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# Relationship between Biotin-Binding Proteins from Chicken Plasma and Egg Yolk

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The plasma of laying hens contains a specific biotin-binding protein that appears to be identical with an egg-yolk biotin-binding protein. Both proteins are saturated with biotin and require elevated temperatures to effect the exchange of [$^{14}$C]biotin for the protein-bound vitamin. The heat-exchange curve in each case is the same and differs sharply from that of avidin, the egg-white biotin-binding protein. On Sephadex G-100 gel filtration, plasma and yolk biotin-binding proteins were each eluted slightly ahead of avidin (mol.wt. 68000), suggesting that they are of similar molecular weight. Plasma and yolk biotin-binding proteins required the same ionic strength to be eluted from a phosphocellulose ion-exchange column. Both the plasma and yolk biotin-binding proteins had a pI of 5; avidin has a pI of 10. Plasma biotin-binding protein cross-reacted with antiserum to yolk biotin-binding protein and showed a precipitin line of identity with purified yolk biotin-binding protein. It is suggested that biotin-binding protein plays an important role in mediating the transport of the vitamin from the bloodstream to the developing oocyte.

As early as 1936 it was suggested that chicken egg-yolk proteins may be derived in a ready-made form from the serum (Roepke & Bushnell, 1936). Much experimental evidence since then has supported the hypothesis that the yolk proteins are synthesized in the liver and transported by the blood stream to the developing oocyte. Williams (1962), for example, showed that the major yolk constituents were identical with serum proteins by means of immunoelectrophoresis, starch-gel electrophoresis and peptide maps. Schjeide *et al.* (1963) injected $^{14}$C-labelled yolk proteins and heterologous serum proteins into the wing veins of laying hens and showed that these proteins were then concentrated in the yolk. They also observed profound changes in the size and secretory ability of the livers from adult hens at the onset of laying and from immature birds after the administration of oestrogenic substances.

The incorporation of serum proteins into the yolk took on an added significance when it was discovered that several of the vitamins necessary for the growth and development of the chicken embryo were protein-bound. The importance of the protein moiety in the deposition of vitamins into the yolk is especially clear in the case of riboflavin. Maw (1954) described a strain of chickens having the hereditary defect riboflavinuria whose affected progeny could survive only when riboflavin was injected directly into the eggs. Winter *et al.* (1967) showed that the source of the problem was the deficiency of a riboflavin-binding protein which existed in identical form in the serum and egg yolk of normal chickens.

Other protein-bound vitamins include vitamin D, in the form of cholecalciferol, and vitamin A (retinol). Vitamin D has a 5–10-fold greater concentration in the yolk than in the bloodstream and is apparently transported by a specific cholecalciferol-binding protein, which is found in chromatographically similar forms in both the yolk and the serum (Fraser & Emtage, 1976). Retinol-binding protein has been isolated and purified from both chicken egg yolk and plasma (Heller, 1976). The binding proteins from each source were similar with respect to molecular weight, electrophoretic mobility, amino acid composition and spectroscopic properties, again indicating a role for the protein in the deposition of vitamin into the developing yolk.

White *et al.* (1976) isolated a biotin-binding protein from egg yolk that displays different characteristics from avidin, the egg-white biotin-binding protein. We now report that a biotin-binding protein exists in the plasma of the laying hen and that similarities between the plasma protein and the yolk biotin-binding protein suggest that they are identical.

## Materials and Methods

### *Collection and preparation of chicken blood and egg yolk*

Chicken blood was obtained from actively laying White Leghorns by means of cardiac puncture. A total of 50ml from five hens was collected into tubes containing heparin (3$\mu$g/ml of blood) and centrifuged for 15min at 5100*g* to remove erythrocytes.

The plasma was removed with a Pasteur pipette. Chicken eggs were obtained commercially, and the egg whites and yolks were carefully separated. The yolks were submerged in water and the adhering egg white was gently removed.

*Delipidation of chicken blood and egg yolk by butan-1-ol*

Chicken plasma and egg yolk were extracted with butan-1-ol. Egg yolk was diluted with water (2:3, v/v) and centrifuged for 1h at 13200*g*. The yolk supernatant and chicken plasma were each made 1M in NaCl. A volume of butan-1-ol equal to 80% of the volume of yolk or plasma was added and the suspensions were centrifuged at 5100*g* for 5min. An aqueous layer and an organic layer were formed as well as a solid intermediate layer. The bottom aqueous layer contains the biotin-binding protein. It was drawn off by aspiration and dialysed overnight against running water. This fraction was used for the studies described below.

*Heat-induced biotin-exchange assay for biotin-binding protein*

Plasma and egg yolk were assayed for biotin-binding activity as described by White *et al.* (1976). Basically, plasma and yolk samples were incubated with 20$\mu$l of D-[*carbonyl*-$^{14}$C]biotin (Amersham/Searle, Arlington Heights, IL, U.S.A.), equivalent to either 0.24$\mu$g (60$\mu$Ci/mg) or 0.24$\mu$g (116$\mu$Ci/mg) at temperatures indicated in the text. The tubes were chilled, 0.3ml of a bentonite suspension (10mg/ml) was added and the assay mixtures were filtered on a Millipore 3025 manifold. The filters were washed three times with 0.05M-potassium phosphate pH7.2, dissolved in 10ml of Instabray (Yorktown Research, South Hackensack, NJ, U.S.A.) and counted for radioactivity in a Beckman liquid-scintillation counter (model LS-3133P).

## Results

*Heat-induced exchange of biotin in chicken plasma*

Egg-white avidin exists primarily in the apoprotein form. Conversely chicken plasma shows very little biotin-binding activity at 24°C suggesting that, like the egg-yolk biotin-binding protein, the biotin-binding sites are almost totally saturated and not easily exchangeable. Raising the temperature, however, does increase the rate of exchange of radioactive biotin for the protein-bound vitamin. The heat-exchange curve obtained for biotin-binding activity in the plasma (Fig. 1) is virtually identical with that for yolk biotin-binding protein (White *et al.*, 1976). Biotin exchange increases with temperature until a maximum is reached at about 70°C; above this temperature denaturation apparently occurs. The thermally induced biotin-exchange reaction of an avidin–biotin complex is markedly different (White *et al.*, 1976) since maximum biotin exchange occurs at 90°C with no significant exchange below 65°C.

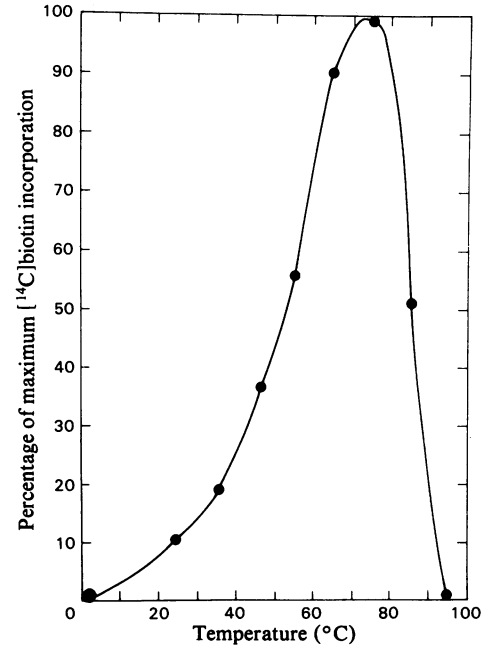


Fig. 1. *Heat-induced exchange of biotin by plasma biotin-binding protein*

Lipid-free chicken plasma was assayed for biotin exchange as described in the Materials and Methods section. Incubation time at each temperature was 15min.

*Gel-filtration chromatography*

A sample of chicken plasma pre-labelled with radioactive biotin and subjected to gel-filtration chromatography shows that the radioactivity is associated with a single peak of a high-molecular-weight component. The elution of a sample containing avidin and radioactively-labelled chicken plasma from a Sephadex G-100 column showed two separate but overlapping peaks (Fig. 2). The plasma biotin-binding activity was eluted slightly ahead of avidin, which has a mol.wt. of approx. 68000. The same behaviour was observed for a mixture of yolk biotin-binding protein and avidin (White *et al.*, 1976), suggesting that the yolk and plasma proteins have similar molecular weights.

*Ion-exchange chromatography*

When egg-yolk biotin-binding protein is applied to a phosphocellulose column and eluted with a

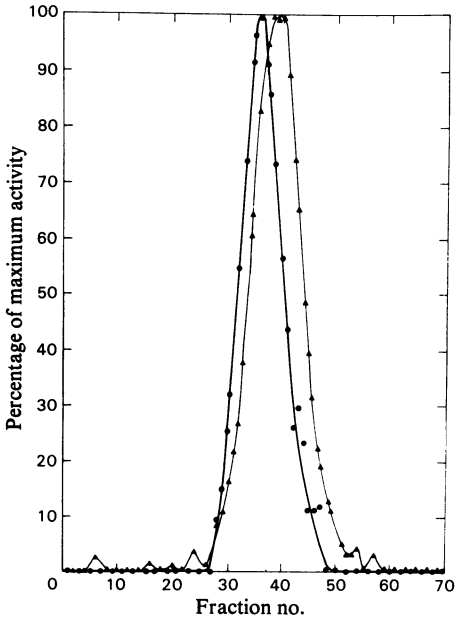


Fig. 2. *Comparison of chicken plasma biotin-binding protein and egg-white avidin by Sephadex G-100 gel filtration*

Plasma biotin-binding protein (●), partially purified by butan-1-ol extraction and phosphocellulose column chromatography, was concentrated in an Amicon Ultrafiltration unit. Sample (0.5ml) of the concentrated binding protein (able to bind $2.5\times10^{-3}$ μg of biotin/ml) was labelled with an excess of [$^{14}$C]-biotin by heat exchange at 65°C and dialysed against 0.5M-$NaHCO_3$, pH8.5. The labelled plasma-binding protein was combined with 0.4mg of commercial avidin (Sigma Chemical Co., St. Louis, MO, U.S.A., 14.1 units/mg) previously saturated with unlabelled biotin (▲). The combined solutions (3ml) were made 10% (w/v) in sucrose and applied to a column (2.5cm×35cm) of Sephadex G-100 equilibrated with 0.5M-$NaHCO_3$, pH8.5. Fractions (1.6ml) were collected and a 0.8ml sample of each fraction was assayed for the radioactively labelled plasma biotin-binding protein. A 0.2ml sample was assayed for avidin activity by the heat-exchange assay at 90°C. At this temperature, the plasma biotin-binding protein is denatured and avidin, which is present in a large excess, can be measured.

potassium phosphate concentration gradient, the major peak of binding activity is eluted from the column at a conductance of $17\times10^3$ ohm$^{-1}$ (Fig. 3*a*). The second peak of activity was identified as avidin from contaminating egg whites as described in the legend to Fig. 3(*a*). The peak of the plasma biotin-binding activity was eluted from the column at the same phosphate concentration as the yolk biotin-binding protein (Fig. 3*b*).

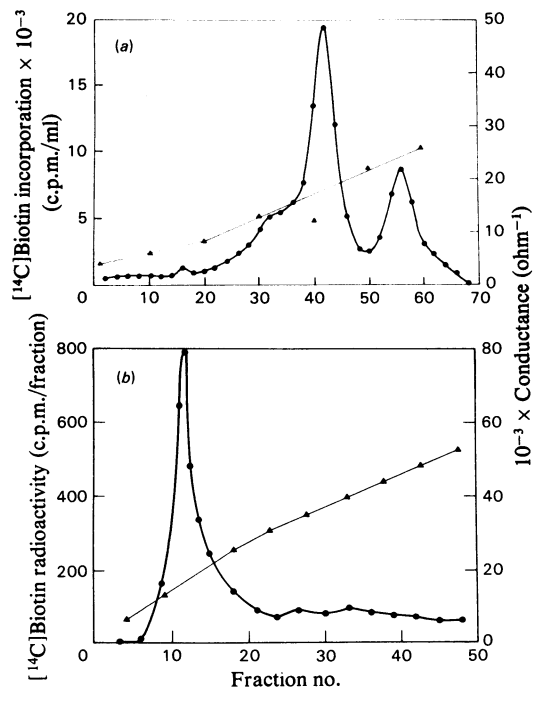


Fig. 3. *Phosphocellulose chromatography of egg-yolk (a) and plasma (b) biotin-binding protein*

(*a*) A lipid-free egg-yolk preparation (1.0litre from 60 eggs) was loaded on to a phosphocellulose column (2.5cm×15cm) equilibrated with 0.05M-potassium phosphate, pH7.2. The column was washed with this buffer until a large peak of protein containing negligible biotin-binding activity (●) was eluted. A linear gradient (▲) (0.05–0.6M-potassium phosphate, pH7.2) was started and 5.0ml fractions were collected. Biotin-binding activity was assayed as described in the Materials and Methods section at a temperature of 65°C. Two peaks of activity were eluted in this preparation. The first peak contained about 80% of the initial biotin-binding activity. The second peak was eluted from the column at the same phosphate concentration needed to elute commercially obtained avidin. This peak also reacts with an antiserum to avidin. The variable appearance of this peak of material was attributed to incomplete separation of egg white and yolk. (*b*) Lipid-free plasma from laying hens (10ml, equivalent to 0.64ng of bound biotin/ml) was labelled with [$^{14}$C]biotin at 60°C for 30min and, after cooling, mixed with 10ml of phosphocellulose equilibrated with 0.05M-potassium phosphate, pH7.2. The column was poured and washed with this buffer until a large peak of protein was eluted. The elution of the column was continued with a phosphate concentration gradient (▲) ranging from 0.05M to 1.0M. Fractions of 2.0ml were collected and 0.5ml of each were assayed for radioactivity (●). Greater than 80% of the chromatographed material was recovered.

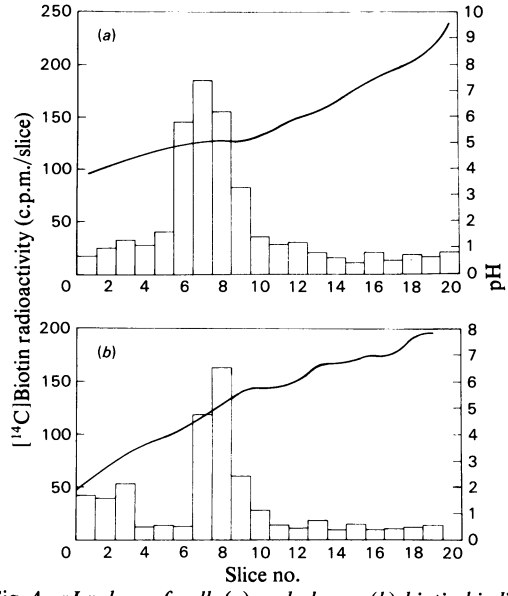


Fig. 4. *pI values of yolk* (*a*) *and plasma* (*b*) *biotin-binding protein*

(*a*) A lipid-free egg-yolk preparation was chromatographed on phosphocellulose as described in Fig. 3. The eluate was dialysed against water and labelled with [$^{14}$C]biotin at 65°C. A 1.26ml sample was incorporated into a 7.5% polyacrylamide isoelectric focusing gel containing pH3–10 ampholytes and focused at 4°C for 6h at a constant power of 4.5W. The gel was sliced into 5mm pieces and soaked overnight in 1.0ml of water to determine pH. The gels were dissolved for scintillation counting by adding 1ml of 30% $H_2O_2$ and incubating for 3h at 80°C (Kobayashi & Harris, 1975). Radioactivity was determined after adding 10ml of Instabray scintillation fluid to the cooled solution. (*b*) Lipid-free chicken plasma was pre-labelled with [$^{14}$C]biotin and treated as described in Fig. 4. The eluate from the phosphocellulose column was dialysed against water and concentrated by freeze-drying. Isoelectric focusing and assay of the gel slices proceeded as described for egg yolk. The peak of radioactivity around pH3 is apparently due to non-protein-bound [$^{14}$C]biotin.

*Isoelectric point*

Plasma and egg-yolk biotin-binding proteins were subjected to isoelectric focusing on polyacrylamide gels. The results (Figs. 4*a* and 4*b*) show that both plasma and yolk biotin-binding proteins have a pI of about 5. The pI of avidin, which is about 10 (Green, 1975), is quite different from the pI of plasma and yolk biotin-binding proteins.

*Immunodiffusion*

In Ouchterlony immunodiffusion tests, antiserum to water-soluble egg-yolk proteins reacts with a partially purified preparation of yolk biotin-binding protein to give several precipitin lines, one of which is a reaction of identity with pure biotin-binding protein from egg yolk. This same precipitin band formed with a partially purified plasma biotin-binding protein. This suggests that the two biotin-binding proteins are immunologically similar (Fig. 5).

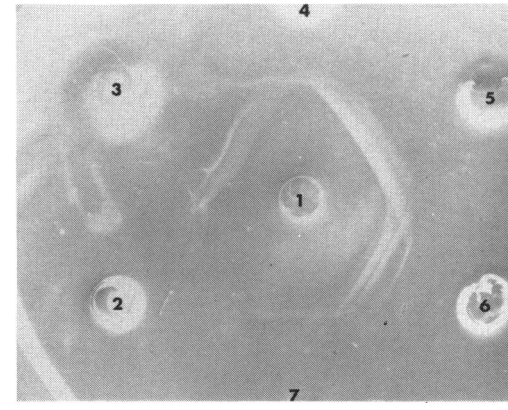


Fig. 5. *Ouchterlony immunodiffusion of avidin* (2), *egg white* (3), *yolk biotin-binding protein* (4 *and* 7), *egg yolk* (5) *and chicken plasma* (6) *against antiserum to water-soluble egg yolk proteins* (1)

Two-dimensional immunodiffusion was run in 1% agar. Samples of 50$\mu$l each were applied to each well. The precipitin bands clearly show a reaction of identity between wells 4, 5, 6 and 7, as well as identity between the several other proteins in wells 5 and 6. Reaction is also observed between wells 1 and 3, demonstrating the presence of several common proteins in both egg white and yolk. No reaction is seen between wells 1 and 2, indicating that avidin is absent from egg yolk.

## Discussion

The existence of apparently identical biotin-binding proteins in the plasma of laying hens and in the yolks of the eggs they lay increases the number of vitamin-binding proteins that have been found in both yolk and blood. This suggests that protein binding may be an important requisite for deposition of these vitamins in the yolk.

The mechanism by which proteins are transported into the developing oocyte is not understood. It appears, however, that specific recognition sites for different yolk proteins may exist on the oocyte plasma membrane. In support of this idea, Cutting & Roth (1973) reported that immunoglobulin G (yolk $\alpha$-livetin) and phosvitin were accumulated at different rates during the early stages of oocyte development.

Later, Yusko & Roth (1976) showed that there were specific binding sites for the serum complex of phosvitin and lipovitellin on the outer surface of chicken oocytes and that unlabelled phosvitin–lipovitellin, but not immunoglobulin G or bovine serum albumin, competed with the binding of the $^{125}$I-labelled complex to the membrane. Other evidence that there may be specific recognition sites for certain yolk proteins was provided by Schjeide *et al.* (1976) in their work on chickens possessing a mutation that suppresses ovulation. The oocytes of these hens incorporated very small amounts of low-density lipoproteins, lipovitellin, phosvitin and other yolk proteins, which then accumulated in the blood at very high concentrations. Plasma albumin, however, seemed to be taken up by the yolks in normal amounts. It was suggested that a possible explanation for this phenomenon was a defect in certain plasma-membrane recognition sites.

With regard to the vitamin-binding proteins then, different recognition processes may be involved in their transport from blood to yolk. Some similarities and differences among the four known vitamin-binding proteins are noteworthy. Yolk biotin-binding protein is a glycoprotein (H. W. Meslar & H. B. White, unpublished work), as are yolk and serum riboflavin-binding proteins (Farrell *et al.*, 1969). This suggests a possible role for the carbohydrate moiety as a recognition site for the uptake of these proteins by the yolk in analogy to the clearance of serum agalacto-glycoproteins by chicken liver (Lunney & Ashwell, 1976). The retinol-binding protein, in contrast, is not a glycoprotein (Heller, 1976). Further, it apparently exists as a complex with pre-albumen and is taken up by the yolk in this form. The carbohydrate content of the vitamin D-binding protein is not known, but it seems to form a complex with $Ca^{2+}$ and phosvitin (Fraser & Emtage, 1976). The formation of a complex with other yolk proteins may therefore play a role in the recognition and uptake of some of these vitamin-binding proteins by the oocyte membrane.

In accord with the induced synthesis of yolk proteins by oestrogenic substances, biotin-binding protein was not found in plasma from immature chickens (R. D. Mandella, unpublished work) using an assay system that could detect the exchange of 4ng of biotin/ml of plasma. In laying hens the amount of protein-bound biotin varied from 7 to 17ng/ml. This is equivalent to 0.1–0.3mg of biotin-binding protein per chicken, assuming a total blood volume of 240ml (Akester, 1971) and that, like avidin, 1mg of biotin-binding protein binds 15$\mu$g of biotin. Chicken egg yolks have been reported to contain 7.4$\mu$g of biotin (Langer & György, 1968) which is equivalent to 0.5 mg of biotin-binding protein. This suggests that turnover of biotin-binding protein in the blood must be fairly rapid to account for the concentration of biotin in the yolk.

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