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RIBOFLAVIN-BINDING PROTEINS

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INTRODUCTION

Perspectives on the Nature of Vitamins and Vitamin Transport Systems

The de novo biosynthetic routes to most water-soluble vitamins were lost in the distant ancestors of the vertebrates. As a consequence, systems for the intestinal absorption, serum transport, and cellular uptake of vitamins un-

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doubtedly exist for all vertebrates, although few have been studied in detail. In addition to these ubiquitous systems, the supply of vitamins to vertebrate embryos presents a special challenge to meet the nutritional needs of the embryo under the constraints of reproductive physiology. This review focuses on soluble, high-affinity, riboflavin-binding proteins that appear to help "carry" or "target" this micronutrient to tissues, with particular emphasis on maternal proteins involved in embryonic development. Other types of proteins, such as membrane-bound transport proteins, are not considered.

Different groups of higher vertebrates have evolved different strategies for supplying nutrients to their embryos. Birds, many reptiles, and a couple of primitive mammals deposit all of the nutrients in eggs before embryonic development begins. Most mammals, however, retain their embryos and continuously supply them with nutrients. Although these two strategies are quite different, the fact that the latter evolved from the former implies that the transition was continuous, innovations were compatible, and that fundamental similarities exist between them. Furthermore, strategies used by egg-laying animals may be utilized by other vertebrates during the earliest phases of pregnancy before the conceptus has implanted in the uterus and established a continuous supply of nutrients. It is not known whether the delivery of vitamins for specific use by the embryo occurs by amplification and modification of the same systems used by other tissues, or by quite separate mechanisms.

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Nomenclature

Riboflavin-binding protein (61, 130, 131) and riboflavin-carrier protein (2, 133) are current names for a protein formerly known as ovoflavoprotein (35) and riboflavin-flavoprotein (135). We prefer the name *riboflavin-binding protein* because it recognizes a property of the isolated protein and is consistent with the nomenclature for other proteins that bind vitamins. The name *riboflavin-carrier protein* emphasizes one function of the protein that, in the case of the apoprotein in egg white, does not apply. Furthermore, the term *carrier protein* has been traditionally applied to protein subunits associated with covalent intermediates of multienzyme complexes, as in acyl carrier protein or carboxyl carrier protein. Unfortunately the abbreviation RBP commonly used for riboflavin-binding protein, is usually associated with retinol-binding protein (43). The suggestion to use FBP for flavin-binding protein (106) does not resolve the problem because it is also used for folate-binding protein. RCP, for riboflavin-carrier protein, distinguishes between riboflavin- and retinol-binding proteins (28), but implies a carrier function that sometimes does not exist. In this review we use the abbreviation RfBP (136) because Rf is a term for riboflavin that is part of the jargon in a number of laboratories.

AVIAN RIBOFLAVIN-BINDING PROTEINS

Function in Chickens

The concentration of riboflavin in the serum of laying hens is about 15 times higher than in males and nonlaying hens (22). Administration of estrogens to immature pullets results in more than a 100-fold increase in serum riboflavin (11). This riboflavin is complexed to a riboflavin-binding protein that was partially purified by Blum (9). He correctly concluded that the protein was similar or identical to riboflavin-binding proteins previously purified from chicken egg white (111) and egg yolk (135). Based on these observations, nutritional studies, and radiotracer analyses, Blum (10) detailed a model, essentially unchanged today, for the role of RfBP in the laying hen. RfBP synthesized in the liver is secreted into the blood stream where it may complex with riboflavin. The vitamin-protein complex is subsequently deposited as part of the yolk in a developing oocyte. Upon ovulation the mature oocyte passes down the oviduct and is encased in albumen secreted by the magnum region of the oviduct. This albumen or egg white also contains a RfBP. The riboflavin bound to RfBP in egg yolk and egg white is derived primarily from the diet but can come from tissues during restricted riboflavin intake provided liver flavin content remains above 50% of normal. Otherwise, egg laying stops. This model is strongly supported by independent genetic evidence.

Unlike the colorless egg white of many bird eggs, the egg white of a normal chicken egg is faint yellow due to riboflavin bound to RfBP. Three related Single Comb White Leghorn hens that laid eggs lacking this typical yellow-colored egg "white" were discovered in a pedigree flock (15, 60). Their eggs were fertile but the embryos in them died of riboflavin deficiency on or near 13 days of incubation. The embryos could be rescued and the strain perpetuated by injecting riboflavin or riboflavin-5'-phosphate (FMN) into the eggs. The affected hens produced riboflavin-deficient eggs even when they were fed riboflavin-supplemented diets. The increased excretion of riboflavin by the affected hens during oogenesis (24) was the basis for naming the condition *riboflavinuria* (25). Subsequently it was shown that the homozygous recessive *(rdrd)* hens were unable to synthesize riboflavin-binding protein (131). Hens heterozygous for the genetic defect produced eggs that had normal hatchability but contained half the riboflavin and RfBP of normal egg yolk and egg white. The defect was similarly expressed in the blood plasma. The mutant strain has been very useful in studying riboflavin metabolism in the laying hen. While the *Rd* gene (and the nature of the mutant allele) remains to be characterized, its product, RfBP, has been purified and characterized from hen plasma, egg yolk, and egg white.

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Chemical and Physical Properties

EGG WHITE RIBOFLAVIN-BINDING PROTEIN RfBP was first purified from egg white (111). Subsequently it has been purified and characterized in many laboratories (5, 33, 90, 96). At a concentration of near 1 mg/ml and constituting slightly less than 1% of the total protein, RfBP in egg white may be the most abundant and readily available of any vitamin-binding protein. As it occurs in egg white, RfBP is not saturated with riboflavin (9, 65, 111). Even when hens are fed diets high in riboflavin, RfBP is less than half saturated (9, 111, 129).

The amino acid sequence of egg white RfBP is shown in Figure 1 (45, 46). It has 219 amino acids and contains several posttranslational modifications. This amino acid sequence has recently been confirmed from the nucleotide sequence of a cDNA clone (137). The nucleotide sequence predicts a 17-residue signal peptide (M-L-R-F-A-I-T-L-F-A-V-I-T-F-S-T-C) at the N-terminus and two arginine residues at the C-terminus not observed in the mature RfBP. The nine disulfide bonds (46) extensively cross-link the protein and may explain its stability to boiling (111). Denaturation occurs between 120 and 130°C (S. Winship and H. B. White, unpublished). One of the disulfide bonds is essential for flavin binding (59, 82). The N-terminal pyroglutamic acid (45) is derived from glutamine (137). There are two complex oligosaccharides (45, 75, 85) attached to asparagines 36 and 147. The structure of these oligosaccharides is yet to be determined; however, their sugar composition, which includes sialic acid, galactose, mannose, and large amounts of N-acetylglucosamine, is distinct from other egg white proteins (86, 112). Of particular interest is a highly anionic region between residues 186 and 199 that contains five glutamate, eight phosphoserine, and one methionine residues (36, 74, 89). The pattern of phosphorylation is similar to that found in caseins (128). A search for homologies with other sequenced proteins (R. F. Doolittle & H. B. White, unpublished) revealed similarity with bovine folate-binding protein, a milk protein that is also a vitamin-binding protein (119). All of the tryptophans and all but one of the nine pairs of cysteine residues in RfBP are conserved in folate-binding protein; this suggests that an aromatic vitamin-binding pocket and disulfide cross-linking are preserved. There is no phosphorylated region in folate-binding protein, but one of the oligosaccharide binding sites has been conserved.

The three-dimensional structure of RfBP has not been determined, but crystals that diffract to 2.8 Å have been obtained (136). Crystallization was achieved only after isolation of a single charged form by isoelectric focusing. The isoelectric point of RfBP is about 4.0 (111). The molecular weight of RfBP based on amino acid, carbohydrate, and phosphate composition is 29,200 (45). This is somewhat less than the values of 30,000 to 36,000

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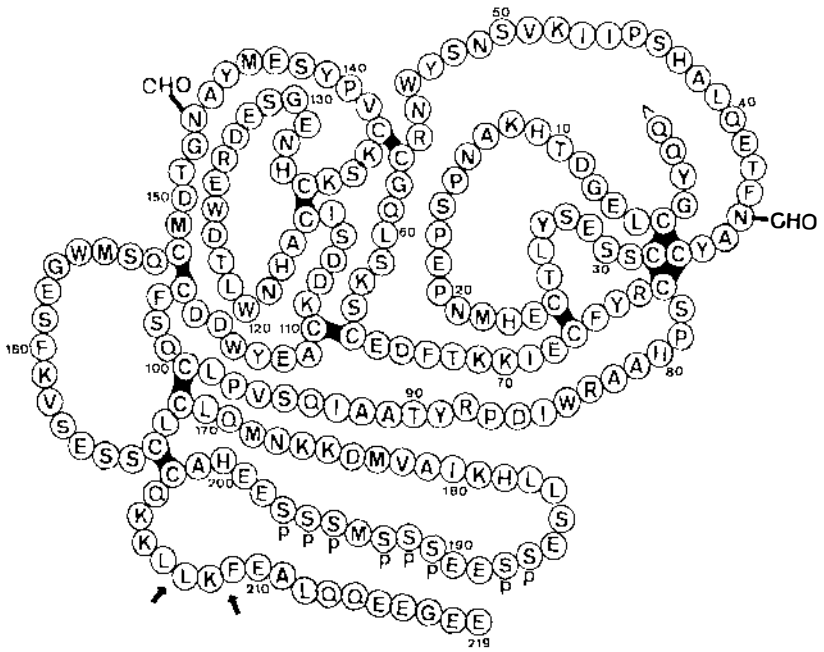

Figure 1 The 219 amino acid sequence of chicken egg white riboflavin-binding protein (45) showing the location of disulfide bonds (46), phosphorylated serines (36, 74, 85), glycosylated asparagines (45), and the N-terminal pyroglutamic acid. Arrows indicate alternate sites of C-terminal peptide cleavage observed in egg yolk RfBP (105). Polymorphism occurs at position 14, with 30–50% of the molecules having lysine instead of the indicated asparagine (45, 105). Single-letter amino acid abbreviations: A = alanine, C = cysteine, D = aspartic acid, E = glutamic acid, F = phenylalanine, G = glycine, H = histidine, I = isoleucine, K = lysine, L = leucine, M = methionine, N = asparagine, Y = tyrosine, P = proline, Q = glutamine, <Q = N-terminal pyroglutamic acid, R = arginine, S = serine, T = threonine, V = valine, W = tryptophan. Modified slightly from Hamazume et al (46) with permission of the authors.

determined by riboflavin-binding (111), sedimentation equilibrium (33, 63, 111) and SDS gel electrophoresis (5, 85).

A dramatic feature of the RfBP:riboflavin complex is that it lacks the characteristic fluorescence of free riboflavin (111) due most likely to stacking of the flavin with aromatic amino acid residues (33). This feature has been exploited in assays for riboflavin and the apoprotein (121). It also has been used by a large number of researchers to evaluate binding of flavin analogs and to probe the structure of the riboflavin-binding site. The flavin-binding properties of egg white RfBP are probably the best studied of any flavoprotein (38). RfBP is unusual among flavoproteins in that it binds riboflavin in preference to the riboflavin-derived coenzymes FMN and flavin adenine

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dinucleotide (FAD) (5, 111, 126). The dissociation constant for riboflavin at 25° was determined to be 1.3 nM and was nearly constant between pH 6 and pH 9 (5). Riboflavin is released from the protein between pH 3 and 4. It can also be removed by methanol extraction without denaturing the protein (55) or by reduction with dithionite and absorption of the reduced riboflavin on charcoal (67). The dissociation constants for FMN and FAD are, respectively, 3 and 4 orders of magnitude larger than for riboflavin (5). No analog has been found that binds more tightly than riboflavin. Charged species do not bind well, and ring and side-chain modifications also decrease binding (19). Modifications at C-2 and N-3 have the least effect on binding and provide the rationale for derivatizing the N-3 position in designing affinity chromatography matrices (8, 80). Based on spectral studies and chemical modification, it has been concluded that one of nine tyrosine and one of six tryptophan residues in RfBP are essential for binding riboflavin (7). Bound riboflavin protects RfBP from inactivation by carbodiimide, which otherwise reacts with a critical carboxyl group presumed to be at the binding site (60).

Circular dichroism measurements on the apo- and holoprotein show little difference in the peptide backbone conformation of RfBP (63, 101, 102). There are changes in the near UV consistent with the involvement of tryptophan and tyrosine residues in the binding of riboflavin. ^{31}P-NMR of the apo- and holoprotein shows no change in the environment of phosphoryl groups (89). There is, however, increased stability of the protein to heat (S. M. Gaug & M. S. Miller, unpublished) and to guanidinium chloride (103) upon binding riboflavin. Monoclonal antibodies do not distinguish between apo- and holo-RfBP (125).

SERUM RIBOFLAVIN-BINDING PROTEIN In contrast to RfBP in egg white, RfBP in the blood plasma of laying hens is a minor protein component requiring more than a thousand-fold purification to homogeneity (85, 112). This combined with the limited availability of large quantities of blood from laying hens makes serum RfBP the least studied form of the protein from chicken. Because the proteins are products of a single gene expressed in different tissues, many, but not all, properties of egg white RfBP are the same as for serum RfBP. For example, the primary sequences are the same (105). However, unlike egg white RfBP, serum RfBP is normally saturated with riboflavin in hens fed adequate amounts of riboflavin (10, 129). A more major difference is the carbohydrate composition, which is more complex (85, 105). There is significantly more sialic acid and galactose than in egg white RfBP and several residues of fucose are present. These additions increase the molecular weight of serum RfBP by a few percent. Comparisons of the sugar compositions of the oligosaccharides attached to asparagines 36 and 147 show them to be very similar, which suggests that their structures may also be

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similar or identical (112). The amino acid sequences around the two attachment sites are remarkably similar as well. Other posttranslational modifications such as phosphorylation and the presence of N-terminal pyroglutamate are the same as for egg white riboflavin-binding protein.

EGG YOLK RIBOFLAVIN-BINDING PROTEIN Although the concentration of RfBP in egg yolk is not much less than it is in egg white (129), the large amounts of lipids and other proteins make purification more difficult. Yolk RfBP has been purified and compared with egg white and hen serum RfBP (72, 90, 107, 135). Since serum RfBP is the precursor to egg yolk RfBP, they would be expected to be very similar, if not identical; however, there is a major difference in amino acid sequence. The C-terminal 11 or 13 residues, which include five glutamate residues, are missing in the egg yolk protein (105). Such limited proteolytic cleavage of proteins deposited in yolk has been previously observed in the conversion of vitellogenin to lipovitellin and phosvitin (20) and for yolk very-low-density lipoprotein (30). There are also indications that some of the peripheral sugars attached to serum RfBP are hydrolyzed during or after incorporation into the yolk (85). This observation is not supported by other work (105) that shows the sugar composition to be unchanged. The difference in C-terminal amino acid sequence may explain the observation that yolk RfBP binds 8-substituted riboflavins slightly less tightly than does egg white RfBP (69).

Synthesis

LIVER The major yolk-specific proteins are produced by the liver during egg laying in response to estrogens, secreted into the blood plasma and subsequently deposited in the oocyte by selective endocytosis (44). This pattern has been demonstrated for RfBP. Normal hen liver (27) and hepatocytes (104) synthesize RfBP while hepatocytes from the riboflavinuric strain do not (104). As a secreted protein, RfBP is synthesized as a larger precursor from which a hydrophobic N-terminal signal peptide is cleaved (29, 137). RfBP has been isolated in small quantities by affinity chromatography from hen liver (42). Perhaps because of compensating posttranslational modifications, the molecular weight of liver RfBP is not detectably greater than serum or yolk RfBP (42, 98). However, the isoelectric point of liver RfBP (3.85) is lower than that of the major serum and yolk forms (4.0) and a minor component found in all three tissues (3.91) (42). Other than these studies, the synthesis of RfBP by the liver has been monitored by the appearance of RfBP in the plasma in response to various hormones (21, 97). The knowledge that in well-fed hens RfBP and riboflavin form a stoichiometric complex means that earlier studies that monitored serum riboflavin (11, 12, 22) reflected RfBP synthesis as well.

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The induction of hepatic RfBP synthesis by estrogen was first studied in roosters and immature chickens (21). Studies on hepatic induction in male birds are convenient because background levels are low and RfBP in the plasma will accumulate to higher levels in the absence of oocytes or an oviduct to remove the protein from the plasma. In contrast to later studies where sensitivity to estradiol induction appears shortly after hatching (27), stimulation was detected beginning at about eight weeks and increasing through 17 weeks. Adult males accumulated RfBP in their plasma within two days of a single injection and RfBP could be detected in plasma for two to three weeks. In hyperthyroid chicks, higher concentrations of estrogen were required to obtain the same response obtained in normal birds (97). This response confirms the observations based on riboflavin accumulation in plasma (23). Induction is specific for estrogen for primary and secondary stimulation (27). Neither progesterone nor the availability of riboflavin have an effect on synthesis (129).

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OVIDUCT Among proteins that are deposited in egg, RfBP is unusual because it occurs in both the yolk and albumen. Most egg proteins are either synthesized in the liver and deposited in yolk or synthesized in the magnum of the oviduct and become part of the egg white. The characteristics of the riboflavinuric hen show that a single gene, *Rd,* coding for RfBP is activated both in the liver and the oviduct (131). This is confirmed by the presence of polymorphism at residue 14 in both egg yolk and egg white RfBP (105).

Studies of the induction of RfBP in the oviduct have found that activation of the *Rd* gene is somewhat different than in liver (27). In the oviduct, as in the liver, primary stimulation of RfBP synthesis is strictly dependent upon estrogen; however, in secondary stimulation, progesterone can substitute for estrogen in the oviduct. Testosterone has no effect on synthesis.

Early studies on the synthesis of major egg white proteins in the oviduct (68) included RfBP peripherally but were the first to demonstrate that RfBP is synthesized by the oviduct. Unpublished immunohistological studies by Dr. Carl W. Nichols indicate that RfBP synthesis is localized to the nongoblet cells of the luminal epithelium of the oviduct magnum. However, this may not be the only source of RfBP in egg white. The unprecedented possibility that egg white RfBP is derived in part from plasma RfBP would be consistent with the unusual presence of sialic acid on egg white RfBP and the unusual presence, among birds, of bound riboflavin in the egg white. However, this possibility seems to be excluded by the observation that no RfBP was detected immunologically in the egg white of riboflavinuric hens that had been injected with 100 mg of RfBP (49). Similarly, ^{125}I from ^{125}I-RfBP
white is not protein bound (83). In retrospect, these experiments would be more convincing if serum RfBP, rather than egg white RfBP, had been used as the tracer. (See also the section on Evolution of Viviparity in Mammals)

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Plasma Clearance and Oocyte Deposition

RfBP synthesized by the liver is the sole source of riboflavin deposited in the oocyte. Free riboflavin is not deposited in the oocyte; therefore, structural features of RfBP must target it for uptake. The striking difference in the oligosaccharide components of plasma RfBP and egg white RfBP (85, 105) and the fact that subtle structural differences in oligosaccharide structure can target glycoproteins for specific cellular uptake (3, 66, 116) suggested that oligosaccharides on plasma RfBP may be recognized by specific oocyte receptors. Numerous studies have been conducted by Miller and coworkers to measure the plasma half-lives and oocyte uptake of various native and modified forms of RfBP.

As a point of reference for these studies, the concentration of RfBP in yolk is about six times that in plasma (129). Based on these concentrations, the rate of laying (1 egg/day), the blood volume of a hen (about 100 ml), the volume of an egg yolk (18 ml), and the assumption that all plasma RfBP is destined to the oocyte, a plasma half-life of approximately 12 h can be estimated for RfBP. Measured plasma half-lives for radioiodinated RfBP in laying hens are 2 h or less (48, 84–87) and 12% or less of the label is deposited in yolk. These results suggest that either plasma RfBP is normally distributed to tissues other than the oocyte or that purified and labeled proteins are not ideal probes.

Egg white RfBP, which has the same amino acid sequence as serum RfBP, has quite a different carbohydrate composition and is not a normal blood component. It is cleared from the plasma more rapidly ($t_{1/2}$ of 81 min) than is serum RfBP ($t_{1/2}$ of 121 min) and is not deposited in yolk as efficiently (4% vs 12%) (85). The difference is probably due to differences in carbohydrate structure but is not attributed to selectivity by an oocyte receptor. Rather, the egg white RfBP is preferentially cleared by a hepatic receptor that recognizes glycoproteins like egg white RfBP with terminal N-acetylglucosamine residues (66, 116). Degradation of injected RfBP and release of low-molecular-weight iodinated compounds was observed in liver, intestine, oviduct, and other tissues within one hour of injection (85).

Studies on the effect of selective modification of carbohydrate structures of egg white and egg yolk RfBP on plasma clearance and uptake suggest that terminal sialic acid residues may play a role in specific yolk deposition (86, 87). Other modifications that do not alter riboflavin-binding ability have rather dramatic effects. Of particular interest is the hydrolysis of phosphoryl residues, which abolishes concentrative uptake to about 12% of the control without greatly altering plasma half-life (84). Successive removal of 1 to 8 phosphoryl groups caused progressive decreases in oocyte uptake. Attempts to restore uptake by restoring negative charges through succinylation were unsuccessful, which further supports a specific role for the clustered phosphoserine residues in recognition and transport (84). In addition to possible roles for carbohydrates and phosphate groups in selective uptake, succinyla-

tion of lysine residues also abolishes concentrative uptake (84). Contrary to biological intuition, RfBP deposition in yolk is not dependent on the presence of bound riboflavin (6, 129).

Attempts to locate a RfBP receptor in the oocyte plasma membrane have been inconclusive (6). As a result, alternative models for concentrative uptake of RfBP have been entertained (130). Vitellogenin, a plasma protein precursor for several yolk proteins (20), contains many phosphoserine residues that associate with calcium ions. It and calcium are concentrated about sixfold in yolk (26, 51). If calcium forms ionic bridges joining phosphorylated regions of vitellogenin and RfBP as occurs in casein micelles (114), RfBP could be deposited selectively as part of an aggregate "parasitizing" the known receptor-transport system for vitellogenin (132). Such a piggy-back transport system has been suggested for deposition of vitamin-D-binding protein in egg yolk (40). However, this transport mechanism suggests RfBP that would be present in yolk granules, which is not the case.

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Metabolism by the Embryo

Very little is known about the metabolism of RfBP and release of riboflavin in the chick embryo. Degradation of egg white RfBP does not become evident before day 13 of development (117, 120), a time that coincides with embryonic death in riboflavin-deficient eggs (70) and the increased synthesis of flavin coenzymes (134). Flavokinase, the first enzyme that acts on riboflavin to convert it to a coenzymatic form, can be purified using immobilized egg white RfBP; hence, a complex may exist in vivo to facilitate utilization of riboflavin when it is released (117b).

All of the RfBP disappears by hatching (120). RfBP in normal eggs appears to prevent the decomposition of riboflavin that occurs in eggs from riboflavinuric hens (47). It appears that yolk RfBP is destroyed rather than being transferred to the embryonic circulation, as occurs for transferrin (41) and immunoglobulins (13).

Avian Riboflavinuria

The availability of the riboflavinuric strain of White Leghorn chickens has made possible a number of definitive experiments relating to riboflavin requirements, riboflavin metabolism, and the role of RfBP. For example, clubbed down has been classically associated with riboflavin insufficiency in chicken embryos; however, most embryos dying in riboflavinuric eggs do not have clubbed down (50). Those that have clubbed down also have a gene for black-colored down. Thus, the condition is manifested as a pleiotropic action of a pigmentation gene and is not due to riboflavin deficiency alone.

Riboflavinuric hens fed normal diets are a continuous source of riboflavin-deficient eggs. These eggs have been used to determine the minimum amount of riboflavin required for normal hatchability (0.7 mg/egg) (14) or the ability

of embryos to use riboflavin analogs such as 7-ethyl-8-methyl flavin (64). The proposal that RfBP deposition was coupled to thiamin deposition in eggs (91) was disproved when it was shown that thiamin deposition by riboflavinuric hens was normal (88). These and other results discussed earlier illustrate the utility of this mutant strain in riboflavin research.

Efforts to characterize a nonfunctional product of the *rd* allele have been thwarted for the most part. A weakly cross-reacting protein was reported in eggs (34). Subsequent work could not demonstrate a cross-reacting protein in egg on plasma but did detect such a protein in liver and oviduct (48, 110), which suggests a defect in cell secretion of the mutant gene product. The molecular weight of the protein was about 27,500 (110) but it occurred in such small quantities that it was not purified to homogeneity. The fact that the protein was only 0.3% as antigenic as native RfBP suggests a grossly altered protein or possibly an artifact that might result from oligosaccharides as antigenic determinants (37). When the normal and mutant genes are characterized, these uncertainties will be resolved.

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Riboflavin-Binding Protein from Other Egg-Laying Vertebrates

RfBP has been detected in the egg white of a number of birds (35, 108). The concentrations and the proportion of apoRfBP vary over a 10-fold range. This may reflect the possibility that RfBP in egg white has antimicrobial functions (122). RfBP has been purified and characterized from the two domestic duck species (1, 92). Less work has been done on yolk RfBP from various bird species; however, the protein is present in a number of species and the concentrations are less variable than in egg white (H. B. White, unpublished).

RfBP has been purified and characterized from the yolk of painted turtle oocytes and python eggs (1). Both are phosphoglycoproteins with riboflavin affinities similar to that of chicken RfBP. The molecular weights are higher (~40,000) because of a greater carbohydrate content. Alligator egg yolk contains a riboflavin-binding protein but none can be detected in alligator egg white (V. A. M. Abrams, T. Kennedy & H. B. White, unpublished). Similar efforts to detect RfBP in fish eggs have been inconclusive. From this fragmentary sampling it is clear that RfBP is widely distributed in the eggs of birds and reptiles and is likely to provide the primary means for depositing riboflavin in oocytes. A related protein appears to be involved in riboflavin transport to the fetus in certain mammals (2).

Riboflavin Transport and the Evolution of Viviparity in Mammals

A very few primitive mammals from Australia (such as the duck-billed platypus and echidna) are oviparous, which suggests that the earliest mammals were oviparous, as were their reptilian ancestors. The transition can be

envisioned as occurring in stages. First the egg would be retained to yield ovovivipary, then the oviduct would differentiate to form a uterus in which continuous nourishment could be provided to the embryo. To retain the nutrient transport mechanisms, nutrients originally destined to the oocyte would have to be redirected to the uterus and placenta. The observation that some mammals have a pregnancy-specific RfBP homologous with egg RfBP in birds (2, 76) supports such a hypothesis.

A curious and still poorly understood aspect of riboflavin deposition in chicken eggs may indicate a propensity for the oviduct to develop nutrient transport abilities analogous to the uterus. Among birds, chickens and a few close relatives are unusual in that there is as much or more riboflavin in the egg white than in the yolk and in both cases is bound to RfBP (35). Because egg white RfBP is synthesized in the oviduct, one might hypothesize that free riboflavin from the blood is scavenged. However, this is not the case because RfBP in egg white is not saturable even at excessive dietary riboflavin levels (10, 129). Furthermore, the amount of riboflavin in the egg white is proportional to *Rd* gene dosage (131) rather than to diet, which indicates that riboflavin deposition is mediated by serum RfBP. Tracer studies with ^{125}I-labeled RfBP in plasma show that RfBP is deposited and degraded in oviduct (85). Since it appears that serum RfBP is not deposited in egg white (48), the most tenable hypothesis is that serum RfBP is recognized by hen oviduct, removed from the blood, and degraded. The released riboflavin would then be scavenged by newly synthesized RfBP in the oviduct (85). Conceptually, this sequence of events could be directly related to the evolution of riboflavin transport to the placenta in mammals.

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MAMMALIAN RIBOFLAVIN-BINDING PROTEINS

Mammals also have a variety of proteins in both tissues and circulation that bind riboflavin (see 79 and 82 for review), but the functions of these RfBP are only beginning to be elucidated.

Albumin

Purified human serum albumin has been reported to bind riboflavin moderately tightly (K_d = 0.77 mM at 30°C) (56–58) and, since albumin is present in high concentrations (~1 mM), might bind approximately half of the riboflavin in plasma. However, it is difficult to account for the greater than 10-fold variation in riboflavin binding by plasma from different human subjects (53) unless there are other factors influencing the binding by albumin. Reanalyses of the dissociation constant for the riboflavin-albumin complex revealed that it is somewhat higher than previously thought (i.e. $\geq$ 3.8 mM) (53); hence, albumin probably binds little more than 13 to 15% of the riboflavin in circulation (53). The presence or absence of other ligands that

normally bind to albumin (such as fatty acids) has little influence on riboflavin binding, nor are there apparent differences among electrophoretically distinct subspecies (82).

Riboflavin 5'-phosphate and the photodegradation products of riboflavin (e.g. lumiflavin and lumichrome) are much more tightly bound and are likely to be associated with albumin (53). Substantial amounts of the photodegradation products, which are potentially antivitaminic (71), are formed when skin is exposed to light (52). Their association with albumin may protect them from glomerular filtration since they are usually only found in trace amounts in urine (18). These compounds are readily formed in dilute solutions of riboflavin, and probably contributed to some of the earlier confusion concerning the affinity of albumin for riboflavin. In fact, while screening plasma samples electrophoretically for riboflavin binding, Cavalli-Sforza et al (16) noted that riboflavin did not bind to albumin except when light was present. This underscores the need for careful purification of riboflavin stocks and protection of samples from light (79).

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Riboflavin-Binding Immunoglobulins

It has long been known that riboflavin is bound by plasma protein fractions other than albumin (39). However, it has only recently been established that the proteins most responsible for individual variations in riboflavin binding are immunoglobulins (53, 54). Analyses of several hundred individuals found a substantial number for whom the majority of the riboflavin in circulation is associated with immunoglobulins. The importance of this interaction is unknown.

The riboflavin-binding immunoglobulins have been purified from normal human plasma by flavin affinity chromatography (73, 77, 78). The type of immunoglobulins obtained depends on the portion of the flavin ring used to prepare the affinity materials; for example, flavins immobilized via position N-3 yielded mainly IgG (78%), whereas IgM (74%) predominated when the flavin was attached via the 7,8-dimethylbenzene ring. All three isolates had a similar subclass distribution, $IG_2 > IG_1 > IG_3 > IG_4$, and had both lambda and kappa chains. The riboflavin-binding immunoglobulins are less than 1% of the total immunoglobulins in circulation.

Since riboflavin binding could be localized to the Fab fragment (78), the complex may represent an antigen-antibody type interaction. It would seem unlikely that riboflavin is the antigen, but antibodies might be formed against a flavoprotein with covalently attached FMN or FAD (many of which are known to occur in many organisms) (79) and some of these might recognize the flavin moiety.

Immunoglobulins with a high affinity for riboflavin have been isolated from the serum of a patient with multiple myeloma (32). This protein, termed IgG^{Gar}, consists of two types of binding sites, one of which binds riboflavin

essentially irreversibly while the other binds riboflavin (K_d=0.6 nM) but reversibly (17). This patient did not show signs of riboflavin deficiency (109). Nonetheless, further investigation of the relations between plasma riboflavin binding levels, the vitamin status of tissues (as indicated by standard indices such as the erythrocyte glutathione reductase activity coefficient), and concomitant urinary excretion levels should be considered.

Pregnancy-Specific Riboflavin-Binding Proteins

Riboflavin-binding proteins have also been implicated in mammalian reproduction. A mammalian RfBP was first isolated by affinity chromatography from the plasma of pregnant cows (76). The bovine RfBP had an apparent molecular weight of approximately 37,000 and bound riboflavin very tightly. Since a comparable protein was not detected in plasma from nonpregnant cows, the authors concluded that mammals, like avian species, might utilize a protein carrier to provide riboflavin for the growing fetus. Interestingly, although cows also have riboflavin-binding immunoglobulins (76), they do not transfer IgG to the fetus (31) and may need another system for this purpose.

The isolation of RfBP from other sources has been reported. The RfBP from pregnant rat serum has an apparent molecular weight of 90,000 (94), and an even larger species (185,00) was isolated from human pregnancy serum (99). More recent studies by this group have described a lower-molecular-weight protein from pregnant bonnet monkeys (36,000) (123) and pregnant human and umbilical cord blood (37,000) (124). These are stated to be structurally similar to the avian RfBP based on competitive assays using monoclonal antibodies to the avian RfBP and rat, monkey, and human sera.

These proteins appear to be crucial for mammalian reproduction because injection of antibodies against the avian RfBP or active immunization of animals with the avian RfBP have been shown to terminate pregnancy in rats (93, 99), mice (100), and the bonnet monkey (115). The fetal degeneration is accompanied by depletion of FAD levels in the fetus and in fetal liver (62, 118).

Others

Riboflavin kinase, which binds riboflavin relatively tightly as a substrate, is the most thoroughly characterized RfBP in tissues (71, 81). A soluble RfBP has also been detected in kidney (82). Other binding proteins surely exist, such as the membrane transport proteins involved in the facilitated uptake of riboflavin by cells (72), but await characterization.

Potential Roles of Binding Protein in the Transport and Disposition of Riboflavin

Despite the likely importance of the various mammalian RfBPs, none have been proven to have a role in the transport or trapping of this vitamin. The

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binding of riboflavin to albumin and immunoglobulins may help conserve body stores by decreasing losses during glomerular filtration. It is tempting to speculate that fetal albumin, immunoglobulins, or specialized proteins account for the apparent "trapping" of riboflavin in fetal circulation at concentrations severalfold higher than in maternal blood (4). The pregnancy-specific RfBP may help carry riboflavin to the fetus (although IgGs are also transported across the placenta by many vertebrates and these antibodies may carry riboflavin). In some animals, they may help provide this micronutrient to the conceptus early in pregnancy, perhaps via uterine secretions as occurs in some mammals such as the pig (95, 113). The mechanism(s) of riboflavin transport to the fetus warrant further study since they may provide clues to some types of infertility and/or birth defects since these are known to occur when the fetus does not get enough riboflavin because of dietary deficiencies (127).

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IMPLICATIONS FOR RIBOFLAVIN REQUIREMENTS OF EMBRYOS

As described in this review, riboflavin delivery to the embryo in birds and, perhaps, in mammals is mediated by maternally produced riboflavin-binding proteins. These proteins determine the upper limit of riboflavin that can be delivered to the embryo, and for at least avian species, dietary riboflavin in excess of the amount required to saturate this system is not available to the embryo. In the case of vitamin allowances of the laying hen, this provides a rational biochemical approach to evaluating the optimal riboflavin requirement of the embryo (130). RfBP and other vitamin-binding proteins are the products of specific genes, the levels of expression of which are the result of millions of years of natural selection. One must presume that these levels correspond to some fitness optimum because both overproduction and underproduction of particular binding proteins could reduce the fitness of an animal. Thus it can be argued that the dietary intake necessary to meet the normal capacity to transport a vitamin to the egg or embryo is a biologically meaningful definition of a recommended allowance during reproduction. This intake for riboflavin is greater than that required for maximum hatchability. When more is known about the mammalian RfBP, this concept may be found to be relevant to mammals as well.

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