20% sucrose in 0.3 M KCl-Buffer B were prepared with a Buchler gradient mixer, Auto-Densi Flow, and Polystyptic pump. Aliquots (0.5 ml) of cytosol or nuclear extracts were layered on gradients and centrifuged for 16 to 24 hours at 0°C at 234,000 x g (average force) with the use of a Beckman L2-65B ultracentrifuge and an SW 50.1 rotor. The gradients were fractionated from the top of the centrifuge tube with the use of the Auto-Densi Flow, and in certain cases the ultraviolet absorbance of each fraction was measured by means of a Gilford 240 spectrophotometer. The fractions were collected in counting vials and were counted in 5 ml of liquid scintillation mixture A (5% Liquifluor in xylene-Triton X-114, 3:1) in a Beckman LS-233 scintillation counter (25% efficiency). Quenching of radioactivity did not vary significantly with the concentration of sucrose in the fractions. Sedimentation coefficients were estimated by comparison with protein markers (chymotrypsinogen, 2.5 S; ovalbumin, 3.7 S; and bovine serum albumin, 4.4 S).

**Agarose Gel Filtration—All chromatographic procedures were carried out at 1-3°C. Agarose beads (Bio-Gel A 0.5m, 100 to 200 mesh from Bio-Rad) were equilibrated with 0.3 M KCl-Buffer B and poured into a column (15 x 85 cm). Samples (0.5 ml) of nuclear extracts or cytosol incubations were applied to the column and 1-ml fractions collected. The optical density of fractions was measured with a Gilford 240 spectrophotometer. Radioactivity in each fraction was measured after addition of 5 ml of scintillation mixture A to each fraction. Efficiency for tritium was 30%. Column flow rates were maintained at 19.5 to 20.5 ml per hour with a Polystyptic pump. The distribution coefficients, Kd, of 1a,25-dihydroxycholecalciferol-binding components were determined according to the equation:

$$K_d = \frac{V_e - V_o}{V_e - V_i}$$

where $V_e$ is the elution volume of the component, $V_o$ is the void volume of the column determined with blue dextran, and $V_i$ is the total column volume determined by substitution of marker proteins.

**Heat and Enzymatic Release Studies—Aliquots (0.3 ml) of nuclear extracts or cytosol incubations were incubated for 30 min at 0°C with (a) no addition, (b) 100 μg of DNase plus 5 μl of 0.3 M magnesium acetate, (c) 100 μg of pronase, (d) 100 μg of protease K, or (e) 100 μg of pronase plus 5 μl of 0.3 M magnesium acetate. In additional experiments, cytosol was incubated at 25°C for various periods of time and then incubated with 1a,25-dihydroxy-3H-cholecalciferol at 0°C. Samples were analyzed for 1a,25-dihydroxycholecalciferol-binding components via sucrose gradient centrifugation.

**DNA Determination—DNA was quantitated by the diphenylamine method (24).**

**Results**

Macromolecular Binding of 1a,25-Dihydroxy-3H-cholecalciferol in Intestinal Nuclei—Following administration of 1a,25-dihydroxy-3H-cholecalciferol (50 μmol) to vitamin D-deficient chicks, macromolecular binding of the sterol was observed in the nuclei of intestinal mucosa (Fig. 1A). Purified chromatin was prepared from the intestine and extracted with 0.3 M KCl-Tris-EDTA buffer which removes some nonhistone proteins from deoxyribonucleoprotein. The nuclear extract was centrifuged through 5 to 20% sucrose gradients containing 0.3 M KCl. The sedimentation coefficient of these 1a,25-dihydroxycholecalciferol-binding molecules was about 4 S.

The interaction of 1a,25-dihydroxycholecalciferol with nuclear-binding components was also observed in vitro. Intestinal mucosa homogenates were incubated with 1a,25-dihydroxy-3H-cholecalciferol (8 nm) at 25°C for 20 min. The chromatin fraction was prepared at 0°C and extracted with 0.3 M KCl. Sucrose gradient analysis of the nuclear extract resulted in a 3.7 S peak of bound 1a,25-dihydroxycholecalciferol (Fig. 1B). When homogenate incubations were carried out at 0°C for 20 min, much less binding of the sterol to the 3.7 S macromolecules was observed. Similar results were observed when
the two components of the 1α,25-dihydroxycholecalciferol receptor system were reconstituted and incubated with labeled hormone (4 nM) for 10 min at 25° or 0° prior to extraction of the nuclear receptors with 0.3 M KCl (Fig. 1C). The specificity of the nuclear-binding components for 1α,25-dihydroxycholecalciferol was confirmed by reduction of the tritium peak following centrifugation of extracts from parallel incubations containing a 200-fold excess of unlabeled hormone (Fig. 1C). The association of 1α,25-dihydroxycholecalciferol with intestinal mucosa chromatin has previously been shown to be very specific for the hormone when the cytoplasmic and nuclear components are reconstituted by this procedure (4, 25).

The binding of 1α,25-dihydroxycholecalciferol to macromolecular components of the nuclear extract is sufficiently tight to permit its resolution on agarose gel columns. Reconstituted cytosol-chromatin was incubated with labeled 1α,25-dihydroxycholecalciferol at 25° for 10 min prior to extraction of the chromatin with 0.3 M KCl. The extract (0.5 ml) was pumped onto the column (1.6 x 65 cm) of agarose A-0.5 m and eluted with 0.3 M KCl-Buffer B (Fig. 2). The first peak of radioactive activity eluted from the column represents materials excluded from the gel since it elutes in the same volume as blue dextran. The major peak of 1α,25-dihydroxycholecalciferol-binding activity has an apparent molecular weight of 47,000, based on estimation from a graph of $M^{1/2}$ versus $K_{d}^{1/3}$ for globular protein standards. The third peak of radioactivity corresponds to the elution position of free $^{3}H$-sterol. The binding of hormone to the nuclear receptor was markedly temperature dependent in the reconstituted receptor system when analyzed by gel filtration. Incubation of 1α,25-dihydroxy$[3H]$cholecalciferol with reconstituted cytosol-chromatin at 0° for 10 min resulted in much less macromolecular-1α,25-dihydroxy$[3H]$cholecalciferol-binding activity in the nuclear extract (Fig. 2).

The selective extraction of the 1α,25-dihydroxycholecalciferol-binding macromolecule from the chromatin with 0.3 M KCl is shown in Fig. 3. The labeled hormone was incubated with the reconstituted receptors in vivo, and the isolated chromatin was extracted with increasing concentrations of KCl in Buffer B. The 1α,25-dihydroxycholecalciferol-binding component could not be detected when the chromatin pellet was extracted with 0.01 M KCl. Maximal extraction of the nuclear component occurs in 0.3 M KCl, although small amounts of macromolecule-1α,25-dihydroxycholecalciferol-binding activity were solubilized in 0.15 M KCl.

Experiments were carried out to determine the chemical nature of the nuclear 1α,25-dihydroxycholecalciferol-macromolecule complex. After preincubation of reconstituted cytosol-chromatin with 1α,25-dihydroxy$[3H]$cholecalciferol at 25° and extraction of the nuclear binding components, incubation at 0° alone or in the presence of pronase, RNase, or DNase was carried out. 1α,25-Dihydroxycholecalciferol-binding activity was then analyzed by sucrose gradient centrifugation. The results in Fig. 4 show that pronase caused a large reduction in the 3.7 S receptor peak, while DNase had a smaller effect. The release of labeled hormone from the receptor in the presence of pronase is indicated by the free radioactivity at the top of the

![Fig. 1. Sucrose gradient centrifugation of nuclear 1α,25-dihydroxycholecalciferol-binding components. A, chicks were sacrificed 30 min following oral administration of 50 pmol of 1α,25-dihydroxy$[3H]$cholecalciferol. B, intestinal mucosa homogenate (3 ml) was incubated with 1α,25-dihydroxy$[3H]$cholecalciferol (8 nM) for 20 min at 25° (●—●) or 0° (O—O). C, intestinal mucosa chromatin and cytosol were prepared and reconstituted (2 ml) prior to incubation with 1α,25-dihydroxy$[3H]$cholecalciferol (4 nM) for 10 min at 25° (●—●) or 0° (O—O). Parallel incubations at 25° contained 800 nM unlabeled 1α,25-dihydroxycholecalciferol (Δ—Δ). Chromatin fractions from each incubation were prepared and extracted with 0.3 M KCl-Buffer B as described under "Materials and Methods." Aliquots of extracts (0.3 ml) were layered on 5 to 20% sucrose gradients (5.0 ml) in 0.3 M KCl-Buffer B. Samples were centrifuged for 20 hours at 0° in a Beckman SW 50.1 rotor at 50,000 rpm. Arrows indicate sedimentation positions of external protein standards: 1, chymotrypsinogen; 2, ovalbumin; and 3, bovine serum albumin.](http://www.jbc.org/)

![Fig. 2. Agarose gel filtration of nuclear 1α,25-dihydroxycholecalciferol-binding components. Intestinal mucosa chromatin and cytosol were prepared and reconstituted (2 ml) prior to incubation with 1α,25-dihydroxy$[3H]$cholecalciferol (4 nM) for 10 min at 25° (●—●) or 0° (O—O). Column elution was carried out with 0.3 M KCl-Buffer B, and 1-ml fractions were collected as described under "Materials and Methods." $V_{0}$ is the void volume, and $V_{t}$ is the total column volume.](http://www.jbc.org/)

![Fig. 3. Sucrose gradient centrifugation of various KCl-chromatin extracts. Intestinal mucosa chromatin and cytosol were reconstituted (2 ml) and incubated with 1α,25-dihydroxy$[3H]$cholecalciferol (2 nM) for 10 min at 25°. Incubations were centrifuged at 30,000 x g for 20 min, and the resulting chromatin pellet was extracted with 0.01 M KCl-Buffer B (Δ—Δ), 0.15 M KCl-Buffer B (O—O), 0.3 M KCl-Buffer B (●—●), or 0.4 M KCl-Buffer B (Δ—Δ). Extracts were layered on gradients and centrifuged for 16 hours at 50,000 rpm as described under "Materials and Methods."](http://www.jbc.org/)
be demonstrated between the nuclear and cytoplasmic components of intestinal nuclei. 1α,25-Dihydroxy[3H]-cholecalciferol (3 nm) was incubated with reconstituted cytosol-chromatin (2 ml) from intestinal mucosa for 10 min at 25°. Chromatin was extracted with 0.3 M KCl-Buffer B, and aliquots of extract (0.3 ml) were incubated in the presence or absence of 100 μg of enzyme at 0° for 30 min. The mixture containing DNA also contained 5 mM magnesium acetate. Following incubation, samples were layered on 5 to 20% sucrose gradients and centrifuged for 20 hours at 0° as described under "Materials and Methods." △—△, pronase; ○—○, DNase; Δ—Δ, RNase; ●—●, no enzyme.

These data indicate that the 1α,25-dihydroxycholecalciferol-binding site on the nuclear receptor is protein in nature, as was previously shown for the cytoplasmic component (4). Whether or not the release of small amounts of hormone from the receptor by DNase indicates the presence of DNA in the receptor complex remains to be determined.

Comparison of Nuclear and Cytoplasmic 1α,25-Dihydroxycholecalciferol Receptor Proteins—Cytoplasmic and nuclear extracts of in vitro incubations with 1α,25-dihydroxy[3H]-cholecalciferol were centrifuged through 5 to 20% 0.3 M KCl-sucrose gradients. In order to obtain maximal resolution of the two receptor proteins, 3-drop fractions were collected. Due to the appearance of free or nonspecifically bound 3H-sterol at the top of tubes following centrifugation of cytoplasmic extracts, specific binding to the cytoplasmic receptor proteins was determined. Parallel cytosol incubations containing a 200-fold excess of unlabeled 1α,25-dihydroxycholecalciferol were centrifuged to identify more precisely the binding profile of the cytoplasmic receptor. Under these conditions, the sedimentation rates (3.7 S) of the nuclear and cytoplasmic components were identical in relation to an internal ovalbumin standard (Fig. 5). Comparison of the cytoplasmic and nuclear 1α,25-dihydroxysterol-binding proteins was also made employing gel filtration on agarose columns in 0.3 M KCl (Fig. 6). As in the case of the nuclear extract (Fig. 2), cytosol 1α,25-dihydroxy[3H]-cholecalciferol-binding activity eluted in three peaks. The major peak represents 3H-sterol binding to a protein of apparent molecular weight 47,000, which is resolved from the major protein peak (eluting in the void volume of the column). No difference could be demonstrated between the nuclear and cytoplasmic components when columns were run under identical, standardized conditions, and both peaks of macromolecule-3H-hormone complexes were abolished when incubations containing excess unlabeled 1α,25-dihydroxycholecalciferol were chromatographed. Thus, the specific nuclear and cytoplasmic 1α,25-dihydroxycholecalciferol receptors could not be distinguished from each other by the ultracentrifugal and chromatographic techniques employed. These data are consistent with the concept that the nuclear receptor originates in the cytosol.

The interaction of 1α,25-dihydroxy[3H]-cholecalciferol with both the nuclear and cytoplasmic components can be reversed at temperatures above 0° in vitro. Incubation at 25° of nuclear or cytoplasmic extracts, which had been preincubated with labeled hormone, results in destruction of hormone-receptor complexes as analyzed by sucrose gradient centrifugation (Fig. 7). The time course of the heat inactivation of the two complexes is very similar. However, warming of the cytosol prior to incubation with 1α,25-dihydroxy[3H]-cholecalciferol resulted in more rapid loss of hormone-binding activity to the cytosol receptor, indicating significant stabilization of the

Fig. 4. Effect of enzymes on 1α,25-dihydroxy[3H]-cholecalciferol-macromolecular complex of intestinal nuclei. 1α,25-Dihydroxy[3H]-cholecalciferol (3 nm) was incubated with reconstituted cytosol-chromatin (2 ml) from intestinal mucosa for 10 min at 25°. Chromatin was then extracted with 0.3 M KCl-Buffer B (●—●). Intestinal mucosa cytosol (prepared in 0.3 M KCl-Buffer B) was incubated with 1α,25-dihydroxy[3H]-cholecalciferol (6 nm) in the presence (Δ—Δ) or absence (○—○) of a 200-fold excess of unlabeled sterol for 30 min at 0°. Centrifugation of nuclear and cytoplasmic extracts was carried out through 5 to 20% sucrose gradients containing 0.3 M KCl-Buffer B for 20 hours at 0° as described under "Materials and Methods." △—△, pronase; ○—○, DNase; Δ—Δ, RNase; ●—●, no enzyme.

Fig. 5. Sucrose gradient centrifugation of cytoplasmic and nuclear 1α,25-dihydroxycholecalciferol-binding components of chick intestine. Reconstituted intestinal cytosol-chromatin (2 ml) was incubated with 1α,25-dihydroxy[3H]-cholecalciferol (6 nm) for 10 min at 25°. The chromatin was then extracted with 0.3 M KCl-Buffer B (●—●). Intestinal mucosa cytosol (prepared in 0.3 M KCl-Buffer B) was incubated with 1α,25-dihydroxy[3H]-cholecalciferol (6 nm) in the presence (Δ—Δ) or absence (○—○) of a 200-fold excess of unlabeled sterol for 30 min at 0°. Centrifugation of nuclear and cytoplasmic extracts was carried out through 5 to 20% sucrose gradients containing 0.3 M KCl-Buffer B for 20 hours at 0° as described under "Materials and Methods." △—△, relative optical density at 280 nm of cytosol cholate.

Fig. 6. Agarose gel filtration of cytoplasmic and nuclear 1α,25-dihydroxycholecalciferol-binding components of chick intestine. Reconstituted intestinal cytosol-chromatin (2 ml) was incubated with 1α,25-dihydroxy[3H]-cholecalciferol (4 nm) for 10 min at 25°. The chromatin was then extracted with 0.3 M KCl-Buffer B (●—●). Five-tenths milliliter of intestinal mucosa cytosol (prepared in 0.3 M KCl-Buffer B) was incubated with 1α,25-dihydroxy[3H]-cholecalciferol (6 nm) for 10 min at 0° (○—○). Column elution was carried out at 0° with 0.3 M KCl-Buffer B, and 1-ml fractions were collected as described under "Materials and Methods." △—△, relative optical density at 280 nm of cytosol cholate.
cytoplasmic component by interaction with the hormone. Heating of the nuclear and cytoplasmic extracts for 10 min at 60° resulted in complete loss of the respective receptor peaks.

**Temperature-dependent Distribution of Nuclear and Cytoplasmic 1α,25-Dihydroxycholecalciferol-binding Components**

Since we were unable to distinguish the cytoplasmic-binding components from the nuclear 1α,25-dihydroxycholecalciferol receptors, the distribution of the two components was determined after incubation in *vitro*, in order to ascertain the origin of the nuclear receptor complex. Reconstituted cytosol-chromatin was incubated with 1α,25-dihydroxy[3H]cholecalciferol at 0° for 5 min, and then incubation was continued at 25° for various periods of time. Following incubation, cytoplasmic and nuclear-binding components were isolated on sucrose gradients (Fig. 8). At zero time, the labeled sterol has formed a complex with the cytoplasmic receptor, and a small amount of this complex has appeared in the nucleus. After incubation of the receptor components at 25°, there is a dramatic increase in the 1α,25-dihydroxy[3H]cholecalciferol bound to the nuclear component, and a concomitant decrease in hormone binding to the cytoplasmic receptors. The loss of cytoplasmic complexes cannot be explained by destruction of the receptors at elevated incubation temperatures because the concentration of cytoplasmic receptor components is not significantly reduced following 5 min of incubation at 25° (Fig. 7).

These results suggest that intestinal mucosa chromatin may contain little 1α,25-dihydroxycholecalciferol receptor prior to exposure to the hormone; and following exposure, the receptor detected in the nucleus results from transfer of the cytoplasmic 1α,25-dihydroxycholecalciferol-receptor complex into the nucleus.

**Incubation of Cytosol and Chromatin from Intestine and Nontarget Tissues with 1α,25-Dihydroxy[3H]cholecalciferol, In Vitro**

In order to examine the specificity of the transfer of cytoplasmic receptors to the nucleus, experiments with cytosol and chromatin from nontarget tissues were carried out. Cytosol fractions from chick liver, kidney, and intestine were prepared and reconstituted with intestinal chromatin. Following incubation with 1α,25-dihydroxy[3H]cholecalciferol, the chromatin was extracted with 0.3 M KCl and analyzed for nuclear complexes on sucrose gradients (Fig. 9). Intestinal cytosol was most efficient in transferring hormone-receptor complex to intestinal nuclei of those tissues tested, although significant
amounts of nuclear receptor complex could be extracted from chromatin incubated with nontarget cytosols. These results were somewhat surprising since the 3.7 S cytoplasmic receptor could not be detected in liver or kidney cytosol, and previous results (Fig. 8, Ref. 4) suggested that the cytosol was the unique source of nuclear 1α,25-dihydroxycholecalciferol receptors. One explanation of this finding is that the 3.7 S receptor is present in intestinal mucosa chromatin prior to exposure to 1α,25-dihydroxycholecalciferol and is detected when this fraction is reconstituted with cytosol from nontarget tissues. Evidence in support of this concept is that no 3.7 S components can be detected in chromatin extracts when liver or kidney cytosol is reconstituted with chromatin from those respective tissues (Fig. 9) and that the 3.7 S receptor can be found in isolated intestinal chromatin, but not liver or kidney chromatin (Fig. 10).

Experiments were performed to determine the specificity of intestinal mucosa chromatin to accept the cytoplasmic 1α,25-dihydroxycholecalciferol-receptor complex. Intestinal mucosa cytosol was reconstituted with chromatin fractions from chick liver, kidney, and intestine and incubated with 1α,25-dihydroxy[3H]cholecalciferol. As shown in Fig. 10, intestinal mucosa chromatin retained more intestinal cytoplasmic receptors than either liver or kidney chromatin. Parallel incubations containing a 200-fold excess of unlabeled 1α,25-dihydroxycholecalciferol showed no macromolecular bound hormone on the sucrose gradients (Fig. 10). Thus the [3H]sterol was specifically bound to the 3.7 S receptor peak following incubation of the labeled hormone with reconstituted intestinal cytosol-nontarget chromatin. Additional experiments were performed in which chromatin fractions were reconstituted with buffer only and incubated with 1α,25-dihydroxy[3H]cholecalciferol. No 3.7 S receptor peak could be detected in the nontarget chromatin following these procedures. Thus the hormone receptor complex which is retained by kidney and liver chromatin apparently originates in the intestinal cytosol. The efficiencies of the liver and kidney chromatin in accepting the cytoplasmic receptor relative to intestinal chromatin are 36 and 27%, respectively. The results in Figs. 9 and 10 indicate that there are two levels of specificity in the interaction of 1α,25-dihydroxycholecalciferol with its target tissue; both intestinal cytosol and chromatin are required for maximal retention of the hormone receptor complex in the chromatin.

**DISCUSSION**

Early studies on the subcellular localization of tritium following administration of [3H]cholecalciferol (vitamin D3) to rachitic chicks demonstrated that a metabolite of vitamin D was specifically localized in the intestinal mucosa chromatin (23, 26). These experiments led to the concept that vitamin D required metabolic activation prior to the alteration of genetic expression in the intestine and eventual stimulation of calcium transport (27). It was further shown that the metabolite could be removed from the chromatin in association with a protein by treatment with 0.3 m KCl-Tris buffer (28). However, due to the low specific activity of the labeled vitamin D, the specificity of the metabolite-protein interaction could not be assessed.

The present results employing the 1α,25-dihydroxy[3H]-cholecalciferol metabolite of high specific activity and in vitro incubations demonstrate that specific 1α,25-dihydroxycholecalciferol-binding macromolecules exist in the small intestine of chicks. Hormone-macromolecule complexes can be isolated from the cytoplasm and nucleus by sucrose gradient centrifugation and agarose gel filtration chromatography in 0.3 m KCl. The nuclear and cytoplasmic components cannot be distinguished from each other when analyzed by the present methods, and the possibility exists that the nuclear 1α,25-dihydroxycholecalciferol-receptor complex represents adsorption of the cytoplasmic component to chromatin under in vitro incubation conditions. However, failure to remove the hormone-binding protein by washing the chromatin with 0.15 m KCl-Tris-EDTA buffer (Fig. 3) and the temperature dependence of the formation of the nuclear receptor complex (Figs. 1, 2, and 8) suggest that its appearance in the nucleus represents more than simply adsorption of the cytoplasmic component.

Initially, 1α,25-dihydroxycholecalciferol receptors exist primarily in the cytoplasm of the intestinal mucosal cell. After incubation of the [3H]hormone in vitro, there is a progressive increase in the concentration of the nuclear-binding component and a concomitant decrease in the cytoplasmic receptor concentration (Fig. 8). Thus, several lines of evidence are compatible with the view that the nuclear hormone-receptor complex arises primarily from a hormone dependent transfer of the cytoplasmic receptor into the nucleus. These include data showing (a) dependence on cytoplasmic sterol binding to achieve maximal nuclear receptor concentration, (b) an inverse relationship between cytoplasmic and nuclear concentrations of receptor, (c) similar binding affinity and specificity of 1α,25-dihydroxycholecalciferol for the two receptors, and (d) their similar physicochemical properties, including apparent molecular weight and thermal instability.

The role of intestinal cytosol in transporting the hormone to
the nucleus was further investigated by incubation of 1α,25-
dihydroxycholecalciferol with reconstitutions of intestinal mu-
cosa chromatin and cytosols from nontarget tissues or buffer
only. In these experiments, significant amounts of nuclear
complex were formed in the absence of the target tissue cytosol.
Since nontarget cytosols contain no detectable 3.7 S receptors
for this hormone, the source of this nuclear complex is
apparently the purified intestinal chromatin, and its presence
may explain the small uptake of hormone by chromatin at 0° in
vitro (Figs. 1, 2, and 8). Binding proteins for progesterone and
estrogen have not been detected in isolated nuclei of their
respective target tissues (14, 29), and the possibility exists that
our results are due to a small amount of adsorption of the
cytoplasmic receptors to chromatin during its preparation.

Experiments employing reconstitutions of intestinal mucosa
chromatins with purified chromatin from nontarget tissues indicate that
there is a greater uptake of the 3.7 S receptor complex at
25° by intestinal chromatin than by liver or kidney chromatin
when these fractions are compared in terms of their DNA
contents. These observations suggest that intestinal chromatin
contains specific acceptor sites with affinity for the 1α,25-dihy-
droxycholecalciferol-receptor complex. The results in Fig. 10
also show significant retention of the cytoplasmic 1α,25-dihy-
droxycholecalciferol-binding component by the nontarget
chromatins. These results may indicate a lower concentration
of “acceptor” sites in these preparations or adsorption of the
intestinal cytoplasmic receptor to chromatin. In either case, the
macromolecular binding of the labeled hormone in the nontar-
get chromatin is specific since it is reduced by excess unlabeled
1α,25-dihydroxycholecalciferol (Fig. 10).

The present results are consistent with the two-step mecha-

nism for steroid hormone binding in target tissues. This
sequence of events involves initial binding of the hormone to
a cytoplasmic receptor protein located only in the respective
target tissue, which is subsequently transported into the
nucleus via a temperature-dependent process. In the case of
estrogen, transformation of the uterine cytoplasmic receptor
from a species sedimenting at 4 S on sucrose gradients to a 5 S
component occurs prior to nuclear binding (12). Such transfor-
mation has not been observed for the progesterone receptor
(14), or as reported here, for the 1α,25-dihydroxycholecalciferol
receptor, although the techniques employed in this study could
not detect small differences in the nuclear and cytoplasmic
forms of the receptor.

A second potential site for specificity in this mechanism is
nuclear acceptor sites for the cytoplasmic receptor complex.
Nontarget chromatins apparently have little capacity for
accepting the oviduct progesterone cytoplasmic receptor (14),
although such a specificity has not been demonstrated for
estrogen (29). In the present experiments, we find that purified
chromatin from kidney or liver show less incorporation of
intestinal 1α,25-dihydroxycholecalciferol receptor complex
than that observed with intestinal chromatin when chromatin
fractions were compared in terms of their DNA contents. Whether or not the binding of the complex to intestinal or
nontarget chromatins affects nuclear RNA synthesis remains
to be determined. Studies are in progress to determine whether
1α,25-dihydroxycholecalciferol receptor complexes are directly
involved in intestinal gene activation and nuclear RNA synthe-
sis.

REFERENCES
Res. Commun. 51, 74–80
2. Tsai, H. C., and Norman, A. W. (1973) J. Biol. Chem. 248,
5967–5975
1231–1237
1258–1262
Nat. Acad. Sci. U.S.A. 71, 2337–2341
6. IUPAC and IUB (1969) Biochemistry 8, 2227
246, 100–101
Biochem. Biophys. 157, 339–347
Sci. U.S.A. 57, 1740–1743
(1971) in The Biochemistry of Steroid Hormone Action (Smelie,
Biol. Chem. 245, 6056–6066
Biol. Chem. 246, 1117–1122
668–695
Biol. Chem. 243, 3849–3856
Mol. Biol. 67, 99–115
1737–1746
21. Lawson, D. E. M., Fraser, D. R., Kodicek, E., Morris, H. R., and
Chem. 243, 4055–4065
24. Dische, F. (1930) Mikrochemie 8, 4–32
Biophys. 118, 145–153
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