Phosvitin, a Phosphoglycoprotein

I. ISOLATION AND CHARACTERIZATION OF A GLYCOPEPTIDE FROM PHOSVITIN*

RUTH SHAINKIN AND GERTRUDE E. PERLMANN‡

From The Rockefeller University, New York, New York 10021

SUMMARY

The purified preparations of phosvitin, the phosphoprotein of hens' egg yolk, contain 6.5% carbohydrate. Analysis showed 6 residues of hexose, 5 of glucosamine (assumed to occur as N-acetyl derivatives), and 2 of sialic acid per molecular weight of 40,000. In order to determine the mode of attachment of the polysaccharide moiety, phosvitin has been digested with pronase. A sequence of fractionation steps that included gel filtration, chromatography on DEAE-cellulose and DEAE-Sephadex afforded isolation of a glycopeptide containing all of the carbohydrate attached to an aspartic acid residue. Edman degradation on this peptide and on a smaller peptide isolated from the tryptic digest of a partially dephosphorylated preparation of the glycopeptide yielded the sequence

Carb-Ser-Asn-Ser-Gly-Psr,-Arg-Ser-Val-Ser-His-His-

In addition to the demonstration of the occurrence of a sequence of 8 phosphoserine residues, at least 3 of the 5 nonphosphorylated serines of phosvitin were located near the single carbohydrate moiety of this protein.

EXPERIMENTAL PROCEDURE

Materials

Phosvitin was isolated from fresh hens' egg yolk by the procedure of Joubert and Cook (3). Pronase, Grade B, from Streptomyces griseus was purchased from Calbiochem. Twice recrystallized, salt-free trypsin (Lot TRSF 6), crystallized disopropylfluorophosphate-treated carboxypeptidase A (Lot CoA DFP SB), chromatographically prepared carboxypeptidase B (Lot CoBC 7KA), and alkaline bacterial phosphatase (Escherichia coli) (Lot BAPC OBA) were products of Worthington.

Hydrolysis of Phosvitin with Pronase

In a typical experiment, phosvitin (200 mg) and Pronase (10 mg) were dissolved in 0.05 N sodium barbital buffer, pH 8.6 (20 ml). Because of the acidic properties of phosvitin the resulting pH of the mixture was pH 7.4. This solution was placed in a constant temperature bath at 37°. An additional, equal amount of pronase was added after 24 hours and the digestion was continued for 72 hours. The reaction mixture contained toluene as a preservative to prevent bacterial growth. The course of digestion was followed by measuring the increase in ninhydrin color in aliquots withdrawn at different time intervals.

Gel Filtration of Peptides

The digest was then applied to a column, 2.5 X 200 cm, of Sephadex G-50 (coarse), equilibrated with 0.005 M NH₄HCO₃, pH 8.3. It was eluted at a rate of 60 ml per hour with the same solvent and 5.0-ml fractions were collected. Peptides were located by subjecting appropriate aliquots of every second effluent fraction to ninhydrin analysis after alkaline hydrolysis (5). The glycopeptides were detected by the orcinol reaction (6) applied to samples of the same fractions. The effluent pattern was divided into regions, as indicated in Fig. 1, and the fractions of each region were combined and lyophilized.

Chromatography on DEAE-cellulose

Further purification of the hexose-containing material of Fig. 1, A and C, was accomplished by chromatography on a column, 1.0 x 15 cm, of DEAE-cellulose pre-equilibrated with 0.005 M NH₄-acetate, pH 6.7. The lyophilized samples were dissolved in distilled water (2 mg per ml) and the solution was adjusted to pH 6.7 with 1.0 M NaOH. Usually amounts of 30 to 40 mg were applied to the column. Gradient elution was initiated. For

* This investigation was aided by Grant GB-8285 from the National Science Foundation.
‡ To whom requests for reprints should be addressed.
this purpose 0.005 M NH₄-acetate, pH 6.7, was placed in a 50-ml mixing chamber and the linear gradient was established by placing 50 ml of 1.0 M NH₄-acetate, pH 6.7, in the second reservoir followed by 50 ml of 3.0 M NH₄-acetate in the same flask. The column was operated at room temperature (23°) at a flow rate of 40 ml per hour. The effluent was collected in 1.0- to 1.5-ml fractions.

Aliquots of each fraction were analyzed by ninhydrin after alkaline hydrolysis (6), and were tested for hexose (6) and phosphorus (7).

For the preparative isolation and purification of the main glycopeptide C, 590 mg of the lyophilized material of Fraction C, originating from 2.2 g of phosvitin, were further fractionated on DEAE-cellulose columns, 2 × 30 cm, as described above. Elution was made with a gradient of 500 ml of 0.005 M NH₄-acetate in the mixing chamber and 750 ml of 0.7 M NH₄-acetate, pH 6.7, in the reservoir, using a flow rate of 150 to 200 ml per hour. Fractions of 3.5 ml were collected and the hexose-containing solutions were pooled, lyophilized, and repeatedly redissolved and freeze-dried to remove the salt.

**Chromatography on DEAE-Sephadex A-25**

A subfraction of C, isolated and denoted as C-3 (140 mg), was dissolved in 0.01 M NH₄-acetate, pH 6.7, and was applied to a DEAE-Sephadex A-25 column, 2 × 30 cm, equilibrated with 0.01 M NH₄-acetate. Gradient elution was used (500 ml of 0.01 M NH₄-acetate, pH 6.7, in the mixing chamber and 1000 ml of 1.0 M NH₄-acetate, pH 6.7, in the reservoir) with a flow rate of 200 ml per hour. Fractions of 4 ml each were collected and treated as described above. The homogeneity of the different peptide fractions was routinely assessed by high voltage electrophoresis.

**Purification of Fraction E on Sephadex G-25**

Of the lyophilized Fraction E, 40 mg (see Fig. 1), originating from 2 g of phosvitin were dissolved in 2 ml of distilled water and applied to a Sephadex G-25 (fine) column, 2 × 33 cm. Water was the eluent and 2-ml fractions were collected at a flow rate of 60 ml per hour. Aliquots of each tube were analyzed as described above and the hexose-containing material was freeze-dried. This material was further purified by high voltage electrophoresis.

**Dephosphorylation**

The glycopeptide, GP-C, was dephosphorylated with alkaline phosphatase in 0.036 M Tris-HCl buffer, pH 8.0, at 37° with a phosphatase to GP-C ratio of 1:50 (w/w). An additional equal amount of enzyme was added after 5 hours and incubation was continued for 21 hours. The extent of the dephosphorylation was followed by measuring the release of inorganic phosphorus (7).

**Enzyme Digestion**

**With Trypsin—**To the partially dephosphorylated glycopeptide (20 mg) dissolved in 1.0 ml of 0.01 M sodium phosphate buffer, pH 7.8, containing 0.01 M CaCl₂, was added trypsin at a trypsin to GP-C ratio of 1:100 (w/w). The solution was kept at 37° for 9 hours. The mixture was then lyophilized and redissolved in a minimal amount of distilled water. This solution was fractionated on a column, 2 × 33 cm, of Sephadex G-25, using water as eluent and a flow rate of 60 ml per hour. The fractions (2.0 to 2.5 ml) were tested with ninhydrin after alkaline hydrolysis and analyzed for hexose. Further purification was achieved by high voltage electrophoresis in pyridine-acetate, pH 6.4 (see below).

**With Pronase—**The phosphorus-containing glycopeptide, GP-C, was further digested with Pronase at a pronase to GP-C ratio of 1:50 (w/w). The digestion was carried out at pH 8.0 and 37° for 7 hours in 0.05 M sodium barbital containing 0.01 M CaCl₂. The digest was lyophilized, dissolved in the minimum amount of distilled water, and chromatographed on Sephadex G-25 and treated as described for the tryptic digest.

**With Carboxypeptidase A—**Enzyme digestion was carried out according to the method of Dopheide, Moore, and Stein (8) by dissolving 2 mg of the glycopeptide in 1.0 ml of 1.0 M KCl buffer, pH 7.8.

**With Carboxypeptidase B—**This was added directly to the glycopeptide (2 mg) dissolved in 1.0 ml of 0.05 M sodium phosphate buffer, 0.1 M NaCl, pH 7.8.

In both cases the amount of enzyme added corresponded to 2% by weight and the digestion at 37° was terminated by the addition of 4.0 ml of 0.2 M sodium citrate buffer, pH 2.2. Aliquots were analyzed for the presence of free amino acids.

**Sequential Degradation of Peptides**

The subtractive Edman method was used along the lines of the modifications described by Konigsberg and Hill (8) and by Dopheide et al. (9). Butyl acetate was used for the extraction of the PTH-amino acids of the glycopeptide, whereas those of the smaller tryptic peptide were extracted with benzene.

**Alkaline Resistance of Carbohydrate-Peptide Linkage in Phosvitin**

A solution of phosvitin in 0.5 N NaOH (10 mg per ml) was kept for 19 hours at 4° after which it was subjected to gel filtration over a column, 2 × 33 cm, of Sephadex G-25 (fine) by the procedure described under "Gel Filtration of Peptides." All of the carbohydrate detected in the effluent by the orcinol reaction was present in the fractions containing the alkali-degraded protein.

**Amino Acid Analysis**

Samples, 0.5 to 1.0 mg, of peptide hydrolyzed in 1.0 ml of 6.0 N HCl in sealed evacuated tubes at 110° ± 1° for 24 hours were analyzed for amino acids according to the procedure of Spackman, Stein, and Moore (10) on an amino acid analyzer equipped with the Spinco resins AA-15 and AA-27 (11) and a range card to increase the sensitivity of the analysis to 0.01 µmole. To obtain accurate values for serine, the values obtained were corrected for 25% loss, a value previously established by Allerton and Perlmann (2) in their work on phosvitin.

**General Chemical Methods**

Phosphorus was determined by the method of Fiske and SubbaRow, using the modification of Bartlett (7) as described by Allerton and Perlmann (2). Hexose was determined by the orcinol reaction (6), using as standard a galactose-mannose solution of equimolar concentration.

For the determination of hexosamine in phosvitin and in glycopeptides, hydrolysis was carried out in 3 N HCl for 4 hours at 100° in stoppered tubes. The color was developed by the Elson Morgan reaction as described by Winzler (cf. Reference 6).

1 The abbreviations used are: PTH, phenylthiohydantoin; Psr, phosphoserine.
Sialic acid was determined after hydrolysis in 0.1 N HCl for 30 min by the thiobarbituric method of Aminoff (12).

High Voltage Electrophoresis

The purity of each peptide fraction isolated was assessed by high voltage electrophoresis in a pH 6.4 pyridine acetate buffer (100 ml of pyridine-4 ml of acetic acid-900 ml of water) on Whatman No. 3MM paper at 22 volts per cm for 90 to 120 min. The peptides were located by spraying with ninhydrin (0.5 g per 100 ml of acetone). The fractions giving a single spot when stained with ninhydrin were used without further purification. The material was eluted from the paper with distilled water and the eluent was tested for hexose and phosphorus.

Nomenclature

Glycopeptides bear the prefix GP. The letters C and E indicate the fractions of the pronase digest from which the glycopeptides were isolated. T-1 and T-2 denote the tryptic peptides of GP-C, whereas Pr-A and Pr-2 are pronase peptides derived from GP-C.

RESULTS

Previous research indicated the presence of hexose and glucosamine in our phosvitin preparations (2). As a prerequisite for the investigations described in this article, the carbohydrate content was further characterized. It was found that phosvitin contains 6.5% carbohydrate, i.e. 2.5 to 2.7% hexose, 2.0 to 2.2% glucosamine, and 1.5 to 1.7% sialic acid. The molar ratios of these components corrected to the integral value correspond to 6 residues of hexose, 5 of glucosamine, and 2 of sialic acid per mole of phosvitin based on the molecular weight of 40,000. Furthermore, since no carbohydrate was released on treatment of the protein with 0.5 N NaOH at 4°C for 19 hours followed by gel filtration on Sephadex G-25, it can be concluded that the point of attachment of the polysaccharide moiety is an asparagine residue.

Having thus established the oligosaccharide composition of phosvitin, the protein was digested with Pronase at pH 7.4 and 37°C. The result obtained on gel filtration of a typical pronase hydrolyzate of phosvitin on Sephadex G-50 is shown in Fig. 1. Of the total carbohydrate (measured as hexose) applied to the column, 95% was present in three zones designated as A, C, and E; i.e. Zone A contained 15%, Zone C 75%, and Zone E 5%. The resolution of the mixtures represented by Peaks A, C, and E is described below. However, at the outset it should be emphasized that many obstacles were encountered in the purification of the peptides. Phosvitin is very resistant to the action of proteolytic enzymes, most likely because of the inhibitory effect of the charged phosphate side chains. Hence, the resulting peptides were nonuniform in size and not all of the final products isolated were completely homogeneous with regard to their amino acid compositions. This complexity became evident when the material of Zones A and C was chromatographed on DEAE-cellulose. As shown in Fig. 2, a and b, four ninhydrin-positive peaks were obtained from each fraction. Their phosphorus and carbohydrate distributions are given in Table I. Only Peaks A-1, A-3, A-4, C-3, and C-4 were carbohydrate-positive. Fraction A-1 contained hexose but was found to be phosphorus-free. Glucosamine, and sialic acid were not detected, thus making it unlikely that this material was a product derived from phosvitin. On testing the Pronase preparation used for carbohydrate, it was found that it contained 2.5% hexose which corresponded quantitatively to that of A-1 if its carbohydrate content is correlated with the ninhydrin color as measure of nitrogenous material. By contrast, on expressing the carbohydrate content of A-3, A-4, C-3, and C-4 in molar ratios of each monosaccharide constituent by taking the number of residues for hexose as 6, the content of hexose, glucosamine, and sialic acid is the same as in the intact protein. It can, therefore, be concluded that phosvitin contains only 1 polysaccharide unit and that A-3, A-4, C-3, and C-4 are peptides of various sizes because of the uneven action of pronase on phosvitin. Also included in Table I is the carbohydrate composition of the intact phosphoprotein and of a peptide isolated from Fraction E, a phosphorus-free peptide consisting of relatively few amino acids.

Attempts at resolution of Fraction C-3 on DEAE-Sephadex A-25 were made. As illustrated in Fig. 3, the carbohydrate-containing material was localized within a small zone and was
Gradient to 3 M
Gradient to 3 M

Effluent volume (ml)

Effluent volume (ml)

Fig. 2. Chromatography on DEAE-cellulose. a, Fraction A of Fig. 1. b, Fraction C of Fig. 1. The column, 1.0 X 15 cm, was equilibrated with 0.005 M NH₄-acetate, pH 6.7. Effluent was collected in 1.5-ml fractions at a flow rate of 40 ml per hour. Peptides were detected by ninhydrin after alkaline hydrolysis on 50-µl aliquots and glycopeptides with the orcinol test on 300-µl aliquots.

Fig. 3. Chromatography of Fraction C-3 of Fig. 2b on DEAE-Sephadex A-25. The column, 2 X 33 cm, was equilibrated with 0.01 M NH₄-acetate, pH 6.7. Effluent was collected in 4.0-ml fractions at a flow rate of 200 ml per hour. Peptides were detected by ninhydrin after alkaline hydrolysis on 100-µl aliquots and glycopeptide by the orcinol test on 300-µl aliquots.

TABLE II
Composition of glycopeptide GP-C and tryptic peptides, T-1 and T-2, derived from GP-C

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Glycopeptide GP-C</th>
<th>Tryptic peptides from partially dephosphorylated GP-C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>T-1a</td>
</tr>
<tr>
<td>Histidine</td>
<td>2.0 (2)</td>
<td>1.5 (2)</td>
</tr>
<tr>
<td>Arginine</td>
<td>1.0 (1)</td>
<td>1.0</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>1.0 (1)</td>
<td>1.0</td>
</tr>
<tr>
<td>Serine</td>
<td>9.0 (&gt;11)</td>
<td>7.2*</td>
</tr>
<tr>
<td>Glycine</td>
<td>1.1 (1)</td>
<td>1.1</td>
</tr>
<tr>
<td>Valine</td>
<td>0.9 (1)</td>
<td>0.9 (1)</td>
</tr>
<tr>
<td>Hexosamine</td>
<td>6.0 (6)</td>
<td>6.0</td>
</tr>
<tr>
<td>Sialic acid</td>
<td>1.8 (2)</td>
<td>None</td>
</tr>
<tr>
<td>Phosphorus</td>
<td>7.7 (8)</td>
<td>None</td>
</tr>
</tbody>
</table>

* Purified by high voltage electrophoresis in pyridine-acetate, pH 6.4.
* Uncorrected.
* Corrected for 10% destruction of nonphosphorylated serine and 25 to 30% destruction of phosphoserine during hydrolysis (cf. Reference 2).

The amino acid composition, phosphorus content, and carbohydrate content of GP-C is shown in Table II. At this point a second difficulty encountered in our work should be pointed out. As reported in an earlier article, destruction of phosphoserine of phosvitin on hydrolysis in 6.0 N HCl at 110° and hence also of peptides rich in phosphoserine is extremely high. Therefore, a correction factor of 25% had to be applied to obtain the right content of serine (cf. Reference 2).

Amino Acid Sequence of Glycopeptide GP-C

The amino acid sequence, phosphorus content, and carbohydrate content of GP-C is shown in Table II. At this point a second difficulty encountered in our work should be pointed out. As reported in an earlier article, destruction of phosphoserine of phosvitin on hydrolysis in 6.0 N HCl at 110° and hence also of peptides rich in phosphoserine is extremely high. Therefore, a correction factor of 25% had to be applied to obtain the right content of serine (cf. Reference 2).

Four steps of the Edman degradation provided evidence on part of the structure of the glycopeptide. The presentation of the results given below follows that introduced by Dopheide et al. (9) in which the amino acid removed at each step is indicated in boldface type.2

2 The figures given here for serine are those experimentally determined and are not corrected for destruction of phosphoserine during hydrolysis in concentrated HCl (cf. Table II and Reference 2).
FIG. 4. Gel filtration of a tryptic digest of partially dephosphorylated GP-C on a Sephadex G-25 column, 2 X 33 cm. Elution was performed with distilled water at a flow rate of 60 ml per hour. Effluent was collected in 2.5-ml fractions. Aliquots of 100 \mu l were taken for ninhydrin determination.

Ser-Asp-Ser-Gly-(His, Arg, Ser, Val)

<table>
<thead>
<tr>
<th>Composition</th>
<th>9.0 1.0 1.1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Step 1:</td>
<td>8.2 0.9 1.1</td>
</tr>
<tr>
<td>Step 2:</td>
<td>8.0 0.5 1.0</td>
</tr>
<tr>
<td>Step 3:</td>
<td>7.5 0.5 0.7</td>
</tr>
<tr>
<td>Step 4:</td>
<td>7.0 0.5 0.5</td>
</tr>
<tr>
<td>Step 5:</td>
<td>6.9 0.5 0.5</td>
</tr>
</tbody>
</table>

No further loss of amino acids occurred after Step 4. The above analysis is consistent with the presence in GP-C of the NH2-terminal sequence, Ser-Asp-Ser-Gly. When GP-C was sub-

FIG. 5. Gel filtration of a Pronase digest of GP-C on a Sephadex G-25 column, 2 X 33 cm. Elution was performed with distilled water at a flow rate of 60 ml per hour. Effluent was collected in 2.5-ml fractions and aliquots of 50 \mu l were taken for ninhydrin determination.

Ser-Val-Ser-His-His

<table>
<thead>
<tr>
<th>Composition</th>
<th>1.9 0.9 1.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Step 1:</td>
<td>0.9 0.9 1.5</td>
</tr>
<tr>
<td>Step 2:</td>
<td>0.9 0.0 1.4</td>
</tr>
<tr>
<td>Step 3:</td>
<td>0.1 1.4 0.7</td>
</tr>
<tr>
<td>Step 4:</td>
<td>0.1 0.5 0.5</td>
</tr>
</tbody>
</table>

In view of the fact that carboxypeptidase B liberated 70% of the theoretical amount of arginine in 2 hours, the position of arginine as the COOH terminus of T-1 was established. Thus, we propose the following amino acid sequence for the glycopeptide GP-C, prior to assignment of the phosphate groups:

Ser-Asp-Ser-Gly-Ser-Arg-Ser-Val-Ser-His-His

Localization of Phosphoserine Residues—As indicated in Table II, the glycopeptide GP-C contained 11 to 12 serine residues but only 8 atoms of organically bound phosphorus. This can be taken as indication that only 8 of the 12 serines were phosphorylated. Therefore, an attempt was made to localize the nonphosphorylated serine residues in GP-C.

Two nonphosphorylated serine residues were located as follows. The glycopeptide, GP-C, was further digested with pronase at pH 7.4 and 37° and the digest was applied to a Sepha-
Table III
Composition of peptides from pronase digest of glycopeptide GP-C and of glycopeptide GP-E

The integral values of the amino acid residues present in each peptide are given in parentheses.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Pronase peptides from GP-C</th>
<th>GP-Ea,b</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pr-2a</td>
<td>Pr-2b</td>
</tr>
<tr>
<td>Histidine</td>
<td>++++</td>
<td></td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>2.0 (2)</td>
<td>0.8 (1)</td>
</tr>
<tr>
<td>Serine</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>Glycine</td>
<td>0.6 (1)</td>
<td></td>
</tr>
<tr>
<td>Alanine</td>
<td>Trace</td>
<td></td>
</tr>
<tr>
<td>Valine</td>
<td>Trace</td>
<td></td>
</tr>
</tbody>
</table>

- Pr-2a: Purified by high voltage electrophoresis in pyridine-acetate, pH 6.4.
- Pr-2b: The carbohydrate composition was 6 hexoses, 5 hexosamines, and 2 sialic acids as referred to 1 mole of aspartic acid.
- GP-Ea,b: The carbohydrate composition was 6 hexoses, 5 hexosamines, and 2 sialic acids as referred to 1 mole of aspartic acid.

Fig. 7: Gel filtration of Fraction E (see Fig. 1) on a Sephadex G-25 column, 2 x 33 cm. Elution was performed with distilled water at a flow rate of 60 ml per hour. 2.0 ml fractions were collected. Aliquots of 100 μl were taken for ninhydrin determination.

Discussion

The evidence presented in this article reveals the existence of a distinct heteropolysaccharide side chain in the phosphoprotein phosvitin. Since there is no loss in polysaccharide after exposure of the protein to dilute alkali, it appears that the carbohydrate-peptide attachment is of the asparagine type. Furthermore, isolation of a glycopeptide from a pronase digest of phosvitin corroborates the fact that this phosphoprotein contains the carbohydrate as one unit. The major factor for this evidence is that the glycopeptide GP-C has 6 residues of hexose, 5 of hexosamine, and 2 of sialic acid per mole, which is identical with the number found in phosvitin. The composition of the different carbohydrates of GP-C corresponds to 2,558 g per mole of peptide, which is in good agreement with the value of 6.5% carbohydrate of the original protein based on a molecular weight of 40,000.

Examination of the composition of the glycopeptide GP-C and the results obtained by Edman degradation, taken together with evidence derived from two smaller peptides isolated from a trypic digest of the partially dephosphorylated GP-C and peptides obtained from a pronase digest of GP-C led us to the conclusion that the glycopeptide has the following amino acid sequence:

Carb

-T-1 Ser-Asn-Ser-Gly-Psy-Arg-Ser-Val-Ser-His-His-

Several points of interest emerge from the structure of GP-C. One of the striking features in this sequence is the occurrence of a stretch of at least 6 but most likely 8 covalently linked phosphoserines. This finding corroborates the suggestion of Williams and Sanger (13) and Belitz (14) that clusters of phosphoserine residues are close to the COOH terminus of the glycopeptide GP-C.

As described in an earlier part of this article, a carbohydrate-containing Fraction E was separated from the pronase digest of phosvitin on Sephadex G-50 (cf. Fig. 1 and Table I). This peptide mixture was passed over a column of G-25 and the material denoted as GP-E (Fig. 7) was further purified by high voltage electrophoresis in pyridine-acetate buffer, pH 6.4. The electrophorogram indicated the presence of several peptides moving cathodically. The material eluted from the paper and denoted as GP-E (Fig. 6) was analyzed for its amino acid composition. As shown in Table III, 1.0 aspartic acid, 0.8 serine, 0.5 glycine, and traces of alanine were present. These results indicate that at least 1 of the serine residues adjacent to the aspartic acid and present in the sequence is not phosphorylated. Because of the scarcity of Fraction GP-E, no further purification was attempted.
residues must be dispersed throughout the phosvitin molecule. The results described in this article, however, represent the first direct experimental evidence for this assumption.

A second point of interest is the occurrence in GP-C of 3 to 4 of the 5 nonphosphorylated serines present in phosvitin. Pronase digestion of GP-C yielded a phosphorus-free peptide with the composition of valine (1.0) and serine (2.0) and thus made it possible to allocate two of the nonphosphorylated serines to the COOH-terminal segment of GP-C, i.e. -Ser-Val-Ser-His-His-. Allocation of a third nonphosphorylated serine residue was derived from the purification of the carbohydrate-containing, phosphorus-free Fraction GP-E containing one serine residue in addition to aspartic acid and glycine, thus making it most likely that at least one, and maybe two, of the nonphosphorylated serines is adjacent to the aspartic acid residue which serves as point of attachment of the polysaccharide to the protein.

A number of difficulties were encountered in this study which lead to some uncertainties. The poor yield in the subtractive Edman degradation of GP-C is one. More serious, however, is the high destruction of phosphoserine during hydrolysis in strong HCl. The use of the correction factor of 25%, established previously for phosvitin (2), led us to the assignment of 8 phosphoserines as part of the glycopeptide GP-C. Allocation of the nonphosphorylated serine residues was also difficult. Although two of these residues could readily be assigned to the COOH terminus of GP-C, it is apparent from Table III that the phosphorus-free but carbohydrate-containing Fraction GP-E1 must have been a mixture of peptides with similar electrophoretic mobilities. It is quite feasible that these peptides originate from the main glycopeptide GP-C and are due to uneven proteolysis by pronase. Thus, GP-E1 may contain two peptides with the serines adjacent to the aspartic acid which serves as point of attachment of the polysaccharide to phosvitin. Should this be the case, the two serines of the NH₂ terminus of GP-C would not be phosphorylated. In conclusion we should like to state that despite the difficulties encountered, the glycoprotein nature of phosvitin has been clearly established. Furthermore, the occurrence in the glycopeptide of the sequence

\[
\text{Carb} -\text{Ser-Asp-Ser-Gly-}
\]

is similar to that found for the Class I glycopeptide of porcine pancreatic ribonuclease (15) and the α-acid glycoprotein (16), thereby adding phosvitin as a further protein with proximity of hydroxyl side chains to the aspartic acid residue which serves as attachment of the carbohydrate (cf. Reference 17).

Acknowledgment—We wish to express our thanks to Mrs. Elizabeth Dharmgrongartama for her expert technical assistance.

REFERENCES

Phosvitin, a Phosphoglycoprotein: I. ISOLATION AND CHARACTERIZATION OF A GLYCOPEPTIDE FROM PHOSVITIN
Ruth Shainkin and Gertrude E. Perlmann


Access the most updated version of this article at [http://www.jbc.org/content/246/7/2278](http://www.jbc.org/content/246/7/2278)

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/246/7/2278.full.html#ref-list-1](http://www.jbc.org/content/246/7/2278.full.html#ref-list-1)