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Oestrogen Induction of Riboflavin-Binding Protein in Immature Chicks

NATURE OF THE SECRETORY PROTEIN

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The riboflavin-binding protein isolated from sera of oestrogen-treated male chicks as well as that synthesized and secreted *in vitro* by the chicken liver have the same molecular size as that of the egg-yolk protein. Functionally the serum and yolk proteins are similar. This is in contrast with the hormone-induced synthesis, secretion and deposition of phosvitin and lipovitellin in the ovary.

In oviparous species, liver is the exclusive site of biosynthesis and secretion of yolk proteins (Heald & McLachlan, 1963; Tata, 1976), and oestrogen can specifically induce these proteins in the adult male and immature animals of either sex (Tata, 1976; Gruber *et al.*, 1976). Both in amphibia and the domestic fowl, the major yolk proteins, namely phosvitin and lipovitellin, are elaborated only as parts of a macromolecular precursor, namely vitellogenin, which is transported through the blood to be split into constituent molecules in the course of or after uptake by the ovary (Deeley *et al.*, 1975; Gruber *et al.*, 1976). It is not known, however, whether this type of precursor processing is obligatory in the elaboration of other yolk proteins.

Riboflavin-binding protein of hen's egg, a unique phosphoglycoprotein of molecular weight similar to that of phosvitin (approx. 34000), is a minor yolk constituent also inducible specifically by oestrogen (Murthy & Adiga, 1977*a*). The kinetics of its induction in oestrogen-treated male chicks follow a pattern very reminiscent of those of vitellogenin, similarity extending from initial lag phase, through the memory effect during secondary stimulation (Murthy & Adiga, 1977*c*) to the half-life in the circulation (Murthy & Adiga, 1977*b*). In the present communication, we show that riboflavin-binding protein, unlike the major yolk proteins, is secreted by the chicken liver and circulates in the blood in a form indistinguishable functionally and with respect to molecular size from that present in the yolk.

Materials and Methods

L-[^{3}H]Lysine monohydrochloride (sp. radioactivity 1.6Ci/mmol), L-[^{3}H]serine (sp. radioactivity 5.4Ci/mmol) and L-[^{3}H]tyrosine (sp. radioactivity 5.6Ci/mmol) were obtained from the Bhabha Atomic Research Centre, Trombay, Bombay, India. Riboflavin-binding protein was isolated in a homogeneous form from egg white (Murthy & Adiga, 1977*a*) and egg yolk (Ostrowski *et al.*, 1962), and a monospecific antibody against the egg-white riboflavin-binding protein was raised in rabbits as described elsewhere (Murthy *et al.*, 1976). Florisil was obtained through Sigma Chemical Co., St. Louis, MO, U.S.A. The sources of other biochemicals are detailed elsewhere (Murthy & Adiga, 1977*a*).

Isolation of riboflavin-binding protein from the serum

The isolation procedure was essentially the same as described for egg-white riboflavin-binding protein (Murthy & Adiga, 1977*a*) with some minor modifications. Pooled serum from 3-month-old White Leghorn male chicks chronically stimulated with oestradiol-17β (10mg/kg body wt. in propane-1,2-diol, intramuscularly daily for 4 days) was dialysed against 0.1M-sodium acetate buffer, pH4.6, and fractionated on a DEAE-cellulose column (2cm×34cm) with 0.5M-NaCl/0.1M-sodium acetate buffer (pH4.6) as eluent. Further purification was achieved by gel filtration on Sephadex G-100. For more details, see the legend to Fig. 1.

Separation of the flavin from the apoprotein

The pH of the protein solution was adjusted to 3.0 and filtered through a column (1.5cm×20cm) of Sephadex G-25 with 0.1M-sodium citrate buffer, pH3.0, as eluent, to separate the yellow flavin from the apoprotein (Ostrowski *et al.*, 1962).

Purification and identification of the flavin

The flavin fraction was purified by adsorption on a Florisil column (1.5cm×20cm) and elution with aq. 10% (v/v) pyridine (Kumar *et al.*, 1968). Pyridine was removed *in vacuo* and the flavin chromatographed on paper (Giri & Krishnaswamy, 1956), with butanol/

acetic acid/water (4:1:5, by vol.) as solvent and riboflavin, FMN and FAD as standard markers.

Flavin–apoprotein interaction

This was monitored spectrofluorimetrically by measuring the quenching of flavin fluorescence (Murthy *et al.*, 1976).

Polyacrylamide-gel electrophoresis

Electrophoresis on analytical polyacrylamide gels (7.5%, w/v) was performed as described by Davis (1964). The gels were stained for protein (Murthy & Adiga, 1977*a*) and for glycoprotein (Zacharius *et al.*, 1969). Labelled riboflavin-binding-protein–antibody complex was analysed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis by the procedure of Palmiter *et al.* (1971). After electrophoresis, the gels were sliced into 2mm-thick discs and individual discs were digested and counted for radioactivity (Young & Fullhorst, 1965).

Labelling of riboflavin-binding protein with ^3H-labelled amino acids in vitro

Livers quickly excised from chicks 24h after a single injection of oestradiol-17β (10mg/kg body wt.) were cut into 1mm-thick slices and approx. 3g of the tissue was incubated with shaking at 37°C for 4h in Eagle's essential minimal medium (pH7.4, 9ml), devoid of unlabelled tyrosine, lysine and serine, but supplemented with streptomycin (100μg/ml) pencillin (100μg/ml), $NaHCO_3$ (2.2mg/ml) and the three radioactive amino acids (10μCi each/ml). After incubation, the medium was separated from the tissue and analysed for secreted labelled riboflavin-binding protein. Washed tissue slices were homogenized in 10vol. of 0.01M-phosphate-buffered saline, pH7.4 (0.9% NaCl in 0.01M-sodium/potassium phosphate buffer, pH7.4), and the homogenates clarified by centrifugation at 140000*g* for 60min. The cytosolic supernatant was analysed for incorporation of ^3H-labelled amino acid into riboflavin-binding protein after immunoprecipitation and sodium dodecyl sulphate/polyacrylamide-gel electrophoresis.

Immunoprecipitation of labelled riboflavin-binding protein

To 0.5ml portions of the tissue homogenate or the incubation media was added 50μg of pure riboflavin-binding protein as carrier followed by an excess (0.2ml) of the monospecific antibody to riboflavin-binding protein and sodium deoxycholate (Luskey *et al.*, 1974) in phosphate-buffered saline to a final concentration of 1% (total volume 1.5ml). After incubation at 37°C for 1h and overnight at 4°C, the immunoprecipitate was washed by centrifugation three times at 2000*g* for 20min with cold phosphate-buffered saline and subjected to sodium dodecyl sulphate/polyacrylamide-gel electrophoresis as described above.

Results and Discussion

Implicit in earlier studies of the mode of elaboration of several polypeptide hormones (Lernmark *et al.*, 1976) and yolk proteins (Deeley *et al.*, 1975) is the basic assumption that the precursors of these proteins share immunological characteristics with their respective products. In fact, the conclusive proof that vitellogenin is the biosynthetic and circulating macromolecular precursor for phosvitin and lipovitellin in amphibia (Berridge & Lane, 1976) and in chicks (Deeley *et al.*, 1975) stems from experiments based on this premise. With this consideration in view, we have ascertained by double-diffusion analysis on agar (Murthy *et al.*, 1976) and using the monospecific antibody to riboflavin-binding protein that there is only one immunoactive species in the sera of oestrogen-treated chicks. This sensitive test was routinely used to monitor the progress of fractionation and to ensure that no cross-reacting material is lost during the process. Since riboflavin-binding protein is a minor yolk constituent as well as a serum constituent, its isolation in significant amounts was considered necessary for its physicochemical and functional characterization. Advantage was taken of several known properties of egg riboflavin-binding proteins (Murthy & Adiga, 1977*a*; Ostrowski *et al.*, 1962), and the pooled sera were processed at 0–4°C to minimize proteolytic activity. Although dialysis against 0.1M-acetate buffer facilitated removal by precipitation of considerable amounts of extraneous serum proteins, during chromatography on DEAE-cellulose more than 80% of the remaining materials were washed off the column. Subsequent elution with 0.5M-NaCl resulted in a protein fraction (Fig. 1*a*) accounting for over 95% of riboflavin-binding protein, as revealed by a quantitative immunoprecipitation procedure. Dialysis of this turbid yellow fraction against water led to further precipitation of nearly 85% of the other inactive proteins, which were removed by centrifugation (10000*g* for 10min). The clear yellow supernatant was shown by disc gel electrophoresis to contain two major glycoproteins in almost equal proportions, one of which had a mobility identical with that of purified yolk riboflavin-binding protein.

Further purification was sought by gel filtration on a Sephadex G-100 column (Fig. 1*b*). Nearly 50% of the material was devoid of immunological cross-reactivity and was of apparently higher molecular weight (Fig. 1*b*, peak A). The protein fraction subsequently eluted with an absorbance at both 280nm and 455nm (Fig. 1*b*, peak B) represented purified riboflavin-binding protein. From 44ml of serum, a yield of 5mg of purified protein was obtained.

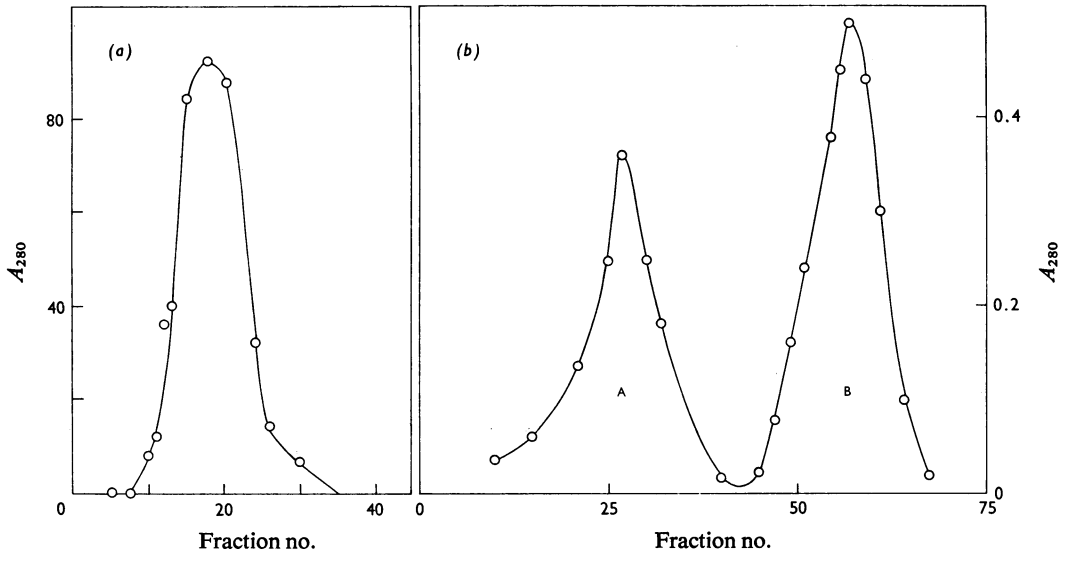

Fig. 1. *Elution of riboflavin-binding protein from sera of oestrogen-treated chicken from a DEAE-cellulose column (a) followed by elution from a Sephadex G-100 column (b)*

(*a*) Chicken sera (44ml), dialysed against two changes of 0.1M-acetate buffer, pH4.6, and clarified, were loaded on a DEAE-cellulose column (2cm×34cm) pre-equilibrated with 0.1M-acetate buffer, pH4.6. The column was washed with the starting buffer (100ml) until the effluent was free of A_{280}-absorbing material and eluted with 0.5M-NaCl; fractions of volume 2ml were collected. Fraction nos. 10–30 were pooled, dialysed against water, clarified by centrifugation and concentrated (crude riboflavin-binding protein). (*b*) Crude riboflavin-binding protein (10mg, 1ml) eluted from DEAE-cellulose was loaded on a Sephadex G-100 column (2cm×63cm) pre-equilibrated and eluted with 0.5M-NaCl in 0.02M-phosphate buffer, pH7.4. Fractions of volume 2ml were collected.

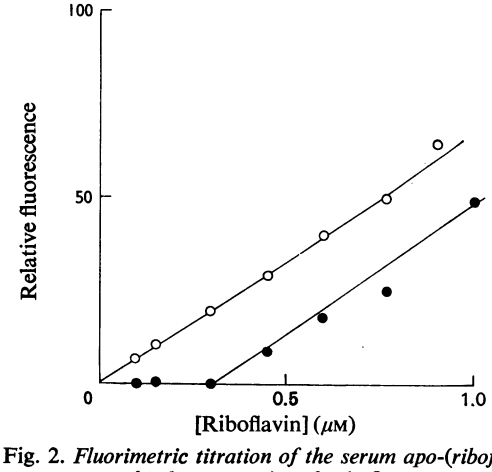

Fig. 2. *Fluorimetric titration of the serum apo-(riboflavin-binding protein) with riboflavin*

Fluorescence of riboflavin was excited at 370nm and analysed at 520nm. The amount of riboflavin at the indicated final concentration was added to 3.5ml of the apoprotein solution (38μg) in 0.1M-phosphate buffer, pH7.0. ○, Buffer alone; ●, apoprotein in the buffer. Fluorescence is given relative to standard riboflavin.

The purified protein on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis exhibited a sharp major band with a mobility identical with that of yolk riboflavin-binding protein (M_r36000) and a minor (<5%) diffuse band of slightly higher molecular weight. Co-electrophoresis could not separate this major protein band from the yolk riboflavin-binding protein.

To study the interaction with riboflavin, the apoprotein was resolved from the flavin by gel filtration on a Sephadex G-25 column as described above. The flavin purified through a Florisil column was identified as exclusively riboflavin by paper chromatography. The apoprotein, when titrated fluorimetrically against increasing amounts of riboflavin, completely quenched the flavin fluorescence and gave a sharp end point (Fig. 2) as for egg-white and egg-yolk riboflavin-binding protein (Rhodes *et al.*, 1959; Ostrowski *et al.*, 1968). Since the molecular weight of the serum riboflavin-binding protein was equal to that of yolk riboflavin-binding protein (36000), it could be calculated that the apoprotein–flavin interaction occurred in 1:1 molar ratio, as it does with egg-yolk (Ostrowski *et al.*, 1968) and egg-white riboflavin-binding protein

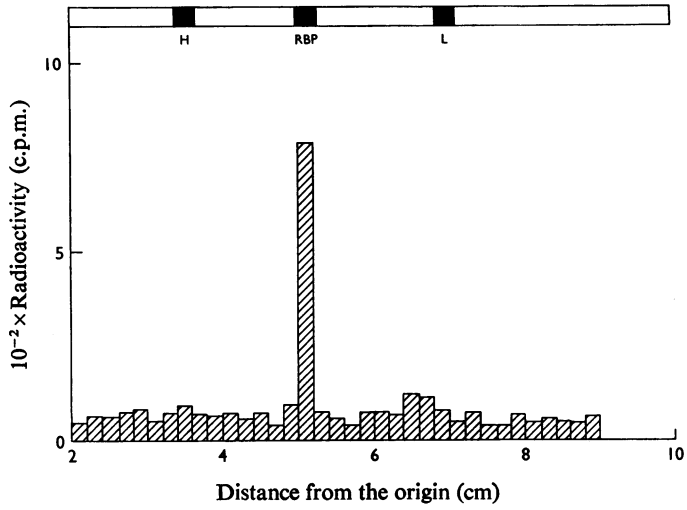

Fig. 3. *Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of the immunoprecipitate*
Liver slices (3 g) from chicks treated with oestrogen for 24 h were incubated for 4 h in the ^{3}H-labelled amino acid mixture as described in the Materials and Methods section. Samples (0.5 ml) of the homogenate were immunoprecipitated with riboflavin-binding protein (RBP) antiserum (0.2 ml) along with 50 μg of pure protein. The immunoprecipitate was washed and electrophoresed on sodium dodecyl sulphate/polyacrylamide gels. H and L refer to heavy-chain and light-chain of immunoglobulin respectively.

(Rhodes *et al.*, 1959). Furthermore, like yolk protein, the protein lost its capacity to bind riboflavin on freeze-drying, probably owing to aggregation (Ostrowski *et al.*, 1968).

Thus, clearly, the riboflavin-binding protein isolated from sera of oestrogen-treated chicken was indistinguishable from yolk riboflavin-binding protein in terms of molecular size and functional characteristics, in contrast with the major oestrogen-induced yolk proteins, namely lipovitellin and phosvitin in amphibia (Tata, 1976) and chicken (Gruber *et al.*, 1976) systems. Isolation of phosvitin directly from the plasma by using lipase is apparently the result of the susceptibility of vitellogenin to a proteolytic contaminant of the lipase (Bergink *et al.*, 1973). Although we have taken care to minimize the degradative processes by conducting the protein isolation at 0–4°C, to substantiate our findings further the nature of newly synthesized and secreted riboflavin-binding protein *in vitro* was examined. Liver slices (freed of blood) from oestrogen-treated birds were incubated *in vitro* with ^{3}H-labelled amino acids and labelled protein in the cytosol and that secreted into the medium was immunoprecipitated and analysed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. Fig. 3 depicts the distribution of radioactivity throughout the gel. Besides stainable bands corresponding to light and heavy chains of the immunoglobulin, the only other protein band, accounting for almost all the radioactivity applied to the gel, corresponded in size to yolk riboflavin-binding protein. Identical patterns were obtained with either the liver cytosol or the labelled protein secreted into the medium, in agreement with qualitative observation made with the sera from oestrogen-treated chicken.

Thus it appears that riboflavin-binding protein is secreted by the liver into the systemic circulation and taken up from there by the ovarian follicles without any gross size and functional modification as for retinol-binding protein of the same source (Heller, 1976). Further, the demonstration that the immunological activity resides entirely with only one molecular species with full biological potency (riboflavin-binding) supports our monitoring the protein in the sera of oestrogen-treated birds by radioimmunoassay (Murthy & Adiga, 1977*a*). This is particularly pertinent, since immunological methods generally do not distinguish between biologically active and inactive or degraded molecules as exemplified by the observations on growth-hormone concentrations in acromegalic patients (Gordon *et al.*, 1976) and parathyroid hormone (Habener, 1976) in the circulation. These data, however, do not preclude the possibility that riboflavin-binding protein is, in fact, synthesized intracellularly as a slightly larger precursor and processed rapidly during translation on polyribosomes and/or transit and packaging through cisternae of endoplasmic reticulum and Golgi bodies, as has been demonstrated for pro-

insulin-insulin conversion and a number of other polypeptide hormones (Lernmark *et al.*, 1976; Campbell & Blobel, 1976) and serum albumin (Judah *et al.*, 1971). Further experiments with purified mRNA specific for the protein and translation in a heterologous cell-free system should clarify this possibility.

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