PURIFICATION OF CHICK 25-HYDROXYCHOLECALCIFEROL BINDING PROTEINS BY AFFINITY CHROMATOGRAPHY

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1. Introduction

Vitamin D (calciferol) and its metabolites have received considerable attention in recent years resulting in a corresponding interest in their binding proteins and hydroxylating enzymes. Purification of these macromolecules has relied [1,2] on conventional and time-consuming chromatographic techniques.

This paper reports the first successful application of affinity chromatography [3] to the vitamin D field. The 25-hydroxycholecalciferol (25-OH-D_3) binding proteins from chick serum and the post-mitochondrial supernatant from chick kidney homogenates have been extensively purified using a simple one-step procedure. The isolation technique can be applied without any preliminary chromatographic purification and losses are minimised. In addition, the highly purified binding proteins led to a marked increase in sensitivity of 25-OH-D_3 assay.

For use as a hapten in affinity chromatography 25-OH-D_3 must be suitably functionalized. If a carboxyl group is incorporated into the metabolite (as a hemisuccinate, for example) aminoalkyl-sepharose can be used as the insoluble support. 25-OH-D_3-3β-hemisuccinate showed appreciable binding to human serum binding protein [4] which suggested this material as a potentially useful hapten.

2. Experimental

Thin layer chromatography was performed on silica plates (Merck, Darmstadt, FRG). Mass spectra were determined on a Finnegan 3200 machine. Ultraviolet spectra were determined in absolute ethanol on a Zeiss PMQ11 Spectrophotometer. Dimethylformamide (DMF) and pyridine were purified by distillation from CaH_2 and KOH respectively.

2.1. *Synthesis of 25-OH-D_3-3β-hemisuccinate (fig.1)*

Treatment of 25-OH-D_3 (50 mg, 0.125 mmol) with succinic anhydride (125 mg, 1.25 mmol) in dry pyridine (1.60 ml) at 37°C for 5 days afforded the 3β-hemisuccinate (62.0 mg, approx. 100%) as a colourless gum. λ max 265 nm (ϵ = 18 330) ν max ($CHCl_3$) 2400–2800 (broad), 1725 cm^{-1}. $M^{\cdot+}$ 500. Titration confirmed the presence of one carboxyl group. R_F 0.24 (MeOH/$CHCl_3$ 1:9).

Tritiated 25-OH-D_3-3β-hemisuccinate was prepared in a similar way.

The stability of the hemisuccinate to pH was studied by thin-layer chromatography and ultraviolet spectroscopy using a solution in 8% ethanol.

2.2. *Coupling of the 3β-hemisuccinate to aminohexyl–Sepharose*

The solid-phase for affinity chromatography consisted of aminohexylsepharose (AH-Sepharose 4B, Pharmacia) containing 6–10 μmol free amino-groups/ml swollen gel.

AH-Sepharose 4B (2.50 g) was allowed to swell in NaCl solution (25 ml, 0.5 M) at room temperature overnight. After washing with NaCl solution (500 ml, 0.5 M) to remove stabilizers and distilled water (100 ml) to remove NaCl, the resulting gel was allowed to settle at 4°C overnight (final vol. 10.0 ml).

A portion of the gel (6.7 ml) was gently stirred

with DMF (11.00 ml) in an ice-salt bath and the pH adjusted to 8.0 by addition of 1 M NaOH solution.

N-Methylmorpholine (17 μl, 0.152 mmol) was added to a stirred solution of tritiated 25-OH-D_3-3β-hemisuccinate (0.152 mmol, determined by ultraviolet absorbance at 265 nm) in dry DMF (9.00 ml) at −10°C. Pivaloyl chloride (19 μl, 0.152 mmol) was added and the solution kept at −10°C for 10 min. The mixture was then added dropwise to the cooled, stirred gel suspension described above. The pH immediately decreased but was restored to 8.0 by addition of 1 M NaOH. The reaction was kept in the ice-salt bath until the pH no longer decreased (15–20 min). The product was transferred to a G3 sinter for the washing procedures which were aided by gentle suction. The product gel was washed with 75% aqueous acetone (2 × 10 ml) followed by acetone (2 × 50 ml). The initial filtrate and washings were combined, except for the second acetone fraction, and all were monitored for radioactivity. The second acetone fraction acted as a check on the efficiency of the previous washes and was always free of recovered hemisuccinate. Finally the gel was suspended in 0.015 M Tris buffer, pH 7.4, and stored in the dark at 4°C in the presence of 0.1% sodium azide (final gel vol. 6.4 ml).

The recovered hemisuccinate had thin-layer chromatographic and spectroscopic properties identical with those of an authentic sample. The incorporation, based on recovered material and determined by absorbance at 265 nm and by disintegrations per minute, was calculated to be 40 μmol hapten (approx. 60–100% coupling yield, based on 10–6 μmol free amino-groups/ml of swollen gel). The product gel showed only a very faint reaction with fluorescamine [5].

2.3. *Removal of 25-OH-D_3 from Sepharose–amino-hexyl-succinyl-25-OH-D_3*

A stirred solution of 25-OH-D_3-3β-hemisuccinate (3.0 μmol) in EtOH (1.00 ml) was treated with aqueous NaOH (1.00 ml, 0.2 M). After 2 h at room temperature hydrolysis was complete, affording pure 25-OH-D_3.

A portion of the Sepharose–aminohexyl-succinyl-tritiated-25-OH-D_3 gel, prepared above (0.4 ml), was washed with acetone and dried in vacuo. The solid (14.9 mg) was stirred with EtOH (1.00 ml) and aqueous NaOH (1.00 ml, 0.2 M) for 2 h. Aliquots were removed for determination of radioactivity, thin-layer chromatographic comparison and ultraviolet spectroscopic measurement. Undamaged 25-OH-D_3 was recovered and the original incorporation estimated to be 37 μmol hapten. After 18 h no further radioactivity had been released.

2.4. *Affinity chromatography*

2.4.1. Chick kidney cytosol

Preparation of the post-mitochondrial supernatant from chick kidney homogenate (cytosol) has been previously reported [6].

A portion of this preparation (2.00 ml, containing 12.7 mg protein) and Sepharose–aminohexyl-succinyl-tritiated-25-OH-D_3 (3.4 ml, 1.5×10^6 dpm) were shaken with 0.015 M Tris buffer, pH 7.4 (3.00 ml) for 24 h at room temperature. The mixture was transferred to a G3 sinter and washed with the incubation buffer (2 × 20 ml). The step-wise elution was continued manually with ammonium acetate solution (20 ml each of 0.030 M, 0.045 M, 0.135 M and 0.405 M, pH 7.0).

In the first experiment (fig.2) using chick kidney cytosol the molarity of the eluting buffer was increased to 5.0.

2.4.2. Chick serum

Two 3-week old vitamin-D deficient chicks were bled by cardiac puncture and the blood pooled, clotted and the serum separated and frozen until assay.

One millilitre of this serum (containing 11.2 mg protein) and Sepharose–aminohexyl-succinyl-tritiated-25-OH-D_3 (3.4 ml, 1.5×10^6 dpm) were shaken with 0.005 M Tris buffer, pH 7.4 (2.00 ml) for 24 h at room temperature. After washing the resulting gel with incubation buffer (2 × 20 ml), the molarity of the eluting buffer was increased (ammonium acetate, 20 ml each of 0.015 M, 0.045 M, 0.135 M, 0.405 M and 1.215 M, pH 7.0).

All fractions were lyophilised and frozen until assay. Protein was estimated by the Folin-Lowry method [7].

2.5. *Assay*

2.5.1. Binding protein dilution-curves

Fractions were dissolved in 1 ml Tris acetate

buffer, pH 8.6, and 500 μl was double-diluted through 6 tubes. Buffer, 500 μl, was then added to all tubes. Binding was estimated by incubating each chromatographic fraction for 18 h at 4°C with 30 000 dpm tritiated 25-OH-D_3 (25-[26-(27)-*methyl*-^{3}H]hydroxycholecalciferol) spec. act. 7.0 Ci/mol (Radiochemical Centre, Amersham). Bound, labelled 25-OH-D_3 was separated from the free material using Dextran T-40 (Pharmacia) coated charcoal (Norit SX2). Supernatant, 500 μl, was counted in a β-counter (Intertechnique) with PPO and toluene, 3:1 with Triton X-100, as scintillator.

2.5.2. Displacement curves

25-OH-D_3 Standard (Roussel, Paris) was serially diluted through 8 tubes, such that tube 1 contained 5 ng/50 μl and tube 8 contained 0.04 ng/50 μl. Eight-point standard-curves were set up using 50 μl of each dilution. Duplicate curves were constructed for each binding protein fraction.

Lyophilised fractions after affinity chromatography were dissolved in 1 ml distilled water and then further diluted in Tris–acetate buffer before assay. Fractions from chick serum and the original serum were diluted 1/200 and fractions from chick kidney cytosol were diluted 1/1000. From each diluted fraction 1 ml was added to the appropriate tubes.

2.5.3. Specificity

Several vitamin D metabolites were tested for binding in this system. 1,25$(OH)_2D_3$, 24*R*,25$(OH)_2D_3$, 1,24*R*,25$(OH)_3D_3$ and D_3 (Sigma) itself were serially diluted from 2 μg/50 μl and incubated with fraction 0.045 M (serum) and 0.030 M (cytosol) and compared to the binding seen with 25-OH-D_3.

2.5.4. Recovery of binding

Tritiated 25-OH-D_3, 300 000 dpm, was incubated with each of the fractions from both serum and kidney cytosol, to determine the amount of total binding found in each fraction and compare this with the total binding of the original material.

3. Results and discussion

25-OH-D_3-3β-hemisuccinate (fig.1) was found to be stable at pH 7.4 in homogeneous solution. Sepharose–aminohexyl-succinyl-25-OH-D_3 was therefore used and stored at this pH. Gel which had been used twice in kidney cytosol experiments and stored as described for 5 months afforded undamaged 25-OH-D_3 when treated with aqueous ethanolic sodium hydroxide.

The DMF : water ratio in the coupling reaction was critical at the hemisuccinate concentration described here. Too much DMF did not sufficiently swell the gel for effective coupling whilst too much water caused precipitation of the lipophilic hemi-succinate. In addition a lower pH reduced the coupling yield. Solvents known to contain traces of peroxides

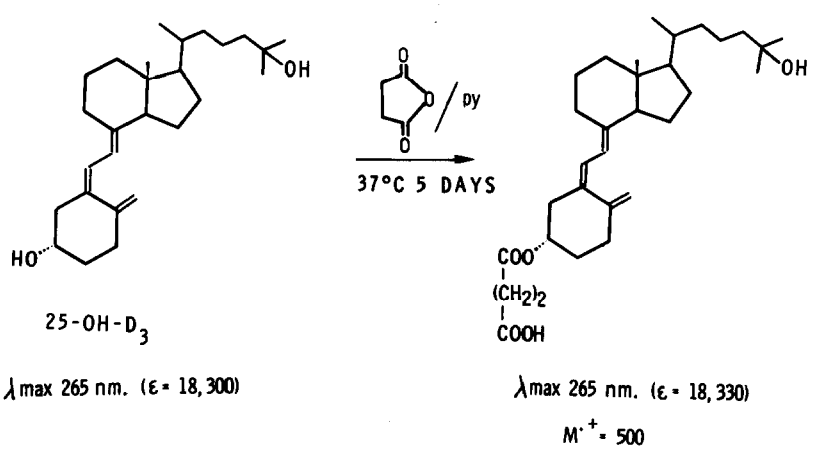

Fig.1. Synthesis of 25-OH-D_3-3β-hemisuccinate. The 25-hydroxy group is inert under the reaction conditions. $M^{\cdot+}$ (mass spectrum) = 500 (molecular ion).

(e.g., Dioxan and THF), which could oxidise the sensitive triene, were avoided. The incorporation was tested by measuring recovered, pure hemisuccinate (which was used in the next preparation) and by measuring the 25-OH-D_3 released on basic hydrolysis of the product gel.

The stability of Sepharose–aminohexyl-succinyl-25-OH-D_3 to peptidases and esterases, expected to be present in the crude biological preparations, was demonstrated using tritiated hapten. (Aliquots removed during 24 h incubation with chick serum and cytosol contained no radioactivity.) This result avoided the necessity for preliminary chromatographic purification and allowed us to explore the limits of the method.

3.1. *Affinity chromatography*

The results of an early experiment with chick kidney cytosol are shown in fig.2. The principal binding protein eluted at 0.045 M ammonium acetate whilst only lower affinity and non-specific binding was observed in the other fractions. In subsequent experiments displacement curves were set up for all fractions as well as the original material (figs 3,4). From the shape and position of these curves fractions containing purest, high affinity binding protein could be localised (figs 5,6).

The 0.030 M (cytosol) and 0.045 M (serum) fractions each contained approximately two-thirds of the total binding protein present before affinity chromatography, with the remainder in adjacent fractions. The purifications obtained (200-fold and 1000-fold for cytosol and serum binding proteins, respectively) were based on protein content before and after chromatography.

Interestingly only 15% (serum)–21% (kidney cytosol) of the protein in the original preparations was eluted by ammonium acetate solution. However, virtually all binding protein present before affinity chromatography was recovered. The adhering protein did not affect the specificity of the hapten as shown

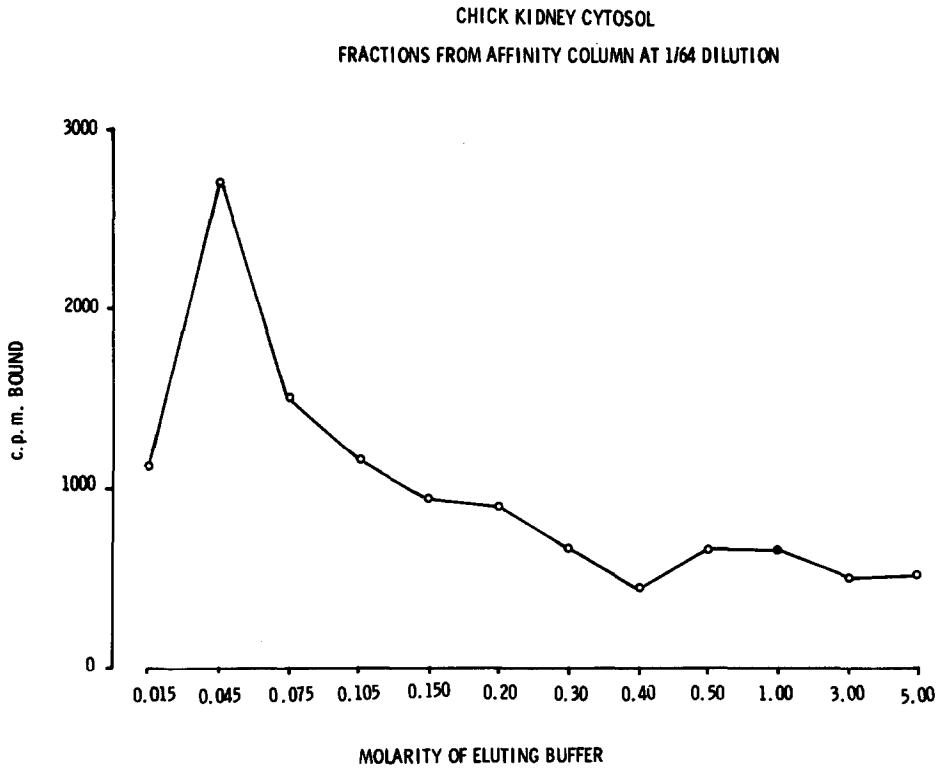

Fig.2. Binding of tritiated 25-OH-D_3 to fractions (1/64 dilution) obtained by affinity chromatography of chick kidney cytosol.

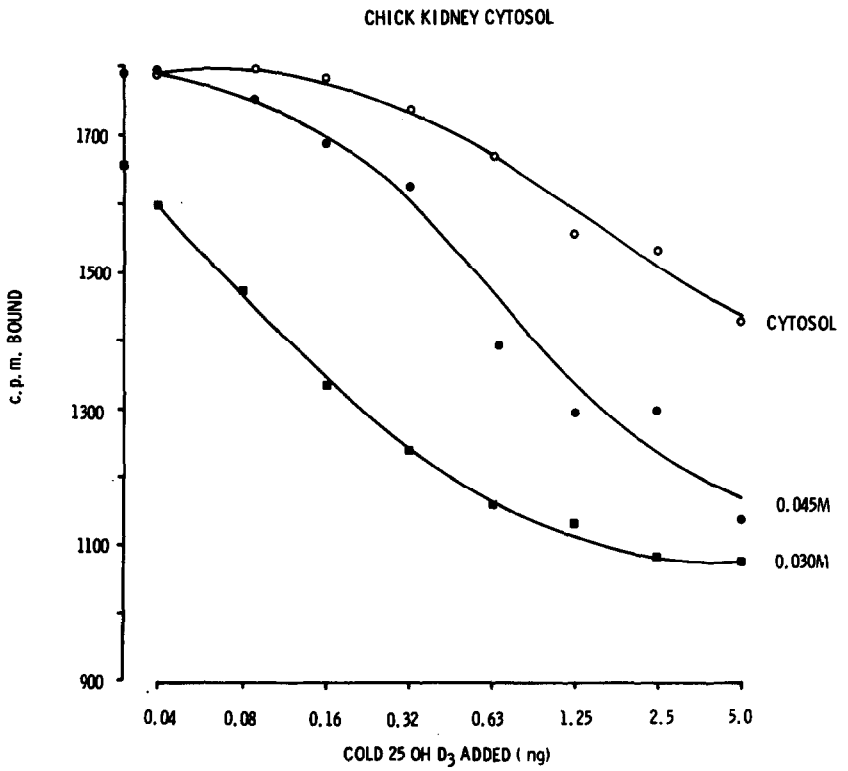
CHICK KIDNEY CYTOSOL
c.p.m. BOUND
1700
1500
1300
1100
900
CYTOSOL
0.045M
0.030M
0.04
0.08
0.16
0.32
0.63
1.25
2.5
5.0
COLD 25 OH D_3 ADDED (ng)

Fig.3

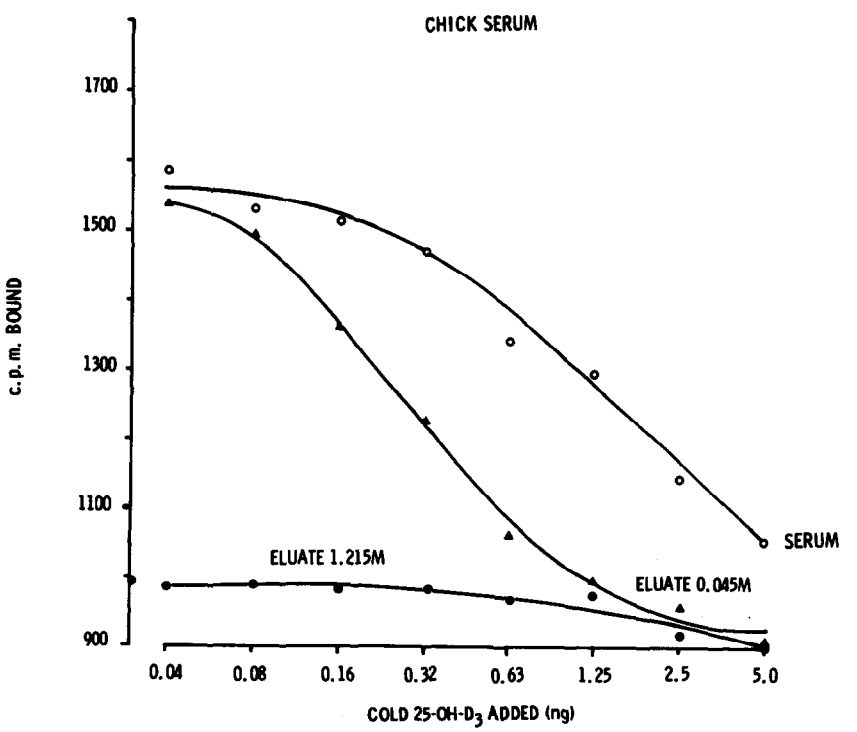
CHICK SERUM
c.p.m. BOUND
1700
1500
1300
1100
900
SERUM
ELUATE 1.215M
ELUATE 0.045M
0.04
0.08
0.16
0.32
0.63
1.25
2.5
5.0
COLD 25-OH-D_3 ADDED (ng)

Fig.4

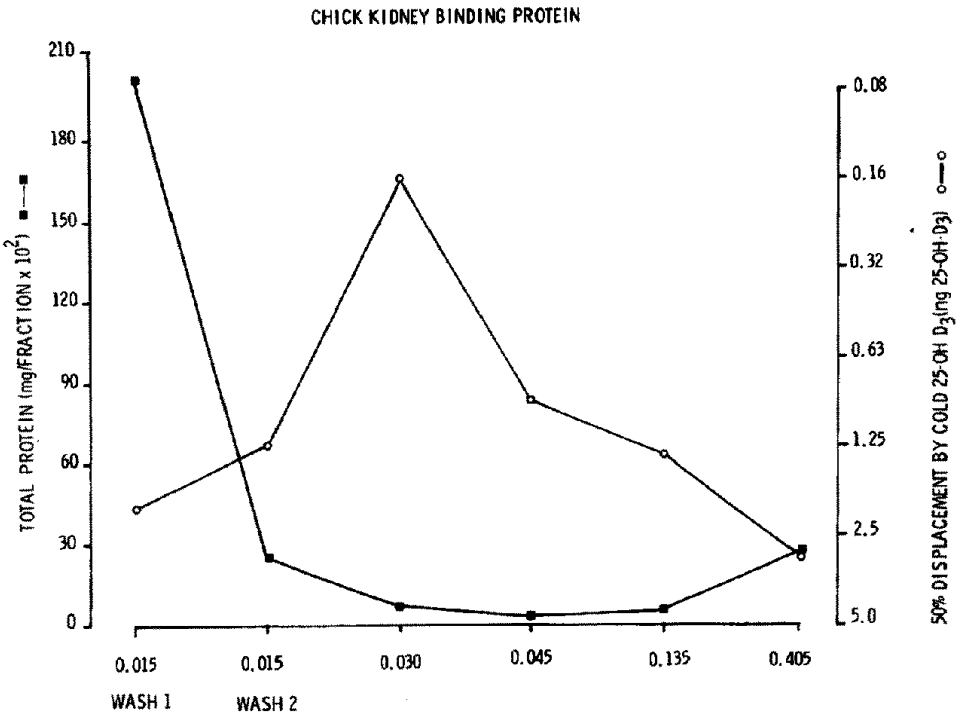
CHICK KIDNEY BINDING PROTEIN
TOTAL PROTEIN (mg/FRACTION x 10^2) ■—■
50% DISPLACEMENT BY COLD 25-OH D_3(ng 25-OH-D_3) o—o
210
180
150
120
90
60
30
0
0.08
0.16
0.32
0.63
1.25
2.5
5.0
0.015
0.015
0.030
0.045
0.135
0.405
WASH 1
WASH 2
MOLARITY OF ELUTING BUFFER

Fig.5

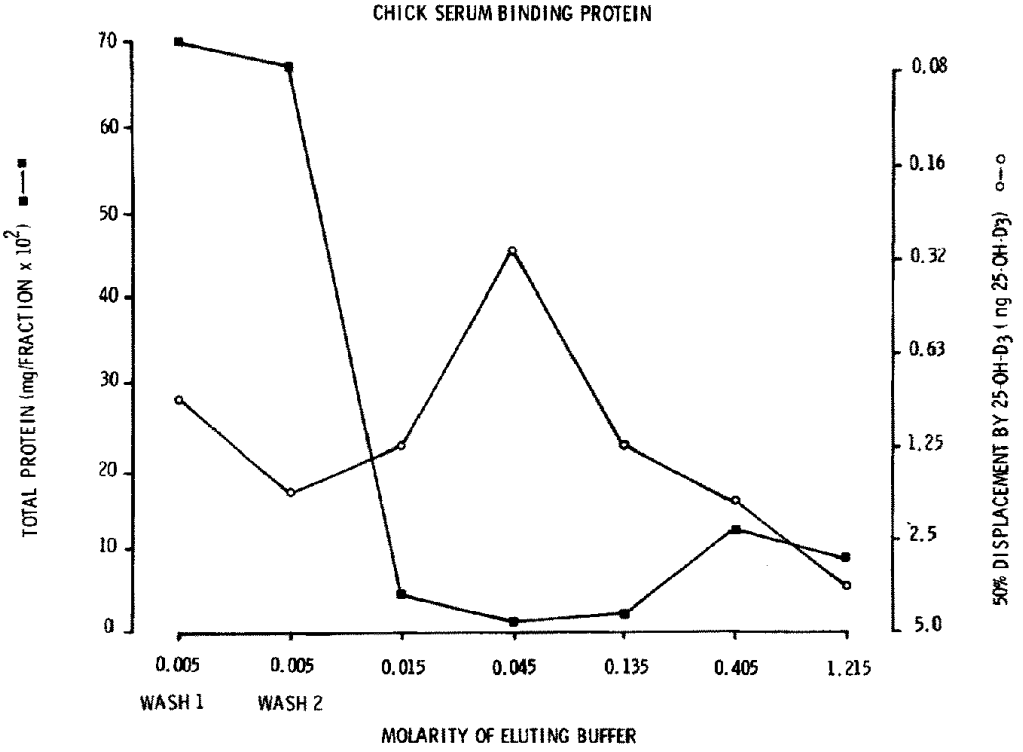
CHICK SERUM BINDING PROTEIN
TOTAL PROTEIN (mg/FRACTION x 10^2) ■—■
50% DISPLACEMENT BY 25-OH-D_3 (ng 25-OH-D_3) o—o
70
60
50
40
30
20
10
0
0.08
0.16
0.32
0.63
1.25
2.5
5.0
0.005
0.005
0.015
0.045
0.135
0.405
1.215
WASH 1
WASH 2
MOLARITY OF ELUTING BUFFER

Fig.6

by subsequent, successful use of the same gel. It is probable that an initial purification (e.g., on Sephadex G-200) would avoid this phenomenon.

Preliminary specificity studies, using the purified binding proteins, showed <0.02% cross-reactivity with vitamin D_3 and 1,24*R*,25$(OH)_3D_3$, 0.1% with 1,25$(OH)_2D_3$, whilst 24*R*,25$(OH)_2D_3$ showed 100% binding. Chick serum, however, is known [2] to contain a cholecalciferol binding protein. (In unpurified serum and cytosol vitamin D_3 showed 1% cross-reactivity.) Our results indicate, therefore, a successful separation of the vitamin D_3 and 25-OH-D_3 binding proteins by affinity chromatography.

The purified binding proteins afforded greater assay sensitivity for 25-OH-D_3 (figs 3,4). For example, using the purified 25-OH-D_3 serum binding protein an approximate 4-fold increase in assay sensitivity could be envisaged. This might be caused by removal of lower affinity binding proteins and other proteins which bind 25-OH-D_3 non-specifically. The increase in sensitivity could not be reproduced by simply diluting the original serum and cytosol. Indeed, 10-fold dilution of serum abolished all binding.

4. Conclusions

Seco-steroids are sensitive molecules and can only the employed as haptens under carefully controlled conditions. Nevertheless successful purification of the chick 25-OH-D_3 binding proteins, used as a model in this study, showed that affinity chromatography in the vitamin D field is entirely practical. The stability of Sepharose–aminohexyl-succinyl-25-OH-D_3 to esterases and peptidases expected to be present in biological preparations, eliminated the necessity for preliminary chromatographic purification. Losses were therefore minimised and the efficiency of the procedure clearly demonstrated by separation of the binding proteins for vitamin D_3 and 25-OH-D_3.

Purification of the binding proteins led to a marked increase in assay sensitivity for 25-OH-D_3. We are currently applying the procedure to the purification of the 25-OH-D_3 human serum binding protein which is routinely used in our assay.

Acknowledgements

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Fig.3. Displacement of tritiated 25-OH-D_3, by cold 25-OH-D_3, from cytosol and fractions (1/1000 dilution) obtained by affinity chromatography. Other curves resemble that for cytosol.

Fig.4. Displacement of tritiated 25-OH-D_3 by cold 25-OH-D_3, from chick serum and the 0.045 M fraction (1/200 dilution) obtained by affinity chromatography. Other curves resemble that for serum.

Fig.5. Affinity chromatography elution profile for chick kidney cytosol. Nanograms of cold 25-OH-D_3 required for 50% displacement (o——o) and total protein (mg × 10^2)/fraction (■——■) are plotted against molarity of eluting buffer. Fraction 0.030 M contained 6.7 × 10^{-2} mg protein (200-fold purification).

Fig.6. Affinity chromatography elution profile for chick serum. Nanograms of cold 25-OH-D_3 required for 50% displacement (o——o) and total protein (mg × 10^2)/fraction (■——■) are plotted against molarity of eluting buffer. Fraction 0.045 M contained 1.1 × 10^{-2} mg protein (1000-fold purification).