Biochem. J. (1976) **157**, 395–400
Printed in Great Britain

Biotin-Binding Protein from Chicken Egg Yolk

ASSAY AND RELATIONSHIP TO EGG-WHITE AVIDIN

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(*Received* 26 *January* 1976)

1. Biotin in chicken egg yolk is non-covalently bound to a specific protein that comprises 0.03% of the total yolk protein (0.8 mg/yolk). This biotin-binding protein is not detectable by the normal avidin assay owing to the biotin being tightly bound. Exchange of [^{14}C]biotin for bound biotin at 65°C is the basis of an assay for this protein. 2. Biotin-binding protein from egg yolk is distinguishable from egg-white avidin on Sephadex G-100 gel filtration, although the sizes of the two proteins appear quite similar. 3. Biotin-binding protein is denatured at a lower temperature and freely exchanges biotin at lower temperatures than does avidin. 4. The biotin-binding protein in egg yolk is postulated to be responsible for the deposition of biotin in egg yolk. D-[*carboxyl*-^{14}C]Biotin injected into laying hens rapidly appears in the egg bound to yolk biotin-binding protein and avidin. Over 60% of the radioactivity is eventually deposited in eggs. The kinetics of biotin deposition in the egg suggests a 25 day half-life for an intracellular biotinyl-coenzyme pool in the laying hen.

The chicken egg contains all the necessary vitamins and minerals for the 21 day development of a chick embryo. The majority of these trace substances are found in the yolk (Romanoff & Romanoff, 1949). The mechanism for the deposition of these vitamins and minerals in the developing yolk is at best poorly understood (Gilbert, 1971*a*; McIndoe, 1971).

The proteins of the yolk differ from those of the white in both the time and place of synthesis. The entire white of an egg is secreted by the magnum of the oviduct within 4–6h after ovulation (Gilbert, 1971*b*). On the other hand, most, if not all, proteins deposited in the yolk are serum proteins synthesized by the liver (Williams, 1962*a*; Schjeide *et al.*, 1963; Cutting & Roth, 1973). The accumulation of 97% of the yolk occurs within a given ovarian follicle in a 1 week period before the ovulation of that follicle (Gilbert, 1971*b*). Despite these differences, there are at least two glycoproteins, one an iron-binding protein, transferrin (conalbumin) (Williams, 1962*b*) and the other a riboflavin-binding protein (Winter *et al.*, 1967*a*), which occur both in egg yolk and egg white.

The importance of riboflavin-binding protein in the transport of riboflavin into the egg is particularly clear. A strain of chickens incapable of concentrating riboflavin in the egg can only be maintained by injection of riboflavin into their riboflavin-deficient eggs (Winter *et al.*, 1967*b*). The defect in these chickens is the lack of a functional riboflavin-binding protein (Winter *et al.*, 1967*a*). These results in particular and the similarity of this system to transferrin, retinol-binding protein (Abe *et al.*, 1975) and vitamin B-12-binding protein (Sonneborn & Hansen, 1970) in chickens suggest that vitamin and mineral transport should be viewed as a problem of protein transport.

In the present paper we enlarge this class of vitamin-transport proteins of the egg to include an egg-yolk biotin-binding protein, a protein whose presence was suggested in 1942 (György & Rose, 1942) but was never studied. A specific radioisotopic assay for this protein is described and evidence is provided that this protein is distinct from egg-white avidin. A preliminary account of the present work has already been published (Meslar *et al.*, 1976).

Materials and Methods

Metabolism of D-[1-^{14}C]*biotin*

Two 14-month-old Buttercup hens (American Poultry Association, 1974) were each injected with 0.5ml of a 0.95% sterile NaCl solution containing 10μCi of D-[1-^{14}C]biotin (45Ci/μmol; Amersham/Searle, Arlington Heights, IL, U.S.A.). The specific radioactivity of the injected biotin was 183μCi/mg.

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One hen was injected intraperitoneally, the other intramuscularly. The total amount of biotin injected (55 μg) is approximately five times the amount of biotin contained in an average chicken egg (György & Langer, 1968). The hens were fed *ad libitum* on a breeder–layer diet (Anon, 1967) throughout the experiment.

Eggs were collected once daily and stored at 4°C until they were assayed for radioactivity in the yolk and in the white. The yolk and white were processed separately. The white was diluted with an equal volume of water and sonicated. The yolk was diluted tenfold in water. Samples (0.10ml) of each solution were then counted for radioactivity in 10ml of Aquasol (New England Nuclear Corp., Boston, MA, U.S.A.) in a Packard refrigerated scintillation counter. The eggs collected before the 36th day of the experiment were counted for 10min and the remainder were counted for 100min. Eggs collected after the 36th day were stored for up to 4 months before being assayed.

Biotin-exchange assay for egg-yolk biotin-binding protein

The procedure for assaying egg-yolk biotin-binding protein is basically the same as the radioactive assay for egg-white avidin (Korenman & O'Malley, 1970) except that an elevated temperature is required. At 65°C the exchange between free and bound biotin is rapid and the protein is stable.

The standard procedure used throughout the present work, unless otherwise noted, is as follows. Egg yolk is diluted 25-fold in 0.05M-potassium phosphate (pH7.2) and a portion (0.5ml) is placed in a test tube (13mm×100mm) at 0°C. A portion (20 μl) of D-[1-^{14}C]biotin (0.03 μg, 61 μCi/mg) is added, mixed and the tube placed in a water bath at 65°C for 15min. The reaction is terminated by returning the tube to 0°C in an ice bath. To the chilled tube 0.3ml of a 10mg/ml suspension of bentonite (Fisher Chemical Co., King of Prussia, PA, U.S.A.) in 0.05M-potassium phosphate (pH7.2) is added and stirred. The assay mixture is then filtered on a Millipore 3025 manifold. Each reaction tube is rinsed three times with the above phosphate buffer, which is added successively to the filter after the previous wash has filtered. After the final wash, the filters (0.45 μm pore-size, 25mm diam.; Millipore Corp., Bedford, MA, U.S.A.) are transferred to scintillation vials, wetted with 100 μl of 10.5% (w/v) trichloroacetic acid to denature protein, and counted with 10ml of Instabray (Yorktown Research, South Hackensack, NJ, U.S.A.) counting solution.

Preparation of an egg-yolk acetone-dried powder

Since diluted egg yolks contain considerable amounts of lipid, which tends to clog Millipore filters, the assay as described above involves a prolonged filtration step. For the purposes of characterizing the assay system, most of the lipids were removed by making an acetone-dried powder of egg yolk as described below. The powder was then resuspended, centrifuged and the supernatant used in the assay.

The yolks from 5 dozen commercial eggs (total volume, approx. 1.0 litre) were blotted free of adhering egg white on paper towels and 500ml of water was added. The yolks were homogenized with a stirring rod and added dropwise via a separatory funnel to 5.2 litres of acetone maintained at −5°C with solid CO_2 in a fume hood. The precipitated protein was collected on a Buchner funnel. The filter cake was resuspended in 4.8 litres of acetone, filtered and suction dried. The white powder was stored at 4°C.

Results

Fate of injected biotin in laying hens

A total of 38 eggs was collected over a 3 month period from the hen injected intraperitoneally with 10 μCi of D-[*carboxy*-^{14}C]biotin. The radioactivity appearing in the yolks and whites of these eggs is shown in Fig. 1. With an average yolk volume of 15ml, an average white volume of 31ml, and a measured 75% counting efficiency, 62% of the injected radioactivity was recovered in the eggs, 39% in the egg yolk and 23% in the egg white. One-half of the injected radioactivity was deposited in six eggs during the first 10 days. The pattern observed for the hen injected intramuscularly was the same as for the hen monitored

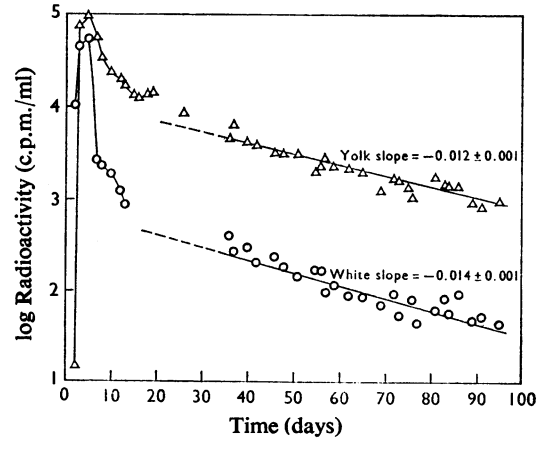

Fig. 1. *Radioactivity recovered in eggs after the injection of [^{14}C]biotin into a laying hen*

Details of the experiment are given in the Materials and Methods section. ○, Egg-white; △, egg yolk.

in Fig. 1, although fewer eggs were collected over a shorter period of time for the former. These results indicate that biotin in a laying hen is primarily deposited in the egg rather than degraded or excreted.

Kinetics of biotin deposition in egg yolk and egg white

The deposition in the egg of injected biotin occurs in three phases. The first phase, characterized by large quantities of radioactive biotin in the egg white, ends for both hens after the egg laid on day 5. During this initial period, the total amount of radioactivity in the egg white exceeds that in egg yolk.

The second phase begins with an abrupt decrease in the radioactivity appearing in the egg white, which results in a distribution of total radioactivity between egg yolk and egg white of about 7 or 8:1. This ratio is maintained for the duration of the experiment and corresponds to the distribution of biotin normally found between egg yolk and egg white (György & Langer, 1968). For both hens, the white from the egg laid on day 6 or 7 contained only 4.7% of the radioactivity in the white of the preceding egg laid on day 5. The transition from the second phase to the final phase is not distinct and is arbitrarily set at about 20 days. Before this time, the amounts of radioactive biotin deposited in both yolk and white are declining rapidly, whereas after this time, the decline occurs more slowly, and at a more-or-less constant rate. The final phase is the period with an exponential decline of radioactive biotin deposition in both egg yolk and egg white ($t_{1/2} = 25$ days).

Nature of biotin in egg yolk

Gel filtration of centrifuged egg-yolk extract derived from an egg containing radioactive biotin reveals a radioactive peak of high molecular weight and no radioactivity in the low-molecular-weight range of biotin (mol.wt. 244). If the egg-yolk extract is boiled and centrifuged, the radioactivity remains in the supernatant fraction and elutes in the low-molecular-weight region on gel-filtration chromatography. These results confirm early reports that biotin egg yolk was non-covalently bound in the high-molecular-weight fraction of egg yolk (György & Rose, 1942). In addition, the symmetrical peak demonstrates that there is probably a single biotin-binding protein in egg yolk. A sample containing both avidin (mol.wt. approx. 68000) and biotin-binding protein was subjected to gel filtration chromatography (Fig. 2). The elution profiles of the two proteins overlap considerably, but the peak of egg-yolk biotin-binding activity slightly precedes that of egg-white avidin.

Assay of the biotin-binding protein of egg yolk

A routine assay for avidin in egg yolk reveals little binding activity (24°C point on Fig. 3*a*), indicating, in the light of the above results, that most of the biotin-binding sites are occupied and non-exchangeable. Increasing the temperature, however, does facilitate an exchange of free radioactive biotin with bound biotin (Fig. 3*a*). The optimum temperature for this exchange is about 70°C; above this temperature denaturation occurs as is demonstrated by the release of bound biotin (Fig. 3*b*). Slightly below the denaturation temperature, the exchange reaction is near equilibrium between 10 and 20 min (Fig. 4). True exchange equilibrium, achieved by heating for up to 24h, results in an additional 10–20% increase in bound radioactive biotin (C. J. Whitney & H. B. White, unpublished work).

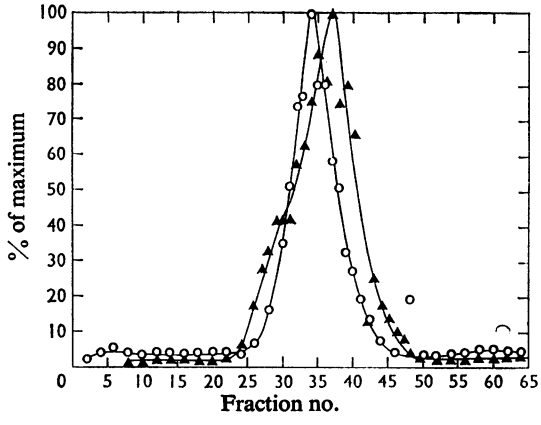

Fig. 2. *Comparison of egg-yolk biotin-binding protein* (○) *and egg-white avidin* (▲) *by Sephadex G-100 gel filtration*

Partially purified egg-yolk biotin-binding protein (1ml, 0.3mg/ml, 0.09 Avidin unit/mg) was heat-equilibrated with an excess of D-[*carboxy*-^{14}C]biotin (Sigma Chemical Co., St. Louis, MO, U.S.A.; 0.24mCi/mol). The protein-bound biotin fraction eluted from this column was combined with 1.0mg of commercial avidin (Sigma Chemical Co.; 11.1 units/mg) previously saturated with unlabelled biotin. The combined solutions (3ml) were made 10% (w/v) in sucrose and applied to a column (2cm×37cm) of Sephadex G-100 equilibrated with 0.5M-$NaHCO_3$, pH9.5. A sample (0.80ml) was removed from each 1.4ml fraction and assayed for radioactivity (egg-yolk biotin-binding protein). Another sample (0.20ml) was mixed with radioactive biotin and heated at 85°C for 15min. The protein-bound radioactivity (egg-white avidin) was measured (Korenman & O'Malley, 1970). Since biotin-binding protein denatures at 85°C and since avidin was in a large excess over biotin-binding protein, both proteins could be monitored in the same fractions.

On the identity of egg-white avidin and the egg-yolk biotin-binding protein

The heat-induced exchange reaction of the egg-white avidin–biotin complex is quite distinct from the biotin complex in egg yolk (Fig. 5). In the former,

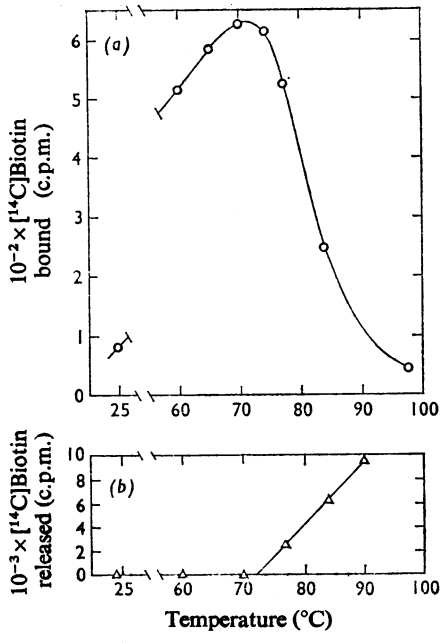

Fig. 3. *Thermally induced (a) exchange of added [^{14}C]biotin with protein-bound biotin in an egg-yolk acetone-dried powder and (b) release of protein-bound [^{14}C]biotin in egg yolk*

(*a*) Acetone powder (20mg) was suspended in 100ml of 0.2M-$NaHCO_3$ (pH8.7) and the solids were removed by centrifugation. Supernatant solution (10.5ml) was mixed with [^{14}C]biotin (32000c.p.m.) and heated at the temperatures indicated for 15min. The protein was absorbed on Bentonite, collected on a millipore filter, and the radioactivity measured by liquid-scintillation counting. (*b*) Radioactive egg yolk (day 13 of Fig. 1) was diluted 1:10 and heated for 15min at the indicated temperatures. Bentonite was added and the solution was centrifuged. Samples of the supernatant were measured by liquid-scintillation counting to determine the amount of biotin released.

significant exchange does not occur below 65°C and denaturation does not become apparent until 95°C. In other words, biotin binds much more tightly to egg-white avidin. Taken with their elution on gel-filtration chromatography (Fig. 2), the properties of egg-white avidin and egg-yolk biotin-binding protein are sufficiently distinct to suggest separate gene products or differentially modified products of a single gene.

Discussion

Relationships among the binding proteins of egg

The discovery of a biotin-binding protein in egg yolk together with the known occurrence of avidin in

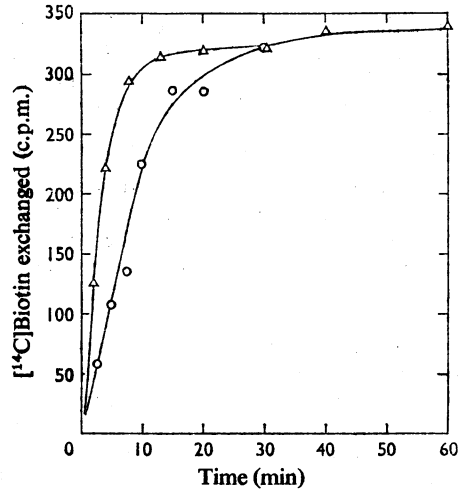

Fig. 4. *Time-course of [^{14}C]biotin exchange with protein-bound biotin in an egg-yolk acetone-dried powder extract at* 50° (○) *and* 60° (△) *C.*

The acetone-dried powder extract was prepared and assayed as described in the legend to Fig. 2. The reaction was terminated at the indicated times by immersion in an ice bath.

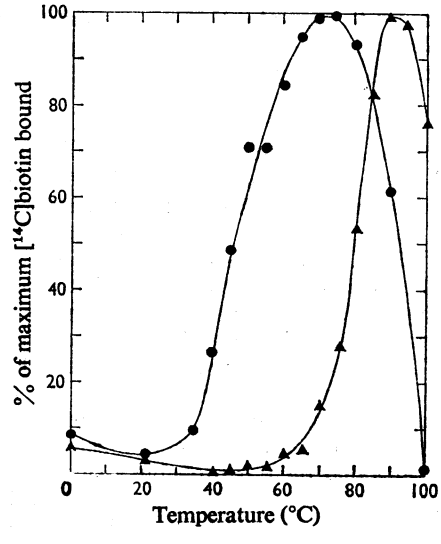

Fig. 5. *Comparison of the thermally induced biotin exchange reaction of egg-white avidin* (▲) *and egg-yolk biotin-binding protein* (●)

Egg-white avidin was mixed with an excess of unlabelled biotin to saturate the binding sites. The unbound biotin was removed on a short desalting column of Sephadex G-25 0.6cm×15cm before the thermal exchange assay of the avidin–biotin complex. The egg-yolk extract was not pretreated with unlabelled biotin, since the biotin-binding protein in these yolks was apparently saturated.

egg white provides a third example of pairs of binding proteins segregating between the yolk and white of chicken eggs. The other two pairs of proteins bind riboflavin (Winter *et al.*, 1967*a*) and iron (Williams, 1962*b*). Certain patterns are the same in all three.

Biotin, riboflavin and iron are trace substances required by the developing embryo (Romanoff & Romanoff, 1949). They are bound to specific proteins in the yolk which appear to be responsible for the transport of these vitamins and iron into the egg yolk. The corresponding proteins in egg white are primarily in their apoprotein form and presumably function as antimicrobial factors by scavenging and binding tightly free biotin, riboflavin and iron that otherwise could support bacterial growth (Board & Fuller, 1974; Green, 1975; Schade & Caroline, 1944).

For the riboflavin-binding proteins and for the transferrins, there is good chemical (Williams, 1962*b*) and/or genetic (Winter *et al.*, 1967*a*; Stratil, 1968) evidence that each pair is coded by a single structural gene. Such evidence does not yet exist for yolk biotin-binding protein and avidin. Their similarity in size (Fig. 2) is consistent with such a view; however, there is a considerable difference in their relative stabilities and biotin affinities (Figs. 3 and 5). Such differences may be caused by post-translational modification such as the nature of an attached carbohydrate moiety.

The difference between yolk biotin-binding protein and avidin in their temperature-dependent biotin-exchange properties is consistent with the physiological functions proposed above. Avidin binds biotin very tightly and will not exchange biotin appreciably at the body temperature of a chicken (41°C). On the other hand, biotin-binding protein from yolk does exchange biotin at this temperature (Fig. 5). This property could be important in that biotin could be made available to a developing embryo without the necessity for proteolysis of the binding protein.

Biotin metabolism in the laying hen

Under the conditions we created by injecting free biotin into a laying hen, egg-white avidin was a major sink for radioactive biotin (Fig. 1). It is doubtful that avidin-bound biotin is critical for the nutrition of an embryo, since only about 15% of the total biotin of an egg is in the white (György & Langer, 1968), the amount of biotin in the yolk alone is sufficient for normal growth and development (Brewer & Edwards, 1972), and avidin-bound biotin is not biologically available without destruction of avidin. The fact that much more than 15% of the recovered radioactivity was found in the egg white shortly after a single injection is undoubtedly due to a difference in specific radioactivity of biotin in the two compartments. During the longer yolk-deposition process, dilution by dietary biotin significantly lowers the specific radioactivity of biotin in the yolk as compared with the white.

Approximately 5% of the recovered radioactivity was deposited over a much longer time and in the ratio expected for the natural distribution of biotin between yolk and white. This radioactivity was incorporated into a biotin pool that exchanges slowly with the serum pool that is the source of biotin in the egg. This pool is probably intracellular biotin, both free and covalently attached to biotin-dependent carboxylases. The 25 day half-life would depend on the turnover of biotinyl-coenzyme and the exchange of circulating biotin with intracellular biotin.

Three biotin-dependent carboxylases in chicken, acetyl-CoA carboxylase, propionyl-CoA carboxylase and pyruvate carboxylase, constitute the bulk of bound biotin (Arinze & Mistry, 1970). These carboxylases are found predominantly in the liver. The half-life of acetyl-CoA carboxylase in chicken liver is 46h (Teraoka & Numa, 1975), or only a fraction of the half-life biotin pool we have detected. Unless the other carboxylases have much slower turnover rates, this suggests that intracellular biotin is conserved and that it is recycled a number of times before it is lost from the cell.

Possibility of other binding proteins in egg

The existence in the egg of binding proteins for biotin, riboflavin and iron suggests that a general mechanism for transporting vitamins and minerals into the egg is via specific protein-bound complexes. We have looked for thiamin and pantothenate-binding proteins in egg yolk without success (J. C. McGuire & H. B. White, unpublished work). Zinc in the yolk appears to be protein-bound (C. A. Kittle & H. B. White, unpublished work), but it is not known whether this binding is to a specific protein.

We thank Dr. Jean S. White and Dr. John C. Wriston for their comments and criticism of the manuscript. This investigation was supported in part by grant GM-19620 from the National Institutes of Health, United States Public Health Service, and a grant from the University of Delaware Research Foundation.

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