Dephosphorylation of Chicken Riboflavin-binding Protein and Phosvitin Decreases Their Uptake by Oocytes

(Received for publication, January 25, 1982)

Mark S. Miller, Marilee Benore-Parsons, and Harold B. White, III‡

From the University of Delaware, Department of Chemistry, Newark, Delaware 19711

Riboflavin-binding protein (RBP) and phosvitin are phosphoglycoproteins transferred from the plasma of laying hens into the yolk of developing oocytes. We have examined the effect of phosphate removal on this yolk deposition process. Unmodified yolk RBP and phosvitin contain, respectively, 8.3 and 109 residues of phosphate/molecule. Complete dephosphorylation of yolk RBP caused a 24-min decrease in the plasma clearance half-life and an 87% decrease in the uptake of the protein into oocytes in vivo. Although partially desialylated, dephospho-yolk RBP was identical with the native protein by several criteria, including riboflavin-binding capacity, mobility on SDS-polyacrylamide gels, and circular dichroism. A series of partially dephosphorylated yolk RBP samples, prepared by limited enzymatic hydrolysis, was indistinguishable from native yolk RBP by all criteria except phosphate content. Removal of the 1st phosphate residue decreased uptake of yolk RBP into oocytes by about 60%. Uptake into oocytes could not be restored to dephospho-yolk RBP by addition of anionic groups by succinylation. However, succinylation of native yolk RBP decreased its deposition into oocytes to the same extent as dephosphorylation. Partial dephosphorylation of phosvitin also had marked effects. The plasma clearance of dephosphophosvitin (76% of phosphate removed) was much faster than native phosvitin. After 4 h, 15% of injected 125I-phosvitin remained in circulation compared with only 3.8% of 125I-dephosphophosvitin. The uptake of dephosphophosvitin into oocytes was 79% less than that of native phosvitin. In vitro, 125I-phosvitin bound specifically to a preparation of oocyte plasma membranes as judged by competition with unlabeled phosvitin but not with RBP. The specific binding of dephosphophosvitin was 8% less than that of native phosvitin and it could be displaced equally well by phosvitin or yolk RBP.

Riboflavin-binding protein (RBP), a phosphoglycoprotein found in egg white, yolk, and blood of laying hens, is necessary for the transport of riboflavin to the egg (Winter et al., 1967; Blum, 1967). We have been interested in determining which features of RBP enable ovarian tissue to recognize it in the plasma and transport it specifically into the yolk of developing oocytes. Since RBP is glycosylated (Farrell et al., 1969; Ostrowski et al., 1968) and since carbohydrates have been implicated in cellular recognition of other glycoproteins (for review see Neufeld and Ashwell, 1980), our efforts have been concentrated on the oligosaccharide moiety of RBP as a possible recognition signal. Supporting this hypothesis is the observation that removal of sialic acid from egg white or yolk RBP decreases the uptake of these proteins into yolk by over 80% (Miller et al., 1981a, 1981b).

Although egg white, yolk, and serum-RBPs are the products of the same gene (Winter et al., 1967) and cross-react with the same antiserum (Farrell et al., 1970), the three proteins differ significantly in their carbohydrate compositions (Miller et al., 1982). We recently tested the carbohydrate recognition hypothesis with these three differently glycosylated proteins and found that while 11.4% of serum RBP and 12.5% of yolk RBP injected into hens could be recovered from the oocytes, only 4.3% of injected egg white RBP was transported to these cells (Miller et al., 1982). However, since plasma clearance times of these proteins differed, as well as their distribution to extraovarian tissues following intravenous injection, it was not possible to support this hypothesis using native proteins.

In addition to being glycosylated, RBP from both egg white and yolk is phosphorylated (Rhodes et al., 1959; Zak and Ostrowski, 1963). Egg white RBP contains 7 to 8 residues of phosphate, all of which can be removed without affecting the riboflavin-binding capacity of the protein (Rhodes et al., 1959). In the present paper, we examined the possible role of phosphorylation in recognition of RBP by ovarian tissue and in deposition of the protein in developing oocytes.

Other yolk proteins, including both phosvitin and lipovitellin, the major yolk proteins, are also phosphorylated (for review, see Taborsky, 1974). Phosvitin is about 10% phosphorylated by weight and contains over 150 residues of phosphate/molecule (Taborsky, 1974). Phosvitin has been shown to bind very specifically to receptors on isolated oocyte plasma membranes (Woods and Roth, 1979). In this report, we consider the involvement of phosphate residues in the interaction of phosvitin with its plasma membrane receptors in vivo, as well as in the plasma clearance and ovarian transport of this protein in vivo. In the case of both RBP and phosvitin, there is an indication that covalently bound phosphate is important for normal yolk deposition of these proteins.

MATERIALS AND METHODS

Experimental Animals and Source of Proteins—The animals used in these experiments were female single Comb White Leghorn chickens (Gallus domesticus) obtained from DeKalb Hatcheries, York, PA, or from Mayer's Chicks, Quakertown, PA. They were maintained for at least 2 weeks at the facilities of the University of Delaware, College of Agriculture and fed a diet of layer feed ad libitum. The hens were used for the experiments when they were between 20 and 30 weeks old and when they were laying at a rate of at least 5 eggs per
Phosphoproteins in Yolk Deposition

week. Egg white RBP was purified from White Leghorn eggs obtained locally by the method of Farrell et al. (1939). The RBP was prepared by addition of riboflavin (Sigma Chemical Co., St. Louis, MO) prior to purification and maintained as the holoprotein throughout its isolation. Yolk and serum RBP were purified by methods described previously (Miller et al., 1981b, 1982). RBP from each of these sources was homogeneous by SDS-polyacrylamide gel electrophoresis (Weber and Osborn, 1969). Circular dichroism (CD) of RBP samples was measured with a Durrum-Jasco model J-10 circular dichroism recorder. The samples for CD were dissolved in distilled water and the concentration adjusted to give an absorbance of 1.0 at 280 nm. CD was measured from 300 to 290 nm using a 1.0-cm path length cell and from 290 to 195 nm using a 0.05-cm cell.

**Dephosphorylation of RBP and Phosvitin**—RBP and phosvitin were dephosphorylated with potato acid phosphatase (Boehringer Mannheim, Indianapolis, IN, grade 1) as described by Rhodes et al. (1959). Holo-yolk RBP or phosvitin (15 mg) was dissolved in 1.5 ml, 0.1 M sodium acetate buffer, pH 5.3, and 50 μl of acid phosphatase (60 units/ml) was added. The mixture was dialyzed against 30 ml of buffer, a water-jacketed beaker at 37 °C. The dialysate was sampled at various intervals and assayed for released phosphate. The dephosphorylated proteins were isolated from the reaction mixture by chromatography on Bio-Gel P-100 (1.5 X 60 cm) in 0.9 M NaCl, dialyzed, and lyophilized. When it was necessary to stop the reaction before complete dephosphorylation had achieved, 1 ml of 0.3 M NaF was added to the incubation mixture to inhibit phosphatase (Hollander, 1971). Partially dephosphorylated yolk RBP samples were prepared by varying the time of dephosphorylation and the amount of acid phosphatase. These samples were purified on small DEAE-cellulose columns (Whatman DE52, 1 × 3 cm) in 0.1 M sodium acetate, pH 5.3. RBP was eluted with 0.5 M NaCl, desalted on Sephadex G-25 (1 × 20 cm) in distilled water, and lyophilized.

**Sucination of RBP**—Yolk RBP and dephosphorylated yolk RBP were succinylated at pH 8.0 by the method of Klapper and Klotz (1969). Succinyl chloride was added in small increments over a 1-h period to 10 ml of protein solution, 1 mg/ml, in distilled water. pH was maintained close to 8.0 by dropwise addition of 0.1 M NaOH. The total amount of succinic anhydride added was in 50-fold molar excess over lysine residues. Following the last addition of succinic anhydride, the protein was dialyzed against distilled water and lyophilized. The number of derivatized lysine residues was determined from the difference between available amino groups on the native and succinylated protein as estimated by the ninhydrin reaction (Moore and Stein, 1954) using a leucine standard.

**Protein Radioiodination**—Protein samples were labeled with 125I by the solid state lactoperoxidase method as described previously (Miller et al., 1982) or with the New England Nuclear (Boston, MA) radioiodination system. The specific activities of the iodinated proteins were typically 50 to 100 μCi/ng. The radiochemical purity of several of the iodinated samples was established by SDS-polyacrylamide gel electrophoresis. The gel was stained and counted in a γ counter (Tracer Analytic, model 1185). The mobility of the radiactive peak was identical with that of companion gels which were stained with Coomassie blue R-250.

**Plasma Clearance and Transport to Oocytes**—Plasma clearance half-life was determined by injection of 25 μg of freshly iodinated protein in 0.5 ml of NaCl into the wing veins of newly hatched birds. Plasma samples (0.2 ml) were taken from the opposite wing at 2-min and 10-min intervals thereafter up to 2 h and placed into heparinized microfuge tubes. Following centrifugation, duplicate 50-μl aliquots of plasma were precipitated with 1 ml of 10% trichloroacetic acid. The precipitates were recovered by centrifugation and counted in a γ counter.

Upake of labeled proteins into developing oocytes was determined with the same half-life values as were used for the clearance studies. The birds were killed 23 h after injection and all ovarian follicles were removed and prepared as described previously (Miller et al., 1982). Radioactivity was determined on washed trichloroacetic acid precipitates of 200-μl aliquots of individual oocyte extracts. In those cases in which we did not measure complete clearance curves before measuring oocyte uptake, we obtained 2-min and 1-h plasma samples which we used as an indication that the birds were turning over the proteins as expected.

**Binding Studies with Oocyte Membranes**—Oocyte plasma membranes were prepared from the ovaries of freshly killed White Leghorn chickens by the procedure of Woods and Roth (1979). Typically we prepared membranes from about 200 oocytes, 1.5 to 2.5 cm in diameter. These were dispersed in 30 ml of incubation buffer, pH 6.0, (Woods and Roth, 1979) and distributed in 2-ml aliquots to screw cap vials, to which were added 200 μl of dimethyl sulfoxide. The membranes were stored frozen at −70 °C. Prior to each membrane-binding experiment, the membranes were thawed, washed 3 times with incubation buffer, and resuspended in 5 ml of incubation buffer.

Membrane-binding studies adapted from those of Woods and Roth (1979) were carried out in 2-ml polypropylene microfuge tubes (W. Sarstedt, Inc., Princeton, NJ). In a total volume of 300 μl of incubation buffer we added 1 mg of 125I-labeled protein, 0 or 100 μg of unlabeled protein, 250 μg of bovine serum albumin, and 100 μl of suspended oocyte membranes. The tubes were incubated for 1 h at 25 °C in a shaking incubator and then transferred to an ice bath prior to pelleting in a microfuge. The membrane pellet was washed twice with ice-cold incubation buffer and counted in the γ counter.

**RESULTS**

**Phosphate Content of RBP**—The phosphate contents of RBP are as follows: yolk RBP, 8.29 ± 0.38 residues; egg white RBP, 7.58 ± 0.17 residues; and serum RBP, 6.71 ± 0.18 residues. All values are based on 36,000 g/mol of RBP (Miller et al., 1982) and have been corrected for protein concentration using holo-yolk RBP, dried 19 h in vacuo at 55 °C as the reference protein. The values for egg white and serum RBP are the mean ± S. D. of 4 determinations; those for yolk RBP represent 9 determinations. The phosphate contents of egg white and yolk RBP are very similar, their means differing by less than 1 residue. The phosphate content of serum RBP is unexpectedly lower than that of yolk RBP, considering that serum RBP is the direct precursor of yolk RBP (Blum, 1967). This could be due to a small difference in the protein determinations which could lead to a larger error in the estimated molar ratios of phosphate. Although it is possible that the chicken plasma contains endogenous phosphatases during the isolation from plasma, this seems unlikely because we have found that small changes in phosphate content cause major effects in clearance and transport (see below) which were not observed with serum RBP (Miller et al., 1982).

**Dephosphorylation of Yolk RBP**—The time course of the enzymatic dephosphorylation of yolk RBP is shown in Fig. 1. This time course is very similar to that obtained by Rhodes et al. (1959) for the enzymatic dephosphorylation of egg white RBP. Dephosphorylation was virtually complete at 4 h and the reaction was stopped after 6 h. Several of the physical properties of dephospho-yolk RBP are compared with native yolk RBP in Table I. The phosphate content of dephospho-yolk RBP indicates that dephosphorylation was over 98% complete. Dephospho-yolk RBP was homogeneous by SDS-polyacrylamide gel electrophoresis (Fig. 2) and had a mobility similar to that of the native protein, demonstrating that proteolysis did not occur during dephosphorylation. However, under nondenaturing conditions, dephospho-yolk RBP was resolved into several bands by polyacrylamide gel electrophoresis (Fig. 2), indicating charge heterogeneity of the protein. Although some of this heterogeneity is probably due to the remaining phosphate groups, this cannot account for all
spectra of native and dephospho-yolk RBP are summarized in Table II. Although the shapes of these spectra were similar, the molar ellipticities of dephospho-yolk RBP at all three peaks in the UV region are less than those of the native protein. However, because of the overall similarity of the spectra, we do not feel there is strong evidence for any gross structural changes accompanying dephosphorylation.

The plasma clearance curves of $^{125}$I-labeled native and dephospho-yolk RBP are shown in Fig. 3A. As we have observed before (Miller et al., 1982), the clearance of these proteins is not a single first order process but can be resolved into two components which obey first order kinetics. The half-

![Fig. 1. Time course of dephosphorylation of yolk RBP (O—O) and phosphin (-----O). The proteins were dephosphorylated with potato acid phosphatase in small dialysis sacs as described under “Materials and Methods.” The dialysate was analyzed for inorganic phosphate at the times indicated above. The percent released was calculated on the basis of the phosphate content of the proteins prior to dephosphorylation.](http://www.jbc.org/content/journal/jbc/257/s1/1526/F1.large.jpg)

**TABLE I**

<table>
<thead>
<tr>
<th>Property</th>
<th>Yolk RBP</th>
<th>Dephospho-yolk RBP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphate content* (residues/molecule)</td>
<td>8.29</td>
<td>0.15</td>
</tr>
<tr>
<td>Sialic acid content* (residues/molecule)</td>
<td>4.24</td>
<td>3.36</td>
</tr>
<tr>
<td>Riboflavin-binding capacity (μg/mg protein)</td>
<td>10.09</td>
<td>10.14</td>
</tr>
<tr>
<td>Plasma clearance*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Slow turnover component</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$t_{1/2}$ (min)</td>
<td>82.3</td>
<td>61.7</td>
</tr>
<tr>
<td>% of total</td>
<td>(69.1)</td>
<td>(68.7)</td>
</tr>
<tr>
<td>2. Rapid turnover component</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$t_{1/2}$ (min)</td>
<td>15.3</td>
<td>13.7</td>
</tr>
<tr>
<td>% of total</td>
<td>(30.9)</td>
<td>(31.3)</td>
</tr>
<tr>
<td>Total transport to oocytes (%)</td>
<td>10.5 ± 1.7</td>
<td>1.36 ± 0.38</td>
</tr>
</tbody>
</table>

* All values are based on 36,000 g/mol of yolk RBP and were corrected for protein concentration using holo-yolk RBP, dried in vacuo at 50 °C, as reference protein. Each value is the average of at least 2 determinations.

The slow turnover half-life was determined from the 60- to 120-min data points from 2 (yolk RBP) or 3 (dephospho-yolk RBP) laying hens. The rapid turnover half-life is the difference between the actual data and the extrapolated slow turnover component at 2 to 60 min.

Mean ± S.D. of total per cent of injected radioactive protein recovered from oocytes 23 h after injection of same birds used for clearance studies.

four bands (Fig. 2 arrows) associated with dephospho-yolk RBP. The other possibility which we considered was partial desialylation of yolk RBP during the course of dephosphorylation. The sialic acid content of dephospho-yolk RBP was 0.9 residues less than that of the native protein. Thus, the observed charge heterogeneity may be attributed to both phosphate and sialic acid heterogeneity.

The riboflavin-binding capacity of dephospho-yolk RBP was indistinguishable from that of the native protein (Table I) both of which were about 97% of the theoretical capacity (10.45 μg of riboflavin/mg of protein based on 1 mol/mole of protein). This observation is in agreement with Rhodes et al. (1959) who found no effect of dephosphorylation on riboflavin binding by egg white RBP. The molar ellipticities of the CD spectra of native and dephospho-yolk RBP are summarized in Table II. Although the shapes of these spectra were similar, the molar ellipticities of dephospho-yolk RBP at all three peaks in the UV region are less than those of the native protein. However, because of the overall similarity of the spectra, we do not feel there is strong evidence for any gross structural changes accompanying dephosphorylation.

The plasma clearance curves of $^{125}$I-labeled native and dephospho-yolk RBP are shown in Fig. 3A. As we have observed before (Miller et al., 1982), the clearance of these proteins is not a single first order process but can be resolved into two components which obey first order kinetics. The half-

![Fig. 2. SDS and nondenaturing polyacrylamide gel electrophoresis of native and dephosphorylated yolk RBP. The molecular weights of yolk RBP (A) and dephospho-yolk RBP (B) were compared by electrophoresis under denaturing conditions on SDS-10% polyacrylamide gels. The charge properties of yolk RBP (C) and dephospho-yolk RBP (D) were compared under nondenaturing conditions on 7.5% polyacrylamide gels at pH 8.9. Nondenaturing gels were run on partially dephosphorylated samples containing 4.0 (E) and 1.9 (F) phosphate residues/molecule. All gels were stained with Coomassie blue R-250.](http://www.jbc.org/content/journal/jbc/257/s1/1526/F2.large.jpg)

**TABLE II**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Phosphate content (residues/molecule)</th>
<th>Molar ellipticity [°]</th>
<th>[°]</th>
<th>[°]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>residues/molecule × (10^{-5})</td>
<td>deg cm² dmol⁻¹ × (10⁻⁴)</td>
<td>deg cm² dmol⁻¹ × (10⁻⁴)</td>
<td>deg cm² dmol⁻¹ × (10⁻⁴)</td>
</tr>
<tr>
<td>Yolk RBP</td>
<td>8.3</td>
<td>8.81</td>
<td>6.79</td>
<td>2.86</td>
</tr>
<tr>
<td>Dephosphorylated yolk RBP</td>
<td>0.15</td>
<td>7.93</td>
<td>6.10</td>
<td>2.37</td>
</tr>
<tr>
<td>Partially dephosphorylated yolk RBP</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a.</td>
<td>7.9</td>
<td>8.55</td>
<td>6.39</td>
<td>2.84</td>
</tr>
<tr>
<td>b.</td>
<td>6.9</td>
<td>9.22</td>
<td>6.45</td>
<td>2.87</td>
</tr>
<tr>
<td>c.</td>
<td>1.9</td>
<td>8.37</td>
<td>6.14</td>
<td>2.84</td>
</tr>
<tr>
<td>Succinylated yolk RBP</td>
<td>8.3</td>
<td>7.98</td>
<td>7.38*</td>
<td>2.83</td>
</tr>
</tbody>
</table>

* [°]_{222 nm}
The plasma clearance rate alone.

Multiple bands (Fig. 2). The sialic acid content of these samples were broader, although they did not resolve into multiples.

The difference between dephospho- and native yolk RBP, the bands indistinguishable from that of yolk RBP. On nondenaturing samples. The phosphate content of these samples ranged from the injected dose (Miller et al., 1982). Since the half-life difference between egg white and yolk RBP was similar to the transport of egg white RBP, which was about 4.3% of the injected dephospho-yolk RBP. This amount can be compared with the transport of egg white RBP, which was about 4.3% of the injected dose (Miller et al., 1982). Since the half-life difference between egg white and yolk RBP was similar to the difference between dephospho- and native yolk RBP, the additional decrease in uptake into oocytes observed with dephospho-yolk RBP cannot be attributed to its increased plasma clearance rate alone.

Sequential Dephosphorylation of Yolk RBP—In order to determine the number of phosphate residues which could be removed from yolk RBP before oocyte uptake is impaired, we prepared a series of partially dephosphorylated yolk RBP samples. The phosphate content of these samples ranged from 1.9 to 7.9 residues/molecule. These samples were homogeneous by SDS-polyacrylamide gel electrophoresis, with mobilities indistinguishable from that of yolk RBP. On non-denaturing gels, the mobilities of the partially dephosphorylated samples were less than that of the native protein and the bands were broader, although they did not resolve into multiple bands (Fig. 2). The sialic acid content of these samples was not significantly different from that of the native protein.

The sample which was most extensively dephosphorylated (1.3 phosphate residues remaining) contained 3.93 residues of sialic acid/molecule. Since none of the samples was exposed to the conditions required for dephosphorylation (37 °C, pH 5.3) for more than 1 h, desialylation was held to a minimum. The CD spectra of the partially dephosphorylated samples were practically indistinguishable from that of native yolk RBP and had very similar molar ellipticities (Table II).

The studies on transport of partially dephosphorylated yolk RBP samples to oocytes are shown in Fig. 5. In this series of experiments, the amount of native yolk RBP which was transported to oocytes was 13.8 ± 3.7% (n = 6) of the injected radiolabeled protein. Removal of less than one phosphate residue/mol reduced uptake by over 50%, and, at the level of 1.4 phosphate residues removed, uptake was reduced by 61%. After the removal of the 1st phosphate residue, uptake decreased at a rate proportional to the number of phosphates removed until an 87% decrease was reached with the removal of 8 residues. Although we did not measure the plasma clearance half-lives of all of these partially dephosphorylated samples, we did measure the per cent of the injected dose remaining in circulation 1 h after injection. This value provides a means of comparison of plasma clearance rates with those of native and dephospho-yolk RBP. The amount of labeled protein remaining in circulation after 1 h was 42% for yolk RBP and 36% for dephospho-yolk RBP (Fig. 3). The values for the partially dephosphorylated samples ranged from 36.8% for the sample with less than 1 residue removed to 33.5% after removal of over 6 residues of phosphate. Thus, removal of the 1st phosphate has profound effects on the uptake of yolk RBP into oocytes but only small effects on plasma clearance.

Succinylation Experiments—In order to determine whether the effects of dephosphorylation on clearance and transport of yolk RBP were due specifically to removal of phosphate or to a more general phenomenon, i.e. removal of anionic groups, we added back negative charges to dephospho-yolk RBP by succinylation. We chose conditions under which the number of succinyl groups added was similar to the

**Fig. 3.** Plasma clearance of 125I-labeled native and modified yolk RBP samples. The upper curves in A represent the per cent of injected labeled protein present in the plasma over a 2-h time course for yolk RBP (O—O) and dephospho-yolk RBP (●—●). The 60- to 120-min values were used to calculate the half-life of a slow turnover component; obtained by linear regression analysis. The difference between the actual data points and the extrapolated values from the slow turnover component yielded the lower series of curves, which were used to calculate the half-lives of the rapid turnover component of each clearance curve. B represents a similar treatment of the data from the clearance of succinylated yolk RBP (O—O) and succinylated dephospho-yolk RBP (●—●). Each point is the mean of individual values taken from 3 different birds (2 for native yolk RBP) at identical time points.

**Fig. 4.** Incorporation of 125I-labeled native (O—O) and dephosphorylated (●—●) yolk RBP into developing oocytes. 23 h after injection with tracer quantities (20 to 55 µg) of the labeled proteins, the same birds which had been used for the plasma clearance studies in Fig. 3A were sacrificed and all oocytes >1 mm in diameter were removed. The points represent the total labeled protein found in each oocyte, expressed as the per cent of injected labeled protein, for the 3 birds injected with 125I-dephospho-yolk RBP and the 2 birds injected with 125I-native yolk RBP plotted as a function of oocyte weight.
the differences in the near UV region indicate a conformation that is associated with riboflavin binding to the apoprotein, both the near and far UV regions (Zak et al., 1972; Nishikimi residues. However, this conformational change is not as large than that of the native protein, while the 272 nm peak is were determined using 2 groups of 3 laying hens.

Comparing the clearance curves of native and dephospho- yolk RBP, the clearance of both succinylated samples is more similar to that of dephospho-yolk RBP. The uptake of the succinylated samples is also similar to or slightly less than that of dephospho-yolk RBP (Table III). Succinylation not only was unable to restore normal oocyte uptake to the dephosphorylated sample, but also it decreased the uptake of native yolk RBP to the same extent as dephosphorylation.

Dephosphorylation of Phosvitin—The phosphate content of phosvitin was found to be 109 residues/molecule, based on 34,000 g/mol of phosvitin (Clark, 1970). This amount of phosphate represents 31% of the total weight of the protein. The time course of the enzymatic dephosphorylation of phosvitin is shown in Fig. 1. The dephosphorylation was slower than that of yolk RBP. The product which was isolated after 24 h was 87% dephosphorylated. However, this protein was completely insoluble in a variety of buffers of different pH and ionic strength. We prepared a partially dephosphorylated phosvitin by addition of NaF to the reaction mixture at the appropriate time. The product was about 70% dephosphorylated. It contained 32 phosphate residues/molecule based on a molecular weight corrected for the loss of phosphate. It was soluble in 0.9% NaCl and in other buffers used for the physiological studies.

The plasma clearance curves of 125I-labeled phosvitin and dephosphosvitin are shown in Fig. 6. Since these curves do not represent first order processes, half-lives cannot be calculated. Contrary to the clearance of the RBP samples, these curves cannot be resolved into 2 components. Qualitatively it is obvious that the clearance of dephosphosvitin proceeds more rapidly than that of native phosvitin. The net effect was that 4 h after injection only 3.8% of the injected dephosphosvitin was still in circulation compared with 15% of the native sample. The uptake of the dephosphorylated sample into the oocytes of these birds was also decreased. Of the injected 125I-labeled phosvitin, a total of 8.72 ± 2.16% was transported to oocytes compared with only 1.87 ± 0.24% of the dephosphorylated protein, a decrease of 79%.

Binding to Oocyte Plasma Membranes—Oocyte membrane-binding studies were done in an effort to measure the effects of dephosphorylation on recognition by oocyte receptors. This system has been characterized by Woods and Roth (1979) and we used the same conditions which they found were optimum for binding of phosvitin. We measured the

![Fig. 5. Incorporation of 125I-labeled native and partially dephosphorylated yolk RBP into developing oocytes.](http://www.jbc.org/)

**Table III**

<table>
<thead>
<tr>
<th>Property</th>
<th>Succinylated native yolk RBP</th>
<th>Succinylated dephospho-yolk RBP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphate content (residues/molecule)</td>
<td>8.29</td>
<td>0.15</td>
</tr>
<tr>
<td>Succinate content (residues/molecule)</td>
<td>7.9</td>
<td>6.7</td>
</tr>
<tr>
<td>Riboflavin-binding capacity (native yolk RBP)</td>
<td>98.5</td>
<td>98.9</td>
</tr>
<tr>
<td>Plasma clearance*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Slow turnover component</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$t_1/2$ (min)</td>
<td>65.8</td>
<td>63.1</td>
</tr>
<tr>
<td>% of total</td>
<td>(69.0)</td>
<td>(80.1)</td>
</tr>
<tr>
<td>2. Rapid turnover component</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$t_1/2$ (min)</td>
<td>10.2</td>
<td>11.6</td>
</tr>
<tr>
<td>% of total</td>
<td>(31.0)</td>
<td>(19.9)</td>
</tr>
<tr>
<td>Total transport to oocytes (%)</td>
<td>1.23 ± 0.17</td>
<td>0.80 ± 0.13</td>
</tr>
</tbody>
</table>

*All values are based on 36,000 g/mol as described in Table I. Succinate was estimated from the difference in molar absorbancy in 3-nitroprusside-reactive groups on native or dephospho-yolk RBP and the corresponding succinylated protein.

* Plasma clearance and transport, as described in Table I, represent the values from 3 determinations with each protein.
are important for the normal yolk deposition of RBP and phosvitin. Complete dephosphorylation of yolk RBP decreased the uptake of the protein into oocytes by almost 90% while removal of only 1 residue of phosphate limited uptake by over 60%. Although identical with native RBP in most respects, the dephosphorylated sample was partially desialylated, which may have contributed to the decreased uptake of this sample. Miller et al. (1981a, 1981b) have previously reported that desialylation of both egg white and yolk RBP reduces oocyte uptake by as much as 90%. The partially dephosphorylated samples, however, contained the same amount of sialic acid as native yolk RBP. These samples were identical with native yolk RBP in structure and activity as well.

Since elimination of either phosphate or sialic acid reduces uptake by oocytes, we considered the possibility of a charge effect on transport rather than specific group recognition. We tested this by succinylation and found that uptake could not be restored by addition of anionic groups to dephospho-yolk RBP. However, succinylation of native yolk RBP also decreased oocyte uptake, which indicated that recognition of portions of the peptide chain itself may be involved in yolk deposition. The recognition feature of yolk RBP may in fact be complex, involving phosphate, sialic acid, and perhaps lysine residues.

We have previously reported that egg white RBP, injected into the bloodstream of laying hens, is deposited in oocytes only about one-third as well as yolk RBP (Miller et al., 1982). Physiologically, egg white RBP never appears in circulation. It is synthesized in the oviduct and secreted directly into the egg white (Mandeles and Ducay, 1962). Egg white RBP has no need for a recognition signal for oocyte uptake. Although we considered the different carbohydrate components of egg white and yolk RBP as the cause of their difference in uptake, we found no strong evidence to support this possibility (Miller et al., 1982). When we found that egg white RBP contains only 1 phosphate residue less than yolk RBP, it appeared that phosphate differences could not be responsible for transport differences either. However, since we found that removal of 1 phosphate residue from yolk RBP decreases transport to a level which is similar to that of egg white RBP, it now seems possible that egg white RBP is missing a single (perhaps unique) phosphate which is recognized by oocyte receptors.

The effect of dephosphorylation on uptake of phosvitin into oocytes was similar to that seen upon dephosphorylation of yolk RBP. The evidence for a specific effect on oocyte recognition in vivo was not as good as that obtained with yolk RBP because of the dramatic increase in the plasma clearance rate of dephosphophosvitin. Two hours after injection, the amount of dephosphophosvitin remaining in circulation was 78% less than that of native phosvitin. At the same time point, the amount of dephospho-yolk RBP in circulation was only 27% less than that of native yolk RBP. The binding of phosvitin to oocyte membranes in vitro was almost totally eliminated by removal of 70% of the phosphate residues, indicating a definite involvement of phosphate in recognition of phosvitin by oocyte plasma membrane receptors. A similar conclusion could not be reached about the binding of RBP since we have not yet established the proper conditions for optimum binding of RBP to oocyte membranes.

The carbohydrate component of glycoproteins has been found to serve as a cellular recognition marker in many

| Table IV
| Oocyte plasma membrane-binding studies with native and dephosphorylated phosvitin and yolk RBP |
| --- | --- | --- |
| 125I-Protein | Binding to oocyte plasma membrane<sup>a</sup> | Specific binding<sup>b</sup> |
|  | Alone  | + Phosvitin  | + RBP  |
| Phosvitin | 1050 ± 100  | 420 ± 90  | 1010 ± 80  | 630 ± 190  |
| Dephosphophosvitin | 132 ± 6  | 106 ± 16  | 110 ± 13  | 26 ± 22  |
| Yolk RBP | 44 ± 14  | 24 ± 2  | 28 ± 6  | 16 ± 20  |
| Dephospho-yolk RBP | 18 ± 2  | 13 ± 1  | 11 ± 1  | 7 ± 3  |

<sup>a</sup> The binding of 125I-labeled phosvitin (1.07 pg), 70% dephosphophosvitin (1.06 pg), yolk RBP (1.00 pg), and dephospho-yolk RBP (1.00 pg) to oocyte plasma membranes was measured alone or in the presence of either 100 pg of unlabeled phosvitin or 100 pg of yolk RBP. The values are the mean ± S. D. of triplicate determinations.

<sup>b</sup> Specific binding is the difference between the amount of labeled protein which bound alone and in the presence of excess phosvitin (for 125I-labeled phosvitin and dephosphophosvitin) or RBP (for 125I-labeled yolk RBP and dephospho-yolk RBP).
Acknowledgments—We wish to thank J. W. Woods and T. P. Roth from the University of Maryland, Baltimore County, for helping us with the oocyte membrane preparation and Robert Alphin for management of the birds used in these experiments.

REFERENCES

Blum, J.-C. (1967) Le Metabolisme de la Riboflavine Chez la Poule: Pondeuse. F. Hoffmann-LaRoche et Cie, Paris

instances (Neufeld and Ashwell, 1980). One of the reasons that we had found the carbohydrate recognition hypothesis attractive was that many of the transport proteins found in chicken blood or yolk are glycosylated. These include not only RBP (Ostrowski et al., 1968) and phospho- (Shainkin and Perlmann, 1971) but also biotin-binding protein (Meslar et al., 1978), transferrin (Williams, 1968), and vitamin B₁₂-binding proteins in serum (Kidroni and Grossowicz, 1969). Thiamin-binding protein is a notable exception since it is not a glycoprotein (Muniyappa and Adiga, 1981). Although it was proposed that thiamin-binding protein complexes with RBP and uses the carbohydrate of RBP as its signal for transport to oocytes (Muniyappa and Adiga, 1979), we have found that normal thiamin transport occurs in the absence of RBP (Miller et al., 1981c). Phosphate on phosphomannosyl residues has been found to be important for the cellular recognition and transport of lysosomal hydrolases to lysosomes (Kaplan et al., 1977). The phosphoprotein nature of the avian transport proteins has not been as well characterized as their glycoprotein nature, nor has the site of attachment of RBP phosphate residues. In a 1974 review on phosphoproteins, the possibility was presented that phosvitin and lipovitellin might be the only phosphoproteins in yolk (Taborsky, 1974). Considering the evidence presented in this paper on the role of phosphate in deposition of RBP and phosvitin, it might be worthwhile to re-evaluate the phosphate compositions of the other yolk transport proteins as candidates for recognition sites by oocyte receptors.
Dephosphorylation of chicken riboflavin-binding protein and phosvitin decreases their uptake by oocytes.

M S Miller, M Benore-Parsons and H B White, 3rd


Access the most updated version of this article at [http://www.jbc.org/content/257/12/6818](http://www.jbc.org/content/257/12/6818)

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at [http://www.jbc.org/content/257/12/6818.full.html#ref-list-1](http://www.jbc.org/content/257/12/6818.full.html#ref-list-1)