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Nature of the thiamin-binding protein from chicken egg yolk

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A simple, rapid and efficient procedure for the purification of thiamin-binding protein from chicken egg yolk was developed. The method involved removal, by exclusion, of lipoproteins from DEAE-cellulose and subsequent elution of water-soluble proteins held on the ion-exchanger with 1 M-NaCl, followed by treatment of the eluted protein fraction with an aqueous suspension of dextran/charcoal to generate apoprotein from the holoprotein. The resultant protein fraction was subjected to bioaffinity chromatography on thiamin pyrophosphate–AE (aminoethyl)-Sepharose. The protein eluted specifically with 10 μM-thiamin at pH 7.0, was homogeneous by the criteria of polyacrylamide-gel disc electrophoresis, had a mol.wt. of 38000 ± 2000 and was not a glycoprotein. The purified thiamin-binding protein specifically interacted with riboflavin-binding protein with no detectable deleterious effect on its [^{14}C]thiamin-binding capacity. The protein bound [^{14}C]thiamin with a molar ratio of 1.0, with dissociation constant (K_d) 0.41 μM. This protein–ligand interaction was inhibited by thiamin analogues and antagonists. The absorption spectrum of the protein in the presence of thiamin exhibited significant hypochromism at the 278 nm band, indicating the involvement of aromatic amino acid residues of the protein, during its binding to the ligand. The protein cross-reacted with the monospecific antiserum to egg-white thiamin-binding protein, showing thereby that thiamin-binding proteins present in chicken egg yolk and white are the products of the same structural gene.

It is now well recognized that, in oviparous species, the proteins of the egg yolk differ from those of the egg white in both the duration and the site of their synthesis (Schjeide *et al.*, 1963; Noble & Moore, 1964). Thus most, if not all, of the yolk proteins are synthesized and secreted by the liver into and transported via the blood stream for deposition in the developing ovarian follicle over a period of several days (Gilbert, 1971), whereas the entire complement of the egg-white proteins is elaborated by the oviducal magnum and directly incorporated into the egg within 4–6 h of ovulation (Gilbert, 1971). Furthermore, many of the major egg proteins are exclusively localized in either the yolk or the white, but not in both. However, among notable exceptions to the latter phenomenon recognized hitherto are the two glycoproteins [namely riboflavin-binding protein (Winter *et al.*, 1967*a*) and transferrin (Williams, 1962)] having a specific micronutrient-carrier function. Even with regard to these two proteins, certain clear-cut differences in terms of size and structural features between the yolk proteins and their respective egg-white counterparts have been recorded (Williams, 1962; Murthy & Adiga, 1978), despite the fact that each pair of these proteins is coded for by a single structural gene (Williams, 1962; Winter *et al.*, 1967*b*). It has been assumed that these subtle differences arise owing to the differential post-translational modifications of the two proteins. Furthermore, in terms of functional significance in embryonic vitamin nutrition, the two yolk proteins fully saturated with their respective ligands should be more important than their counterparts in the egg white, which are primarily apoproteins (Schade & Caroline, 1944; Board & Fuller, 1974).

Recently we described the isolation and physicochemical characterization of thiamin-binding protein for the first time from chicken egg white and provided immunological evidence for the existence of similar proteins in the yolk and oestrogenized

Abbreviations used: AE-Sepharose, aminoethyl-Sepharose; SDS, sodium dodecyl sulphate.

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chicken sera, thus enlarging the class of vitamin-carrier proteins present in the chicken egg (Muniyappa & Adiga, 1979, 1980). An intriguing aspect of these investigations was the discovery that thiamin- and riboflavin-binding proteins specifically interact with each other, thus affording the development of a specific protein-affinity-chromatographic procedure to isolate thiamin-binding protein from the egg white. On the basis of these observations, it was surmised that this specific protein–protein interaction, if it exists in the yolk, may have important implications in terms of recognition at the ovarian plasma-membrane-receptor sites as a prerequisite for adequate thiamin-binding-protein deposition in the yolk to subserve embryonic development. To substantiate these findings and to compare the characteristics of the putative yolk thiamin-binding protein with those of egg-white protein, isolation and characterization of the thiamin-binding protein from yolk were considered essential. The present paper deals with these aspects and some features of protein–ligand interaction.

Materials and methods

Fresh eggs from White Leghorn hens were procured from the Poultry Farm of the University of Agricultural Sciences, Hebbal, Bangalore, India. [*thiazole*-2-^{14}C]Thiamin was obtained from The Radiochemical Centre, Amersham, Bucks., U.K., and had a specific radioactivity of 14 Ci/mol. CNBr was synthesized by the procedure of Hartman & Dreger (1931). DEAE-cellulose (0.88 mequiv./g), thiamin, thiamin monophosphate, pyrithiamin, thioproline, dextran and Norit A charcoal were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. The sources of other chemicals and biochemicals have been detailed previously (Muniyappa & Adiga, 1979). Apo-(riboflavin-binding protein) from the egg yolk was purified by the procedure developed previously (Murthy *et al.*, 1979).

Coupling of thiamin pyrophosphate to Sepharose 4B

The methods of preparation of AE-Sepharose and coupling of thiamin pyrophosphate to the Sepharose derivative were the same as described for the egg-white thiamin-binding protein (Muniyappa & Adiga, 1979).

Polyacrylamide-gel disc electrophoresis

Electrophoresis on analytical polyacrylamide gels (7.5%) was performed as described by Maurer (1968). The gels were stained for protein and glycoprotein and destained as described previously (Muniyappa & Adiga, 1979).

Molecular-weight determination

The molecular weight of the protein was estimated by SDS/polyacrylamide-gel electrophoresis on 7.5% gels (Weber & Osborn, 1969) as detailed for egg-white thiamin-binding protein (Muniyappa & Adiga, 1979). The following marker proteins, of known molecular weights, were used: cytochrome *c* (13700), chymotrypsinogen (25000), ovalbumin (44000), bovine serum albumin (68000) and phosphorylase *a* (94000). The molecular weight of thiamin-binding protein under non-denaturing conditions was also determined by gel filtration (Andrews, 1965) on a column (1.6 cm × 96 cm) of Sephadex G-100 with 0.1 M-sodium phosphate buffer/0.5 M-NaCl as the eluent. In this experiment, the same marker proteins of known molecular weight as mentioned above were used.

[^{14}C]*Thiamin binding*

Thiamin-binding activity of the isolated protein and its affinity constant were determined by equilibrium dialysis in a Perspex (Lucite) dialysis cell as described previously (Muniyappa & Adiga, 1979). The binding of [^{14}C]thiamin in the presence of thiamin analogues and antagonists was also performed by equilibrium dialysis. Assay samples contained the apo-(thiamin-binding protein) (200 μg) in 0.2 ml in one compartment and in the other compartment, 0.1 ml (0.5 μCi) of [^{14}C]thiamin plus 0.1 ml of the appropriate unlabelled compound at specified concentrations were included.

Interaction of thiamin-binding protein with riboflavin-binding protein

Equal amounts (2 mg) of riboflavin-binding protein and [^{14}C]thiamin-saturated thiamin-binding protein were incubated at 30°C for 1 h and subjected to gel-filtration chromatography on a Sephadex G-75 column in the absence of NaCl to demonstrate specific interaction leading to complex-formation. Each fraction was monitored for A_{230} and [^{14}C]thiamin. The radioactivity in 0.1 ml samples was measured by liquid-scintillation spectrometry as detailed previously (Muniyappa & Adiga, 1979). Similarly, the nature of interaction between these proteins was ascertained by incubating them in the presence of 1 M-NaCl/0.1 M-sodium phosphate buffer followed by chromatography on Sephadex G-75, which was pre-equilibrated with the same buffer in the presence of the salt.

Immunological techniques

Ouchterlony (1958) double-diffusion analysis was performed with the antibody raised against the egg-white thiamin-binding protein as detailed previously (Muniyappa & Adiga, 1979).

Spectral measurements

Changes in absorption spectra were recorded with a Cary 14 spectrophotometer at 28°C. The concentrations of thiamin in the samples were progressively increased by the addition of very small volumes ($\leq 10\mu l$) of a concentrated solution of thiamin to the apoprotein ($263\mu M$) solution in the experimental cuvette and also to the buffer solution (0.1 M-sodium phosphate buffer, pH 7.0) in the reference cuvette.

Protein determination

Protein concentrations were measured as described by Lowry *et al.* (1951), with bovine serum albumin as standard.

Results

Purification of thiamin-binding protein from the egg yolk

Unless otherwise stated, all the steps were carried out at 4°C. The yolks, physically separated from the whites, were homogenized with 5 vol. of 0.1 M-sodium phosphate buffer/0.2 M-NaCl, pH 7.0. This yolk preparation was then centrifuged at 23000 *g* for 30 min. The clarified homogenate was loaded on a DEAE-cellulose column pre-equilibrated with the above buffer. The column was washed extensively until the effluent was free of 280 nm-absorbing material. The proteins retained on the column were eluted with 1 M-NaCl/0.1 M-sodium phosphate buffer, pH 7.0. The protein concentration in the eluate was monitored by the method of Lowry *et al.* (1951). The protein-rich fractions were pooled and dialysed extensively against water. The sample was then stirred with an aqueous suspension of dextran/charcoal (500 mg of charcoal/50 mg of dextran in 100 ml of water) for 24 h to remove endogenously bound ligand. The yolk preparation was centrifuged at 23000 *g* for 20 min to sediment dextran/charcoal. The supernatant thus obtained represented processed crude water-soluble yolk proteins.

The clarified protein solution was dialysed to remove salt and was then applied to a column (1.2 cm × 8 cm) of thiamin pyrophosphate–AE-Sepharose and extensively washed with the equilibration buffer (0.1 M-sodium phosphate buffer, pH 7.0) until the effluent was free of 280 nm-absorbing materials. The proteins retained on the column were then eluted with 1 M-NaCl/0.1 M-sodium phosphate buffer, pH 7.0 [which dislodges riboflavin-binding protein (Muniyappa & Adiga, 1979)] and and then $10\mu M$-thiamin/0.1 M-sodium phosphate buffer, pH 7.0. The protein concentration in the effluent at this stage could not be accurately monitored because of thiamin interference with quantitative determination either by the method of Lowry *et al.* (1951) or by measurement of A_{280}. An alternate elution procedure was attempted (Muniyappa & Adiga, 1979), but again the protein obtained had very weak [^{14}C]thiamin-binding capacity. Hence specific elution with thiamin was preferred. The protein eluted from the affinity matrix with thiamin was dialysed against three 2-litre changes of water, concentrated to a small volume against Aquacide and treated with a dextran/charcoal suspension to remove firmly bound thiamin as described above. From 100 egg yolks a yield of 12 mg of homogeneous thiamin-binding protein could be obtained. The protein in solution in 0.1 M-sodium phosphate buffer (pH 7.0) stored at 4°C for 4–5 days was stable in terms of its vitamin-binding capacity.

Criteria of purity

The resultant apo-(thiamin-binding protein) exhibited a single stainable protein band on analytical polyacrylamide-gel disc electrophoresis at pH 4.3. However, when stained for glycoproteins (Zacharius *et al.*, 1969), it did not take up the stain, showing the absence of any covalently attached carbohydrate. Further evidence for its homogeneity was obtained by SDS/polyacrylamide-gel electrophoresis, which exhibited a single stainable band corresponding to an apparent mol.wt. of 38000 ± 2000, with no detectable subunits. On immuno-double-diffusion analysis on agar against the antiserum to the homogeneous chicken egg-white thiamin-binding protein, the purified yolk thiamin-binding protein exhibited a single precipitin line, showing homogeneity by this criterion also (Fig. 1).

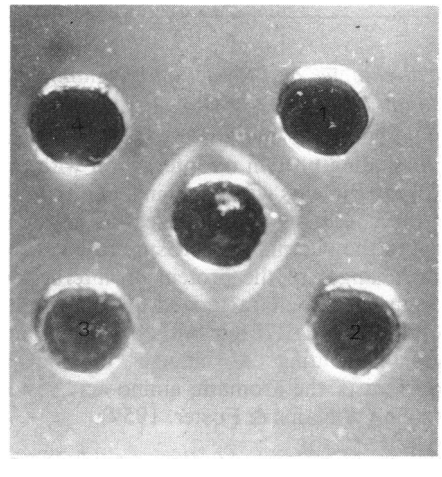

Fig. 1. *Ouchterlony immuno-double-diffusion analysis of thiamin-binding protein from egg white and yolk*
The central well contained antiserum to egg-white thiamin-binding protein. Peripheral wells; 1 and 2, egg-white thiamin-binding protein ($20\mu g$); 3 and 4, egg-yolk thiamin-binding protein ($20\mu g$)

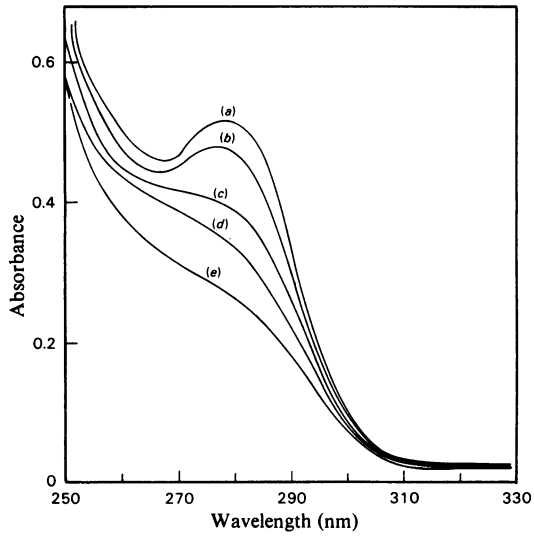

Fig. 2. *Absorption spectral changes produced by the interaction of thiamin with thiamin-binding protein*
The protein (263 μM) was dissolved in 1.0ml of 0.1 M-sodium phosphate buffer (pH 7.0)/0.1 M-NaCl. The spectral changes were recorded at room temperature (28°C). Increasing concentration of thiamin was obtained by the addition of small volumes (5 μl or multiples thereof) of concentrated thiamin (6 mM) solution. The same amount of thiamin was added each time to the reference cuvette also. Corrections for the volume changes have been made. (*a*) Apo-(thiamin-binding protein) alone in 0.1 M-sodium phosphate buffer (pH 7.0)/0.1 M-NaCl; (*b*) as (*a*) plus 30 μM (5 μl)-thiamin; (*c*) as (*a*) plus 90 μM (15 μl)-thiamin; (*d*) as (*a*) plus 120 μM (20 μl)-thiamin; (*e*) as (*a*) plus 180 μM (30 μl)-thiamin.

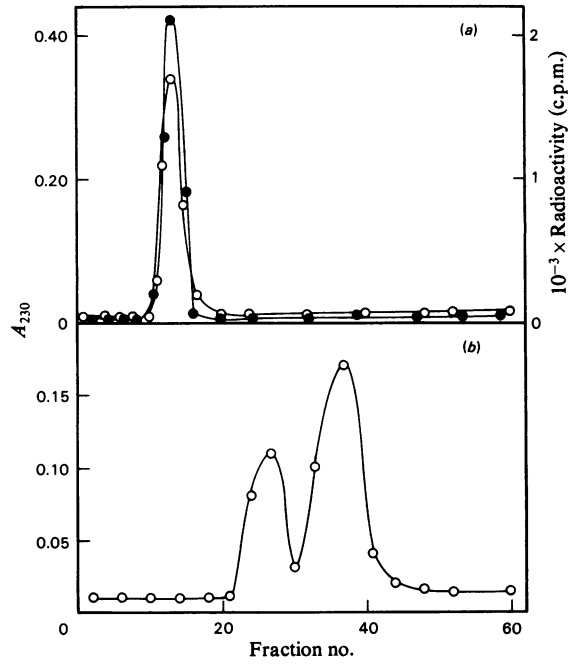

Fig. 3. *Chromatography of the riboflavin-binding protein and the thiamin-binding protein on Sephadex G-75*
(*a*) Riboflavin-binding protein and [^{14}C]thiamin-saturated thiamin-binding protein (2 mg each) were mixed and incubated at 37°C for 1 h in 0.1 M-sodium phosphate buffer (pH 7.0)/1 M-NaCl. The column (1 cm × 80 cm) was pre-equilibrated and eluted with the above buffer and the eluate was collected in 1.5 ml fractions. ○, A_{280}; ●, radioactivity. (*b*) Samples (2 mg) of thiamin- and riboflavin-binding proteins were mixed and incubated at 37°C in 0.1 M-sodium phosphate buffer (pH 7.0)/1 M-NaCl for 1 h. The column (1 cm × 80 cm) was pre-equilibrated and eluted with the same buffer and the eluate was collected in 1.5 ml fractions.

Spectral changes on interaction of thiamin with the apo-(thiamin-binding protein)

In Fig. 2 are depicted the u.v.-spectral changes of thiamin-binding protein on interaction with thiamin. Control experiments with thiamin alone showed that, under the conditions used, there was no detectable damage to the vitamin on exposure to u.v. light. The spectral changes of the protein on interaction with the ligand are characterized by a significant hypochromism at 278 nm without a shift in band position, indicating a change in the micro-environment of the aromatic amino-acid residues of the protein (Williams & Foster, 1959).

Interaction of thiamin-binding protein with riboflavin-binding protein

On the basis of previous findings (Muniyappa & Adiga, 1979) that thiamin-binding protein of egg white interacts specifically with riboflavin-binding protein in 1:1 molar ratio, it was surmised that the complex thus generated facilitates the ovarian uptake of a (non-glyco)protein (thiamin-binding protein) at the level of the oocyte plasma membrane through the specific recognition of the complexed glycoprotein (riboflavin-binding protein) before deposition in the developing ovarian follicle. In conformity with the above postulate is the finding that, in the absence of 1 M-NaCl, the two yolk vitamin-binding proteins emerged from the column as a single peak of apparently higher molecular weight with tightly bound [^{14}C]thiamin still associated with the complex (Fig. 3*a*). In the presence of 1 M-NaCl, the two proteins were resolved as two distinct though overlapping peaks (Fig. 3*b*). These results amply demonstrate that the yolk thiamin-binding protein, in common with its counterpart in

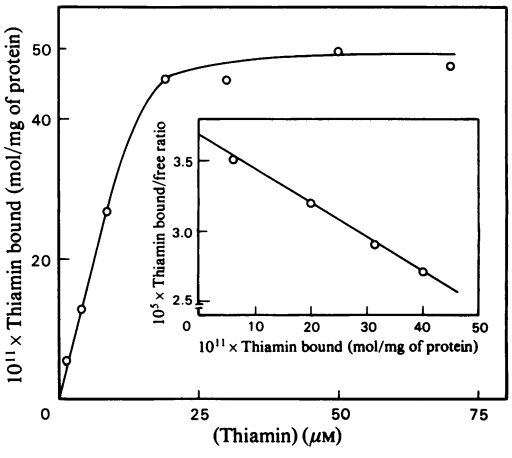

Fig. 4. *Binding of [^{14}C]thiamin to apo-(thiamin-binding protein) under equilibrium conditions*
For experimental details, see the text. The amount of the apoprotein used was 20 μM. The inset shows the replotting of the data as described by Scatchard (1949).

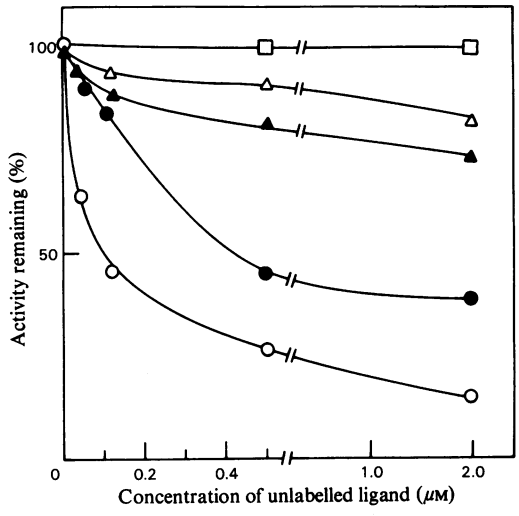

Fig. 5. *Inhibition of [^{14}C]thiamin binding by thiamin analogues and antagonists to thiamin-binding protein*
The assay samples (0.2 ml) were prepared by combining 0.1 ml of [^{14}C]thiamin (0.5 μCi; 35 nmol) plus 0.1 ml of the indicated unlabelled compound at specified concentrations in one compartment and 0.2 ml (200 μg) of the apo-(thiamin-binding protein) in another compartment. For further details, see the text. ○, Unlabelled thiamin; ●, thiamin monophosphate; ▲, thiamin pyrophosphate; △, pyrithiamin; □, thioproline.

the white, is capable of tight association with riboflavin-binding protein and that such an interaction does not measurably alter the pattern of apo-(thiamin-binding protein)–[^{14}C]thiamin interaction.

[^{14}C]*Thiamin binding*

When a fixed concentration of purified thiamin-binding protein was allowed to equilibrate with [^{14}C]thiamin in a dialysis cell, it avidly bound the labelled ligand. When the amount of protein-bound [^{14}C]thiamin was plotted against the concentration of thiamin, a saturation type of curve was obtained (Fig. 4). Furthermore, it could be calculated that this protein–ligand interaction has a dissociation constant (K_d) 0.41 μM and occurs with a 1:1 molar ratio. These values are comparable with that of egg-white thiamin-binding protein (Muniyappa & Adiga, 1979).

The patterns of competition between unlabelled thiamin, thiamin monophosphate, thiamin pyrophosphate, pyrithiamin and thioproline on the one hand, and [^{14}C]thiamin, on the other, for interaction with apo-(thiamin-binding protein) are illustrated in Fig. 5. The data clearly show that each of these unlabelled compounds, except thioproline, inhibits the binding of [^{14}C]thiamin to the apoprotein, though to different extents, at all concentrations tested. The sequential addition of phosphate moieties to the vitamin leads to a progressive decrease in the ability of the vitamin derivatives to compete with [^{14}C]thiamin for binding to the protein. It is significant that pyrithiamin, the well-known physiological antagonist (Naber *et al.*, 1954) of the vitamin having only a substituted pyridine ring, but lacking the thiazole moiety, was also effective, though marginally. Curiously enough, thioproline, with a substituted thiazole ring, had no effect, even at very high concentrations, on this protein–ligand interaction.

Discussion

Consistent with the immunochemical evidence provided previously (Muniyappa & Adiga, 1979), the data above amply demonstrate that thiamin-binding protein can be isolated in a pure form from the egg yolk as a distinct molecular entity with an affinity for the vitamin high enough to classify it as a functional vitamin carrier. These observations thus strengthen the concept that prior interaction with a specific protein carrier is an essential prerequisite for adequate vitamin deposition in the egg (Gilbert, 1971). Moreover, the finding that the purified yolk thiamin-binding protein is indistinguishable from its egg-white counterpart in several physicochemical, immunological and functional characteristics, not only expands the list of vitamin-binding proteins

present in both the compartments of the avian egg, but also strongly suggests that both these proteins are products of a single structural gene. It is significant that similar conclusions have been arrived at in the case of riboflavin-binding protein (Winter *et al.*, 1967*b*) and transferrin (Williams, 1962).

One of the major impediments routinely encountered in the purification of water-soluble proteins of the egg yolk is the interference due to the massive [33% by weight (Vadehra *et al.*, 1977)] amount of lipids present, over 90% of which are in the form of lipoproteins (McIndoe, 1971). The conventional procedures of lipid removal, such as extraction with diethyl ether (Morton, 1955) or butan-1-ol (Meslar & White, 1979), are cumbersome and are often unsatisfactory, either owing to incomplete extraction at low temperatures, resulting in subsequent colloid formation, or to significant protein denaturation at elevated temperatures. To circumvent these difficulties, an alternate, efficient, yet mild, procedure adopted in the present investigation stems from a previous observation (Raju & Mahadevan, 1975; Murthy *et al.*, 1979) that the lipoproteins are almost entirely excluded from DEAE-cellulose and the water-soluble proteins firmly held on the ion-exchanger are easily recovered by elution with 1 M-NaCl. This exclusion-chromatographic step afforded nearly 10-fold enrichment of the water-soluble yolk proteins. Furthermore, since the yolk vitamin-binding proteins (unlike the corresponding egg-white proteins) are mostly saturated with their respective ligands (White *et al.*, 1976), necessity arose for the adoption of a mild procedure to disassociate the firmly-bound vitamin before protein isolation by affinity chromatography. Towards this end, the observation that high-affinity steroid binding to intracellular receptors/antibodies can be interfered with by prolonged treatment with dextran/charcoal (Rousseau *et al.*, 1972) was successfully exploited to generate yolk apo-(thiamin-binding protein). The protein purified by affinity chromatography followed by specific elution with thiamin was homogeneous, had a mol.wt. of 38000 ± 2000 and, interestingly enough, is not a glycoprotein. These indistinguishable characteristics of the egg-yolk and the egg-white thiamin-binding proteins are indicative of the absence of any discernible gross post-translational modifications of the vitamin carrier such as glycosylation etc., in contrast with the situation that occurs with riboflavin-binding protein (Murthy *et al.*, 1979) and transferrin (Williams, 1962).

It is also clear that, in terms of affinity to interact with the apo-(thiamin-binding protein), unmodified thiamin is clearly the most efficient among the vitamin compounds tested (Fig. 5). At equimolar concentrations the decreased ability of the vitamin derivatives to compete with [^{14}C]thiamin for binding to the apo-(thiamin-binding protein) seems to be directly proportional to the extent of vitamin phosphorylation. This is in marked contrast with the situation expected of coenzyme–enzyme interaction, wherein the coenzyme derivative of the vitamin is clearly a preferred ligand (Krampitz, 1969; Koike *et al.*, 1976). Among the anti-vitamins used, only pyrithiamin exhibited marginal, yet significant, competition, whereas the thiazole analogue, i.e. thioproline, even at higher concentrations, was completely ineffective in this respect. On the basis of these observations it is envisaged that the pyrimidine moiety of the vitamin is relatively more important than the thiazole portion with regard to the ligand–protein interaction. Significant hypochromism exhibited by the u.v. spectra during titration of the apoprotein with thiamin may be indicative of changes in the microenvironment of aromatic residues of the protein. This interpretation is in line with the observations of Heinrich *et al.* (1972) regarding thiamin pyrophosphate–transketolase interaction and of Nose *et al.* (1976) with *Escherichia coli* thiamin-transport protein; in both these cases, involvement of tryptophan residues of the protein in ligand–protein interaction has been implicated.

It is intriguing that the thiamin-binding protein in the yolk remains non-glycosylated and retains its capacity to interact specifically with riboflavin-binding protein in 1:1 molar ratio, as in the case of corresponding egg-white protein. Of particular significance is that this protein–protein interaction has no detectable adverse effect on [^{14}C]thiamin binding (Fig. 3*a*). Furthermore, the fact that, under identical conditions of complex-formation, the ability of riboflavin-binding protein to interact with the flavin is also unimpaired, as monitored spectrofluorimetrically (Murthy *et al.*, 1976), clearly favours a distinct physiological significance to this interaction in the yolk. This, then, lends credence to the hypothesis put forward previously (Muniyappa & Adiga, 1979) that the complex-formation between riboflavin-binding protein and thiamin-binding protein is related to recognition of the latter as a complex with riboflavin-binding protein at the appropriate receptor site on the oocyte plasma membrane. This may facilitate preferential protein uptake and deposition in the ovarian follicle (Gilbert, 1971) to ensure adequate vitamin availability to the prospective embryo.

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