Thiamin deposition in eggs is not dependent on riboflavin-binding protein

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The thiamin content of eggs laid by hens possessing no, one or two functional genes for riboflavin-binding protein is unaffected by the genotype. This does not support the hypothesis of Muniyappa & Adiga [*Biochem. J.* (1979) 177, 887–894] that the deposition of thiamin-binding protein is coupled to the deposition of riboflavin-binding protein.

Egg yolk is a rich source of vitamins. Most of these vitamins are bound to specific proteins sequestered from the plasma of laying hens during yolk deposition by the ovarian follicles. Since the oligosaccharide moiety is often the recognition signal for transporting glycoproteins into cells (for review see Neufeld & Ashwell, 1980), and since many plasma proteins are glycoproteins (for review see Schwick *et al.*, 1977), it is tempting to hypothesize that the oligosaccharide moiety of vitamin-binding proteins targets these proteins for the ovarian follicle (Miller, 1976; Muniyappa & Adiga, 1979). With the single exception of retinol-binding protein (Heller, 1976), the other vitamin- and mineral-binding proteins that have been characterized from the yolk are glycosylated, including riboflavin-binding protein (Ostrowski *et al.*, 1968), biotin-binding protein (Meslar *et al.*, 1978), transferrin (Williams, 1968) and phosvitin (Shainkin & Perlmann, 1971).

Thiamin-binding protein was first detected in the chicken egg by Muniyappa & Adiga (1979), who reported that immunologically indistinguishable forms of the protein occur in both egg white and yolk. They purified the egg-white protein to homogeneity in a single step by affinity chromatography on thiamin pyrophosphate coupled to aminoethyl-Sepharose. Unexpectedly, riboflavin-binding protein was also retained by this matrix. It was shown that the two proteins form a complex and that immobilized riboflavin-binding protein could also be used to purify egg-white thiamin-binding protein.

Among the properties of egg-white thiamin-binding protein reported by Muniyappa & Adiga (1979), the lack of interaction with a carbohydrate-specific gel stain indicated that this protein, unlike riboflavin-binding protein, is not a glycoprotein. To circumvent the problem of transporting a non-glycosylated protein to the yolk, Muniyappa & Adiga (1979) suggested that the physical association with riboflavin-binding protein was physiologically important in a coupled transport system which

recognizes the oligosaccharide moiety of riboflavin-binding protein.

We have tested and rejected this hypothesis by using a mutant strain of White Leghorn chickens (*rdrd*) which do not produce riboflavin-binding protein and lay eggs practically devoid of riboflavin (Maw, 1954; Winter *et al.*, 1967). Eggs from normal hens (*RdRd*) contain approximately twice as much riboflavin-binding protein and riboflavin as do eggs from heterozygotes (*Rdrd*) (Winter *et al.*, 1967). Embryos in eggs from *rdrd* hens die unless they are rescued with free riboflavin injected into the egg (Maw, 1954). The fact that thiamin is not also needed to overcome the genetic defect suggests that sufficient thiamin is deposited in these eggs. We have confirmed this directly by showing that the amount of thiamin in chicken eggs is independent of the riboflavin-deficient genotype.

Materials and methods

The eggs used in this study were obtained from genetically defined lines of Single Comb White Leghorn chickens maintained by the Poultry Science Department of the Pennsylvania State University. The pedigree of the null gene for riboflavin-binding proteins (*rd*) may be traced to the birds originally studied by Maw (1954). Each egg examined was laid by a different hen.

Yolks from normal (*RdRd*), heterozygous (*Rdrd*) and homozygous recessive (*rdrd*) hens were separated from albumen, washed with distilled water and weighed. Each yolk was diluted to 40 ml with 0.5 M-NaCl, homogenized with a glass rod, and samples were removed for thiamin determinations.

Thiamin was determined as its fluorescent derivative, thiochrome, by the procedure of Edwin (1979) with modifications as noted. Diluted yolk samples were mixed with 9 vol. of 0.1 M-HCl, which dissociates protein-bound thiamin. Since thiamin occurs as the non-phosphorylated vitamin in yolk, the dephosphorylation step was eliminated. Thiamin

was adsorbed on to 0.1 g of bentonite (Mickelsen & Yamamoto, 1958). The bentonite was then sedimented by centrifugation (3000 g for 5 min) and washed twice with nearly boiling distilled water. Thiamin bound to bentonite was oxidized with HgCl_2 in NaOH, extracted into 2-methylpropan-1-ol, and quantified by fluorescence as described by Edwin (1979). Standard thiochrome solutions were prepared by oxidizing a series of standards containing 0.1–0.5 μg of thiamin hydrochloride (Merck, Rahway, NJ, U.S.A.) with HgCl_2 and extracting as above. The fluorescence emission spectra of the samples and the standard thiochrome solutions excited at 365 nm were indistinguishable between 390 and 550 nm, with a peak near 430 nm. Riboflavin, which would also fluoresce under these conditions, is removed by the extraction procedure and thus does not interfere with the assay.

Results and discussion

The thiamin contents of yolks from eggs laid by hens carrying no, one or two functional genes for riboflavin-binding protein are presented in Table 1. The average thiamin contents are respectively 2.55, 2.89 and 2.39 $\mu\text{g/g}$ of yolk. Also included in Table 1 are the riboflavin contents of yolks from hens with the same genotypes (Winter *et al.*, 1967). There is no correlation between the thiamin and riboflavin contents of these yolks.

These data unambiguously show that the transport of thiamin into the egg yolk is not dependent on the presence of riboflavin-binding protein. Since binding proteins found in the yolk are fully saturated with their ligands (Winter *et al.*, 1967; Meslar *et al.*, 1978), the concentration of thiamin probably reflects the amount of thiamin-binding protein in yolk. Therefore, thiamin-binding protein must contain its own recognition signals for uptake by ovarian receptors and not rely on its putative association with riboflavin-binding protein for coupled uptake.

Table 1. *Thiamin and riboflavin contents of egg yolks from hens carrying no, one or two functional genes (Rd) for riboflavin-binding protein*

Genotypes: normal (*RdRd*), heterozygous (*Rdrd*) and homozygous recessive (*rdrd*) for the riboflavin-binding-protein-deficient trait. Results for thiamin are means $\pm$ S.D. for *n* eggs. Data for riboflavin are from Winter *et al.* (1967). The riboflavin content of yolks was not measured in this study; however, the genotype of each egg can be visually determined by the intensity or absence of yellow colour (riboflavin) in the albumen fraction.

Genotype	Thiamin content ($\mu\text{g/g}$ of yolk)	Riboflavin content ($\mu\text{g/ml}$ of yolk)
<i>RdRd</i>	2.39 ± 0.38 (<i>n</i> = 8)	3.8 ± 1.1
<i>Rdrd</i>	2.89 ± 0.08 (<i>n</i> = 6)	2.2 ± 0.68
<i>rdrd</i>	2.55 ± 0.33 (<i>n</i> = 8)	0.03 ± 0.07

Since the completion of our experiments, Muniyappa & Adiga (1981) have reported the purification of thiamin-binding protein from chicken-egg yolk. The single protein band associated with thiamin-binding protein does not react with the periodic acid–Schiff reagents, a staining procedure frequently used to detect glycoproteins. This observation would seem to preclude a role for oligosaccharide recognition in thiamin-binding-protein uptake and concomitant thiamin transport. A role for oligosaccharide recognition in the uptake of other nutrient-binding proteins by the ovarian follicle remains an attractive hypothesis. Consistent with this idea is the observation that riboflavin-binding protein in the white of domestic duck eggs is non-glycosylated, whereas it is glycosylated in the egg yolk (Muniyappa & Adiga, 1980). Furthermore, the carbohydrate compositions of chicken egg-yolk and egg-white riboflavin-binding protein are different (Miller, 1976), as are those of yolk and white transferrins (Williams, 1968), also suggesting a role of oligosaccharides in uptake.

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