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Vitamin D in the Avian Egg

ITS MOLECULAR IDENTITY AND MECHANISM OF INCORPORATION INTO YOLK

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The chemical identity of vitamin D in the egg of the domestic fowl was studied by analysing radioactivity in eggs from hens injected with [^{3}H]cholecalciferol. Labelled molecules were found throughout the egg, but the concentration of total radioactivity in albumin was only 5–7% of that in yolk. In lipid extracts of yolk, more than 90% of the radioactivity was as unchanged cholecalciferol and 5% as 25-hydroxycholecalciferol. Only about 3% of the radioactivity in albumin was choloroform-soluble, and of this, 40% was 25-hydroxycholecalciferol and 15% was cholecalciferol. Evidence is presented to support the idea that the specific transport of cholecalciferol into yolk is mediated by a cholecalciferol-binding protein in blood. This protein forms a complex with yolk proteins in transit from liver to ovary via the blood. A cholecalciferol-binding protein, chromatographically similar to that from blood, was found in egg yolk. It is postulated that cholecalciferol forms part of a complex with its specific binding protein, Ca^{2+} and the yolk phosphoprotein, phosvitin. This complex is then incorporated into yolk by the thecal cells of the ovarian follicle.

As deduced from its antirachitic potency, egg yolk produced by the domestic fowl (*Gallus domesticus*) contains a considerable quantity of vitamin D. The physiological concentration is equivalent to 1–2 μg of cholecalciferol per egg (approx. 50–100 ng/g of yolk) and this can be raised further by increasing the intake of vitamin D of the laying hen (Romanoff & Romanoff, 1949). This concentration in yolk is 5–10 times higher than that of total vitamin D plus its metabolites in blood plasma of normal chickens and 50–100 times higher than the concentration in any other tissue (Fraser, 1975). Thus when yolk is deposited in the oocyte, vitamin D is selectively accumulated from plasma, presumably to meet the needs during development of the prospective chick embryo.

Because several biologically active metabolites of vitamin D as well as the unchanged molecule are present in plasma, the molecular species of vitamin D which passes into yolk cannot be determined just from measurement of the antirachitic activity. With the usual input of vitamin D into the hen, either from u.v. irradiation of 7-dehydrocholesterol in skin or from dietary supplementation, the major vitamin D substance in plasma is 25-hydroxycholecalciferol. Therefore consideration of the vitamin D-derived molecules available in plasma would perhaps suggest that 25-hydroxycholecalciferol is the most likely form to be accumulated by yolk.

The identity of the biologically active vitamin D in yolk was investigated by analysing the labelled molecules in eggs from hens that had been given [1,2-^{3}H]cholecalciferol. From the results reported here it is concluded that cholecalciferol itself is the molecular form in egg yolk. To explain how the small proportion of unmetabolized cholecalciferol in plasma becomes concentrated in yolk, one of the two cholecalciferol-binding proteins found in chicken plasma (Edelstein *et al.*, 1973) was postulated to be involved. Support for this idea came from the extensive survey of Hay & Watson (1976*b*), who found that of 19 species of birds only four possessed both these plasma proteins: the domestic goose (*Anser anser*), domestic turkey (*Melleagris gallopauo*), domestic fowl (*Gallus domesticus*) and the ringdove (*Columba p. polumbus*). These all have a capability for continuous, rather than seasonal, egg production.

One of these cholecalciferol-binding proteins *in vivo* binds mainly 25-hydroxycholecalciferol, whereas the other binds mainly cholecalciferol (Edelstein *et al.*, 1973). In the present studies it is shown that the protein with specificity for cholecalciferol complexes with egg-yolk proteins during their transit from liver

to ovary via the plasma. Hence by being attached to proteins that are themselves being transported into yolk by the thecal cells of the ovarian follicle, the cholecalciferol-binding protein specifically transfers cholecalciferol from plasma into yolk.

Experimental

Materials

[1-^{3}H]Cholecalciferol (2Ci/mmol) and [1,2-^{3}H]-cholecalciferol (276.9mCi/mmol) were kindly prepared by Dr. P. A. Bell and Dr. B. Pelc by the methods described by Callow *et al.* (1966) and Lawson *et al.* (1971). [4-^{14}C]Cholecalciferol (32.3mCi/mmol) and 25-hydroxy[26,27-^{3}H]cholecalciferol (11Ci/mmol) were obtained from The Radiochemical Centre, Amersham, Bucks. HP7 9LL, U.K. All these radiochemicals were purified before use by t.l.c. on silica gel GF_{254} (Merck, Darmstadt, Germany) with ethyl acetate/heptane (1:1, v/v) as developing solvent. Unlabelled crystalline 25-hydroxycholecalciferol was obtained from Philips–Duphar N.V., Amsterdam, The Netherlands, and unlabelled crystalline cholecalciferol from Peboc Ltd., Belvue Road, Northolt, Middx., U.K.

Triton X-100, 2-mercaptoethanol, Tris base and oestradiol-17β 3-benzoate were purchased from Sigma (London) Chemical Co., Norbiton Station Yard, Kingston-upon-Thames, Surrey KT2 7BH, U.K. Other reagents were obtained from BDH Chemicals, Poole, Dorset BH12 4NN, U.K.

Methods

Measurement of radioactivity in aqueous samples was done by using a Triton X-100 scintillator solution (Edelstein *et al.*, 1973).

Protein concentrations were determined by a biuret method (Gornall *et al.*, 1949) against a standard of bovine serum albumin (Armour Pharmaceutical Co., Eastbourne, Sussex, U.K.).

Incorporation of labelled cholecalciferol into eggs. Laying hens were fed on a commercial pelleted diet (120 Laying Pellets; Labsure Animal Foods, Agrarian House, Castle Street, Poole, Dorset BH15 1HL, U.K.) containing 31.25μg of cholecalciferol/kg. Two hens, a Rhode Island Red (2.1kg) and a White Leghorn (1.6kg), were given respectively 35.8 and 56.8μg of [1,2-^{3}H]cholecalciferol (276.9mCi/mmol) in propylene glycol by single subcutaneous injections. One White Leghorn hen (1.4kg) was injected in the same way with a mixture of 26.1μg of [4-^{14}C]-cholecalciferol (32.3mCi/mmol) and 28.9μg of 25-hydroxy[26,27-^{3}H]cholecalciferol (123.9mCi/mmol). Eggs were collected and stored at 4°C until processed. At the end of the experiment, up to 24 days after injection, the birds were killed and blood serum was collected.

Analysis of egg radioactivity. The yolk and albumin fractions of each egg were separated and their volumes were measured. They were then diluted with water to 25 and 50ml respectively and dispersed by stirring with a glass rod. Total radioactivity was measured in 0.5ml volumes of diluted yolk and albumin by using Triton X-100 scintillator.

The chloroform-soluble radioactivity was extracted by shaking the yolk or albumin preparations with 15vol. of chloroform/methanol (2:1, v/v) in separating funnels. The chloroform layer was run off after adding 3vol. of water, and the upper aq.-methanol layer was re-extracted with 100ml of chloroform. The combined chloroform extracts from each preparation were reduced in volume by rotary evaporation under vacuum at 30°C. Yolk lipid was then made up to 50ml and albumin lipid was made up to 10ml with chloroform. Radioactivity was measured in 0.25–0.5ml and 0.05–0.1ml volumes respectively from these chloroform extracts. The same extraction procedure was applied to the blood serum.

The radioactive substances in the lipid extracts were analysed by t.l.c. Lipid samples containing about 1000d.p.m. of ^{3}H were applied in chloroform, together with unlabelled cholecalciferol (20μg) and 25-hydroxycholecalciferol (20μg), to 0.5mm thin layers of silica gel GF_{254}. The chromatograms were developed in ethyl acetate/heptane (1:1, v/v), and cholecalciferol (R_F 0.50) and 25-hydroxycholecalciferol (R_F 0.35) were identified as quenched spots against a fluorescent background when viewed in u.v. light. The silica gel was divided into ten ascending regions, which were scraped from the plate, eluted with diethyl ether/ethanol (3:1, v/v), and the radioactivity was measured. Recovery of radioactivity from t.l.c. was 90–100%.

Studies on the mechanism of vitamin D transport into yolk. (*a*) Effect of oestradiol treatment on plasma binding capacity. Rhode Island Red×Light Sussex female chickens from the National Institute for Research in Dairying (Shinfield, Reading, Berks., U.K.) were raised in the absence of u.v. light and fed, from 1 day of age, on a vitamin D-deficient diet (Table 1). From 8 to 18 days of age, six chickens were given daily intramuscular injections of 1mg of oestradiol-17β 3-benzoate in 0.1ml of arachis oil. On day 19, blood from the oestradiol-treated chickens and from six untreated chickens was collected into heparinized tubes and pooled plasma was prepared for each group.

The specific binding capacity for 25-hydroxycholecalciferol was determined for both plasma samples by a competitive-saturation analysis method. Plasma diluted 200 times with 0.01M-Tris/HCl, pH7.4, was added in 1ml volumes to tubes containing, in 10μl of ethanol, 13000d.p.m. of 25-hydroxy-[26,27-^{3}H]cholecalciferol plus unlabelled 25-hydroxycholecalciferol to give a final concentration range of

Table 1. *Vitamin D-deficient chicken diet*

Vitamin mixture (mg/kg of diet): retinyl acetate, 6; DL-α-tocopheryl acetate, 25; menaphthone, 2; nicotinamide, 100; calcium pantothenate, 20; thiamine hydrochloride, 9; riboflavin, 9; pyridoxine hydrochloride, 9; folic acid, 3; biotin, 0.3; cyanocobalamin, 0.02. Salt mixture, supplied by Arthur H. Cox and Co., Brighton, Sussex, U.K. (g/kg of diet): $CaCO_3$, 12.3; $CaHPO_4$, 19.5; Na_2HPO_4, 11.1; KCl, 12.3; $MgSO_4$, 4.2; $MnSO_4$, 0.27; ferric citrate, 0.261; $ZnCO_3$, 0.045; $CuSO_4$, 0.0225; KIO_3, 0.0015.

	Amount (g/kg of diet)
Cornflour	602
Acid-precipitated casein	180
Gelatin	100
Salt mixture	60
Arachis oil	50
L-Cystine	3
DL-Methionine	3
Choline chloride	1.5
Vitamin mixture	0.18332

5–50ng/ml. After standing for 1.5h at 0°C, 1ml of a suspension of dextran-coated charcoal (Edelstein *et al.*, 1973) was added with mixing to each tube to remove the unbound 25-hydroxycholecalciferol. The protein-bound 25-hydroxycholecalciferol was measured by the method of Edelstein *et al.* (1973). The specific binding capacities were calculated graphically by the procedure of Rosenthal (1967).

(*b*) Chromatography of plasma proteins. The plasma samples, diluted 1:20 with 0.02M-Tris/HCl, pH7.6, were added to 25-hydroxy[26,27-^{3}H]cholecalciferol (11Ci/mmol) so that there was 20% more 25-hydroxycholecalciferol than that which would bind to the total specific sites as calculated by competitive-saturation analysis. These preparations from control and oestradiol-treated chickens contained 886 and 410μg of protein respectively and were kept at 4°C for 12h before chromatography. Ion-exchange chromatography was done at 4°C on columns (15cm×1.5cm) of DEAE-Sephadex A-50 (Pharmacia Fine Chemicals, Uppsala, Sweden), equilibrated with 0.02M-Tris/HCl, pH7.6, and eluted with a linear gradient of 0–0.6M-NaCl in the same buffer. In some experiments, ion-exchange chromatography was done with eluting solutions containing 1mM-2-mercaptoethanol and 0.1% Triton X-100.

Chromatographic separation of 25-hydroxy[26,27-^{3}H]cholecalciferol-bound proteins in the plasma of oestradiol-treated chickens was also done on columns (41cm×1.5cm) of Sephadex G-200 at 4°C. The plasma was diluted, and the columns were eluted with NaTEMT buffer.*

* Abbreviation: NaTEMT buffer, 0.02M-Tris/HCl, pH7.6, containing 0.2M-NaCl, 1.5mM-EDTA, 1mM-2-mercaptoethanol and 0.1% Triton X-100.

All these chromatographic procedures were modifications of those described by Edelstein *et al.* (1973).

(*c*) Polyacrylamide-gel electrophoresis. Plasma from control and oestradiol-treated chickens was diluted 1:20 with 0.02M-Tris/HCl, pH7.4, and was labelled with 25-hydroxy[26,27-^{3}H]cholecalciferol as for Sephadex chromatography. Samples (20μl) containing 33 and 18μg of plasma protein respectively from the control and oestradiol-treated chickens were run on 7.5% (w/v) polyacrylamide gels as described by Davis (1964). Each plasma sample was run in duplicate, and, after completion, one gel was stained with Coomassie Brilliant Blue, and the other was sliced into 1.6mm segments and the radioactivity measured (Edelstein *et al.*, 1973).

(*d*) Cholecalciferol binding by protein of egg yolk *in vitro*. The yolk from untreated eggs was diluted 1:6 with NaTEMT buffer. This yolk preparation was added to [1-^{3}H]cholecalciferol (2Ci/mmol) in the ratio 10.9ng of cholecalciferol/mg of yolk protein. This concentration was equivalent to 16.5μg of cholecalciferol per whole yolk, i.e. about 10 times higher than the usual endogenous vitamin D content. After standing at 4°C for 6 days, the yolk preparation was centrifuged at 2000*g* for 30min at 4°C to yield a clear yellow supernatant and a white precipitate. The precipitate was dissolved in the original volume of NaTEMT buffer. These supernatant and pellet fractions were run separately on columns (55cm×2.5cm) of Sephadex G-200 in NaTEMT buffer at 4°C.

Radioactive eluates from the columns were dialysed for 2 days against large volumes of 0.038mM-EDTA (pH7.5)/1mM-2-mercaptoethanol. The volumes of the dialysis sacs were decreased in a blast of air at 4°C.

(*e*) Preparation of ^{14}C-labelled cholecalciferol-binding proteins from chicken plasma. Vitamin D-deficient chickens, 3 weeks of age, weighing 150–160g were injected intracardially with 3μg of [4-^{14}C]-cholecalciferol in 0.1ml of propylene glycol. After 4h, blood was collected into heparinized tubes and the plasma was dialysed against 0.038mM-EDTA (pH7.5)/1mM-2-mercaptoethanol.

Results

Analysis of radioactive cholecalciferol molecules incorporated into eggs in vivo

Radioactivity was found in both the yolk and the albumin of eggs laid after hens had been injected with [1,2-^{3}H]cholecalciferol. The concentration of ^{3}H-labelled molecules in yolk increased in eggs produced up to 6–8 days after injection, and then gradually declined (Fig. 1). The concentration of ^{3}H-labelled molecules in albumin was about 3–7% of that in yolk, but because the albumin volume is greater than that of the yolk, the proportion of total ^{3}H in the egg,

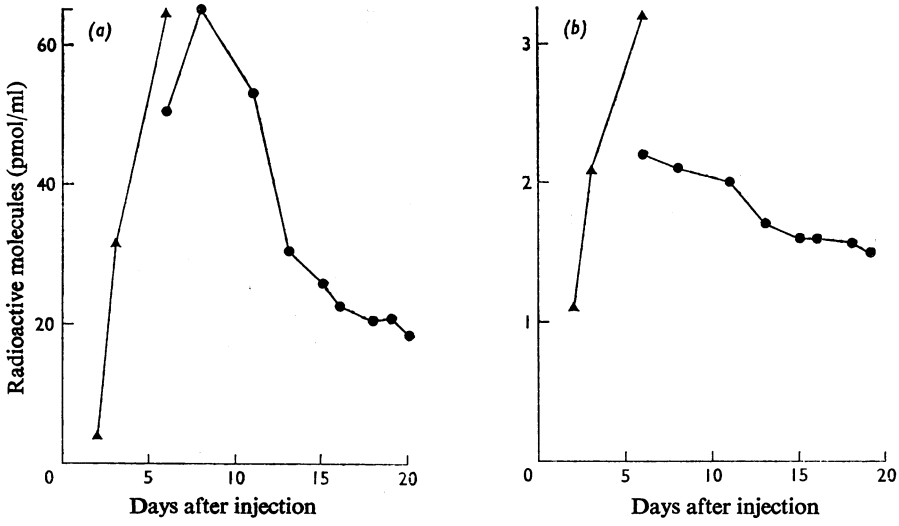

Fig. 1. *Total ^3H-labelled molecules in eggs from hens given [1,2-^3H]cholecalciferol*

Hen 1 and hen 2 were given respectively 35.8 and 56.8 μg of [1,2-^{3}H]cholecalciferol. The concentration of total radioactive molecules (hen 1, ▲; hen 2, ●) is shown for (*a*) yolk and (*b*) albumin fractions in the sequence of eggs laid after injection.

Table 2. *Proportion of total egg radioactivity found in albumin*

Hen 1 and hen 2 were injected respectively with 35.8 and 56.8 μg of [1,2-^{3}H]cholecalciferol.

	Day after injection that egg was laid	^{3}H concentration in albumin (% of that in yolk)	Percentage of total egg ^{3}H in albumin
Hen 1	2	28.3	29.6
	3	6.8	12.0
	6	5.0	7.6
Hen 2	6	4.3	6.7
	8	3.2	8.4
	11	3.7	8.7
	13	5.5	11.8
	15	6.2	13.6
	16	7.1	15.1
	18	7.7	15.0
	19	7.4	14.7
	20	10.0	16.1

found in the albumin fraction, varied mainly between 7 and 15% (Table 2). In comparison with the yolk, the ^{3}H content of albumin remained relatively constant. Consequently as the ^{3}H in yolk declined, there was an increase in the proportion of total egg ^{3}H in the albumin. Moreover, since the albumin is secreted after the yolk, a considerable proportion of egg ^{3}H was found in albumin of the egg laid 2 days after injection. In this case, yolk formation was almost complete at the time of injection and relatively little ^{3}H had been incorporated. On the other hand, all the albumin was secreted during the time when the blood ^{3}H concentration was rising, so that 29.6% of the total ^{3}H in this egg was in the albumin. In the next egg (day 3) there had been a longer opportunity for ^{3}H uptake by yolk and thus the proportion of ^{3}H in the albumin had fallen to 12%.

The chloroform-soluble radioactivity was closely related to the total ^{3}H in the yolk, with recoveries of 86.8% (S.D. 4.9) in the extracts. However, only a small amount of ^{3}H in the albumin was soluble in chloroform, recovery in these extracts being 3.1% (S.D. 0.6) of the total. Thus the chloroform-extractable radioactivity from albumin was about 0.5% of that from the yolk.

Chromatography of the lipid extracts revealed that most of the radioactive molecules in yolk were cholecalciferol (Fig. 2). Eggs laid up to 6 days after injection of [1,2-^{3}H]cholecalciferol contained about 90% [^{3}H]cholecalciferol and 5% as the metabolite 25-hydroxy[1,2-^{3}H]cholecalciferol. Thereafter the proportion of cholecalciferol in the labelled molecules decreased, so that the egg laid on day 20 contained 74% cholecalciferol and 18% 25-hydroxycholecalciferol. Over all time-intervals, 3.7% (S.D. 1.7) of label was not associated with either cholecalciferol or 25-hydroxycholecalciferol on the chromatograms. Most of the fall in yolk radioactivity with time was explained by a fall in the quantity of [^{3}H]cholecalciferol. The 25-hydroxy[^{3}H]cholecalciferol concentration did not decrease until 15 days after the injection.

Since the ratio of 25-hydroxycholecalciferol to cholecalciferol in serum was about 3:1 at both 10 and 24 days after injection (Table 3), it was apparent that yolk was accumulating cholecalciferol in preference to 25-hydroxycholecalciferol. The fall with time in the proportion of labelled cholecalciferol in yolk (Fig. 2) is partly the effect of dilution with incoming unlabelled cholecalciferol from the diet and partly a reflexion of the differential decline of cholecalciferol and 25-hydroxycholecalciferol in plasma. Thus with the normal daily input of cholecalciferol into the laying hen, the proportion of total cholecalciferol molecules in the yolk as unchanged cholecalciferol would be maintained at greater than 90%.

In contrast, the small amount of chloroform-extractable radioactivity from albumin was mainly 25-hydroxy[^{3}H]cholecalciferol (40%) rather than [^{3}H]cholecalciferol (15%). About 40–50% of the chloroform-soluble radioactivity remained at the origin on t.l.c. Hence 98% or more of the total ^{3}H in albumin (of which 97% is chloroform-insoluble) was present as polar metabolites of cholecalciferol or 25-hydroxycholecalciferol. The chemical identity of this material was not investigated further.

To examine which molecules would be taken into yolk when equivalent quantities of cholecalciferol and 25-hydroxycholecalciferol were available, a hen was injected with 68nmol (26.1μg) of [4-^{14}C]cholecalciferol and 72.3nmol (28.9μg) of 25-hydroxy[26,27-^{3}H]-cholecalciferol. In yolk from eggs laid on days 3 and 4, 100 and 56% respectively of the labelled molecules were derived from 25-hydroxy[26,27-^{3}H]-cholecalciferol (Fig. 3). This indicated that 25-hydroxycholecalciferol, when present in sufficient concentration in blood, could be used instead of cholecalciferol for incorporation into yolk. Nevertheless, in eggs laid on days 5, 7 and 8 after injection, label derived from [4-^{14}C]cholecalciferol predominated in yolk. Here the ^{14}C-labelled molecules followed the same pattern as in the previous experiments, and over this period more than 85% were present as unchanged cholecalciferol. The serum from this hen on day 7 contained 8.1 and 6.7pmol of 25-hydroxycholecalciferol/ml, derived respectively from 25-hydroxy[26,27-^{3}H]cholecalciferol and [4-^{14}C]-cholecalciferol, and it contained only 1.3pmol of unchanged [4-^{14}C]cholecalciferol/ml (Table 3). Therefore cholecalciferol was still being selected despite a more than 10 times higher concentration of 25-hydroxycholecalciferol in blood.

It should be noted that yolk formation takes place over several days. Romanoff & Romanoff (1949) state that the most rapid increase in yolk size occurs over the 6 days before ovulation, when the diameter

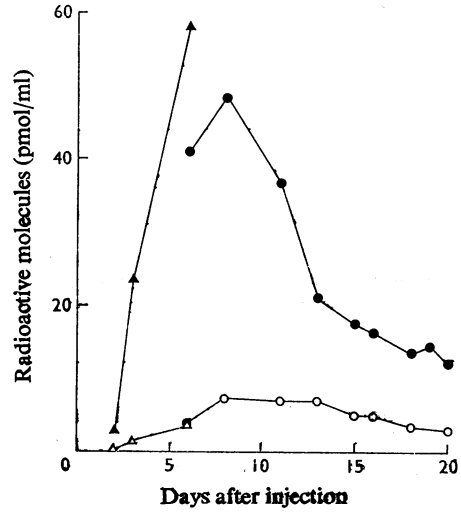

Fig. 2. *Radioactive cholecalciferol and 25-hydroxycholecalciferol in egg yolk from hens given* [1,2-^{3}*H*]*cholecalciferol*

Hen 1 and hen 2 were given respectively 35.8 and 56.8μg of [1,2-^{3}H]cholecalciferol. The concentration of [^{3}H]cholecalciferol (hen 1, ▲; hen 2, ●) and 25-hydroxy[^{3}H]-cholecalciferol (hen 1, △; hen 2, ○) is shown in the sequence of eggs laid after injection.

Table 3. *Composition of serum radioactivity in laying hens*

Hen 1 and hen 2 were injected respectively with 93.2 and 147.9nmol of [1,2-^{3}H]cholecalciferol. Hen 3 was injected with 68nmol of [4-^{14}C]cholecalciferol and 72.3nmol of 25-hydroxy[26,27-^{3}H]cholecalciferol.

Day after injection ...	Hen 1 10		Hen 2 24		Hen 3 7				
					^{3}H		^{14}C		Total ^{3}H+^{14}C
	(%)	(pmol/ml)	(%)	(pmol/ml)	(%)	(pmol/ml)	(%)	(pmol/ml)	(pmol/ml)
Cholecalciferol	18.4	3.4	23.6	1.3	0	0	11.6	1.3	1.3
25-Hydroxycholecalciferol	65.1	12.1	60.3	3.3	55.2	8.1	58.7	6.7	14.8
Other	16.5	3.1	16.1	0.9	44.8	6.6	29.7	3.4	10.0

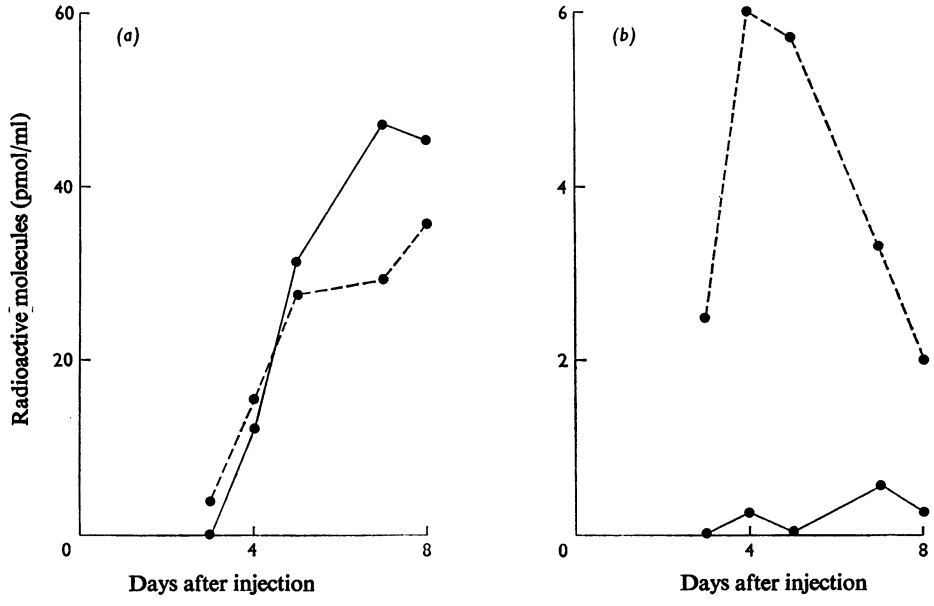

Fig. 3. *Total labelled molecules in eggs from hen given* [4-^{14}*C*]*cholecalciferol and* 25-*hydroxy*[26,27-^{3}*H*]*cholecalciferol*

A hen was injected with a mixture of 26.1 μg of [4-^{14}C]cholecalciferol and 28.9 μg of 25-hydroxy[26,27-^{3}H]cholecalciferol. The concentration of total molecules derived from [4-^{14}C]cholecalciferol (●——●) and from 25-hydroxy[26,27-^{3}H]cholecalciferol (●-----●) is shown for (*a*) yolk and (*b*) albumin fractions in the sequence of eggs laid after injection.

expands from 6 to 35 mm. During this time, when there is a 200-fold increase in volume, most of the labelled cholecalciferol would be incorporated. Thus in the hens injected with [1,2-^{3}H]cholecalciferol, the maximum radioactivity in yolk was found in the eggs where ovulation had taken place 6 days after injection (Fig. 1). Since plasma radioactivity was falling from 24–48 h onwards, there was a proportional decline in yolk radioactivity when ovulation occurred more than 6 days after injection.

In eggs from the hen given ^{3}H- and ^{14}C-labelled molecules, the ratio of ^{3}H and ^{14}C found in the yolk was related to the changing pattern of plasma metabolites over 6–7 days before each egg was complete. During this time the composition of ^{14}C-labelled molecules in plasma was changing from being mainly cholecalciferol to being mainly 25-hydroxycholecalciferol (Table 3) and the concentration of 25-hydroxy[26,27-^{3}H]cholecalciferol was gradually declining. The total radioactivity in albumin, in contrast with that in yolk, was predominantly ^{3}H in all the eggs with only 10–15% present as ^{14}C in eggs laid on days 7 and 8 (Fig. 3). This indicated that the polar metabolites in albumin were further derivatives of 25-hydroxycholecalciferol rather than non-25-hydroxylated metabolites of cholecalciferol. This view is supported also by the relative constancy, compared with that in yolk, of the total concentration of radioactivity in albumin, which resembles the slow decline of the 25-hydroxycholecalciferol concentrations in plasma and in yolk.

Effect of oestradiol treatment on the cholecalciferol-binding proteins in chicken plasma

In seeking a mechanism for the uptake of cholecalciferol into yolk, the possibility was considered that a cholecalciferol-specific binding protein might play a role. Such a protein is present in the plasma of immature chickens (Edelstein *et al.*, 1973). Since oestradiol treatment of young chickens will induce the premature synthesis of yolk proteins by liver and their secretion into plasma (Schjeide & Urist, 1960), the effect of such treatment on the concentrations of the cholecalciferol-binding proteins in plasma was investigated.

Plasma from vitamin D-deficient control chickens was found to have a total specific binding capacity of 722 ng of 25-hydroxycholecalciferol/ml as determined graphically by saturation analysis (Table 4). When chromatographed on DEAE-Sephadex with all the specific sites saturated with 25-hydroxy-[26,27-^{3}H]cholecalciferol, this plasma demonstrated two peaks of eluted ^{3}H, representing the 25-hydroxy-

Table 4. *Specific capacities of cholecalciferol- and 25-hydroxycholecalciferol-binding proteins in plasma from control and oestradiol-treated chickens*

	Control		Oestradiol-treated	
	(ng of 25-hydroxycholecalciferol/ml)	(pmol/ml)	(ng of 25-hydroxycholecalciferol/ml*)	(pmol/ml*)
Total binding capacity				
By saturation analysis	722	1805	1076	2690
By Sephadex chromatography	534	1335	883	2208

	Control		Oestradiol-treated	
	% of specific binding sites	Binding capacity for each protein (pmol/ml)	% of specific binding sites	Binding capacity for each protein (pmol/ml)
Cholecalciferol-binding protein	31.3	418	24.6	720
25-Hydroxycholecalciferol-binding protein	68.7	918	75.4	2208

* These values were derived from the supernatant after precipitation of yolk material and therefore relate to the 25-hydroxycholecalciferol-binding protein only.

cholecalciferol- and cholecalciferol-binding proteins (Fig. 4*a*). The calculated capacity for each protein is given in Table 4.

Plasma from the oestradiol-treated chickens was viscous and milky in appearance. When diluted with Tris/HCl a flocculent precipitate formed which readily pelleted on centrifugation at 2000*g*. The specific binding capacity of the supernatant was 1076ng of 25-hydroxycholecalciferol/ml. However, only the 25-hydroxycholecalciferol-binding protein was found on DEAE-Sephadex chromatography (Fig. 4*b*).

The absence of the cholecalciferol-binding protein from the plasma supernatant from the oestradiol-treated chickens was also shown by gel electrophoresis (Fig. 5). Here the control had two peaks of binding activity, whereas the experimental plasma had only one. When 0.5ml of control plasma was mixed with 0.1ml of experimental plasma before dilution and centrifugation, the cholecalciferol-binding protein was removed from the electrophoretic profile of the control plasma (Fig. 5*c*).

It was evident that yolk material, precipitated on dilution of the plasma from oestradiol-treated chickens, was also bringing down the cholecalciferol-binding protein, but was leaving the 25-hydroxycholecalciferol-binding protein in solution. Therefore an attempt was made to recover cholecalciferol-binding protein from the plasma of the oestradiol-treated chickens. Because of the lipaemic nature of this plasma, Triton X-100 was added to the diluting buffer to aid dispersion of yolk lipoprotein. A precipitate was still formed on diluting with the detergent-containing buffer, but the concentration of protein remaining in solution, under the influence of Triton X-100, had risen from 18.2 to 45.4mg/ml of original plasma. Nevertheless, although the 25-hydroxycholecalciferol-binding protein appeared as usual, no cholecalciferol-binding protein was found on DEAE-Sephadex chromatography run with Triton X-100 and 2-mercaptoethanol in the eluting solution.

Since precipitation of yolk material occurred on dilution, even in the presence of detergent, it was likely that this resulted from the lowered ionic strength of the solution. Thus a diluting buffer (NaTEMT) was devised which contained 0.2M-NaCl to maintain ionic strength, and 2-mercaptoethanol and EDTA, which are reported to prevent aggregation of the cholecalciferol-binding protein (Edelstein *et al.*, 1973). Dilution of the experimental plasma with NaTEMT buffer gave a clear opalescent solution with no precipitate. The protein concentration in the original plasma was, from this solution, calculated to be 60mg/ml. After saturation of the known binding sites with 25-hydroxy[26,27-^{3}H]cholecalciferol this plasma was run on a column of Sephadex G-200 with NaTEMT buffer. A radioactive peak was eluted at the void volume, containing an excess of 25-hydroxy-[26,27-^{3}H]cholecalciferol in lipid micelles, and a later peak (K_D 0.35) contained 25-hydroxy[^{3}H]cholecalciferol bound to protein. The fractions in this peak were combined and the volume was decreased to 3.7ml. Chromatography on DEAE-Sephadex now gave two peaks of radioactivity, representing the 25-hydroxycholecalciferol- and cholecalciferol-binding proteins in the ratio 75.4:24.6 (Fig. 6). From this, the relative binding capacities of the two proteins in the original plasma were calculated (Table 4). In comparison with the control plasma, oestradiol treatment had increased the concentration of 25-hydroxycholecalciferol-binding protein by 141% and that of the cholecalciferol-binding protein by 73%.

The observation that oestradiol treatment lowered

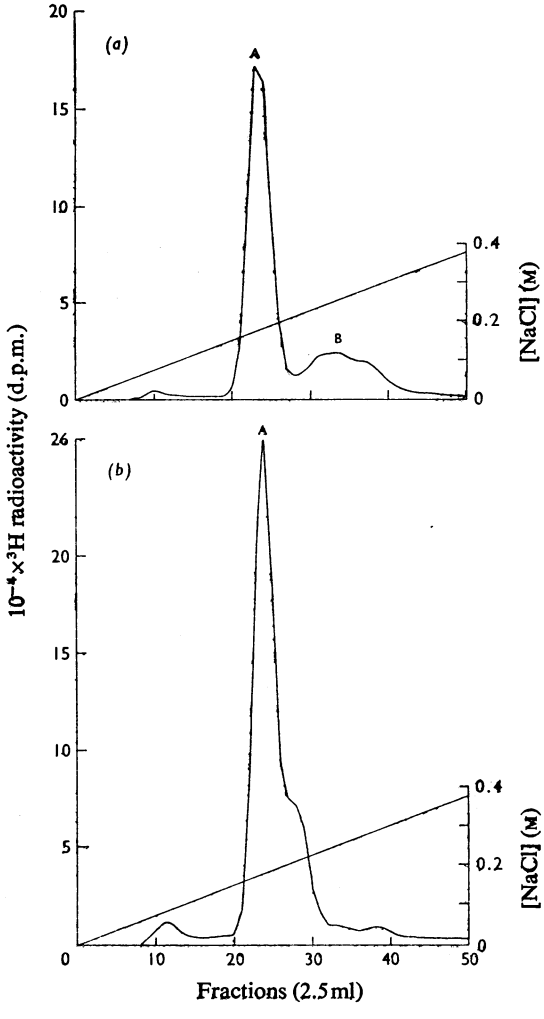

Fig. 4. *Ion-exchange chromatography of plasma from (a) control and (b) oestradiol-treated chickens*

The plasma was diluted 1:20 with 0.02M-Tris/HCl, pH 7.6, and was added to 25-hydroxy[26,27-^{3}H]cholecalciferol to saturate the specific cholecalciferol-binding proteins (for details see the text). The samples were chromatographed on DEAE-Sephadex with a linear gradient of NaCl as indicated. The void volume is demonstrated as a peak of unbound 25-hydroxycholecalciferol at about fraction 10. A, 25-Hydroxycholecalciferol-binding protein; B, cholecalciferol-binding protein.

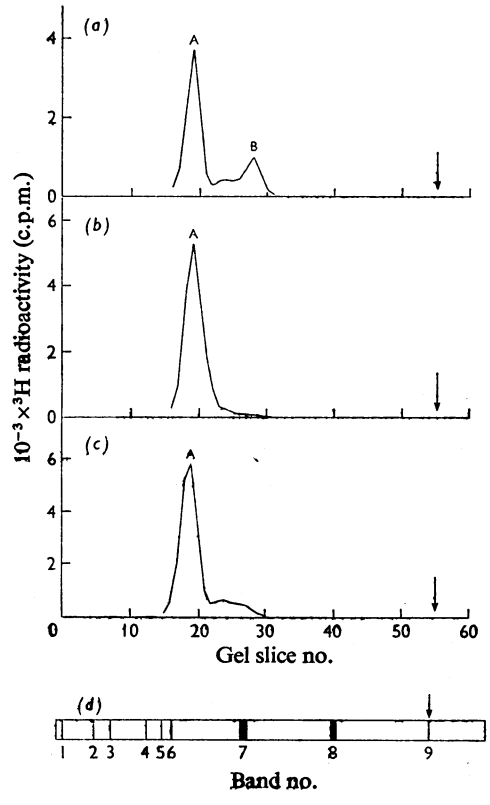

Fig. 5. *Gel electrophoresis of plasma from (a) control and (b) oestradiol-treated chickens*

Plasma was diluted and added to 25-hydroxy[26,27-^{3}H]-cholecalciferol as for DEAE-Sephadex chromatography (for details see the text). Samples (20μl) containing 33 and 18μg of protein from plasma respectively of control and oestradiol-treated chickens were applied to gels. The ^{3}H-distribution pattern along the gels is shown. In one experiment, (*c*), 0.1ml of experimental plasma was added to 0.5ml of control plasma before preparation for electrophoresis (for details see the text). A diagram of the stained protein bands after electrophoresis of control plasma is shown in (*d*). Band 8 is plasma albumin. Bands no. 4 and 7 were absent from plasma of oestradiol-treated chickens. The positions of the tracker dye, Bromophenol Blue, are indicated by arrows. A, 25-Hydroxycholecalciferol-binding protein; B, cholecalciferol-binding protein.

the concentration of the normal plasma proteins from 32.8 to 18.2mg/ml is in agreement with the findings of Schjeide & Urist (1960). By difference, therefore, the yolk protein concentration in the plasma of the oestradiol-treated chickens was 41.8mg/ml. Thus a decrease in the concentration of plasma albumin and globulin proteins (Schjeide & Urist, 1960) was associated here with an absolute increase in the two cholecalciferol-binding proteins.

A discrepancy was observed in the total binding capacity estimated by two different methods. The values obtained by competitive-saturation analysis for control and experimental plasma were respectively 35 and 22% higher than those obtained more

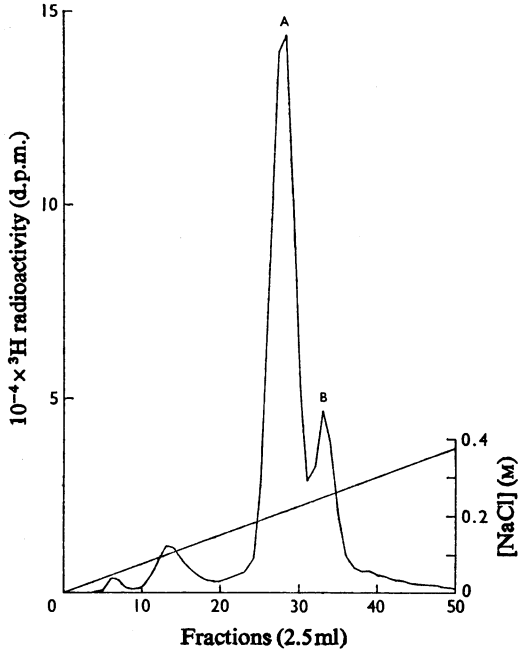

Fig. 6. *Ion-exchange chromatography of the radioactive peak obtained from Sephadex G-200 chromatography of plasma from oestradiol-treated chickens*

Plasma was diluted with NaTEMT buffer before being added to 25-hydroxy[26,27-^{3}H]cholecalciferol and running on Sephadex G-200 in the same buffer. The fractions eluted at K_D 0.35 were combined, dialysed and applied here to DEAE-Sephadex with a linear gradient of NaCl as indicated. Two peaks of unbound radioactivity were eluted, one at the void volume (fraction 7) and another associated with residual Triton X-100 (fraction 13). A, 25-Hydroxycholecalciferol-binding protein; B, cholecalciferol-binding protein.

directly by Sephadex chromatography. It is possible that competitive saturation may have included some low-specificity sites and thus overestimated the specific-binding capacity. On the other hand, there may have been dissociation of 25-hydroxycholecalciferol during chromatography, which would result in underestimates of the total specific sites. Both methods, however, demonstrated the enhanced binding capacity produced by oestradiol treatment.

Search for cholecalciferol-binding protein in egg yolk

As NaTEMT buffer had been effective in solubilizing the yolk protein and lipid in plasma and had revealed the presence of the cholecalciferol-binding protein, a similar procedure was tried with egg yolk itself. Although much of the yolk was soluble in

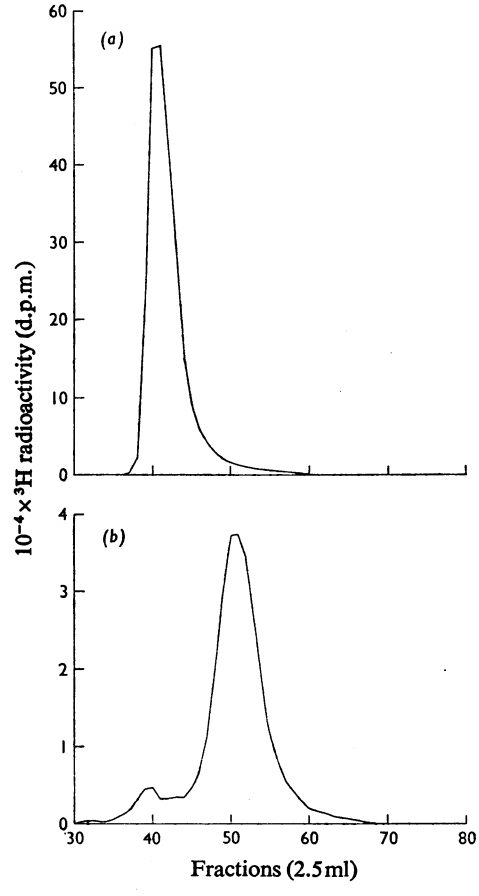

Fig. 7. *Chromatography on Sephadex G-200 of fractions from egg yolk mixed in vitro with [1-^{3}H]cholecalciferol*

Egg yolk was diluted with 6vol. of NaTEMT buffer before being added to [1-^{3}H]cholecalciferol. Supernatant and precipitate fractions were obtained by centrifugation. Chromatography on Sephadex G-200 columns was done with (*a*) the supernatant and (*b*) the resolubilized precipitate fractions (see the text for details). Void volume for these columns was at fraction 41.

NaTEMT buffer, centrifugation at 2000*g* for 30min at 4°C gave a clear yellow supernatant and a white pellet. However, after removal of the supernatant yolk material, the pellet was soluble in the original volume of NaTEMT buffer. When 1ml of diluted yolk (25mg of protein) was added to 272ng of [1-^{3}H]-cholecalciferol, 89.3% of the ^{3}H was found in the supernatant and 10.7% was in the precipitate.

On chromatography on columns of Sephadex G-200 in NaTEMT buffer, all the radioactivity in the yolk supernatant fraction came off at the void volume. In contrast, the resolubilized pellet fraction gave two

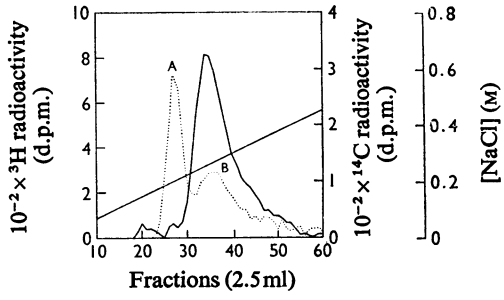

Fig. 8. *Ion-exchange chromatography of egg-yolk protein bound in vitro to [1-^{3}H]cholecalciferol and plasma protein labelled in vivo with [4-^{14}C]cholecalciferol*

[1-^{3}H]Cholecalciferol-bound yolk protein was obtained from the radioactive peak eluted at K_D 0.16 on Sephadex G-200 (Fig. 7*b*). For details of the ^{14}C-labelled plasma protein preparation see the Experimental section. Proteins from yolk (130μg) and plasma (7.3mg) were co-chromatographed on DEAE-Sephadex with a linear gradient of NaCl as indicated. Elution profiles for ^{3}H (——) and ^{14}C (· · · ·) are shown. Peaks A and B represent plasma binding proteins for 25-hydroxycholecalciferol and cholecalciferol respectively.

peaks of radioactivity, one at the void volume and one with K_D 0.16 (Fig. 7). The combined fractions of this latter peak produced a white precipitate on dialysis, but 92% of the radioactivity stayed bound to protein in solution.

This soluble preparation, containing protein-bound [1-^{3}H]cholecalciferol, was mixed with dialysed chicken plasma labelled *in vivo* with [4-^{14}C]cholecalciferol. The differently labelled proteins from yolk and plasma were then co-chromatographed on DEAE-Sephadex. The ^{14}C profile gave the two peaks of the 25-hydroxycholecalciferol- and cholecalciferol-binding proteins of plasma (Fig. 8). The ^{3}H came off in just one peak, which coincided with the plasma cholecalciferol-binding protein. It is therefore concluded that the cholecalciferol-binding protein found in egg yolk is similar to, if not identical with, that of plasma. Assuming that all endogenous cholecalciferol had been displaced, it can be calculated from the quantity of [1-^{3}H]cholecalciferol bound to this yolk protein that the binding capacity in 15ml of total yolk would be 1.9μg of cholecalciferol. This value agrees well with the usual estimate of vitamin D concentration in yolk.

Discussion

From these experiments, the conclusion is drawn that cholecalciferol is transported into yolk, bound to a specific plasma protein, which associates with yolk protein during its passage from liver to ovary. Four pieces of evidence are adduced to support this suggested mechanism.

First, the binding protein for cholecalciferol in plasma, but not that for 25-hydroxycholecalciferol, forms a complex with yolk material secreted in response to oestradiol. Secondly, although this binding protein has a higher affinity for cholecalciferol than for 25-hydroxycholecalciferol, it will nevertheless effectively bind the latter (Edelstein *et al.*, 1973). When present in high concentration, 25-hydroxycholecalciferol can displace cholecalciferol from the protein. This property accords with the finding that when large amounts of 25-hydroxycholecalciferol are given to a laying hen, it is well incorporated into yolk (Fig. 3). A third piece of evidence is that a cholecalciferol-binding protein can indeed be found in yolk which has similar chromatographic properties to the binding protein from plasma. Fourthly, oestradiol treatment increases the apparent concentration of the two cholecalciferol-binding proteins in plasma, thus suggesting that they may have a role in processes associated with reproduction.

This last observation raises the question of why the cholecalciferol-binding protein is present in the plasma of immature and of male chickens if its function is to do with transport of vitamin D into yolk. Both male and female immature chickens respond to oestradiol treatment by secreting egg-yolk protein into plasma (Greengard *et al.*, 1965), and therefore both sexes have the genetic ability to demonstrate an aspect of female reproductive biochemistry. By the same argument, the genetically determined proclivity for egg production of the domestic fowl may be expressed also by the permanent presence of the cholecalciferol-binding protein in the plasma of both sexes. Another indication that this may be so comes from the observation that this protein is apparently only found in the plasma of species of birds with high fecundity (Hay & Watson, 1976*b*). This idea would be further substantiated if the protein were to be discovered in plasma of less-fecund species, either during the egg-laying period or after artificial induction with oestradiol.

The finding that oestradiol treatment increases the concentration of both binding proteins in plasma suggests that during egg production, cholecalciferol-transport mechanisms would be enhanced because of an increased utilization of vitamin D. This would concern not only the incorporation of vitamin D into the egg but also the requirements for regulation of calcium transport in intestine and bone and the deposition of calcium in the egg shell. It is possible that the so-called 25-hydroxycholecalciferol-binding protein in chicken plasma is the homologue of the single binding protein found in other animal species (Edelstein *et al.*, 1973; Hay & Watson, 1976*a*,*b*). Its role

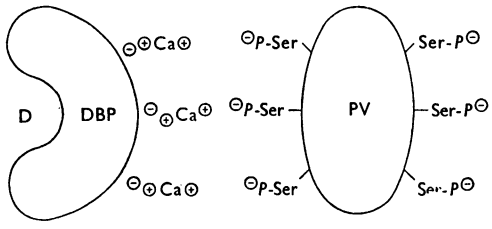

Fig. 9. *Schematic representation of proposed complex to explain the association of cholecalciferol with yolk protein*

The scheme suggests how a bivalent positive ion may interact with and cross-link two negatively charged proteins. D, Cholecalciferol; DBP, cholecalciferol-binding protein; PV, phosvitin; Ser-*P*, phosphoserine residues.

would therefore be to transport in blood both cholecalciferol and 25-hydroxycholecalciferol, the precursors of the homoeostatic hormone, 1,25-dihydroxycholecalciferol.

The yolk component considered most likely to complex with the cholecalciferol-binding protein in plasma is the phosphoprotein, phosvitin. This is composed of 50% serine, almost half of which is present as phosphoserine, giving an overall phosphorus content of 10% (Schjeide & Urist, 1960). In its natural state phosvitin contains about 7% calcium, but when diluted with water much of the calcium dissociates to expose numerous negative charges. The precipitate that forms on dilution of plasma from oestradiol-treated chickens is a complex of phosvitin, yolk lipoglycoprotein, yolk lipid and calcium. In the present studies this electrostatic association was prevented by the presence of NaCl and Triton X-100 in the NaTEMT buffer. As phosvitin has a mol.wt. of about 21000 (Mecham & Olcott, 1949) and the two cholecalciferol-binding proteins have mol.wts. in the range 50000–60000 (Edelstein *et al.*, 1973), chromatography on Sephadex G-200 removed phosvitin, thus enabling the cholecalciferol-binding protein to be revealed on subsequent ion-exchange chromatography.

In the yolk granules of the completed egg, phosvitin and lipoglycoprotein are present in a giant complex, which differs from that produced by diluting the plasma, in that it retains a higher percentage of calcium (Schjeide & Urist, 1960). It is proposed that cholecalciferol is deposited in yolk complexed with cholecalciferol-binding protein and phosivitin, which in turn forms part of the giant aggregate. This aggregate was disrupted when yolk was diluted with NaTEMT buffer and the cholecalciferol-binding protein was then separated by chromatography on Sephadex G-200. In this case it is probable that some of the yolk protein was still associated with the cholecalciferol-binding protein, for it emerged from the column earlier (K_D 0.16) than the corresponding protein from plasma (K_D 0.35). Moreover, on dialysis of this fraction against EDTA, a white precipitate of yolk protein separated from the cholecalciferol-binding protein, which remained in solution, and this latter protein then co-chromatographed with the cholecalciferol-binding protein of plasma on DEAE-Sephadex (Fig. 8).

Another feature of the plasma cholecalciferol-binding protein is its ability to self-aggregate, a process that can be prevented by EDTA (Edelstein *et al.*, 1973). This instability is a further example of the tendency for the cholecalciferol-binding protein to undergo ionic interaction with other charged molecules. However, from its electrophoretic mobility and the degree of retention on DEAE-Sephadex chromatography, it can be deduced that this protein has a net negative charge. Hence in order for it to aggregate with other negatively charged molecules, such as phosvitin, the mediation of a positive charge is needed. The bivalent Ca^{2+} meets this requirement for an intermediary cationic linking agent. Therefore the primary association of cholecalciferol with yolk protein is thought to be in the form of a complex of cholecalciferol, its specific binding protein, calcium and phosvitin, shown schematically in Fig. 9. Precipitation of phosvitin by the addition of water is the first step in the isolation of this protein from either plasma (Heald & McLachlan, 1964) or egg yolk (Mecham & Olcott, 1949). Thus the precipitate that brought down the plasma cholecalciferol-binding protein in the present experiments also contained phosvitin with its associated Ca^{2+}. The cholecalciferol-binding protein could not be released from this precipitate by Triton X-100, but it was found in solution after treatment with EDTA. These properties indicate the formation of the type of complex illustrated in Fig. 9. Such a complex would be dissociated or prevented from forming by chelating the Ca^{2+} with EDTA or by masking the negative charges on the proteins with Na^+.

From the point of view of comparative biology it is noteworthy that cholecalciferol, rather than 25-hydroxycholecalciferol, is the form of vitamin D available to the chick embryo. In contrast, the vitamin D supply to the mammalian foetus is mainly 25-hydroxycholecalciferol, delivered across the placenta during gestation (Haddad *et al.*, 1971; Weisman *et al.*, 1976). The chick embryo, unlike its mammalian counterpart, is independent of maternal regulation of its chemical environment. Thus the selective mechanism incorporating cholecalciferol into yolk gives the chick embryo the opportunity to control its own 25-hydroxycholecalciferol supply through having to produce it itself from the cholecalciferol precursor.

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