The Transporting Proteins of Cholecalciferol and 25-Hydroxycholecalciferol in Serum of Chicks and Other Species

PARTIAL PURIFICATION AND CHARACTERIZATION OF THE CHICK PROTEINS

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Chick serum contains two cholecalciferol-binding proteins, one of which binds mainly cholecalciferol (cholecalciferol-binding protein) and the other binds 25-hydroxycholecalciferol (25-hydroxycholecalciferol-binding protein). By means of Cohn fractionation, $(\text{NH}_4)_2\text{SO}_4$ precipitation, gel filtration on Sephadex G-200, ion-exchange chromatography on DEAE-Sephadex and an additional gel-filtration step on Sephadex G-100, these two binding proteins were purified. Both proteins possess β -globulin mobility on analytical polyacrylamide-disc-gel electrophoresis, a sedimentation coefficient of 3.5S and approximate molecular weights of 60000 for the cholecalciferol-binding protein and 54000 for the 25-hydroxycholecalciferol-binding protein. Sera obtained from rat, pig, human and monkey were shown to contain a single binding protein that is responsible for the transport of both cholecalciferol and 25-hydroxycholecalciferol. In the toad the lipoproteins are used for the transport of these two steroids.

There have been several reports on the nature of the protein in plasma to which cholecalciferol and 25-hydroxycholecalciferol are bound. In the earliest studies (Thomas *et al.*, 1959; De Crousaz *et al.*, 1965) the plasma proteins containing the cholecalciferol compounds were submitted to starch-gel electrophoresis and the cholecalciferol was located on the electrophoretogram by assaying for anti-rachitic activity. Subsequently the fate of the cholecalciferol was followed by ^{14}C or ^3H labelling (Chalk & Kodicek, 1961; Chen & Lane, 1965). Although the identity of the protein was not established these investigators found that in man, rat and dog the radioactivity was mainly associated with α_2 -globulin and albumin. The association of the radioactivity with the α -globulin occurred rapidly *in vivo*, 80% being so bound within 15 min of an intravenous dose of radioactive cholecalciferol. Four other protein bands observed after gel electrophoresis also contained radioactivity. These bands were or contained lipoproteins, but as the proportion of the radioactivity associated with them decreased with time it was concluded that plasma contained a specific cholecalciferol-binding globulin (Rikkens *et al.*, 1969). In a study in which [^{14}C]-cholecalciferol was administered intravenously to rachitic baboons and the plasma proteins were fractionated by gel electrophoresis, 70% of the total plasma radioactivity was associated with an α_1 -globulin and 30% with albumin (Rosenstreich *et al.*, 1971).

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It is now known that cholecalciferol is metabolized to 25-hydroxycholecalciferol and then to 1,25-dihydroxycholecalciferol, which is believed to be the metabolically active form of the vitamin. In most species, including man (Mawer *et al.*, 1971) and chickens (Lawson *et al.*, 1969), the anti-rachitic activity of the plasma is due almost entirely to 25-hydroxycholecalciferol and cholecalciferol, with less than 5% of the activity being due to 1,25-dihydroxycholecalciferol. In the rat a higher proportion of 1,25-dihydroxycholecalciferol may be present (Lawson *et al.*, 1971).

Since the above findings were reported there have been renewed attempts to characterize the α -globulin binding of cholecalciferol and 25-hydroxycholecalciferol. Haddad & Chyu (1971) gave two subjects a dose of [25,26- ^3H]25-hydroxycholecalciferol and fractionated the serum on DEAE-cellulose columns. Most of the radioactivity was associated with the α -globulins. In other experiments (Haddad & Chyu, 1971) in which the plasma was incubated with the [25,26- ^3H]25-hydroxycholecalciferol *in vitro*, 85% of the radioactivity was associated with a protein possessing α -globulin mobility after gel electrophoresis; the molecular weight of this protein was estimated by its elution volume from a Sephadex G-100 column to be between 40000 and 50000.

Peterson (1971) purified a protein from human plasma by $(\text{NH}_4)_2\text{SO}_4$ precipitation and chromatography on Sephadex G-200, DEAE-Sephadex, sulphoethyl-Sephadex and finally Sephadex G-100. This protein was recognized by its high affinity for

cholecalciferol *in vitro*. The molecular weight appeared to be about 53000 and it was found in human serum in concentrations of approx. 5 µg/ml. Smith & Goodman (1971) have reported that 25-hydroxycholecalciferol is associated with a protein of molecular weight 50000–60000.

Although from the above investigations it was clear that both cholecalciferol and 25-hydroxycholecalciferol are bound to protein, no systematic study has been made to establish whether in any species there are specific proteins for both these steroids. In a previous study (Edelstein *et al.*, 1972) we have bound both cholecalciferol and 25-hydroxycholecalciferol to the plasma proteins of chicks *in vivo* and have fractionated the proteins. These two steroids were associated with two proteins, one of which is specific for 25-hydroxycholecalciferol, whereas the other binds both steroids (Edelstein *et al.*, 1972). The present paper reports a procedure for purifying these two proteins and describes some of their characteristics. The nature of the binding proteins in other species is also described.

Experimental

Serum

(a) *Chicks*. 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 a vitamin D-deficient diet as described previously (Lawson *et al.*, 1969) and used after 4 weeks, by which time they were vitamin D deficient. The radioactive solutions in propylene glycol were administered intracardially and the chicks killed after 16h.

(b) *Rats*. Piebald weanling rats were raised on the vitamin D-deficient diet of Numerof *et al.* (1955). After 2–3 weeks the test material in propylene glycol was administered intracardially and the rats were killed 16h later.

(c) *Pigs*. Two pigs, weighing 8 and 12 kg, that had been raised on a vitamin D-deficient diet (E. Widdowson, personal communication) for 8 weeks were injected with 4.96 mCi (9.1 mg) of [^3H]cholecalciferol and killed 24h later. A third vitamin D-deficient pig, weighing 8 kg, was injected with 1.2 mCi (1.83 mg) of [^3H]ergocalciferol and killed 24h later.

(d) *Man*. Plasma was obtained from three volunteers with the co-operation of Dr. T. C. B. Stamp from the University College Hospital Medical School.

Subject 1 was a 60-year-old female with primary hyperparathyroidism and associated osteitis fibrosa (plasma anti-rachitic activity 0.75 i.u./ml). She received 10 µCi (19.2 µg) of [^3H]cholecalciferol and plasma was obtained at 4 and 8h. Subject 2

was a 57-year-old male with anticonvulsant osteomalacia (plasma anti-rachitic activity 0.28 i.u./ml) who received 10 µCi (19.2 µg) of [^3H]cholecalciferol and 2 µCi (23.8 µg) of [^{14}C]cholecalciferol. The plasma sample was obtained 12h later. Subject 3 was an 11-year-old male with pseudo-vitamin D-deficient rickets (plasma anti-rachitic activity 1.33 i.u./ml). He received 10 µCi (19.2 µg) of [^3H]cholecalciferol and 2 µCi (23.8 µg) of [^{14}C]cholecalciferol and plasma was obtained at 24h.

These investigations were carried out with the informed consent of the patients concerned and with the approval of the Ethical Committee of the University College Hospital and the Medical Research Council.

(e) *Toads*. Vitamin D-deficient toads (*Xenopus laevis*) were raised for 2 years on ox heart and kept in the dark at 25°C. Test material in propylene glycol was administered into the dorsal lymph sac.

(f) *Monkeys*. A New World monkey (*Cebus albifrons*) was maintained for several months on a synthetic vitamin D-deficient diet (R. Fiennes, personal communication). [^3H]cholecalciferol in propylene glycol was administered intravenously and serum obtained 24h later. An Old World monkey (*Erythrocebus patas*) was given the stock diet *ad lib*.

Column chromatography

Gel filtration was carried out on 2.5 cm × 45.0 cm columns of Sephadex G-200 (Pharmacia Fine Chemicals Ltd., London W5 1BR, U.K.) or 1.5 cm × 40.0 cm columns of Sephadex G-100 equilibrated with either 0.05 M-sodium phosphate buffer, pH 7.6, or 0.05 M-sodium phosphate buffer containing 0.2 M-NaCl, 1 mM-2-mercaptoethanol and 1.5 mM-EDTA (buffer A). Ion-exchange chromatography was carried out on a 1.0 cm × 20 cm column of DEAE-Sephadex (Pharmacia Fine Chemicals Ltd.) equilibrated with 0.02 M-sodium phosphate buffer, pH 7.6. All column chromatography was carried out in a cold-room at 4°C. All column eluates were monitored continuously for absorption of light at 280 nm with an LKB Uvicord II Absorptiometer as a measure of protein content.

Electrophoresis

All electrophoretic analysis was performed on polyacrylamide-disc gels with a continuous buffer system (Hjerten *et al.*, 1965). Two samples of the serum or purified proteins were run simultaneously. At the completion of the run one gel was stained with Naphthalene Black, whereas the other was frozen with solid CO₂ and 1 mm segments were sliced with a macrotome (Macrotome GTS-1; Yeda R & D Co. Ltd., Rehovot, Israel). Radioactivity was extracted from the gel slices with NCS, a tissue solubilizer (Searle Co. Ltd., High Wycombe,

Bucks., U.K.), as described by Hellung-Larsen (1971).

Sucrose-gradient ultracentrifugation

Portions (0.5ml) of the radioactive serum were layered on to 13ml of a continuous 5–20% (w/v) sucrose gradient in 0.05M-sodium phosphate buffer, pH7.6. After centrifugation at 36000g for 20h in an SW 36 rotor in a Beckman L2-65B centrifuge, the gradient was pumped out and 0.5ml fractions were collected. All operations were carried out at 4°C. The position of the radioactivity in the gradient was established by using bovine serum albumin and ovalbumin as molecular markers.

Measurements 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 0.03% 1,4-bis-(5-phenyloxazol-2-yl)benzene and 0.5% 2,5-diphenyloxazole. The extracts of the gel slices were counted for radioactivity in a toluene–dioxan–ethanol solution (Hellung-Larsen, 1971). Quenching was corrected for by using the automatic external standardization and correlation curves for ^{14}C or ^3H , either individually or combined.

Competitive displacement studies

Competitive displacement studies of protein-bound [26,27- ^3H]25-hydroxycholecalciferol and [1,2- ^3H]cholecalciferol by various amounts of the corresponding non-labelled steroid were carried out to assess the affinity of the two serum-binding proteins for these steroids. The separation of the protein-bound steroid from that unbound was achieved 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.) as discussed by Baulieu *et al.* (1971). The charcoal was coated with dextran, which absorbs the free steroid, as described by Murphy (1967). The serum-binding proteins used were those obtained after the ion-exchange-chromatographic step that resolves the cholecalciferol- and the 25-hydroxycholecalciferol-binding proteins. Both protein fractions were diluted with 0.02M-sodium phosphate buffer, pH7.6, until 50% of the added radioactive 25-hydroxycholecalciferol was bound; 1ml was pipetted into each tube. Ethanolic solutions (70μl) containing 4000 d.p.m. of [26,27- ^3H]25-hydroxy-

cholecalciferol and various amounts of non-labelled steroid were added. The tubes were shaken, cooled to 4°C and 1ml of the dextran-coated charcoal was added. After centrifugation 1ml was taken from the clear supernatant, containing the bound steroid, for radioactivity counting. Each value was obtained in triplicate.

In those attempts to obtain a displacement curve with cholecalciferol, the ^3H -labelled cholecalciferol and the various amounts of the non-labelled material were dissolved in either dimethyl sulphoxide or ethanol. Otherwise the procedure was as described for the 25-hydroxycholecalciferol.

Other methods

Cohn fractionation of serum was carried out by method 10 of Cohn *et al.* (1950). Proteins were determined by the method of Lowry *et al.* (1951) with bovine serum albumin (Armour Pharmaceutical Co. Ltd., Eastbourne, U.K.) as standard.

Materials

[4- ^{14}C]Cholecalciferol was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. (sp. radioactivity 32.3mCi/mmol). The preparation of the ^3H -labelled substances has been described previously: [1,2- ^3H]cholecalciferol of sp. radioactivity 508mCi/mmol (Lawson *et al.*, 1971); [1- ^3H]ergocalciferol of sp. radioactivity 1.26Ci/mmol (Pelc & Kodicek, 1971); [26,27- ^3H]25-hydroxycholecalciferol of sp. radioactivity 11Ci/mmol (Bell & Scott, 1973). [1- ^3H]Cholecalciferol of sp. radioactivity 211mCi/mmol (Bell, 1968) was prepared by Dr. B. Pelc.

Results

The existence of two proteins in chick serum can be readily demonstrated by polyacrylamide-gel electrophoresis of serum to which either cholecalciferol or 25-hydroxycholecalciferol has been bound *in vitro*. When the binding occurs after a dose of radioactive cholecalciferol *in vivo* and the binding proteins are separated and analysed for the nature of the sterol attached to them, it is found that 83% of the radioactivity attached to one of the proteins is due to 25-hydroxycholecalciferol and only 10% is due to cholecalciferol. The recovered radioactivity of the second protein consists mainly of cholecalciferol (55%) and to a lesser extent of 25-hydroxycholecalciferol (20%) (Edelstein *et al.*, 1972). The application of the usual techniques for protein purification as outlined in Scheme 1 results in a highly purified preparation of cholecalciferol-binding

Scheme 1. Scheme of purification of cholecalciferol-binding protein and 25-hydroxycholecalciferol-binding protein

protein and of 25-hydroxycholecalciferol-binding protein.

Cohn fractionation and (NH₄)₂SO₄ precipitation

Cohn fraction V was prepared from sera of chicks that had each been given a dose of 3.2 μ Ci (2.5 μ g) of [1,2-³H]cholecalciferol and was found to contain 82% of the serum radioactivity (Table 1). This fraction was dialysed extensively against water, freeze-dried and redissolved in buffer A (see the Experimental section). Solid (NH₄)₂SO₄ was added with continuous stirring to give 50% saturation. The fraction was left at 4°C overnight and the precipitate collected by centrifugation. The remaining supernatant was adjusted to 80% saturation with (NH₄)₂SO₄ and treated as described above. The precipitate that formed, containing 69% of fraction V radioactivity, was redissolved in 3 ml of buffer A.

Gel filtration and column chromatography

The radioactive precipitate in buffer A was fractionated on a column of Sephadex G-200 equilibrated with buffer A and the elution profiles of protein and radioactivity were recorded (Fig. 1). The fractions containing radioactivity were combined and further chromatographed on a DEAE-Sephadex column. Two radioactive peaks were eluted (Fig. 2). As shown previously (Edelstein *et al.*, 1972), peak 1 contained the 25-hydroxycholecalciferol-binding protein and peak 2 contained the cholecalciferol-binding protein.

The fractions within each peak were combined, dialysed against water and freeze-dried. The material of peak 1 was redissolved in 1.5 ml of 0.05 M-sodium phosphate buffer, pH 7.6, and that of peak 2 in 1.5 ml of buffer A. The combined material of each peak was applied to a column of Sephadex G-100

Table 1. *Distribution of radioactivity in Cohn fractions of chick sera*

Serum (5ml) was collected 8h after intracardial injection of 3.2μCi (2.5μg) of [1,2-³H]cholecalciferol to vitamin D-deficient chicks, and was immediately fractionated by the method of Cohn *et al.* (1950). Results are expressed as percentages of the ³H radioactivity in the fresh serum.

Cohn fraction	Distribution of ³ H radioactivity (%)
1+II+III	8
IV	9
V	82
VI	1

Fig. 1. *Gel filtration on Sephadex G-200 of protein fraction from chick serum obtained by Cohn fractionation and (NH₄)₂SO₄ precipitation*

Fractions (2.5ml) were collected. For other details see the text. —, E₂₈₀; . . . ., ³H radioactivity.

previously equilibrated with the buffer in which the protein was dissolved. Fractions (1.2ml) were collected and counted for radioactivity. A single radioactive peak was eluted from each column. The radioactive fractions from each peak were combined, dialysed against water, freeze-dried and redissolved in 1.0ml of the corresponding buffer. Polyacrylamide-gel electrophoresis of these two preparations showed a single band after staining for protein. The 25-hydroxycholecalciferol-binding-protein (100μg) and the cholecalciferol-binding

Fig. 2. *Ion-exchange chromatography on DEAE-Sephadex of the radioactive fractions obtained by gel filtration*

Fractions 36–58 of the large radioactive peak eluted from the Sephadex G-200 column (Fig. 1) were combined and dialysed extensively against 0.02M-sodium phosphate buffer, pH 7.6, before chromatography. Fractions (2.5ml) were collected. For other details see the text. ●—●, E₂₈₀; . . . ., ³H radioactivity; —, [NaCl].

protein (65μg) were obtained with a 30 and 20% yield respectively.

Physical properties of the binding proteins of the chick

Comparison of the electrophoretic mobility of these two proteins with known proteins such as albumin and α-, β- and γ-globulins established that both proteins had β-globulin mobility. In the sucrose gradient, from the position of the radioactivity from [1-³H]cholecalciferol-labelled plasma proteins the sedimentation coefficient seemed to be identical with that of ovalbumin. The approximate sedimentation coefficient of these binding proteins is then given as 3.5S. The molecular weight of these proteins as estimated by chromatography on a calibrated column of Sephadex G-100 was 60000 for the cholecalciferol-binding protein and 54000 for the 25-hydroxycholecalciferol-binding protein. No special precautions had to be taken to prevent denaturation of the 25-hydroxycholecalciferol-binding protein during the isolation procedure. The other protein, however, was unstable and required the presence of EDTA and mercaptoethanol to prevent aggregation. The protein was then stable over several months when stored in the frozen state. In the absence of EDTA and mercaptoethanol the

aggregated cholecalciferol-binding protein was eluted with the void volume from Sephadex G-200 columns.

Species distribution of the cholecalciferol-binding proteins

Chick. Polyacrylamide-disc-gel electrophoresis analysis of serum from rachitic chicks that had been incubated with radioactive 25-hydroxycholecalciferol showed that the 25-hydroxycholecalciferol and the cholecalciferol-binding proteins take up this steroid (Plate 1a). However, if the serum was incubated with radioactive cholecalciferol and analysed in the same way the radioactivity was associated mainly with cholecalciferol-binding protein and lipoproteins, and to a smaller extent with 25-hydroxycholecalciferol-binding protein and albumin (Plate 1b).

Experiments have been carried out to assess the role that each of these two proteins plays in the transport of these steroids in plasma. The reversible nature of the binding between steroids and proteins results in the accumulation of the steroid on proteins with a high affinity for them, although binding in the first instance may occur with low-affinity proteins. However, with cholecalciferol this process takes several hours *in vivo* (Rikkers *et al.*, 1969) and would be even longer if it occurred at 4°C. Again the incubation of serum proteins for several hours at 37°C may result in denaturation of the proteins. As a consequence studies in which these proteins were incubated with these steroids at 4°C may not reveal the specific nature of the binding. In addition it has been shown that other substances, e.g. retinol (Muto *et al.*, 1972), are only released into the bloodstream bound to their specific binding protein.

The transport of cholecalciferol and its 25-hydroxy metabolite was studied by giving a group of rachitic chicks a dose of [26,27-³H]25-hydroxycholecalciferol and collecting serum after 8 h. A second group of chicks was given a dose of [4-¹⁴C]cholecalciferol and 3 h later, when most of the ¹⁴C was still present as the unchanged sterol, serum was again collected.

The two samples of sera were mixed and subjected to gel filtration on Sephadex G-200 and ion-exchange chromatography on DEAE-Sephadex (Fig. 3). ³H-labelled 25-hydroxycholecalciferol was bound to both proteins in approximately equal proportions, whereas [¹⁴C]cholecalciferol was bound only to the cholecalciferol-binding protein. T.l.c. of the lipid extracts of these two proteins showed the 25-hydroxy-[4-¹⁴C]cholecalciferol to be distributed between the 25-hydroxycholecalciferol-binding protein and the cholecalciferol-binding protein in the ratio of 9:1. The addition of 25-hydroxycholecalciferol to plasma either directly into the blood stream

or *in vitro* results in immediate binding to the two high-affinity binding proteins in chick plasma in approximately equal proportions. The relatively low solubility of 25-hydroxycholecalciferol in water ensures that this process occurs instantaneously.

The contrast in the distribution of 25-hydroxycholecalciferol between these two proteins according to whether the binding occurred *in vivo* or *in vitro* strongly suggests that 25-hydroxycholecalciferol synthesized in the liver is released into the bloodstream bound to its specific binding protein and that some redistribution of this steroid to other proteins of high affinity occurs during the chromatographic procedures. That high-affinity binding is involved was demonstrated by analysis of the displacement curves (Figs. 4a and 4b) obtained with both proteins for 25-hydroxycholecalciferol (Scatchard, 1949). The association constant of this steroid with the 25-hydroxycholecalciferol-binding protein was 3.0×10^8 litres/mol and with the cholecalciferol-binding protein was 4.3×10^8 litres/mol.

Binding could not be obtained between the 25-hydroxycholecalciferol-binding protein and cholecalciferol, demonstrating the high specificity of the

Fig. 3. Ion-exchange chromatography of mixed samples of serum of chicks given a dose of either [26,27-³H]25-hydroxycholecalciferol or [4-¹⁴C]cholecalciferol

Serum (1 ml) of rachitic chicks given a dose of 0.5 µg of 25-hydroxycholecalciferol was mixed with 3 ml of serum from chicks given a dose of 2.5 µg of [4-¹⁴C]cholecalciferol. The serum was chromatographed on Sephadex G-200 and this was followed by ion-exchange chromatography. For full details see the text and Fig. 2. ○, ¹⁴C radioactivity; ●, ³H radioactivity. A, 25-hydroxycholecalciferol-binding protein; B, cholecalciferol-binding protein.

EXPLANATION OF PLATE I

Distribution of ^3H and protein on the polyacrylamide-disc-gel electrophoretogram of chick serum incubated with radioactive 25-hydroxycholecalciferol (a) and cholecalciferol (b)

Serum (0.25 ml) obtained from rachitic chicks was incubated for 16 h at 4°C with 1.5 ng of [26,27- ^3H]25-hydroxycholecalciferol or 10 ng of [1,2- ^3H]cholecalciferol. A sample (20 μl) was applied to the gel. For further details see the text.

EXPLANATION OF PLATE 2

Distribution of protein and ^3H on the polyacrylamide-disc-gel electrophoretogram of serum of Cebus monkey (a) and Erythrocebus patas monkey (b) incubated with radioactive 25-hydroxycholecalciferol as described in Plate 1

For details see the text.

Fig. 4. Competitive displacement of [26,27-³H]25-hydroxycholecalciferol from the 25-hydroxycholecalciferol-binding protein (a) and of [26,27-³H]25-hydroxycholecalciferol and [1,2-³H]cholecalciferol from the cholecalciferol binding protein (b) by increasing amounts of the two non-labelled steroids

For details see the text. Each point is the mean of three determinations. ○, 25-Hydroxycholecalciferol; ●, cholecalciferol.

binding sites on this protein. However, limited binding of radioactive cholecalciferol to its binding protein could be demonstrated *in vitro* but the displacement with increasing quantities of unlabelled cholecalciferol was small (Fig. 4b). A high association constant of cholecalciferol with its binding protein has not been obtained.

This difficulty is a consequence of the virtual insolubility of cholecalciferol in water, whereas 25-hydroxycholecalciferol with its additional polar group has sufficient solubility to allow binding to occur. Attempts to overcome this problem with cholecalciferol have involved the use of other means of separating bound from free steroid, e.g. Sephadex G-25 chromatography and other adsorbents such as Florisil, and solvents other than ethanol to maintain the cholecalciferol in solution (see the Experimental section). Although the radioactive cholecalciferol is held in solution by the ethanol or dimethyl sulphoxide, the dextran-coated charcoal failed to remove all the cholecalciferol from the water. This suggests that this steroid is held in solution such that interaction between this steroid and the cholecalciferol-binding protein is limited.

It was considered that the relative inactivity of ergocalciferol in chicks compared with cholecalciferol may be due to decreased affinity by the binding proteins for this series of sterols. However, after an

intracardial dose of [1-³H]ergocalciferol the two serum proteins contained the same proportion of ergocalciferol and its 25-hydroxy metabolite as was observed after a dose of cholecalciferol.

Rat and pig. In confirmation of the results mentioned above, the single binding protein seen on polyacrylamide-disc-gel electrophoresis of animal sera showed α -globulin mobility. Attempts were again made to establish conclusively that only one binding protein is present for both cholecalciferol and 25-hydroxycholecalciferol. Therefore two groups of rachitic rats were injected with [4-¹⁴C]-cholecalciferol and [26,27-³H]25-hydroxycholecalciferol and killed after 3 and 8 h respectively. The mixed sera were again chromatographed on DEAE-Sephadex but only a single peak of radioactivity was observed (Fig. 5) and the ³H:¹⁴C ratio was constant throughout the peak. Labelling of the sera of these two species with radioactive 25-hydroxycholecalciferol *in vitro* followed by disc-gel electrophoresis gave only a single peak of radioactivity with α -globulin mobility.

Man. Human plasma in which either radioactive cholecalciferol or 25-hydroxycholecalciferol was the major component was also analysed.

Plasma was obtained from Subject 1 at 4 and 8 h after a dose of 10 μ Ci of [³H]cholecalciferol. At these time-intervals the proportions of cholecalciferol to

Fig. 5. Ion-exchange chromatography of mixed samples of serum of rats given a dose either of $[26,27\text{-}^3\text{H}]25\text{-hydroxycholecalciferol}$ or $[4\text{-}^{14}\text{C}]cholecalciferol$

Serum (1 ml) from rachitic rats given a dose of $0.025\text{ }\mu\text{g}$ of $[26,27\text{-}^3\text{H}]25\text{-hydroxycholecalciferol}$ 16h previously was mixed with 3 ml of serum from rachitic rats given a dose of $0.5\text{ }\mu\text{g}$ of $[4\text{-}^{14}\text{C}]cholecalciferol$ 3h earlier. The mixed serum was subjected to chromatography on Sephadex G-200 and DEAE-Sephadex as before. For details see Fig. 2 and the text. $\bullet$, E_{280} ; $\circ$, ^{14}C radioactivity; $\blacksquare$, ^3H radioactivity.

25-hydroxycholecalciferol were 5.4:1 and 2:1, respectively (T. C. B. Stamp, personal communication). Cohn fractionation of these plasma samples showed that 47% of the radioactivity at 4h was in Cohn fraction IV, which contains the lipoproteins. At 8h the proportion in this fraction was 41.6%. The globulin fraction at 4 and 8h contained 20% and 25% respectively. The radioactivity in the 12h-plasma samples from Subject 2 was due mainly to 25-hydroxycholecalciferol. In this case 37.5% of the radioactivity was found in fraction IV and 50% in the α -globulin fraction. However, a 24h sample of plasma obtained from Subject 3, with a cholecalciferol/25-hydroxycholecalciferol ratio of 6:1, gave a single peak of radioactivity after chromatography on Sephadex G-200 and DEAE-Sephadex.

New- and Old-World monkeys. Analytical electrophoresis was carried out on sera from a vitamin D-deficient monkey, *Cebus albifrons* (New World) and from an *Erythrocebus patas* (Old World) monkey, both of which sera had been previously incubated with radioactive 25-hydroxycholecalciferol. As with

other animals the binding protein of Old-World monkeys showed α -globulin mobility, whereas for the New-World monkey the radioactivity was associated with a protein showing albumin mobility (Plate 2). The same mobility was observed with serum obtained from a *Cebus* monkey 24h after a dose of $1\text{ }\mu\text{Ci}$ of $[1\text{-}^3\text{H}]cholecalciferol$. The latter serum was fractionated by the Cohn method and 87% of the radioactivity was recovered in the albumin fraction, fraction V. An attempt to separate a carrier protein from the albumin by chromatographing this fraction on Sephadex G-200 followed by DEAE-Sephadex was unsuccessful. It remains to be shown whether albumin itself is the carrier protein for cholecalciferol in New-World monkeys.

Toad. Specimens of vitamin D-deficient *Xenopus laevis* were each given a dose of $1.6\text{ }\mu\text{Ci}$ ($1.25\text{ }\mu\text{g}$) of $[1\text{-}^3\text{H}]cholecalciferol$ and 5 days later serum was collected. Analysis of the serum by t.l.c. showed that the radioactivity was due equally to cholecalciferol and 25-hydroxycholecalciferol. Disc-gel electrophoresis of the serum showed the radioactivity to be near the origin in an area staining heavily with

Table 2. *Distribution of binding proteins among different species and their electrophoretic mobility*

Sera obtained from the different species either after a dose of radioactive cholecalciferol or after incubation with radioactive 25-hydroxycholecalciferol (for details see the text) were subjected to polyacrylamide-disc-gel electrophoresis. The gels were sliced (1 mm) and counted for radioactivity.

Species	Number of binding proteins	Electrophoretic mobility
Human	1	α -Globulin
Rat		
Pig		
Monkey (<i>Erythrocebus patas</i>)		
Chick	2	β -Globulin
Monkey (<i>Cebus albifrons</i>)	1	Albumin
<i>Xenopus</i>		α -Lipo-protein

Oil-Red O. After precipitation of the α -lipoproteins with heparin and $MnCl_2$ the radioactivity remained in the supernatant. Electrophoresis of the supernatant fraction showed the radioactivity to be still near the origin in an area staining heavily with Oil-Red O, suggesting that the radioactivity was associated with α -lipoproteins. Cohn fractionation of the serum from *X. laevis* provided further evidence that α -lipoproteins transport cholecalciferol and its 25-hydroxy derivative in this species. It was found that 80% of the radioactivity was recovered in Cohn fraction IV-1, which contains the α -lipoproteins.

The electrophoretic properties of the binding proteins from the animals studied are summarized in Table 2.

Discussion

It seems clear from the above studies that the chick is the only species so far examined that has more than one binding protein for cholecalciferol and its metabolites. These proteins have been extensively purified by treatment of the chick serum by the procedure of Cohn *et al.* (1950), followed by $(NH_4)_2SO_4$ precipitation, gel filtration and ion-exchange chromatography. This treatment results in two protein fractions, each of which gives only one band on gel electrophoresis. In addition, with radioactive 25-hydroxycholecalciferol bound to both fractions only one symmetrical peak of radioactivity was observed in each case after both gel electrophoresis and sucrose-gradient analysis.

The smaller of the two proteins specifically binds 25-hydroxycholecalciferol with an association constant of 10^8 litres/mol. This we have called the

25-hydroxycholecalciferol-binding protein, since binding with cholecalciferol could not be detected *in vitro* and 25-hydroxycholecalciferol formed in the liver is released bound only to this protein. However, it should be noted that this protein is present in the blood of rachitic chicks. The other, larger, protein, cholecalciferol-binding protein, appears to bind only cholecalciferol *in vivo* but the displacement curve obtained *in vitro* does not support this high specificity. However, it is probable that the difficulties encountered are due to the lipophilic nature of the cholecalciferol which, under the conditions used in these investigations to effect solubility in water, hindered the binding to its specific protein.

Only one carrier protein for both cholecalciferol and 25-hydroxycholecalciferol has been detected in man, Old-World monkeys, pig and rat. If two proteins are normally circulating in the plasma their properties must be very similar. The properties of the carrier proteins in the chick and New World monkeys, however, are such as readily to allow a distinction to be observed between them and the carrier proteins in other animals. Thus the electrophoretic mobility of the protein of animals examined in this study is that of an α -globulin, whereas that of chick is of a β -globulin type and that of the New-World monkey has albumin mobility. However, the proteins are of a similar size, since Peterson (1971) found the molecular weight of purified human binding protein to be 53000. This compares with approximate molecular weights of 54000 and 60000 for the two chick proteins which we report.

The samples of human plasma used in the present study showed significant binding of the two steroids to lipoproteins. This was most noticeable at the early time-intervals after an intravenous dose of the cholecalciferol. The proportion associated with the lipoproteins fell slowly so that by 24h virtually all of the cholecalciferol and its metabolites were associated with the α -globulin. This binding of cholecalciferol to lipoproteins was unrelated to body stores of the vitamin, since Subject 2 was osteomalacic owing to an inadequate tissue content of vitamin D, and yet only 50% of the cholecalciferol was associated with the α -globulin. Subject 3, however, had previously received large doses of cholecalciferol but at 24h only the α -globulin was labelled.

It is probably not a coincidence that the two species, chick (Chen & Bosman, 1964) and New-World monkeys (Hunt *et al.*, 1967), that cannot use ergocalciferol as efficiently as cholecalciferol have different carrier proteins from those animals that use these two steroids equally well. For the chick the explanation may be that the two proteins require a higher degree of hormone specificity than the single protein, which must carry both cholecalciferol and

25-hydroxycholecalciferol. The determination of the affinities of these two steroids for the various proteins is necessary before this question can be answered.

Vitamin D is required not only by higher animals but also by amphibians (Bruce & Parkes, 1950). The amphibians are the first evolutionary group that requires an efficient regulation of calcium absorption and excretion in order to maintain calcium homeostasis. If the toad is typical of the amphibia group, then they do not appear to have 'developed' a specific binding protein but rather depend on the non-specific lipoproteins for the transport of cholecalciferol and its 25-hydroxy metabolite.

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