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Isolation and Characterization of Thiamin-Binding Protein from Chicken Egg White

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A thiamin-binding protein was isolated and characterized from chicken egg white by affinity chromatography on thiamin pyrophosphate coupled to aminoethyl-Sepharose. The high specificity of interaction between the thiamin-binding protein and the riboflavin-binding protein of the egg white, with a protein/protein molar ratio of 1.0, led to the development of an alternative procedure that used the riboflavin-binding protein immobilized on CNBr-activated Sepharose as the affinity matrix. The thiamin-binding protein thus isolated was homogeneous by the criteria of polyacrylamide-gel disc electrophoresis, double immunodiffusion and sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, had a mol.wt. of 38000±2000 and was not a glycoprotein. The protein bound [^{14}C]thiamin with a molar ratio of 1.0, with dissociation constant (K_d) 0.3 μM.

The mode of deposition in adequate amounts of some of the vitamins and other micronutrients in the chicken egg for the 21-day development of the prospective embryo involves the participation of carrier proteins specific for each of these nutrients. This is exemplified by the discovery in recent years of the binding proteins for biotin (Eakin *et al.*, 1940; White *et al.*, 1976), riboflavin (Rhodes *et al.*, 1959; Murthy & Adiga, 1977), vitamin B_{12} (Sonneborn & Hensen, 1970), retinol (Abe *et al.*, 1975) and cholecalciferol (Fraser & Emtage, 1976). An intriguing aspect of these proteins is the higher specificity and avidity of their interaction with the free vitamins than with the respective active metabolites/coenzyme forms. This property is presumably advantageous to the embryo in terms of the control of its supply of active metabolites, since this enables the embryo to produce the metabolites itself at rates and in forms physiologically most appropriate for proper growth and development.

Thiamin (vitamin B_1), a normal constituent of the chicken egg, participates in a number of biochemical reactions vital to embryonic development and survival (Coates, 1971). Deprival of this vitamin results in the accumulation of lactate and pyruvate in embryonic tissues (Polin *et al.*, 1962). The importance of the adequate deposition of this vitamin in the egg is further demonstrated by the high rate of embryonic mortality on administration of anti-thiamin drugs either directly into hatching eggs or by dietary supplementation to the laying hens (Naber *et al.*, 1954). However, the mode of transport of this essential nutrient from the maternal system to the developing oocyte has not been examined so far. By analogy with the transport of other vitamins, it is most likely that thiamin deposition in the avian egg is also carrier-protein-mediated, notwithstanding a study in which the existence of a binding protein for this vitamin could not be demonstrated (White *et al.*, 1976). In the present paper we report the isolation of a thiamin-binding protein from the chicken-egg white by the technique of affinity chromatography and some of its physicochemical properties.

Abbreviation used: SDS, sodium dodecyl sulphate.

Materials and Methods

Fresh eggs from White Leghorn hens were procured from the Poultry Farm of the University of Agricultural Sciences, Hebbal, Bangalore, India. Sepharose 4B, 3-(3-dimethylaminopropyl)-1-ethylcarbodi-imide, thiamin pyrophosphate, thiamin, riboflavin, cytochrome *c*, chymotrypsinogen, ovalbumin and yeast alcohol dehydrogenase were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. *NNN'N'*-Tetramethylethylenediamine was a product of Canalco, Bethesda, MD, U.S.A. Acrylamide and *NN'*-methylenebisacrylamide were purchased from Koch–Light Laboratories, Colnbrook, Bucks., U.K. Sephadex was from Pharmacia Fine Chemicals, Uppsala, Sweden. CNBr was synthesized by the procedure of Hartman & Drager (1931). Aquacide was a product of California Foundation for Biochemical Research, Los Angeles, CA, U.S.A. [*thiazole*-2-^{14}C]Thiamin was obtained from The Radiochemical Centre, Amersham, Bucks., U.K., and had a specific radioactivity of 14 mCi/mmol.

Apo-(riboflavin-binding protein) from the egg white was purified by the procedure of Murthy & Adiga (1977). Other chemicals and reagents were of analytical grade.

Coupling of thiamin pyrophosphate to Sepharose 4B

Sepharose was activated with CNBr by the procedure of Marsh *et al.* (1974). Briefly, to the washed gel (10ml) suspended in an equal volume of 2M-Na_2CO_3, CNBr in distilled acetonitrile was added to a final concentration of 200mg/ml of the gel. After vigorous stirring at 4°C for 2min, the slurry was filtered and washed successively with cold 0.1M-$NaHCO_3$ (pH9.5), water and then with 0.2M-$NaHCO_3$ (500ml each). The moist cake was stirred for 12h at 4°C with 6mM-ethylenediamine in an equal volume of 0.2M-$NaHCO_3$. The gel was then washed thoroughly with 500ml each of 0.1M-sodium acetate buffer (pH4.0), 2M-urea and 0.1M-$NaHCO_3$ (pH9.5); all these solutions contained 0.5M-NaCl. The presence of the amine bound to the gel was ascertained by reaction with picrylsulphonic acid (Cuatrecasas, 1970). To the resulting aminoethyl-Sepharose suspended in water at pH6.4, a mixture of 100mg of thiamin pyrophosphate and 300mg of 3-(3-dimethylaminopropyl)-1-ethylcarbodi-imide was added over a period of 10min. After stirring for 16h at 4°C, the gel was extensively washed with cold water and then with 0.1M-sodium phosphate buffer (pH7.0) and used directly (Matsuura *et al.*, 1973).

Immobilization of apo-(riboflavin-binding protein) to Sepharose 4B

Apo-(riboflavin-binding protein) (40mg) was covalently linked to CNBr-activated Sepharose (10ml) with gentle stirring at 4°C for 18h in 10ml of 0.1M-$NaHCO_3$ buffer, pH9.5. Glycine (100mg) was then added to block the unoccupied reactive sites and stirring continued for 10h. The gel was then washed successively with 500ml each of 0.1M-sodium acetate buffer, pH4.0, 2M-urea and 0.1M-$NaHCO_3$ buffer. About 3mg of apo-(riboflavin-binding protein) was covalently linked to 1ml of Sepharose, as ascertained by the amount of riboflavin bound to the immobilized protein. The flavin-binding capacity was determined by saturating the gel-bound protein (used as a column, 5ml bed volume) with riboflavin. After the excess of flavin was washed off completely with 0.1M-sodium phosphate buffer (pH7.0)/1M-NaCl, that held by the immobilized protein was eluted with 0.1M-sodium citrate buffer, pH3.0 (Murthy & Adiga, 1978), and quantified spectrophotometrically by using $\varepsilon = 1.25 \times 10^4 M^{-1} \cdot cm^{-1}$ (Whitby, 1953) and by assuming a 1:1 molar stoicheiometry of the ligand–protein interaction (Murthy *et al.*, 1976).

Preparation of the crude egg-white protein for affinity chromatography

Unless otherwise stated, all the steps were carried at 4°C. The egg white, physically separated from the yolk, was sonicated for 2min at 4mA at 4°C by using a Branson sonifier (model S 75), and to the dispersed material (250ml) was added solid $(NH_4)_2SO_4$ to a final saturation of 90%. The precipitated protein was recovered by centrifugation at 15000*g* for 20min, redissolved in 250ml of 0.05M-sodium phosphate buffer, pH7.0, and dialysed extensively against water. The pH of the protein solution was then lowered with continuous stirring to 3.5 with 1M-acetic acid and the protein dialysed against 8vol. of 0.1M-sodium acetate buffer (pH3.5). After dialysis the pH of the protein fraction was re-adjusted to 7.0 with 1M-NaOH.

Polyacrylamide-gel disc electrophoresis

Analytical gel electrophoresis was carried out by the procedure described by Maurer (1968) on 7.5% (w/v) gels at 4mA/tube for 2h at 25°C. For analysis of thiamin-binding protein, 0.035M-β-alanine/acetic acid buffer (pH4.5), and for riboflavin-binding protein, 0.025M-Tris/glycine buffer (pH8.3), was used. The proteins on the gels were stained with Coomassie Brilliant Blue R-250 and destained with methanol/acetic acid (3:2, v/v). Staining for the glycoproteins was carried out as described by Zacharius *et al.* (1969).

Immunochemical techniques

Antibodies to the purified thiamin-binding protein were raised in albino rabbits. The protein dissolved in 0.01M-sodium phosphate buffer (pH7.2)/0.15M-NaCl (1mg/ml) and emulsified with an equal volume of Freund's complete adjuvant (Difco Laboratories, Detroit, MI, U.S.A.) was injected subcutaneously at multiple points at weekly intervals. After 4 weeks, a booster dose of 0.5mg of protein in 0.01M-sodium phosphate/0.15M-NaCl was administered. After 5 days, the rabbits were bled through the ear vein, and serum was prepared. Double immunodiffusion analysis on agar was performed as described by Ouchterlony (1958).

Molecular-weight determination

The molecular weight of the protein was estimated by gel filtration (Andrews, 1965) on Sephadex G-100 by using 0.1M-sodium phosphate buffer (pH7.0)/1M-NaCl as the eluent. Electrophoresis on SDS/polyacrylamide gels as described by Weber & Osborn (1969) was also used for this purpose. The following proteins of known molecular weight served as markers in both the procedures: cytochrome *c* (13700), chymotrypsinogen (25000), bovine serum albumin (68000, monomer), ovalbumin (44000) and yeast alcohol dehydrogenase (125000).

[^{14}C]Thiamin binding

Thiamin-binding activity of the isolated protein was measured by equilibrium dialysis in a Perspex

(Lucite) dialysis cell. For this, 1.0ml of the sample protein (1mg) in 0.1M-sodium phosphate buffer, pH7.0, was allowed to equilibrate for 48h at 30°C with various known concentrations of [^{14}C]thiamin in the same buffer. After the establishment of equilibrium, the radioactivities in both the compartments of the dialysis cell were quantified by liquid-scintillation spectrometry by using a Beckman LS-100 instrument (Murthy & Adiga, 1978). Protein was measured by the method of Lowry *et al.* (1951), with bovine serum albumin as the standard.

Results

Affinity chromatography on thiamin pyrophosphate–Sepharose

When the processed crude protein from egg white was applied to a thiamin pyrophosphate–Sepharose column and the column was washed extensively with the equilibration buffer (0.1M-sodium phosphate buffer, pH7.0) until the effluent was free of material absorbing at 280nm, the vast majority of the proteins were excluded from the affinity matrix as monitored spectrophotometrically. The proteins adsorbed on the column were then eluted stepwise with 0.1M-sodium phosphate buffer containing 1M- and 4M-NaCl, followed by the buffer with 4M- and 8M-urea (Fig. 1). A major protein peak (peak A) representing nearly 75% of that held on the affinity matrix was eluted with 1M-NaCl. Increasing the salt concentration to 4M or subsequent elution with 4M-urea failed to dislodge any additional protein. Only when the urea concentration was raised to 8M did a second symmetrical protein peak emerge (peak B). After dialysis and concentration to a small volume, only peak-B protein, but not peak-A frac-

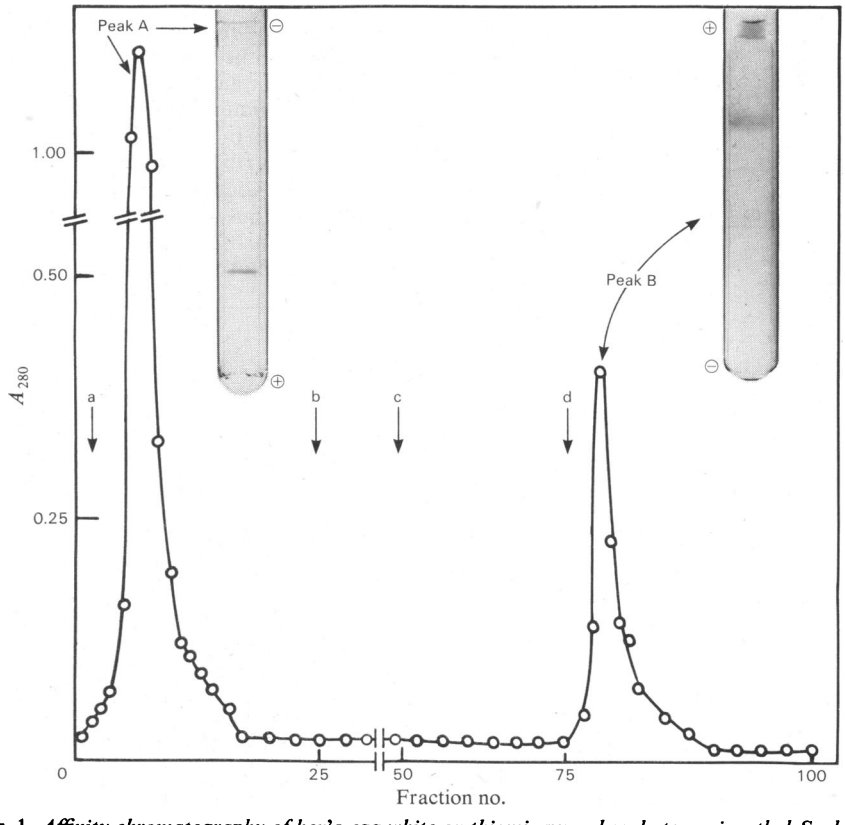

Fig. 1. *Affinity chromatography of hen's-egg white on thiamin pyrophosphate–aminoethyl-Sepharose*
The column (1.2cm×7cm) was equilibrated with 0.1M-sodium phosphate buffer, pH7.0. Crude egg-white protein solution (250ml) was applied on the column at a flow rate of 30ml/h. The column was washed with the same buffer until the effluent was devoid of material absorbing at 280nm. At the indicated intervals, the following eluting agents were used and 2ml fractions collected: a, 1M-NaCl in 0.1M-sodium phosphate buffer, pH7.0; b, 4M-NaCl in 0.1M-sodium phosphate buffer, pH7.0; c, 4M-urea in 0.1M-sodium phosphate buffer, pH7.0; d, 8M-urea in 0.1M-sodium phosphate buffer, pH7.0. Inset: polyacrylamide-gel electrophoresis of peak A (left) and peak B (right).

tion, was able to bind a significant amount of [^{14}C]-thiamin as determined by equilibrium dialysis with an apparent $K_a = 28\,mM^{-1}$, assuming the mol.wt. of the protein to be 38000±2000 (see below). Elution with 0.1M-sodium acetate buffer, pH3.5, instead of urea yielded a symmetrical protein peak (Fig. 2, peak B) that bound the radiolabelled thiamin with an affinity comparable with that obtained by the urea-elution method. Thus it is clear that although a specific thiamin-binding protein could be identified in the egg white, its affinity for the ligand was very much lower than those reported for protein–ligand interaction of other egg-white vitamin-binding proteins, riboflavin-binding protein, $K_a = 77\,\mu M^{-1}$ (Murthy *et al.*, 1976) or avidin, $K_a = 1\,fM^{-1}$ (Green, 1963). Among the several possibilities considered, it appeared likely that the rather drastic conditions used to dissociate the protein from the affinity matrix might have resulted in partial denaturation leading to an apparent low K_a of the protein for its binding to [^{14}C]thiamin.

Polyacrylamide-gel disc electrophoresis of peak-A and -B fractions revealed a single protein band in both cases, indicating the absence of cross-contamination (Fig. 1, insert).

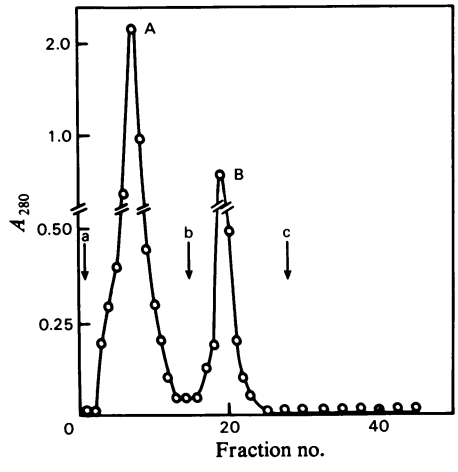

Fig. 2. *Elution profile of hen's-egg-white protein from thiamin pyrophosphate–aminoethyl-Sepharose*

The column (1.2cm×8cm) was pre-equilibrated with 0.1M-sodium phosphate buffer, pH7.0. Crude egg-white-protein solution (250ml) was applied and the column was washed with the same buffer until there was no material absorbing at 280nm. The column was eluted successively with a, 1M-NaCl in 0.1M-sodium phosphate buffer, pH7.0, b, 1M-NaCl in 0.1M-sodium acetate buffer, pH3.5, and c, 8M-urea in 0.1M-sodium phosphate buffer, pH7.0, and the effluent collected in 2ml fractions.

Identification of peak-A protein as riboflavin-binding protein

The symmetrical protein peak eluted from the thiamin pyrophosphate–Sepharose column by 1M-NaCl (Fig. 1) was pale yellow in colour and exhibited significant absorbance at 445nm (in addition to that at 280nm), indicative of the presence of bound flavin (Murthy & Adiga, 1978). As mentioned above, it was homogeneous by the criterion of polyacrylamide-gel disc electrophoresis, in which it moved with a mobility identical with that of the purified egg-white riboflavin-binding protein (Murthy & Adiga, 1977); in fact, co-electrophoresis could not separate the two proteins. The following additional evidence unequivocally established its identity with riboflavin-binding protein: (1) it could be stained positively for glycoprotein (Zacharius *et al.*, 1969) after gel electrophoresis, as was the case with riboflavin-binding protein; (2) on double-immunodiffusion analysis on agar against monospecific antiserum to riboflavin-binding protein (Murthy & Adiga, 1977) it gave a single precipitin line confluent with that corresponding to the purified riboflavin-binding protein (Fig. 3); (3) the flavin could be dissociated by exposure to sodium citrate buffer (pH3.0) and separated from the apoprotein by gel filtration on Sephadex G-25 and identified as riboflavin by paper chromatography (Murthy & Adiga, 1978). The apoprotein thus obtained could quench flavin fluorescence when a fixed concentration of the protein was titrated spectrofluorimetrically (Nishikimi & Kyogaku, 1973) against

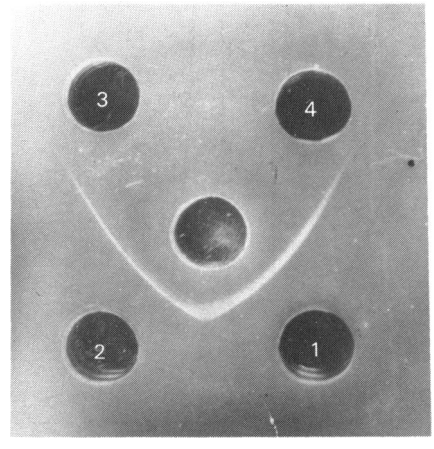

Fig. 3. *Double-immunodiffusion analysis of riboflavin-binding protein*

The centre well contained antiserum to riboflavin-binding protein. Peripheral wells contain: 1, authentic riboflavin-binding protein; 2, peak-A sample from thiamin pyrophosphate–aminoethyl-Sepharose; 3, thiamin-binding protein; 4, ovalbumin; 20μg of protein was applied in each case.

various concentrations of riboflavin, with a K_a comparable with that reported (Murthy *et al.*, 1976) for the purified egg-white riboflavin-binding protein (results not shown).

The above observations clearly showed that, among the several egg-white proteins, only two apparently pure proteins, namely the thiamin-binding entity and the riboflavin-binding protein, are specifically retained on thiamin pyrophosphate–Sepharose and that, as expected, the latter was held less tenaciously than the thiamin-binding protein. The observation that 1M-NaCl could readily dislodge the riboflavin-binding protein from the affinity matrix suggested that the association of the riboflavin-binding protein with the affinity matrix was not direct, but through interaction with the thiamin-binding protein. The specificity of this association was further established by gel filtration on Sephadex G-75. In the presence of 1M-NaCl, the two proteins mixed in nearly equal proportions were eluted from the gel as two distinct, though overlapping, peaks (Fig. 4*b*), whereas in the absence of the salt they emerged from the gel as a single complex of much higher molecular weight (Fig. 4*a*). Since the molecular weights of the two individual proteins are very close (riboflavin-binding protein 32000±2000, thiamin-binding protein 38000±2000; see below), it is suggested that they interact in 1:1 molar ratio to form a tight complex.

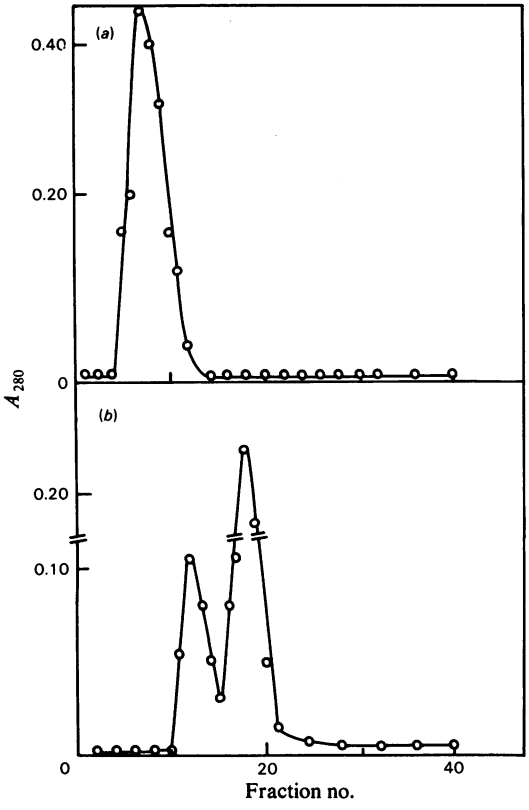

Fig. 4. *Chromatography of the riboflavin-binding protein and the thiamin-binding protein on Sephadex G-75*
Samples (2mg) of each of the proteins were mixed and incubated at 37°C in 0.1M-sodium phosphate buffer, pH7.0, for 1h. The column (1cm×80cm) was pre-equilibrated with the same buffer and the eluate collected in 1.5ml fractions. Eluents: (*a*) 1M-NaCl in 0.1M-sodium phosphate buffer (pH7.0); (*b*) 0.1M-sodium phosphate buffer (pH7.0) without NaCl.

Isolation of thiamin-binding protein by affinity chromatography on riboflavin-binding-protein–Sepharose

The high specificity of complex-formation between the two proteins and the ease with which this protein–protein interaction could be curtailed led us to explore an alternative but milder procedure for the isolation of the thiamin-binding protein. Purified riboflavin-binding protein from egg white (Murthy & Adiga, 1977) was coupled to CNBr-activated Sepharose and the processed egg-white proteins were passed through the affinity matrix used as a column. After removal of extraneous protein by extensive washing with the equilibration buffer, the proteins still held on the gel were then eluted with 1M-NaCl/0.1M-sodium phosphate buffer (pH7.0). The elution profile exhibited a single sharp protein peak (Fig. 5). However, polyacrylamide-gel disc electrophoresis of this

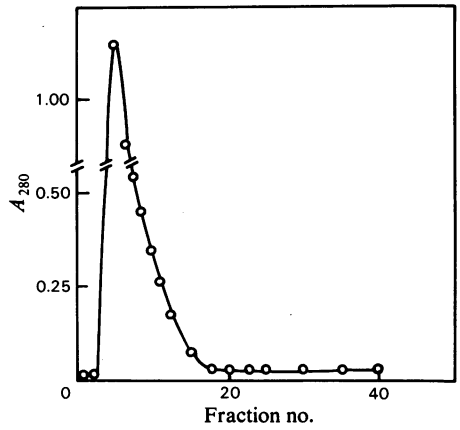

Fig. 5. *Chromatography of crude egg-white protein on riboflavin-binding-protein–Sepharose*
The column (1.2cm×12cm) was equilibrated with 0.1M-sodium phosphate buffer, pH7.0. Processed egg-white protein (250ml) was applied on the column with a flow rate of 30ml/h. All non-specific proteins were washed off with the same buffer and the column was then eluted with 1M-NaCl in 0.1M-sodium phosphate buffer, pH7.0, at a flow rate of 10ml/h; 2ml fractions were collected.

protein revealed two protein bands, one of which corresponded to the thiamin-binding protein isolated from the thiamin pyrophosphate–Sepharose. Further purification of the component corresponding to thiamin-binding protein could be achieved by gel filtration on Sephadex G-75 (Fig. 6, peak B). The nature of the protein peak A, apparently of higher molecular weight, is not known at present.

The protein corresponding to peak B, after concentration over Aquacide to a small volume, was tested for its identity with the thiamin-binding protein isolated by the method using thiamin pyrophosphate–Sepharose. On analytical disc electrophoresis it exhibited a single compact band identical in mobility with that eluted by 8M-urea from thiamin pyrophosphate–Sepharose. When stained on the gel as described by Zacharius *et al.* (1969) it did not take up any stain, showing that, unlike the riboflavin-binding protein, it was not a glycoprotein. Gel filtration on Sephadex G-100 or electrophoresis on SDS/polyacrylamide gels revealed that the protein was homogeneous by these criteria also and had a mol.wt. of 38000 ± 2000 with no detectable dissociation into subunits. When analysed by double immunodiffusion by using a potent antiserum raised against the purified protein, it gave a single precipitin line, whereas the purified riboflavin-binding protein and ovalbumin were devoid of any detectable cross-reactivity against the antiserum (Fig. 7).

When a fixed concentration of isolated protein was allowed to equilibrate with [^{14}C]thiamin in a dialysis cell, it avidly bound the labelled ligand. When the amount of the protein-bound thiamin was plotted against the concentration of thiamin (Fig. 8) a

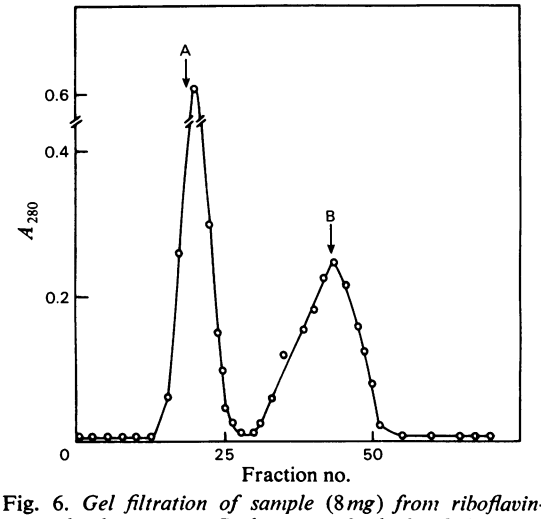

Fig. 6. *Gel filtration of sample (8mg) from riboflavin-binding-protein–Sepharose on Sephadex G-75*
The column (1.2cm×80cm) was equilibrated with 0.1M-sodium phosphate buffer, pH7.0, at a flow rate of 10ml/h; 2ml fractions were collected.

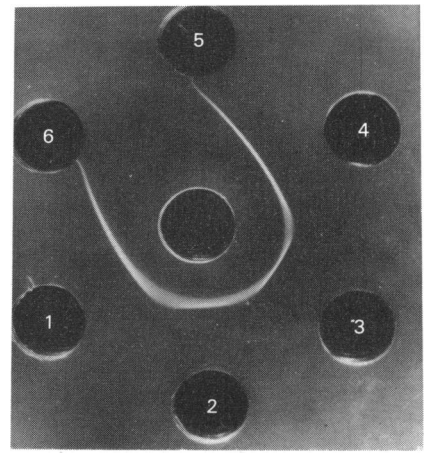

Fig. 7. *Double-immunodiffusion analysis of the thiamin-binding protein*
The centre well contained antiserum to thiamin-binding protein. Peripheral wells contained (25μg of protein): 1, thiamin-binding protein from thiamin pyrophosphate–Sepharose; 2, thiamin-binding protein from riboflavin-binding-protein–Sepharose; 3, crude yolk proteins; 4, oestrogen-stimulated immature-chicken serum; 5, riboflavin-binding protein; 6, ovalbumin.

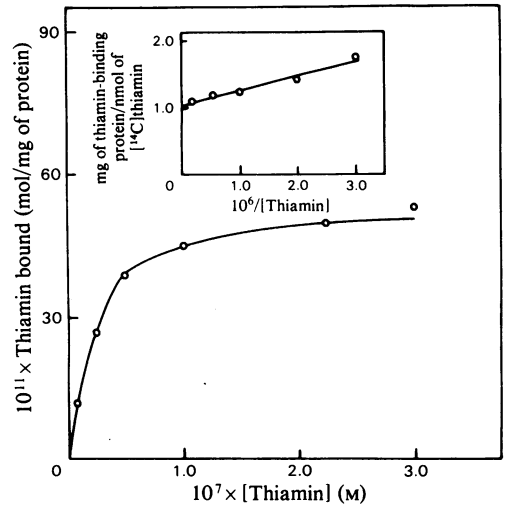

Fig. 8. *Binding of [^{14}C]thiamin to apo-(thiamin-binding protein) under equilibrium conditions*
For experimental details see the text. The amount of the apoprotein used was 1mg. The inset shows the amount of [^{14}C]thiamin bound per unit amount of the protein plotted against the amount of [^{14}C]thiamin used for equilibrium dialysis.

saturation-type curve was obtained. Furthermore, it could be calculated that this protein–ligand interaction had $K_d = 0.3\,\mu M$ and occurred with 1:1 molar ratio (Fig. 8, inset). Thus it is clear that the protein-affinity-column procedure for isolation has enhanced the ligand affinity of the thiamin-binding protein in comparison with the ligand-affinity procedure used for isolation.

Discussion

It is now well established that the binding proteins specific for riboflavin (Ostrowski *et al.*, 1962; Murthy & Adiga, 1978), biotin (White *et al.*, 1976) and iron (Williams, 1962) are present in both the yolk and the white of the chicken egg; although they are fully saturated with their respective ligands in the yolk, they occur primarily as apoproteins in the white (Feeney & Allison, 1969). The less lipoidal egg white was preferred as the starting material in view of the fact that thiamin in the egg is almost exclusively localized in the yolk (Long, 1961), and the technique of affinity chromatography on immobilized thiamin was the method of choice in our first attempts towards the isolation of the putative thiamin-binding protein. Before affinity chromatography, precipitation with $(NH_4)_2SO_4$ at 90% saturation was found highly desirable as a means of concentrating the proteins in a non-gelatinous state, free from extraneous soluble substances including free thiamin, if any. Furthermore, on the basis of our previous experience with riboflavin-binding protein from egg white (Murthy *et al.*, 1976) it was considered that prior exposure of the crude proteins to low pH would facilitate the dissociation of the protein-bound thiamin, if any, and generate more of the apoprotein.

It is thus clear from our data that the thiamin-binding protein existing as a distinct entity in the egg white is similar to the riboflavin-binding protein only in terms of molecular size and the affinity to bind to the respective ligands. Among the apparent differences between the two is the non-glycoprotein nature of the thiamin-binding entity, which is apparently responsible for its lesser stability on exposure in isolation to acidic pH on thiamin pyrophosphate–Sepharose compared with riboflavin-binding protein (Murthy *et al.*, 1976). The finding that immunologically they could be clearly distinguished suggests that they do not share sequence homology and are therefore apparently different gene products.

For the transferrins present in both the chicken egg yolk and the white, there is sufficient chemical and genetic evidence to suggest that they are coded individually by a single structural gene (Williams, 1962). A similar situation exists with regard to the riboflavin-binding protein (Winter *et al.*, 1967). However, the sites of synthesis are different; the yolk proteins are synthesized and secreted by the liver and transported via blood to the ovarian follicles (Heald & McLachlan, 1963; Tata, 1976), whereas the egg-white proteins are elaborated by the oviduct and incorporated into the egg directly (Gilbert, 1971). The only exception to this phenomenon so far seems to be the biotin-binding protein of the yolk and white, which appear to be different gene products (Mesler *et al.*, 1978). The availability of a potent monospecific antiserum for the egg-white thiamin-binding protein facilitated an examination of the yolk extract for the presence of an immunologically similar protein species. Analysis of the suitably processed soluble yolk extracts as well as the blood of laying hens by double immunodiffusion against the monospecific antiserum revealed that, in fact, similar protein species exist in these extracts, with strong immunological cross-reactivities with the egg-white thiamin-binding protein. Preliminary experiments also revealed that the blood of oestrogen-treated immature male chickens, but not that of untreated birds, also exhibits similar immunological cross-reactivity when tested by immunodiffusion (Fig. 7). Thus it would appear that the thiamin-binding protein is more akin to the riboflavin-binding protein than the biotin-binding protein in this respect.

The most significant aspect of the present study is the intriguing finding that there exists a high degree of specificity with which the thiamin- and riboflavin-binding proteins associate to form a 1:1 complex. This property has been successfully exploited as an alternative but milder affinity procedure to purify the thiamin-binding protein from the egg white, with considerable improvement in the affinity for its ligand. The physiological significance of this specific protein–protein interaction is not clear at present. One distinct possibility, that this may be related to ovarian uptake of the thiamin-binding protein, becomes apparent when the suggested modes of transport of some of other vitamin-binding proteins of egg yolk are considered. There is now evidence to show that there are specific recognition sites on the outer surface of the chicken oocyte plasma membrane for some of the yolk-specific glycoproteins (Cutting & Roth, 1973), which might explain the differential rate of uptake of some of the yolk constituents by the developing oocyte (Yusko & Roth, 1976). Among the known vitamin-binding proteins, biotin- (Mesler *et al.*, 1978) and riboflavin-binding proteins (Murthy & Adiga, 1977) are glycoproteins, whereas the retinol-binding protein (Heller, 1976) is not, and is taken up by the yolk as a complex with pre-albumin. Furthermore, although it is not known whether cholecalciferol-binding protein is a glycoprotein, its transport is presumed to be mediated in the form of a complex with Ca^{2+} and phosvitin (Fraser & Emtage, 1976), which is a glycoprotein (Tata, 1976). Thus it would seem that the carbohydrate moieties of the yolk

glycoproteins play an important role as recognition sites for preferential ovarian uptake of these proteins, by analogy with the clearance of serum agalactoglycoproteins by the chicken liver (Lunney & Ashwell, 1976). If in fact this is the case, then specific and tight interaction with the yolk riboflavin-binding protein might greatly facilitate the transport of thiamin-binding protein for deposition in the developing oocyte. The validity of this premise should await the actual demonstration of the existence of this specific protein–protein interaction in the blood and the yolk of a laying hen.

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