Induced Formation of Phosphoprotein in Tissues of Cockerels in Vivo and in Vitro*

OLGA GREENGARD, A. SENTENAC, AND G. ACS

From the Institute for Muscle Disease, Inc., New York, New York 10021

(Received for publication, September 21, 1964)

The appearance of phosphoprotein in the plasma of male birds treated with estrogen has been reported by other investigators (1, 2). Earlier studies from this laboratory have indicated that this process reflects synthesis de novo of a protein fraction normally absent in cockerels and that the initial action of the hormone requires an intact ribonucleic acid-synthesizing system (3). In the present study of the mechanism of this phenomenon, attempts were made to identify the organ responsible for the induced synthesis of these proteins. However, administration of diethylstilbestrol did not appear to cause a detectable increase in the incorporation of radioactive phosphorus in vivo into any of the tissues studied. This observation suggested that the secretion of phosphoproteins into the blood stream may be so rapid that their concentration in the appropriate organs remains low at any time. The present results obtained with tissue slices indicate that livers of cockerels which have been treated with diethylstilbestrol in vivo can synthesize a phosphoprotein fraction.

The identification of phosvitin by paper electrophoresis of unfractioned material is applicable to egg yolk (4) but not to plasma, where phosphoproteins are more extensively complexed with lipoproteins, lipids, and Ca$^{2+}$ ions (5). Therefore, only after the elaboration of a suitable fractionation procedure was the phosphoprotein in the plasma of laying hens identified, by Heald and McLachlan (6), as phosvitin. The plasma of cockerels after treatment with estrogen, as well as the soluble fraction of incubated liver slices from such animals, has now been subjected to a similar procedure for liberating the material from the complex. Column chromatography and electrophoresis of the resulting preparation showed that the phosphoprotein produced by liver slices, or present in the plasma of estrogen-treated cockerels, or in the plasma of laying hens, is identical.

**Experimental Procedure**

White Leghorn cockerels, or laying hens, 1 to 2 months old, were employed. An aqueous suspension of diethylstilbestrol, 5 mg/100 g of body weight, was injected subcutaneously. When indicated, radioactive inorganic phosphate ($^{32}$P, 40 μc/100 g of body weight) was administered subcutaneously 2 hours before the animals were bled by cutting the jugular vein. Isolation of plasma proteins and estimation of their radioactivity and alkali-labile phosphorus content have been previously described (3).

Isolation of Phosphoprotein from Plasma—$^{32}$P was administered to the animals 2 hours before bleeding. The method used for fractionation of plasma was based on that of Heald and McLachlan (6).

To 85 ml of cold plasma, 8.5 ml of calcium chloride solution (20 mg ions of Ca$^{2+}$ per ml) were added, diluted to 850 ml with cold water, and allowed to stand for 2 hours at 0°. After centrifugation at 8,000 × g for 15 minutes, the precipitate was dissolved by stirring with 70 ml of 0.25 M NaCl for 1 hour at room temperature. The yellow suspension was centrifuged at 40,000 × g for 20 minutes and the precipitate was discarded. The supernatant was diluted to 700 ml with water and stored for 3 hours at 0°. The precipitate formed was collected by centrifugation at 8,000 × g for 15 minutes and redissolved at room temperature in 50 ml of 0.2 M NaCl by stirring for 15 minutes. This solution was exhaustively dialyzed against EDTA (0.05 M, pH 7.4) and against water.

Removal of Lipids—Of the dialysate, 10 ml were adjusted to pH 8.5 and incubated for 1 hour with 5 mg of pancreatic lipase (Nutritional Biochemicals Corporation) at 37°. Then 90 ml of 0.4 M ammonium sulfate in 0.05 M sodium acetate buffer (pH 4.0) and 100 ml of ether were added and shaken vigorously for 5 minutes. After standing for 2 hours at room temperature, the yellow ether layer was discarded and the water layer saved. The gelatinous interphase was extracted for a second time with 50 ml of the above ammonium sulfate solution and 25 ml of ether. The two aqueous phases were combined, dialyzed against H$_2$O, and lyophilized.

This material will be referred to as the phosphoprotein preparation.

Paper Electrophoresis—An aliquot of the preparation was dialyzed against borate buffer (0.05 M, pH 8.6), and 0.05 ml of the preparation, containing approximately 0.25 mg of protein, was applied across a 3-cm width of Whatman No. 3MM paper subjected to electrophoresis in the borate buffer, pH 8.6, at 7 volts per cm for 5 hours (7). The paper was dried and cut into segments 2 cm wide, and the total radioactivity of these segments was estimated. Commercially purified egg phosvitin that had been subjected to electrophoresis simultaneously was stained as previously described (8).

Chromatography—Another aliquot of the phosphoprotein preparation was dialyzed against Tris buffer (0.005 M, pH 8.0), chromatographed on a DEAE-cellulose column (25 × 1 cm), and eluted with a gradient of NaCl in the Tris buffer, as previously described (6).

Treatment of Liver Slices—Liver slices, approximately 0.35 mm thick, were placed in Warburg vessels containing Krebs-Ringer-bicarbonate medium, glucose (0.05 M), and the indicated radioactive precursor. The vessels were incubated at 37° in an atmosphere of 95% O$_2$-5% CO$_2$ with continuous shaking. After

* This investigation was supported by the Muscular Dystrophy Associations of America, Inc., by United States Public Health Service Grant CA-07037-01, and by Grant P-299 from the American Cancer Society.
incubation the content of the Warburg flask (medium plus slices) was homogenized and centrifuged for 50 minutes at 100,000 $\times$ g. The resulting particulate fraction was freed of nonprotein material, and its radioactivity was measured at infinite thinness in a gas flow counter, as previously described (3). An aliquot of the soluble fraction was treated in the same way, and the remainder was used for isolation of the phosphoprotein preparation. Precipitation by calcium chloride and dilution, employed for plasma, were avoided owing to the expected low amount of phosphoprotein in the slices. Therefore, free $^{32}$P was removed by dialysis against two changes of water, followed by sequential dialysis against 0.2 M sodium phosphate buffer (pH 7.4), water, and 0.001 M borate buffer (pH 5.6). The removal of lipids was similar to that described for plasma preparations except that solid ammonium sulfate and more concentrated sodium acetate (1 M) were used to avoid excessive dilution. The electrophoretic and column chromatographic procedures were the same as those described for plasma preparations.

**RESULTS**

The aim of this study was to correlate the estrogen-induced appearance of phosphoprotein in the plasma of cockerels with the ability of the liver to synthesize phosphoprotein, and to compare some properties of the synthesized material with that normally found in the plasma of laying hens. The first part of this section describes studies on the phosphoprotein formed in vivo, and the second part on that formed by liver slices in vitro.

**Phosphoprotein Formed in Plasma of Estrogen-treated Cockerels and Laying Hens in Vivo**—Fig. 1 depicts the estrogen-induced appearance of phosphoprotein in the plasma of cockerels. Diethylstilbestrol was administered once, at zero time. As in previous studies in vivo, the rate of incorporation of $^{32}$P and the alkali labile phosphorus content of plasma protein were taken as a measure of the rate of synthesis and concentration of phosphoprotein, respectively. Both measurements begin to give significant values 24 hours after administration of the hormone.

The rate of synthesis of the phosphoprotein reaches a maximum 2 days after hormonal treatment and remains high for another 5 days. The amount continues to rise for several days and begins to decline on the 7th day. This decline occurs earlier than would be expected from the rate of $^{32}$P uptake, and may indicate an increased rate of removal of the phosphoprotein from the blood stream.

Plasma of cockerels which had received injections of diethylstilbestrol 3 days previously was used for the isolation of the phosphoprotein fraction. Two hours before bleeding, $^{32}$P was administered to three cockerels. The radioactivity of the total isolated plasma protein was 310 c.p.m. per mg of protein (cf. Fig. 1) before fractionation, and that of the phosphoprotein preparation obtained from this plasma was 4500 c.p.m. per mg of protein, indicating more than a 15-fold enrichment; 30% of the total (protein-bound) counts were recovered in this fraction. This preparation and a similar one obtained from laying hens were subjected to paper electrophoresis. The identity of the phosphoprotein which appears in cockerels after treatment with estrogen is similar to that normally present in laying hens. The peak of radioactivity (Segments 6 and 7) coincides with that to which

\[ \begin{align*} 
\text{C.M. per mg} & \quad \text{C.P.M. per mg} \\
0 & \quad 0 \\
1 & \quad 100 \\
2 & \quad 200 \\
3 & \quad 300 \\
4 & \quad 400 \\
5 & \quad 500 \\
6 & \quad 600 \\
7 & \quad 700 \\
8 & \quad 800 \\
9 & \quad 900 \\
10 & \quad 1000 \\
\end{align*} \]

\[ \begin{align*} 
\text{C.M. per mg} & \quad \text{C.P.M. per mg} \\
0 & \quad 0 \\
1 & \quad 100 \\
2 & \quad 200 \\
3 & \quad 300 \\
4 & \quad 400 \\
5 & \quad 500 \\
6 & \quad 600 \\
7 & \quad 700 \\
8 & \quad 800 \\
9 & \quad 900 \\
10 & \quad 1000 \\
\end{align*} \]

\[ \begin{align*} 
\text{C.M. per mg} & \quad \text{C.P.M. per mg} \\
0 & \quad 0 \\
1 & \quad 100 \\
2 & \quad 200 \\
3 & \quad 300 \\
4 & \quad 400 \\
5 & \quad 500 \\
6 & \quad 600 \\
7 & \quad 700 \\
8 & \quad 800 \\
9 & \quad 900 \\
10 & \quad 1000 \\
\end{align*} \]

\[ \begin{align*} 
\text{C.M. per mg} & \quad \text{C.P.M. per mg} \\
0 & \quad 0 \\
1 & \quad 100 \\
2 & \quad 200 \\
3 & \quad 300 \\
4 & \quad 400 \\
5 & \quad 500 \\
6 & \quad 600 \\
7 & \quad 700 \\
8 & \quad 800 \\
9 & \quad 900 \\
10 & \quad 1000 \\
\end{align*} \]

\[ \begin{align*} 
\text{C.M. per mg} & \quad \text{C.P.M. per mg} \\
0 & \quad 0 \\
1 & \quad 100 \\
2 & \quad 200 \\
3 & \quad 300 \\
4 & \quad 400 \\
5 & \quad 500 \\
6 & \quad 600 \\
7 & \quad 700 \\
8 & \quad 800 \\
9 & \quad 900 \\
10 & \quad 1000 \\
\end{align*} \]
commercial phosvitin migrated. In these experiments it was not necessary to include preparations from untreated cockerels as controls, since they do not incorporate detectable amounts of \(^{32}P\) into their plasma proteins (cf. Fig. 1).

Fig. 3 illustrates the behavior upon column chromatography of two phosphoprotein preparations obtained in two different experiments with estrogen-treated cockerels. The main peak of radioactivity occurs in Fractions 52 to 54, i.e., when the eluate is 0.3 m with respect to NaCl. With the same elution technique, our sample of pure, commercially obtained phosvitin was also concentrated in tubes 52 to 54. This is in agreement with Heald and McLachlan (6), who eluted purified phosvitin at 0.3 m NaCl.

To ascertain that the eluted radioactive material was phosphoprotein and not other phosphorus-containing substances such as oligonucleotides or low molecular weight ribonucleic acid, the following test was carried out. The total radioactivity in tubes 52 to 54, was precipitable by trichloroacetic acid. Alkaline hydrolysis of this precipitate, by a previously described method (3), releases 95% of the radioactivity, which, in the form of the ammonium phosphomolybdate complex, is extractable into isobutyl alcohol.

The sum of the counts in tubes 52 to 54 represents 60% of the counts placed on the column. Since the phosphoprotein preparation to be chromatographed contained 20% of the total counts in plasma, this phosvitin peak (cf. Fig. 3) represents a minimum of 20% of the induced phosphoprotein in blood.

Synthesis of Phosphoprotein in Vitro—To investigate whether the liver may be responsible for the synthesis of the plasma phosphoprotein described above, cockerels which had been treated by injection with diethylstilbestrol 3 days previously were used, since at this time the rate of \(^{32}P\) incorporation into the plasma proteins was high (Fig. 1). Initial studies showed that slices from estrogen-treated cockerels incorporate more \(^{32}P\) into both their soluble and particulate protein fraction than slices from untreated cockerels.

The problem that \(^{32}P\) uptake can represent an exchange reaction by preformed phosphoprotein and AT\(^{32}P\), rather than by synthesis de novo, did not arise in studies in vivo since the plasma of untreated cockerels was devoid of phosphoprotein and that of cockerels treated with estrogen did not incorporate \(^{32}P\) if puromycin had been administered (3). However, Table I illustrates that puromycin, an inhibitor of protein synthesis, in concentrations at which it prevented amino acid uptake (cf. Columns 2 and 4), did not completely inhibit \(^{32}P\) incorporation into the proteins of liver slices (cf. Columns 1 and 3); this finding indicates that some of this incorporation reflects an exchange reaction between phosphoproteins and labeled ATP formed during incubation. Therefore, only that portion of \(^{32}P\) uptake (last column of Table I) that was prevented by puromycin was considered to reflect synthesis de novo. By this criterion, liver slices of untreated cockerels and those treated with estrogen synthesized particulate phosphoprotein to a similar extent, whereas the synthesis of soluble phosphoprotein is much higher in the case of slices from treated cockerels. In a typical experiment, shown in Table I, the puromycin-sensitive incorporation of \(^{32}P\) into the soluble protein of liver slices of untreated cockerels was only 5 c.p.m. per mg, whereas that into the soluble protein of slices of diethylstilbestrol-treated cockerels was 72 c.p.m. per mg. In two other experiments, hormonal treatment increased this incorporation into the soluble protein fraction from 0 to 88 and from 42 to 321 c.p.m. per mg, respectively.

Having shown the ability of liver slices of cockerels treated with estrogen to synthesize soluble phosphoproteins, we next compared the resulting material with that formed in the plasma of the same animals. For this purpose, incubation experiments...
The phosphoprotein preparation from liver slices of estrogen-treated cockerels was subjected to column chromatography. The similarity between Curve 1 in Fig. 5 and the curves in Fig. 3 further suggests that the phosphoprotein, which appears in the

![Table II](#)

**Induced Synthesis of Phosphoprotein**

Liver slices of a normal cockerel and of one that had been given diethylstilbestrol 2 days previously were incubated with 9 µc of 32P, with and without 5 mg of puromycin, in 8 ml of medium for 2 hours. After homogenization and centrifugation, the particular fraction was discarded. The radioactivity of the washed, fractionated protein content of one aliquot of the soluble fraction was measured; the phosphoprotein preparation was isolated from the remainder of the soluble fraction, and its radioactivity was measured. The values given were arrived at by subtracting the radioactivity of the preparation obtained from the puromycin-containing flask from that of the puromycin-free flask. A and B refer to two separate experiments.

<table>
<thead>
<tr>
<th>Source of liver slices</th>
<th>Difference in radioactivity</th>
<th>Radioactivity (c.p.m/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total soluble protein</td>
<td>Phosphoprotein preparation</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>Normal cockerel</td>
<td>84</td>
<td>40</td>
</tr>
<tr>
<td>Diethylstilbestrol-treated cockerel</td>
<td>250</td>
<td>105</td>
</tr>
</tbody>
</table>

Similar to those in Table I but on a larger scale, were carried out, and the resulting soluble phase was subjected to a fractionation procedure similar to that employed for plasma. To determine the extent of purification achieved, the specific radioactivity of the preparation was compared with that of the starting material, the total soluble protein fraction. In Table II, which illustrates two such experiments, the results are expressed as a difference between the puromycin-free and the puromycin-containing incubation mixtures, since only that fraction of 32P incorporation was considered to reflect phosphoprotein synthesis inhibited by puromycin. The specific radioactivity of the phosphoprotein preparation of the experimental slices is 20 to 40 times higher than that of the starting material. Thus, the purification procedure used for plasma is applicable to liver, which indicates a similarity between the phosphoprotein fractions in the two tissues. In both experiments of Table II, the difference between liver slices derived from normal and estrogen-treated cockerels was further exaggerated by the subfractionation procedure, since the extent of purification was less in the case of the former.

The four phosphoprotein preparations of Experiment A (see Table II), after dialysis against borate buffer, were subjected to electrophoresis. Fig. 4 presents the original data rather than the difference between the puromycin-free and puromycin-containing material. The radioactive peak of the phosphoprotein preparation of normal cockerels (Fig. 4) was no greater when obtained from slices incubated without puromycin than it was with it; this suggests the absence of significant synthesis de novo of this protein. Fig. 4 shows clearly the radioactive peak obtained when the phosphoprotein preparation of incubated liver slices derived from estrogen-treated cockerels is subjected to electrophoresis, and illustrates that, when the slices are incubated in the presence of puromycin, this peak is much lower. A comparison of Fig. 4 with Fig. 2 shows that the migratory properties of this phosphoprotein fraction and of that obtained from the plasma of the same animals or from the plasma of untreated laying hens are identical.
plasma of treated cockerels, can be synthesized in vitro by liver slices of the same animal and is identical with phosvitin.

Curve 2 in Fig. 5 illustrates that the phosphoprotein synthesized by liver slices, as well as that present in plasma (5, 6), exists in a complexed form and that analysis by column chromatography requires prior removal of lipids by treatment with lipase and extraction with ether.

In liver slices of the same animal and is identical with phosvitin. The relationship between the hormonal stimulus and response.

In hens, the synthesis of phosvitin, which circulates in plasma before its transfer to the egg, is under hormonal control. Cockerels, which have no detectable amount of plasma phosphoprotein unless treated with estrogen, are eminently suited to the study of the relationship between the hormonal stimulus and response. In 48 hours the rate of incorporation of 32P into the protein of cockerels changes from an insignificant one to one as extensive as that in laying hens (3). The accumulation of protein-bound, alkali-labile phosphorus continues for several days (Fig. 1). Puromycin is inhibitory if administered at any time during the rapid phase of accumulation of plasma phosphoprotein, whereas the preventive action of actinomycin is detectable only if it is administered together with, or very shortly after, diethylstilbestrol (3). Thus, the RNA species initially synthesized remains stable, and the phase of the induction process now under study does not require an intact RNA-synthesizing system but requires an adequate capacity to synthesize protein de novo.

In consideration of the liver as the possible organ in which estrogen induces plasma phosphoprotein synthesis, the incorporation of 32P into protein was no longer a sufficient parameter. The liver of untreated cockerels, as that of other animals, contains significant amounts of phosphoprotein, and thus, unlike the case of plasma, estrogen treatment can be expected to cause only a quantitative increase in the total phosphoprotein concentration. Therefore, a fractionation procedure was required to identify the appropriate phosphoprotein component. A further complication arose from the exchange, catalyzed by a liver enzyme, between the phosphate groups of ATP and phosphoproteins. For this reason the studies in vitro were routinely performed with and without puromycin, and only that portion of the 32P uptake was considered to reflect synthesis de novo which was prevented by puromycin.

The incorporation of 32P into the particulate protein fraction of liver slices from estrogen-treated cockerels, and its partial inhibition by puromycin, were similar to observations in preparations of untreated cockerels, which suggests that the hormone did not affect this group of phosphoproteins. In contrast, the uptake of 32P into the soluble fraction, and its sensitivity to puromycin, were significantly greater in liver slices of estrogen-treated cockerels (Table II). This difference becomes increasingly obvious with two further fractionation steps, i.e., isolation of a highly radioactive subfraction of the soluble phase and the resolution of this subfraction by paper electrophoresis or by chromatography. Thus, Fig. 5 shows that only liver slices of estrogen-treated cockerels yield a radioactive material the formation of which is prevented by puromycin. However, some of this material is apparently also present in liver of untreated cockerels. This suggests the possibility that the male chicken has some capacity for the synthesis of this phosphoprotein in vitro but that this capacity is small to be manifested in vitro or to result in the release into the blood of detectable amounts of phosphoprotein in vivo.

The amino acid composition, phosphate content, and chromatographic behavior of the phosphoprotein isolated from the plasma of laying hens are identical with those of egg phosvitin (6). The present studies show that the blood of estrogen-treated cockerels and the liver slices after incubation contain the same material. Whether this represents a single homogeneous protein is not certain; purified egg phosvitin itself has been resolved into different species by the chromatographic procedures of Connell and Taborsky (9).

In general, the synthetic capacity of tissue slices is much less than that of the corresponding organ in vivo. It is impossible to judge what fraction of the synthesis in vivo is represented by the low rate of phosphoprotein synthesis in slices in the present experiments. Therefore, we do not know whether the synthetic functioning of the liver accounts for the total amount of phosphoprotein in the blood, and the possibility that organs other than the liver contribute significantly cannot be excluded. Since estrogen-treated cockerels or immature pullets (10) are as able as laying hens to form plasma phosphoprotein, it is unlikely that sex organs represent an important site of synthesis. In harmony with this view, recent work by Heald and McLachlan, an abstract (11) of which appeared in the course of the present studies, indicates that liver slices of laying hens can synthesize phosvitin.

SUMMARY

The rate of synthesis of a phosphoprotein fraction in the plasma of cockerels was found to be maximal 2 to 5 days after administration of diethylstilbestrol. Liver slices of such animals, when incubated with 32P, produce a soluble protein fraction that is highly labeled. This process is inhibited by puromycin.

A fraction rich in phosphoprotein was isolated, by a similar method, from plasma of cockerels treated with estrogen and from the particle-free fraction of the liver slices after incubation. On paper electrophoresis the column chromatography, the two preparations were indistinguishable from each other and from that obtained from the plasma of laying hens. The results suggest that the liver is the organ, or one of the organs, responsible for the estrogen-induced synthesis of phosphoprotein in cockerels and that much of this phosphoprotein may be identical with the egg protein, phosvitin.

REFERENCES