# Intranuclear Localization and Receptor Proteins for 1,25-Dihydroxycholecalciferol in Chick Intestine

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1. The intranuclear distribution of cholecalciferol and its metabolites was studied in the intestine of rachitic chicks. 2. At high doses of cholecalciferol the nuclei contain the vitamin and its 25-hydroxy metabolite, but over 80% of this is localized on the nuclear membranes. The hormone, 1,25-dihydroxycholecalciferol, is found within the cell nuclei irrespective of the intake of cholecalciferol, but significant amounts could not be found with chromatin isolated free of nuclear membranes. 3. 1,25-Dihydroxycholecalciferol is associated in the nucleus with an acidic protein. Since one of the actions of 1,25-dihydroxycholecalciferol is to control the synthesis of mRNA for calcium-binding protein it was to be expected that the hormone would be bound to chromatin, as with the other steroid hormones. It is suggested that the hormone–receptor complex exists as part of an equilibrium mixture of the complex bound to the DNA and in a free form. 4. A protein extract of nuclei was obtained, which when incubated at 4°C for 1h took up the 1,25-dihydroxycholecalciferol. The nature of this binding was studied. 5. There appear to be two nuclear proteins able to bind the hormone one of which is the intestinal nuclear receptor. The binding sites on this protein are saturable with the hormone, have an association constant of $2 \times 10^9 \, M^{-1}$ and show a high chemical specificity for the 1,25-dihydroxycholecalciferol. The number of nuclear binding sites for the hormone provided by this receptor is similar to the maximum intestinal hormone concentration so far observed. Its sedimentation coefficient is 3.5S, and is very close to that observed for the nuclear protein to which is attached the 1,25-dihydroxycholecalciferol formed *in vivo* from vitamin D. 6. The cytoplasmic protein has an association constant of $1 \times 10^9 \, M^{-1}$ and a sedimentation coefficient of 3.0S, but its relation with the nuclear receptor is not yet clear.

The intracellular localization of hormones has long been used to support theories on their molecular action and consequently much effort has been expended on obtaining a complete understanding of their cellular localization. This approach also has been taken by several groups studying the recently discovered steroid hormone 1,25-dihydroxycholecalciferol. As with all the other steroid hormones this substance is found primarily in nuclei and cytoplasm, with very little occurring in the mitochondrial and microsomal fractions (Lawson *et al.*, 1971). Fractionation of the nuclei to more exactly define the localization of 1,25-dihydroxycholecalciferol has resulted in conflicting views.

Thus Haussler *et al.* (1968) have concluded that chromatin is the major location of the 1,25-dihydroxycholecalciferol, whereas Stohs & DeLuca (1967), on the basis of the effect of Triton X-100 on nuclei, concluded that the hormone was associated with the outer nuclear membrane. We found that both findings can be readily reproduced, but that the effect of Triton X-100 was not confined to removing the outer nuclear membrane, and suggested that chromatin was

the more likely site of 1,25-dihydroxycholecalciferol localization (Lawson *et al.*, 1969a). In further studies DeLuca's group showed that the chromatin preparation of Haussler *et al.* (1968) was contaminated with membranes and that preparations of homogeneous intestinal chromatin from chicks and rats did not contain a significant proportion of the total tissue 1,25-dihydroxycholecalciferol (Chen *et al.*, 1970). More recently the same group found that the intranuclear distribution differed according to the origin of the 1,25-dihydroxycholecalciferol; it was claimed that pure chromatin contains 1,25-dihydroxycholecalciferol only after doses of the hormone and not of the precursors, vitamin D and 25-hydroxycholecalciferol (Chen & DeLuca, 1973), but they did not offer an explanation for this observation. The chromatin was found to contain about 50% of the 1,25-dihydroxycholecalciferol in the crude nuclear fraction from which it was derived, with the remainder associated with a low-density lipoprotein fraction.

Few thorough studies have been carried out on the nature and characteristics of the intestinal receptors of 1,25-dihydroxycholecalciferol. The evidence

available suggests that in both the nuclei and the cytoplasm the hormone is attached to a protein. The association constant for the uptake of the hormone by the cytoplasmic receptor has been found to be $10^7 M^{-1}$ and to show a high specificity (Tsai & Norman, 1973). It was further claimed by Tsai & Norman (1973) that the hormone could only become attached to the intestinal nuclei if presented to this organelle when bound to the cytoplasmic receptor.

We report here our findings on the intranuclear distribution of 1,25-dihydroxycholecalciferol and of the nature and some of the characteristics of the binding components for this steroid hormone in the nuclei and cytoplasm of intestine. A brief account of some of these results has been published (Lawson & Emtage, 1974).

## Experimental

### Animals

Rhode Island Red × Light Sussex chicks (1 day old) were obtained from the National Institute for Research in Dairying (Shinfield, Reading, U.K.). They were fed on the following diet *ad lib.*: maize starch, 60% (w/w); vitamin-free casein, 18% (w/w); gelatin, 10% (w/w); salt mixture, 6% (w/w); arachis oil, 5% (w/w); cysteine, 0.3% (w/w); methionine, 0.3% (w/w); and vitamin mixture. The salt mixture and vitamin mixture have been described (Lawson *et al.*, 1969a).

After about 3 weeks on this diet the chicks were vitamin D-deficient. Radioactive solutions in propylene glycol were administered intracardially and the chicks were killed at 16h unless otherwise stated.

### Materials

25-Hydroxy[26-³H]cholecalciferol (sp. radioactivity 11 Ci/mmol) was prepared as described previously (Bell & Scott, 1973). [1-³H]Cholecalciferol (sp. radioactivity 211 mCi/mmol) (Bell, 1968) was prepared by Dr. B. Pelc (M.R.C. Mineral Metabolism Unit, General Infirmary, Leeds, U.K.). [4-¹⁴C]-Cholecalciferol (sp. radioactivity 32.5 mCi/mmol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. [4-¹⁴C,1-³H]Cholecalciferol was obtained by mixing the two singly labelled preparations so as to give a ³H/¹⁴C ratio of approx. 5.0. 1-Hydroxy- and 1,25-dihydroxy-cholecalciferol were prepared by Dr. B. Pelc also. The preparation of the 25-hydroxy-5,6-*trans*-cholecalciferol has been described (Lawson & Bell, 1974). 1,25-Dihydroxy[26-³H]cholecalciferol was prepared by Dr. D. R. Fraser (Dunn Nutritional Laboratory, Milton Road, Cambridge, U.K.) by incubating 25-hydroxy[26-³H]cholecalciferol with kidney homogenates (Fraser & Kodicek, 1970). All enzymes were

obtained from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K.

### Intestinal cell fractionation

(a) *Pure nuclei*. These were prepared as described previously (Lawson *et al.*, 1969a).

(b) *Nuclear membranes*. Pure nuclei isolated as above were treated with DNAase* (50 µg/2 mg of nuclear protein) for 14h and the nuclear pellet was collected by centrifugation at 1200g for 10 min. The pellet was extracted twice with 0.5 M-MgCl₂ for 15 min and the nuclear membranes were collected by centrifugation at 27000g for 15 min (Berezney *et al.*, 1970). Recovery of nuclear membranes was assessed by measurement of the Mg²⁺-stimulated ATPase activity of nuclei (Zbarsky *et al.*, 1969).

(c) *Cytosol*. Intestinal mucosal scrapings were weighed and homogenized in 3 vol. of 10 mM-Tris–HCl buffer, pH 7.5–1 mM-mercaptoethanol–1.5 mM-EDTA (TEM buffer). The homogenate was centrifuged at 100000g for 1h and the clear supernatant removed (cytosol).

(d) *Chromatin*. For the isolation of chromatin the starting material was always crude nuclear fraction prepared by homogenizing the intestinal mucosal scrapings in 0.32 M-sucrose in 50 mM-Tris–HCl, pH 7.5–25 mM-KCl–5 mM-MgCl₂ (TKM buffer) and centrifuging the homogenate at 800g for 10 min. The pellet was washed in the 0.32 M-sucrose–TKM buffer and extracted with a solution of 80 mM-NaCl–20 mM-EDTA, pH 6.2, as described by Dingman & Sporn (1964).

(e) *Extraction of nuclear proteins*. Unless specified the procedure adopted as a routine was to homogenize intestinal mucosal scrapings of the chick in 3 vol. of TEM buffer diluted to give a 10% (w/v) homogenate before filtering through nylon bolting cloth. The filtrate was centrifuged at 800g for 10 min and the pellet was washed twice with an equal volume of buffer and then centrifuged at 800g for 10 min. Proteins were extracted from the pellet by suspending in TEM buffer containing 0.4 M-KCl. After 1h at 4°C the suspension was centrifuged at 100000g for 1h and the clear supernatant removed. The protein content of this extract was measured and the extract diluted with TKM buffer containing 0.4 M-KCl to 1 mg/ml for studying the binding characteristics for 1,25-dihydroxycholecalciferol.

### Competitive displacement studies

Competitive displacement studies of bound 1,25-dihydroxy[26-³H]cholecalciferol by various amounts of the non-labelled steroid or its various isomers and

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* Abbreviations: DNAase, deoxyribonuclease; ATPase, adenosine triphosphatase; RNAase, ribonuclease.

analogues were carried out to assess the affinity and specificity of intestinal cytosol and of protein extracts of nuclei for this hormone. The separation of the protein-bound steroid from that remaining unbound was done with charcoal (Norit GSX; Hopkin and Williams Ltd., Chadwell Heath, Essex, U.K.) coated with dextran of mol.wt. 60000–90000 (Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K.). The charcoal was coated with dextran as described by Murphy (1967) and diluted 1:10 in TEM buffer. The displacement assay was carried out by adding 1 ml of the protein solution to each tube containing an ethanolic solution (40 $\mu$l) of 3000 d.p.m. of 1,25-dihydroxy[26-$^3$H]cholecalciferol with appropriate amounts of the non-labelled steroid. The tubes were shaken and left for 1 h at 4°C and then 1 ml of the dextran-coated charcoal was added and the mixture left for 10 min. The tubes were then centrifuged for 10 min at 1000 g and 1 ml was taken from the clear supernatant, containing the bound steroid, and counted for radioactivity.

## Column chromatography

Gel filtration was carried out on columns (2.5 cm × 45 cm) of Sephadex G-150 (Pharmacia Fine Chemicals Ltd., London W5 1BR, U.K.) equilibrated with TEM buffer. Column chromatography was done in a cold-room at 4°C and the $E_{280}$ of the eluates was monitored continuously with an LKB Uvicord II Absorptiometer as a measure of protein content.

## Sucrose-gradient ultracentrifugation

Portions (0.5 ml) of the nuclear extracts or cytosol with radioactive 1,25-dihydroxycholecalciferol bound to them were layered on to 5 ml of a continuous 5–20% (w/v) sucrose gradient in TEM buffer. For analysis of the nuclear extracts the buffer also contained 0.4 M-KCl. After centrifugation at 50000 rev./min for 20 h in an SW50 rotor in a Beckman L2-65B centrifuge, the gradient was pumped out through an absorptiometer to measure protein content and collected in equal fractions of approx. 0.2 ml.

## Measurement of radioactivity

All radioactivity measurements were carried out in a Packard Tri-Carb automatic liquid-scintillation spectrometer no. 3375. Aqueous samples were counted for radioactivity in a solution of Triton X-100 in toluene (1:2, v/v) containing 0.028% (w/v) 1,4-bis-(5-phenyloxazol-2-yl)benzene and 0.33% (w/v) 2,5-diphenyloxazole. Lipid samples were counted for radioactivity in a solution of 100 mg of 1,4-bis-(5-phenyloxazol-2-yl)benzene and 4.0 g of 2,5-

diphenyloxazole/litre of toluene. Quenching was corrected by using the automatic external standardization and correlation curves for $^{14}$C or $^3$H, either individually or combined.

## Other methods

Protein was determined by the method of Warburg & Christian (1941).

## Results

### Isolation of membranes of intestinal nuclei

To obtain more detailed information on the intranuclear localization of 1,25-dihydroxycholecalciferol it was of obvious advantage to have nuclei containing the maximum possible amount of the radioactive hormone. Consequently the concentration of the hormone in the intestine was measured in rachitic chicks, which had received increasing amounts of radioactive cholecalciferol. The intestinal concentration of 1,25-dihydroxycholecalciferol increased to a maximum of 7.5 pmol/g of tissue with doses of cholecalciferol up to 2.5 $\mu$g and could not be increased above this value irrespective of the amount of vitamin given to the birds. The maximum concentration of 1,25-dihydroxycholecalciferol in the intestinal nuclei is reached 8 h after dosing with the vitamin and remains unchanged for up to 24 h (Lawson & Emtage, 1975). In these studies nuclei were isolated for convenience 16 h after dosing the rachitic chicks with 2.5 $\mu$g of [4-$^{14}$C,1-$^3$H]cholecalciferol, and then were further treated with DNAase and $Mg^{2+}$ so as to obtain a pure nuclear membrane preparation. Almost half (45.4%) of the nuclear radioactivity was recovered in the membrane fraction (Table 1), and on chromatographic analysis of this radioactivity the proportion of cholecalciferol and metabolites was: cholecalciferol, 65.5%; 25-hydroxycholecalciferol, 10%; and 1,25-dihydroxycholecalciferol, 18%. The radioactivity in the other two fractions was primarily 1,25-dihydroxycholecalciferol, accounting for 87.2% of the DNAase-solubilized radioactivity and 65% of the $Mg^{2+}$-solubilized material.

On decreasing the dose of radioactive cholecalciferol to the chicks to 0.5 $\mu$g, at which intake 90% of the nuclear radioactivity is in 1,25-dihydroxycholecalciferol, it was found in two experiments that 6.5 and 14.3% of the nuclear radioactivity was recovered with the nuclear membranes. The $Mg^{2+}$-stimulated ATPase activity of nuclei is reported to be confined to the nuclear membranes and, by using this enzyme as a marker, it was found that the membrane preparations isolated in the course of this work contained over 40% of the

Table 1. *Effect of dose of radioactive cholecalciferol on the distribution of radioactivity in the intestinal nuclei*

Groups of four rachitic chicks received an intracardial dose of [4-$^{14}$C, 1-$^3$H]cholecalciferol and after 16h the birds were killed and pure intestinal nuclei prepared. Nuclear membranes were then isolated and the radioactivity in their lipid extracts was measured. Details are given in the text. Results are expressed as the percentage of nuclear radioactivity recovered at each stage of the isolation of nuclear membranes (mean±S.D. for the number of determinations given in parentheses, where appropriate).

| Dose (µg of cholecalciferol) | Proportion of total nuclear radioactivity | | |
|---|---|---|---|
| | Released by DNAase (%) | Released by Mg$^{2+}$ (%) | Nuclear membranes (%) |
| 2.5 | 43.0±4.6 (5) | 11.8±6.7 (5) | 45.4±6.4 (5) |
| 0.5 | 77.7 | 15.8 | 6.5 |
| 0.5 | 46.4 | 39.3 | 14.3 |

Table 2. *Recovery of radioactivity in preparations of chromatin from tissues of rachitic chicks*

The intestinal chromatin preparations were prepared from four groups of three rachitic birds that had received an intracardial injection of 0.5µg of [4-$^{14}$C]cholecalciferol 16h previously. The birds used for the preparations of bone and kidney chromatin had received 50ng of 25-hydroxy[26-$^3$H]cholecalciferol 16h previously. Chromatin was prepared by the method of Dingman & Sporn (1964) and results are expressed as d.p.m./mg of DNA (mean±S.D., where appropriate).

| Tissue fraction | Recovery of radioactivity | | |
|---|---|---|---|
| | Intestine | Kidney | Bone |
| Whole homogenate | 93.0±10.5 | 34540 | 10700 |
| Crude nuclei | 43.0±14.3 | 8000 | 1500 |
| Chromatin | | | |
| Solubilized | 36.8±45.0 | 5770 | 990 |
| Precipitated | 6.5± 7.0 | 130 | 360 |

initial total nuclear enzyme activity. It seems clear therefore that the nuclear 1,25-dihydroxycholecalciferol is not associated with either of the two nuclear membranes. However, the effect of the DNAase treatment in the membrane isolation procedure suggests that it is associated in some way with the DNA.

*Isolation of intestinal chromatin*

In confirmation of the findings of Chen *et al.* (1970) it was found that chromatin prepared by the procedure of Haussler *et al.* (1968) was heavily contaminated with brush-border membranes and, as expected from the findings of these two groups, such preparations contained over 60% of the 1,25-dihydroxycholecalciferol present in the crude nuclear fraction from which chromatin was obtained.

Almost all procedures for the preparation of chromatin are basically modifications of the method of Zubay & Doty (1959). One well-described variation, which has been applied to tissues from a number of sources, is that of Dingman & Sporn, (1964). Examination of the chick intestinal chromatin, by light and electron microscopy, prepared by this procedure showed it to be homogeneous with only a trace of membranous material. The preparation of chromatin results in a loss of nuclear proteins as well as in the removal of the nuclear membranes and consequently, if 1,25-dihydroxycholecalciferol is associated with chromatin, the concentration of this hormone should be related to DNA and not to protein. A number of analyses were carried out and although there was a variation between experiments certain features are apparent (Table 2). After treatment of the crude nuclear

fraction from intestine, kidney or bone with a solution of EDTA and NaCl to yield a preparation of impure chromatin a significant loss of total radioactivity was always observed. The chromatin in this fraction is now solubilized by shearing to give a much purer chromatin fraction, as shown by comparison of the $E_{280}$ and $E_{260}$ (Dingman & Sporn, 1964). The almost quantitative precipitation of the chromatin in this extract can be achieved by adjustment of the NaCl concentration (precipitated chromatin). Throughout this procedure the total radioactivity at each stage decreased as did, in most cases, the concentration expressed as d.p.m./mg of DNA (Table 2). A maximum of 10% of the radioactivity in the nuclear fraction was detected in the precipitated chromatin fraction from which it was derived.

Intestinal chromatin also has been prepared by the method of Marushige & Bonner (1966) from birds dosed with 0.5µg of [4-$^{14}$C]cholecalciferol. As with the other procedures the initial pellet containing the nuclei had about 50% of the radioactivity in the whole homogenate. Treatment with EDTA and NaCl resulted in a loss of over 80% of this radioactivity, and after purification of the chromatin by centrifugation through 1.7M-sucrose only 7% of the initial nuclear radioactivity was found in the pure chromatin fraction. To overcome the problem of the low level of radioactivity in the intestinal mucosal scrapings after dosing with [4-$^{14}$C]cholecalciferol and also the problem of a possible effect of proteolytic enzymes (see below), chromatin was isolated from kidney and bone of birds dosed with 25-hydroxy[26-$^3$H]cholecalciferol. Again significant amounts of 1,25-dihydroxy[26-$^3$H]-cholecalciferol could not be shown to be present in these pure chromatin preparations (Table 2).

Table 3. *Effect of enzymes on release of 1,25-dihydroxy-cholecalciferol from intestinal nuclei*

Nuclei were prepared from intestines of rachitic chicks dosed with 50ng of 25-hydroxy[26-$^3$H]cholecalciferol. Nuclear preparations containing 3000d.p.m. of $^3$H and 0.7mg of DNA were incubated with 0.5mg of each enzyme in 1ml of 0.32M-sucrose-TKM buffer, pH7.5, at 28°C for 15min. The incubation solutions were centrifuged at 1000g for 10min and then the radioactivity in each pellet and supernatant was measured. Results are expressed as the percentage of nuclear radioactivity in the supernatant.

| Enzyme | Proportion of 1,25-dihydroxycholecalciferol released |
|---|---|
| DNAase | 28.5 |
| RNAase | 15.1 |
| Hyaluronidase | 10.6 |
| Phospholipase | 10.9 |
| Control | 3.3 |

It was noticed that in these and other experiments there was a variation in the amount of 1,25-dihydroxycholecalciferol found in nuclei. This observation was also made in studies on the effect of enzymes on the concentration of nuclear 1,25-dihydroxycholecalciferol (Table 3). Phospholipase, hyaluronidase and RNAase released little of the 1,25-dihydroxycholecalciferol from the nuclei whereas DNAase released significant amounts, in some experiments as much as 40% in 15min. The release of this steroid appeared to stop after 60min (Stohs & DeLuca, 1967), although DNA hydrolysis continued. It is noteworthy that up to 30% of the 1,25-dihydroxycholecalciferol can be released from the nuclei kept at 4°C in the absence of enzymes. Pure intestinal nuclei, isolated from vitamin D-deficient chicks injected with 0.125μg of 25-hydroxy[26-$^3$H]-cholecalciferol, were therefore incubated in 0.32M-sucrose in TKM buffer at 4°C and the time-course of the release of this steroid was followed. In one typical experiment it was found that 24% of the 1,25-dihydroxycholecalciferol was released over 20min. Other studies (see next section) showed that the steroid was attached to a protein and it was therefore possible that a trace of the potent intestinal proteolytic enzymes was responsible for this release. Some proteolytic enzyme activity was detected in the nuclear preparations, although they had been obtained by centrifugation through 2.4M-sucrose. Inhibitors of proteolytic enzymes such as di-isopropyl fluorophosphate, phenylmethylsulphonyl fluoride or 0.2mM-Pb$^{2+}$ decreased this release by about 50%, with the most effective inhibitor being 0.2mM-Pb$^{2+}$. Intestinal chromatin was therefore isolated with 0.2mM-Pb$^{2+}$ added to all solutions, but again very little radioactivity was found in the isolated chromatin.

## Nuclear receptors for 1,25-dihydroxycholecalciferol

(*a*) *Studies in vivo*. The first studies on the characteristics of the intestinal receptors were carried out on intestinal fractions from vitamin D-deficient chicks, which 16h previously had received 50ng of 25-hydroxy[26-$^3$H]cholecalciferol. It was necessary first to establish the conditions under which the nuclear 1,25-dihydroxycholecalciferol-receptor complex could be solubilized. Pure intestinal nuclei obtained from such birds were extracted with salt solutions of varying ionic concentration and with buffers over the range pH3-11. Solutions of univalent cations such as K$^+$ or Na$^+$ extracted the radioactivity from the nuclei efficiently; concentrations of 0.6M-KCl extracted 80% of the radioactivity (Fig. 1*a*). However, less efficient extractions resulted when equivalent concentrations of MgCl$_2$ were used. The maximum solubilization (55%) of the 1,25-dihydroxycholecalciferol receptors achieved with a range of buffers was obtained at pH9.0 (Fig. 1*b*). In subsequent studies the nuclear extracts were prepared from crude nuclei by treatment with 0.4M-KCl, which solubilized at least 50% of the nuclear 1,25-dihydroxycholecalciferol with less contamination from the other nuclear proteins.

The protein nature of the nuclear receptor complex was indicated by the finding that the proteolytic enzymes effected a significant release of the hormone from the receptor into the incubation solution (Table 4). Further, the radioactivity in the extracts (>75%) was precipitated by half-saturation with (NH$_4$)$_2$SO$_4$ and it was eluted at the void volume from columns of Sephadex G-25. The behaviour of the 1,25-dihydroxycholecalciferol-receptor complex from crude nuclei was examined on columns of Sephadex G-150 and on linear 5-20% (w/v) sucrose gradients. It was found that recoveries of radioactivity in both systems and reproducibility of the results were improved in the presence of 3mM-EDTA and 1mM-mercaptoethanol in the 0.01M-Tris buffer, pH7.4. The elution pattern of the radioactivity from the Sephadex G-150 column showed three peaks (Fig. 2). One peak was eluted from the column with the void volume (peak 1) and would appear to be due to aggregation of the other proteins. The last peak to be eluted from the column (peak 3) was unstable and was not observed in KCl extracts stored for 2-3 days. With the disappearance of this peak there was a concomitant increase in peak 1 eluted with the void volume, suggestive of the formation of the latter by aggregation of the low-molecular-weight peak.

The radioactivity in the nuclear extract was also analysed by centrifugation through a sucrose gradient. Measurements of the radioactivity distributed throughout the gradient showed the hormone to
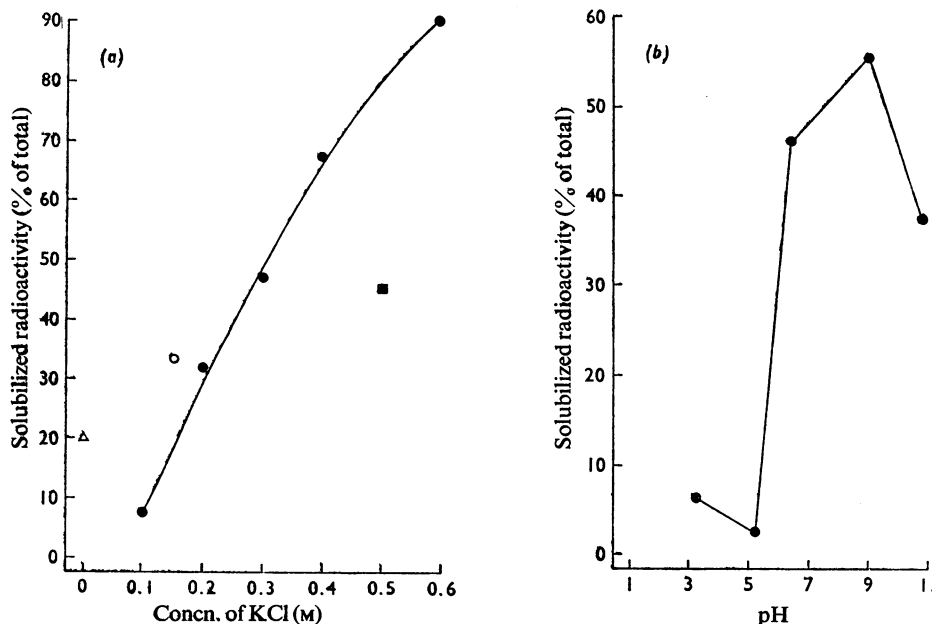
Fig. 1. *Extraction of 1,25-dihydroxycholecalciferol receptor proteins from chick intestinal nuclei*

Rachitic chicks were dosed with 125ng of 25-hydroxy[26-³H]cholecalciferol and pure intestinal nuclei isolated. The nuclei were treated for 1 h at 4°C with: (*a*) ●, KCl in TEM buffer; ○, NaCl in TEM buffer; ■, MgCl₂ in TEM buffer; △ H₂O; or (*b*) with buffers over the range pH 3–11 in the absence of KCl. The suspension was then centrifuged for 1 h at 100000g and the radioactivity in the supernatant measured.

Table 4. *Effect of enzymes on release of 1,25-dihydroxy-cholecalciferol from KCl extract of intestinal nuclei*

Pure nuclei were isolated from chicks dosed with 50ng of 25-hydroxy[26-³H]cholecalciferol and extracted with 0.4M-KCl in TKM buffer, pH7.5. The extracts were incubated with 0.5mg of enzyme in 1.0ml of 0.32M-sucrose-TKM buffer at 28°C for 15min. The incubation solutions were then passed through a column (5cm × 15cm) of Sephadex G-25 equilibrated in the same buffer, and the radioactivity in the void volume was measured. Results are expressed as the percentage of the radioactivity in the extract.

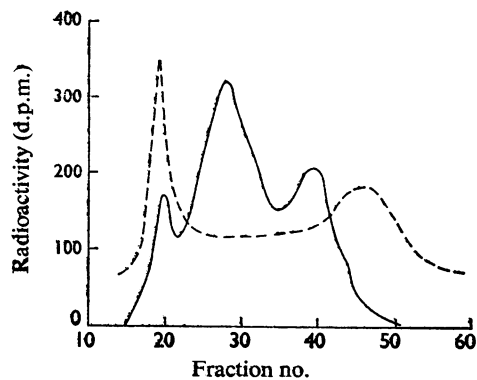| Enzyme | Proportion released (%) |
|---|---|
| DNAase | 21.0 |
| Trypsin | 61.3 |
| Chymotrypsin | 80.7 |
| Control | 20.0 |



Fig. 2. *Chromatography on Sephadex G-150 of the 0.4M-KCl extract of nuclei preloaded with 1,25-dihydroxy[26-³H]-cholecalciferol*

Rachitic birds were dosed with 125ng of 1,25-dihydroxy-[26-³H]cholecalciferol and the intestinal nuclear-debris fraction was prepared and extracted with 0.4M-KCl in TEM buffer. The extract was chromatographed on Sephadex G-150 and the column developed with TEM buffer (see the Experimental section). Radioactivity in the fractions (3ml) was measured. - - - -, $E_{260}$; ——, radioactivity.

be associated with a protein having a sedimentation constant of approx. 3.2S.

(*b*) *Studies in vitro.* The analysis of the intestinal receptors of 1,25-dihydroxycholecalciferol was in part hindered owing to the low amounts of radioactivity bound to these receptors. The uptake of
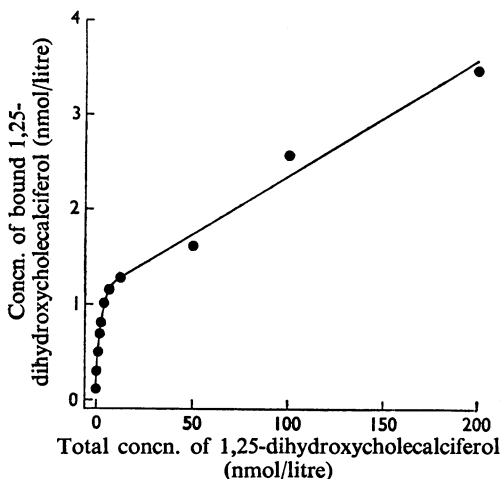
Fig. 3. *Uptake of radioactive 1,25-dihydroxycholecalciferol by chick intestinal nuclear extracts*

The 0.4M-KCl extract of chick intestinal nuclei was incubated for 1 h at 4°C with increasing amounts of 1,25-dihydroxy[26-³H]cholecalciferol. The mixture was treated with charcoal coated with dextran and the bound radioactivity in the supernatant measured.



Fig. 4. *Displacement of 1,25-dihydroxy[26-³H]cholecalciferol from intestinal nuclear extracts by increasing amounts of the unlabelled hormone*

The 0.4M-KCl extracts of intestinal nuclear-debris fractions were incubated with 4000 d.p.m. of 1,25-dihydroxy[26-³H]cholecalciferol containing increasing amounts of the unlabelled steroid. The incubation was carried out for 1 h at 4°C and then free hormone was removed by addition of charcoal coated with dextran. The radioactivity in the supernatant was measured and was taken as the amount of bound hormone.

the hormone by the KCl extracts of the intestinal nuclei was therefore studied *in vitro*.

The crude nuclear fraction of chick intestine was extracted with 0.4M-KCl in TEM buffer and incubated with 1,25-dihydroxy[26-³H]cholecalciferol in 4% (v/v) ethanol for 1 h at 4°C. After removal of the unbound steroid by addition of charcoal coated with dextran the KCl extract was found to have taken up a significant proportion of the hormone. The characteristics of this binding activity of the KCl extract were investigated in a series of experiments. It was found that the proportion of 1,25-dihydroxycholecalciferol bound to the nuclear extract was related to the protein concentration in the incubation mixture such that about 40–50% was bound with a protein concentration of 0.5–1.0 mg/ml of incubation mixture. The concentration of 1,25-dihydroxycholecalciferol bound by 1 mg of protein from the KCl extract was measured with increasing amounts of the hormone. As the concentration of steroid in the incubation mixture increased the amount of the bound fraction increased in a biphasic manner, but at any given protein concentration the binding sites were not saturable (Fig. 3).

The affinity of the protein in the KCl extracts for the 1,25-dihydroxycholecalciferol was measured by recording the displacement of the bound radioactive steroid by increasing amounts of the non-labelled hormone (Fig. 4). Analysis of such a curve by the method of Scatchard (1949) also gave a curve
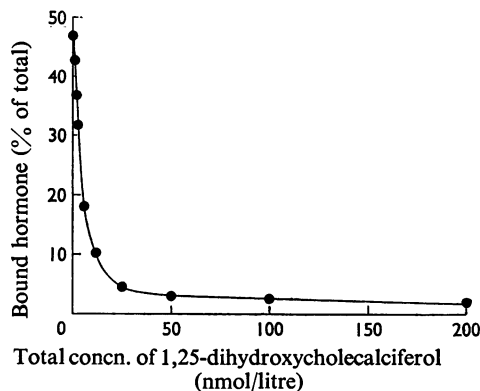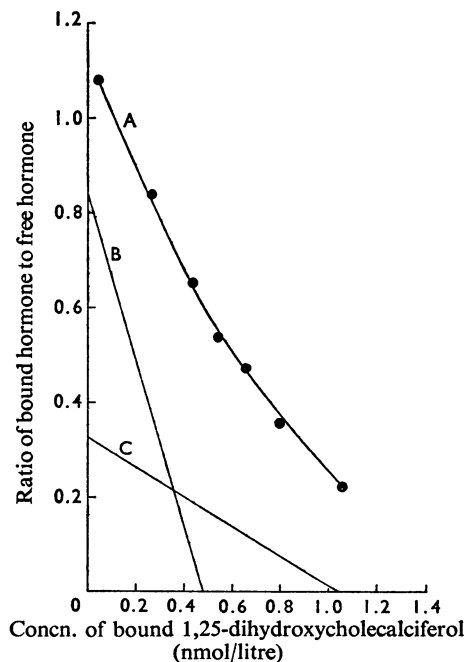


Fig. 5. *Analysis of the 1,25-dihydroxycholecalciferol displacement curve obtained with intestinal nuclear extract*

Displacement curves similar to that shown in Fig. 4 were re-expressed by the method of Scatchard (1949), and the resulting curve was analysed by the method of Danchin & Gueron (1970).
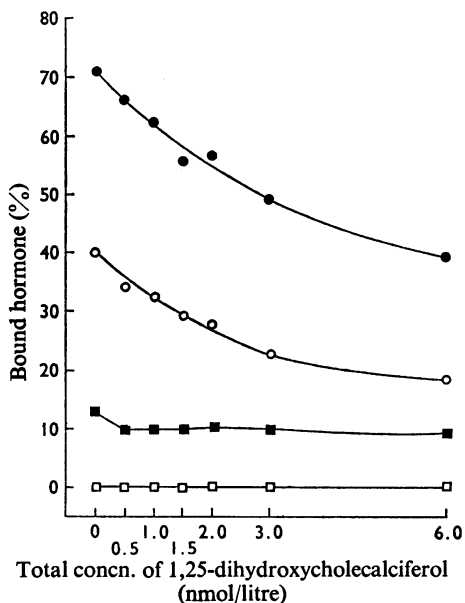
Fig. 6. *Displacement curves for 1,25-dihydroxycholecalciferol obtained with nuclear extracts of chick tissues*

Nuclear fractions were obtained of the intestine, kidney, liver and muscle of chicks and extracted with 0.4M-KCl in TEM buffer. The extracts were incubated for 1h at 4°C with 1,25-dihydroxy[26-³H]cholecalciferol and various amounts of the unlabelled steroid. Displacement curves were constructed as before. ●, Intestinal extract; ○, kidney extract, ■, liver extract, □, muscle extract.

(curve A in Fig. 5) indicating the presence of more than one binding site. This curve was analysed by the procedure of Danchin & Gueron (1970) and was shown to be a consequence of two binding classes in the KCl extract for 1,25-dihydroxycholecalciferol (Fig. 5, curves B and C). The association constants were calculated to be $1.7 \times 10^9 M^{-1}$ and $3.1 \times 10^8 M^{-1}$ and the number of sites for 1,25-dihydroxycholecalciferol due to the two classes was 2.4pmol/g of tissue and 5.3pmol/g of tissue respectively. This high-affinity binding activity was observed in KCl extracts of crude nuclei from both rachitic and normal birds.

Other tissues were examined for the presence of similar nuclear receptors for 1,25-dihydroxycholecalciferol. Crude nuclear-debris fractions of intestine, liver, kidney and muscle were prepared and extracted with 0.4M-KCl in TEM buffer. The extracts were incubated for 1h at 4°C with 1,25-dihydroxy-[26-³H]cholecalciferol and increasing amounts of the non-labelled steroid, and displacement curves were constructed (Fig. 6). Only the intestinal and renal nuclear extracts bound a high proportion of the radioactive hormone, and this bound radioactivity

was displaceable by low concentrations of the unlabelled hormone. The association constants for the two classes of intestinal binding sites were $1.5 \times 10^9 M^{-1}$ and $4.5 \times 10^8 M^{-1}$ and for the kidney binding protein the value was $5.8 \times 10^8 M^{-1}$. Liver nuclear extracts bound only 10% of the radioactive hormone, and this bound fraction could not be displaced by amounts of the steroid that were effective in displacing the bound radioactivity from the intestine and kidney nuclear extracts. Binding activity could not be detected with the KCl extracts of the crude nuclear fraction of skeletal muscle.

The features of the 1,25-dihydroxycholecalciferol molecule essential for binding to the KCl extracts of intestinal nuclei were determined by measuring the displacement of the hormone by structures such as 1-hydroxy- and 25-hydroxycholecalciferol. Neither steroid was found to effect a displacement of the bound 1,25-dihydroxycholecalciferol, showing the necessity for all three hydroxyl groups for binding activity.

The nuclear binding proteins for 1,25-dihydroxycholecalciferol were analysed by centrifugation in a linear 5–20% sucrose gradient. The position of the 1,25-dihydroxy[26-³H]cholecalciferol in the gradient was compared with that of serum albumin. Only one peak of radioactivity was found, with a sedimentation coefficient of 3.5S.

These studies were carried out on 0.4M-KCl extracts prepared from the crude nuclear fraction of the intestine. When similar extracts were prepared from pure intestinal nuclei isolated by centrifugation through 2.4M-sucrose they did not take up 1,25-dihydroxy[26-³H]cholecalciferol during incubation under the standard conditions. It was found that binding activity in the KCl extracts of pure nuclei could only be observed when the pellets of the pure nuclei were first suspended in 100ml of TEM buffer for 10min. The swollen nuclei were then sedimented again by centrifugation at 1000g for 10min and extracted with 0.4M-KCl in TEM buffer. This extract, containing 0.88mg of protein, bound 44.3% of 1,25-dihydroxy[26-³H]cholecalciferol. Analysis by the method of Scatchard (1949) of the displacement curve showed in this case only one class of binding sites, with an association constant of $4.1 \times 10^8 M^{-1}$.

Although the stability of the nuclear binding components has not been studied systematically, some observations on this aspect have been made. The role of EDTA in the buffer seems to be that of facilitating the extraction of the binding proteins from the nuclei, whereas the mercaptoethanol decreases the rate at which affinity of the protein for the 1,25-dihydroxycholecalciferol declines. Storage of the KCl extracts of nuclei at 4°C results in a complete loss of binding activity over 3–4 days, although some activity can still be

observed after storage at −20°C for 7 days. The addition of 20% (w/v) glycerol to the TEM buffer decreases the rate of decline of this binding activity.

### Cytosol receptors for 1,25-dihydroxycholecalciferol

Accounts of the experiences of other groups with this fraction have been published and are consistent with our experiences, showing for example the protein nature of the cytosol receptor (Tsai & Norman, 1973). The uptake of 1,25-dihydroxycholecalciferol by the cytosol is directly related to the amount of cytosol used in the incubation mixture. Thus with protein concentrations ranging from 10 to 17mg/ml of incubation solution the proportion of 1,25-dihydroxycholecalciferol bound ranged from 20 to 35%. As expected with such high concentrations of protein, displacement of 1,25-dihydroxy[26-$^3$H]cholecalciferol was only observed with high concentrations of the non-labelled hormone. Nevertheless there again appeared to be two classes of binding sites, one of which had an association constant of $1 \times 10^9 M^{-1}$/mg of protein and the other of $8 \times 10^7 M^{-1}$/mg of protein. The binding activity due to the high-affinity site declined rapidly with time, whereas that due to the low-affinity site was more stable. This binding activity of the cytosol was found in the intestines of both normal and rachitic birds.

Because of the need to use large amounts of protein to show binding activity for 1,25-dihydroxycholecalciferol, competition studies with other related steroids could not be carried out effectively. Thus 25-hydroxycholecalciferol did not compete with the labelled hormone, in confirmation of the finding of Tsai & Norman (1973), but this may be because of the presence in the intestinal cytosol of a protein with a high affinity for 25-hydroxycholecalciferol (D. E. M. Lawson & P. W. Wilson, unpublished work). This latter protein is present in much larger quantities than the binding protein for 1,25-dihydroxycholecalciferol, and therefore displacement or competition cannot be shown with the protein concentrations of 10mg/ml of incubation mixture used in these studies and in those of other groups. Resolution of these two binding proteins was achieved by centrifugation through a linear 5–20% sucrose gradient. The 25-hydroxycholecalciferol-binding protein with the radioactive steroid bound to it gave a single peak with a sedimentation coefficient of 5.0S. On the other hand the position in the gradient of the radioactivity after centrifugation of the 1,25-dihydroxy[26-$^3$H]cholecalciferol bound to the chick intestinal cytosol proteins showed the hormone to be attached to a much smaller protein with a sedimentation coefficient relative to albumin of 3.0S. Further, there was a shoulder on the side of the main peak of radioactivity, suggesting that the hormone was bound to two components, but it has not been possible to

effect a more complete separation. It has been shown that the treatment of the uterine cytosol receptor for oestradiol with KCl decreases the size of the receptor so that it is indistinguishable from that of the nuclear receptor (Jensen et al., 1969). Treatment of the intestinal cytosol receptor for 1,25-dihydroxycholecalciferol with KCl did not bring about any change in the size of the receptor as measured by sucrose-gradient analysis.

### Discussion

1,25-Dihydroxycholecalciferol has many of the characteristics of steroid hormones, e.g. its production is controlled by another hormone, parathyroid hormone (Garabedian et al., 1972; Rasmussen et al., 1972; Fraser & Kodicek, 1973), it is accumulated only in its target tissues (Lawson & Emtage, 1974) and it is localized in the nuclei of these tissues (Haussler & Norman, 1967; Stohs & DeLuca, 1967; Lawson et al., 1969b).

A number of studies support the view that the main function of steroid hormones is the control of the expression of some of the genetic information in the target tissues of the hormones (Stein et al., 1974). The mechanism of action of 1,25-dihydroxycholecalciferol resembles in many respects the action of the other steroid hormones. An intestinal calcium-binding protein has been characterized and its presence in the intestine is recognized to be dependent on an adequate intake of cholecalciferol. It has been shown that cholecalciferol stimulates the synthesis de novo of calcium-binding protein and that this is brought about by the induction of mRNA activity for calcium-binding protein in the intestinal polyribosomes (Lawson & Emtage, 1975). The accumulation of the hormone on the chromatin of the target tissues, as reported for the other steroid hormones, is consistent with their involvement in the control of DNA transcription. Although the proteins involved in the accumulation process have been partially characterized the question is still unresolved as to whether they play an active part in the regulation of the activity of the genome responsive to the hormone. Consequently it was of concern that intestinal chromatin when prepared free of nuclear membranes did not contain significant quantities of the 1,25-dihydroxycholecalciferol. This present study indicates a possible explanation of this finding.

At high doses of cholecalciferol animals accumulate in the intestine both the vitamin and its 25-hydroxy metabolite, but these steroids are found on membranous cell fractions such as the endoplasmic reticulum (Kodicek, 1965; Norman & DeLuca, 1964) and the nuclear membrane (see Table 1 and the Results section). However, irrespective of the size of the vitamin dose, less than 20% of the nuclear

1,25-dihydroxycholecalciferol is found with the nuclear-membrane fraction. On the other hand, little 1,25-dihydroxycholecalciferol is found associated with the isolated DNA or chromatin, but the explanation for this may well lie in the nature of the association of the hormone–receptor complex with the DNA. The means by which the hormone can be solubilized and the effect of enzymes on the radioactivity both in the nuclei and in the nuclear extracts strongly suggest that the 1,25-dihydroxycholecalciferol is attached to an acidic protein within the cell nuclei. At least a portion of the 1,25-dihydroxycholecalciferol is readily released from the nuclei either by DNAase treatment or on incubation of the nuclei at either 4° or 37°C, suggesting that this portion of the hormone–protein complex exists in a soluble form within the nucleus. An equilibrium may therefore exist between this free form of the hormone–protein complex and a DNA-bound form. Damage to the nuclear membrane, caused for example by changes in osmotic pressure, may result in a rapid loss of the unbound portion of the hormone–receptor complex followed by a slower loss of the bound form as it dissociates. Such an explanation is in keeping with all the observations reported so far. The fate of the hormone–receptor complex would be dependent on the state of the nuclear membranes, e.g. under conditions in which the membrane was completely solubilized the hormone–receptor complex would behave as a light lipoprotein fraction as reported by Chen & DeLuca (1973).

A number of reports have appeared showing that 1,25-dihydroxycholecalciferol is bound to proteins within the cytoplasm (Tsai & Norman, 1973; Chen & DeLuca, 1973; Lawson & Emtage, 1974) and the nucleus (Chen & DeLuca, 1973; Lawson & Emtage, 1974), but little information is available on the physical properties and binding characteristics of these proteins, particularly the nuclear protein.

In the present study conditions were found for the solubilization of the nuclear protein so that after incubation with the 1,25-dihydroxy[26-³H]cholecalciferol for 1h at 4°C significant amounts of the hormone were protein-bound. The criteria for proteins to be designated receptors are low capacity, high affinity and high chemical specificity for the hormone (reviewed by Baulieu et al., 1971). The findings reported in the present paper show the presence of a receptor for 1,25-dihydroxycholecalciferol in the KCl extract of the intestinal nuclei. Analysis of the type of binding involved was made from the relation between hormone concentration and the proportion that was bound at that concentration by a constant amount of protein (Fig. 3), and also from the extent of the displacement of radioactive hormone by increasing amounts of the unlabelled steroid (Fig. 5). In both cases the shape of the curves suggests that two proteins may be

involved in the uptake of the hormone by the nuclear extracts. The break in the curve in Fig. 3 indicates that one of these proteins, which takes up the hormone at low concentration, is saturable and has an association constant of $1.7 \times 10^9 \, \text{M}^{-1}$, whereas the other is not saturable and has an association constant of $3.1 \times 10^8 \, \text{M}^{-1}$. At low steroid concentrations 1,25-dihydroxy[26-³H]cholecalciferol is only replaced by the hormone itself and not by either 25-hydroxy- or 1-hydroxy-cholecalciferol, suggesting that the binding sites on the protein have a very high chemical specificity and require the presence of all three hydroxyl groups on the molecule. This is the first reported isolation from pure nuclei of a receptor protein able to specifically bind its hormone in vitro. With other hormones there appears to be obligatory step involving attachment first to the cytoplasmic protein. This latter protein is converted, by an unknown mechanism in vivo and by treatment with KCl in vitro, into the much smaller nuclear receptor (Baulieu et al., 1971). Although 1,25-dihydroxycholecalciferol is attached to a protein in the cytoplasm we have found that it is, if anything, smaller than the nuclear receptor and does not undergo any change in size after treatment with KCl.

Attempts also have been made with some success to show that the nuclear protein that takes up the 1,25-dihydroxycholecalciferol in vitro is the same as that on which the hormone formed in vivo was found. Thus the radioactivity bound to the two extracts sedimented to very similar positions in a sucrose gradient after centrifugation, in both cases showing only one peak with sedimentation coefficients between 3.2 and 3.5 S. Also the number of binding sites in the nucleus corresponding to the component with a high affinity would give maximum nuclear hormone concentration close to that found from studies in vivo. However, a full investigation of this problem is inhibited by the instability of the high-affinity component. Although the instability is decreased by low temperatures (<20°C), analysis carried out over long periods at 4°C makes interpretations of the results difficult.

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