CONCANAVALIN A REACTIVITY AND CARBOHYDRATE STRUCTURE OF BLATTELLA GERMANICA VITELLIN*

JOSEPH G. KUNKEL†, GARY L. SHEPARD†, ROBERT A. MCCARTHY†, DAVID B. ETHER† and JOHN H. NORDIN†

Departments of Zoology† and Biochemistry†, University of Massachusetts, Amherst, MA 01003, U.S.A.

(Received 26 March 1980)

Abstract—The carbohydrate moiety of Blattella germanica vitellin has been investigated. A glycopeptide fraction obtained by protease digestion and gel filtration has a Man : GlcNAc ratio of approximately five and is enriched in aspargine and phosphate. Further structural characterization indicates only a single oligosaccharide species is present. It contains ten to eleven mannosyl units and is linked through a chitobiosyl-asparagine linkage to peptide. There are approximately twelve to sixteen of these oligosaccharide chains per mole of vitellin and they are located in the 100,000 mol. wt subunit.

A mixture of glycopeptides derived from radiolabelled vitellogenin and unlabelled vitellin was digested with β N-acetyl glycosaminidase H and the oligosaccharides submitted to gel filtration. Coincident elution of radioactivity and carbohydrate suggests that no change occurs in the size of the oligosaccharide between the time the glycoprotein is secreted from the fat body and one day after its uptake by the oöcyte.

Experiments with Concanavalin A, anti Blattella antibodies and a number of ligands indicate (a) multiple lectin binding sites occur on vitellin, (b) a portion (but possibly not all) of the oligosaccharide chains of vitellin are located on the exterior of the vitellin molecule and (c) the oligosaccharide is not a determinant group of vitellin.

Key Word Index: Blattella germanica, vitellin, vitellogenin, oligosaccharide structure, Concanavalin A reactivity, vitellin carbohydrate composition, glycopeptides

INTRODUCTION

In the development of the eggs of many animal species, certain serum proteins, termed vitellogenins* are the major source of yolk proteins. They are synthesized in, and secreted from, other maternal organs (the liver in vertebrates and fat body in insects) entering the circulation to be subsequently sequestered by the developing egg. Ultrastructural and chemical studies by Roth and Porter (1964) showed that both the mosquito and chicken oöcyte use adsorptive endocytosis to facilitate uptake. This process is highly selective; vitellogenin is concentrated some twenty to thirty times in the developing oöcyte, relative to serum, while other serum proteins are essentially excluded from uptake (Roth et al., 1976). The mechanism controlling this selectivity is unknown. However, in the cockroach it may involve a pinocytotic vesicle specific for vitellogenin (Anderson, 1970).

Insect vitellins, as a class, are multimeric phosphoglycolipoproteins (see Hagedorn and Kunkel, 1979). Although comparative studies of the amino acid compositions of some of these vitellins have been reported (Kunkel and Pan, 1976; Hagedorn and Kunkel, 1979) very little is known about their associated lipid and carbohydrate components. The carbohydrate constituents of a few vitellogenins and vitellins have been reported (Yamasaki, 1974; Chen et al., 1978; Mundall and Law, 1979). In addition, vitellin from Locusta migratoria has been shown to bind Con A (Gellison et al., 1976).

Evaluation of any potential role that the associated carbohydrate portions may play in the sequence of events between vitellogenin synthesis in the fat body and vitellin utilization by the embryo will require knowledge of their structures. Furthermore, data permitting structural comparisons between vitellins in the class Insecta and other glycoproteins in the animal kingdom are lacking at present.

Reactivity of vitellin from Blattella germanica with Con A and chemical studies of the carbohydrate portion of the molecule have been conducted with the aim of determining certain aspects of its structure and location in vitellin. A preliminary report of this work has been published (Kunkel et al., 1978).

MATERIALS AND METHODS

Animals

A closely inbred line of B. germanica was used for these
studies (fifteen generations of brother–sister matings). The parents of the systematically inbred strain were a cross of the Orlando Strain which has been in laboratory culture for several decades (Ross and Cochran, 1975) with the New York Strain in culture since 1962 (Kunkel, 1966). Animal development and synchrony of cultures were controlled by regulation of food availability (Kunkel, 1966).

Materials
Sodium [3H]borohydride and d-[1-14C]mannose

13.2 Ci/mmol, and d-[2-3H]-mannose, 1 Ci/mmol, were obtained from New England Nuclear Co. The borohydride was stored at −85°C (as a solution in 0.1 M NH4OH, 1 MCI/100 μl). Disodium p-nitrophenyl phosphate, p-nitrophenyl α-mannopyranoside, ovalbumin, (Grade V), phenylmethylsulphonyl fluoride and Saccharomyces cerevisiae mannan were purchased from Sigma Chemical Co. Proteinase K was purchased from Beckman Industries and pronase from Calbiochem. Ion exchange resins Dowex AG-50W-X2, AG-50X-16 and AG-1-X10, (all 200–400 mesh) BioCell P-2 and P-6 (both minus 400 mesh), TEAE-cellulose, and electrophoresis grade agarose were purchased from Bio-Rad Laboratories. Amberlite IR-120 (H+ ) and IR-45 (OH−) were purchased from Mallincrodt. Highly purified Concanavalin a was obtained from Pharmacia Fine Chemicals. ECNNS-M (3% w/w) on Gas Chrom P (80–100 mesh) was obtained from Applied Sciences Laboratories. Highly purified endo H was a gift from Dr. P. W. Robbins, Biology Department, Massachusetts Institute of Technology.

Paper chromatography
Mono- and oligo-saccharides were analyzed by descending chromatography using Whatman No. 1 paper, with solvent A, pyridine: ethyl acetate: water (2:5:7, by vol, upper phase); and solvent B, butan-1-ol pyridine, water (4:3:4, by vol).

General methods
For routine work, protein concentrations were measured by 280 nm absorbance, or by the procedure of Lowry et al. (1951). However, 280 nm absorbance was used for purified vitellogenin or vitellin (E280nm = 7.46). Phosphorous was determined by the method of Bartlett (1959) and neutral hexose with phenol and sulphuric acid (Dubois et al., 1956). Reducing sugars were detected using the assay of Park and Johnson (1949) and N-acetyl hexosamines by the method of Reissig et al. (1955). Radioactivity was estimated by liquid scintillation spectrometry.

Protein isolation
Vitellogenin and vitellin from B. germanica, vitellogenin from Blattella (sp. near humbertiana) and two non-vitellogenic serum proteins, designated SP-1 and SP-2, from B. germanica were purified by TEAE cellulose chromatography and sucrose gradient centrifugation (Kunkel and Pan, 1976) from animals raised in synchronous cultures. Batches of 4–8 g of eggs were routinely used (always within 24 hr of ovulation) for vitellin isolation. Eggs (4 g) were suspended in 2 vol of distilled water and disrupted in a loose fitting Dounce homogenizer. After 5 min, during which time the yolk platelet membranes lysed, the mixture was made 0.2 M with respect to NaCl, further homogenized and centrifuged for 20 min at 8500 g at 4°C. The gelatinous lipid overlaying the carotenoid coloured supernatant solution was carefully penetrated and the latter removed and adjusted to 0.15 m NaCl and chromatographed at 4°C on a 2.5 cm i.d. × 125 cm TEAE cellulose column equilibrated with 0.01 m sodium phosphate, 0.1 M NaCl (pH 6.5). The column was washed sequentially with 200 ml each of equilibration buffer fortified to 0.15 and 0.3 M NaCl. Vitellin elutes in the buffer containing 0.3 M salt.

In vivo radiolabelling of vitellogenin
The oligosaccharide of vitellogenin was labelled in vivo with [3H]-mannose using the technique described by Kunkel and Pan (1976). Briefly, twenty adult females, ovariectomized as larvae, were each injected with 30 μCi of isotope two days after feeding and bled 3 hr later through an autotomized limb into 0.9% NaCl containing 0.05 volume of 6% PMSF. Five microliters of haemolymph were collected from each. Approximately 100 μl were purified as described above on a 0.3 cm i.d. × 3 cm TEAE cellulose column and yielded 3 mg of vitellogenin (2900 dpm per μg). For final purification, the vitellogenin fraction was layered onto a 5–25% glycerol gradient in PBS. The gradient was centrifuged in an SW 41 Spinco rotor at 20,000 g for 15 hr at 4°C and the 17 S peak concentrated by vacuum dialysis.

SDS acrylamide gel electrophoresis
The subunit compositions of proteins were examined using SDS polyacrylamide gel electrophoresis (Weber et al., 1972). For determination of radioactivity, a 1 mm gel slice was digested in 0.5 ml of a Protosol:water mixture (20:1 v/v), and counted after the addition of 5 ml of a toluene based scintillation fluid. Recovery of the radioactivity (as dpm) applied to each gel was monitored by using a [3H]-toluene standard and correcting for quenching of radioactivity by the Protosol:water mixture. Counting efficiency was 25%.

Immunological techniques
Ouchterlony double diffusion tests and immuno-electrophoresis were performed as previously described (Kunkel and Lawler, 1974). Photometric precipitin assays were performed using Con A and purified vitellin from B. germanica. A Con A–vitellin precipitin curve was established first. Serial dilutions of purified vitellin in 1 ml of PBS were incubated at 25°C with 2.0 ml of a solution of Con A (0.21 mg/ml) for 18 hr by which time the reaction had reached equilibrium. The light scattering absorbance was recorded at 420 nm. Since vitellin itself absorbs appreciably at 420 nm, similar dilution series lacking Con A were prepared to serve as blanks. Net readings were plotted vs the dilution of vitellin to give a precipitin curve. A solution with 2 mg/ml vitellin gave the highest precipitin absorbance and was used in subsequent inhibition assays.

For the inhibition assay the proper amount of vitellin was added to serial dilutions of inhibitors in a total volume of 1.0 ml and mixed. Con A was added and the absorbance was measured at intervals of up to 18 hr to assure that equilibrium was reached. The percentage precipitation inhibition was calculated using the expression $100 \times \frac{(A - B) \times 102}{A}$, where $A$ equals the absorbance without inhibitor added and $B$ equals the absorbance with inhibitor added.

Isolation of vitellin glycopeptides
Purified vitellin (100 mg) containing 4–5% neutral hexose (Kunkel and Pan, 1976) was delipidified (Kunkel and Pan, 1976) and then digested in water with proteinase K employing a vitellin to enzyme ratio of 100:1 (w/w) for 36 hr at 55°C. The mixture initially formed a turbid suspension which usually completely dissolved during the course of proteolysis. Digestion was terminated by the addition of 1/20 vol of 6% (v/v) phenyl methylsulphonyl fluoride. Occasionally, a small proteinaceous precipitate formed during the digestion but this was found to be devoid of neutral sugar. It was removed by centrifugation of the digest at 1000 g for 5 min. The supernatant solution was lyophilized, the residue dissolved in 2 ml of water and the solution chromatographed on a Bio-Gel P-2 column (1.6 cm i.d. × 100 cm, minus 400 mesh) maintained at 50°C (using a flow rate of approx. 20 ml per hr. The carbohydrate-containing fractions (called the P-2 glycopeptide fraction) were pooled, concentrated to 10 ml and applied to a Dowex AG-50-X16(F+1) column (2.2 cm i.d. × 38 cm). The carbohydrate rich fraction eluted with water, was neutralized with NaOH and lyophilized (AG 50 glycopeptides).
**Amino acid analyses**

A sample of the AG 50 glycopeptide (1 mg) was hydrolyzed for 24 hr in 500 μl of conc. HCl: proionic acid (1: 1 v/v). The acid was removed under reduced pressure at 40°C and approx. 0.2 mg of hydrolysate was examined using an automatic amino acid analyzer.

**Carbohydrate analyses**

Conditions were developed for the acid hydrolysis of vitellin glycopeptides utilizing both ovalbumin and vitellin glycopeptides as well as with mixtures of pure N-acetyl glucosamine and mannose. Results of these experiments showed that glycopeptide hydrolysis under N₂ and 2 M HCl (H⁺) column and again concentrated under reduced pressure. Final resolution of the sample was obtained by Bio-Gel P-6 column chromatography as described above. Paper chromatography of the product in solvent system B for 16 hr revealed only a single radioactive spot with an Rf of 0.12. Acid hydrolysis of a specimen of the oligosaccharide alcohol followed by paper chromatography in solvent system A indicated the presence of a single radioactive component with a mobility identical to a glucosaminitol standard.

**Size comparison of vitellogenin and vitellin oligosaccharides**

Samples of unlabelled vitellin (approx. 700 μg neutral hexose) and vitellogenin labelled in vivo with [3H] in the mannansy residues (76,000 cpm) were mixed and delipified. The preparation was then converted to endo H oligosaccharides as described in Materials and Methods. The mixed oligosaccharides were chromatographed on a 1.6 cm i.d. × 100 cm Bio-Gel P-6 and both neutral sugar (vitellin) and radioactivity (vitellogenin) contents of each fraction monitored. (The amount of carbohydrate in the vitellogenin specimen was negligible and did not contribute to the sugar content of the mixture.)

**Phosphatase assays**

Both protease K and endo H were tested for the possible presence of contaminating phosphatase activity. Assays were conducted in 0.05 M sodium acetate and 0.10 M sodium chloride buffer (pH 6.0) with O-nitro-phenyl-phosphate as substrate (HOLLANDER, 1971). Protease K was adjusted to a concentration of 5.0 mg/ml in buffer. Enzyme solution (0.2 ml) was added to 50 μl substrate and incubated at 22°C for 2 hr. The reaction mixture was then made alkaline with 3.0 ml or 0.2 M Na₂CO₃ and the absorbance read at 405 nm. Potential contaminating phosphatase activity in the endo H preparation was tested using 2 μl of enzyme in 250 μl of citrate buffer. pH 5.5 with 50 μl of substrate. The reaction mixture was incubated for 30 hr at 37°C under toluene vapour. An aliquot (50 μl) was made alkaline with 5.0 ml of 0.2 M Na₂CO₃ and absorbance read at 405 nm.

**RESULTS**

**Preparation of vitellin glycopeptides**

Vitellin digested with protease K was purified initially by Bio-Gel P-2 column chromatography (Fig. 1). Greater than 95% of the carbohydrate was routinely recovered. There is no evidence of free monosaccharides (elution position at V₁) indicating the protease K used is free of exoglycosidases. Extended digestion of the vitellin or a second digestion of the pooled P-2 glycopeptides did not change the carbohydrate elution profile. Fractions 12-65 were pooled and lyophilized. Analysis showed the product contained 15-18% neutral hexose by weight. The P-2 glycopeptides were further purified by passage through an AG 50 column. Recovery of the carbohydrate was always quantitative with a further two-fold purification of the product to 38% neutral hexose.

Carbohydrate analyses of an acid hydrolysate of this glycopeptide fraction was carried out using GLC.
Proteinase K Digest of Delipidated Vitellin on Bio-Gel P-2 (100 × 1.6 cm)

Fig. 1. Elution profile of vitellin glycopeptides on Bio-Gel P-2 (minus 400 mesh). Delipidified Vt (approx. 100 mg, 4.5% neutral hexose) was digested exhaustively with proteinase K at 55 °C for 36 hr in water and the products chromatographed at 50 °C on a 100 × 1.6 cm i.d. column of Bio-Gel P-2 (minus 400 mesh) with water as eluant. Solid line carbohydrate; dashed line, ninhydrin positive material. Tubes 12-65 were pooled. V, void volume; V included volume (elution volumes of glycogen and D-mannose standards respectively).

Comparison of retention times for vitellin sugars with standards of Man, Gal, Glc, GlcNac, GalNac and ManNac plus co-chromatography with mixed standards of Man and GlcNac, indicated Man and GlcNac are the two sugar components. Mixed standards consisting of various ratios of Man and GlcNac alditol acetates were also chromatographed and their molar response values used to calculate the amount of each sugar present in vitellin (SWEET et al., 1975). Colourimetric assays were used as an independent measure of the sugar composition of the glycopeptide fraction. The results of these experiments are summarized in Table 1. Comparative amino acid analyses (Table 1) of vitellin and the glycopeptide fraction show a significant enrichment of aspartic and residues in the latter relative to the former, the mole percentages reported represent major residues only.

Precipitin reactions in agarose gel

Ouchterlony tests with Con A demonstrate that both B. germanica vitellin and vitellogenin as well as Blattella (sp. near humbertiana) vitellogenin possess reactive groupings presumably α-D-mannopyranosyl. Immunoelectrophoresis was performed on B. germanica purified vitellogenin, whole larval serum (which contains both S-I and S-II) and purified S-I. The proteins were separated electrophoretically, their positions identified with anti-sera characterized previously and their activity with Con A determined (KUNKEL and PAN, 1976; KUNKEL and LAWLER, 1974). Precipitin bands were formed between both S-I (lower arc) and S-II (upper arc) and the anti larval serum, as well as between anti vitellin and vitellogenin. However, Con A does not react with S-II but does form a precipitin arc with vitellogenin and purified S-I (Fig. 2). Formation of precipitin band suggests multiple Con A combining sites on the protein. Chicken ovalbumin, which has only one mannose containing carbohydrate chain per 43,000 mol. wt in the native protein (SHARON, 1966) behaved like hapten and did not form a precipitate with Con A in either the Ouchterlony, Oudin or immunoelectrophoresis assays. However, the ability of ovalbumin to react with Con A as a hapten was demonstrated and utilized in the following precipitation inhibition test.

**Photometric precipitation inhibition assays**

To characterize the Con A reactive residues on vitellin, an inhibition study of Con A–vitellin precipitation was carried out. Visible precipitates are formed between Con A and vitellin with maximal reactivity at 0.21 mg/ml Con A and 2.4 mg/ml vitellin (data not shown). At the peak of precipitation there is approximately molar equivalence between Con A (0.19 μmoles) and vitellin (0.3 μmoles). The relative strengths of inhibitors of the peak precipitation calculated on a molar basis are summarized in Table 2. Vitellin glycopeptide is the most effective hapten while ovalbumin is one order of magnitude more effective than yeast mannan in inhibiting the precipitation. All of the other sugars used were more than four orders of magnitude less effective than mannan. Only ovalbumin and vitellin glycopeptide approach inhibiting the Con A-vitellin reaction in amounts that are equimolar with the lectin.

In a separate experiment, it was found that the AG 50 glycopeptide did not inhibit the quantitative precipitin reaction between vitellin and anti B. germanica vitellin. This indicates that the oligosaccharide moities on vitellin are not major immunodeterminants.

**Protein-oligosaccharide linkage**

Digestion of the AG 50 glycopeptide fraction with

| Table 1. Compositions of vitellin and the AG 50 glycopeptide fraction |
|------------------------|-----------------|-------------------|
| Amino acid | Vitellin | AG 50 Glycopeptide |
| Asx | 12.2 | 22.6 |
| Glx | 10.9 | 16.2 |
| Ser | 9.1 | 13.0 |
| Thr | 5.7 | 9.8 |
| Leu | 8.6 | 6.6 |
| Val | 8.5 | 5.8 |
| Phe | 4.4 | 5.6 |

Man : Glc NAc Ratio

<table>
<thead>
<tr>
<th>Method</th>
<th>Observation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colourimetric</td>
<td>ND 5.7, 5.5, 5.0 (5.4)*</td>
</tr>
<tr>
<td>Gas-liquid chromatography</td>
<td>ND 4.4, 4.5, (4.5)*</td>
</tr>
</tbody>
</table>

ND = not determined.

* Average.
Fig. 2. Immunoelectrophoretic demonstration of Con A reactivity of cockroach serum proteins. Electrophoresis was carried out at pH 8.6 in agarose as described previously (KUNKEL and LAWLER, 1974). Aliquots of larval serum (L), purified serum protein I (SI) and vitellogenin (V) were placed in sample wells. After electrophoresis of duplicate slides, one was stained to visualize the separated proteins and one had troughs cut in it for immunodiffusion. Anti Blattella germanica larval serum (anti Bg) (KUNKEL and LAWLER, 1974) and anti B. germanica vitellin (anti BgV) (KUNKEL and PAN, 1976) were added to the left hand troughs and Con A (3 mg/ml) to the right hand troughs. The reactivity of S-I and S-II and vitellogenin with each was then visualized and the duplicate slides superimposed for the photographic record. The anode is at the bottom of the figure.
Table 2. Equilibrium inhibition titres for concanavalin A–vitellin coprecipitation

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Mol. wt</th>
<th>50% inhibition titre in mMoles</th>
<th>95% CI</th>
<th>Moles inhibitor/mole vitellin at 50% inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitellin P2 glycopeptide*</td>
<td>4700</td>
<td>0.25</td>
<td>0.00031</td>
<td>0.3</td>
</tr>
<tr>
<td>Ovalbumin</td>
<td>43,000</td>
<td>4.5</td>
<td>0.0030</td>
<td>3.1</td>
</tr>
<tr>
<td>S. cerevisiae mannan</td>
<td>28,000</td>
<td>1.17–1.35</td>
<td>0.045</td>
<td>44.3</td>
</tr>
<tr>
<td>Trehalose</td>
<td>343</td>
<td>55–81</td>
<td>195</td>
<td>1.93 × 10^4</td>
</tr>
<tr>
<td>Methyl-α-d-mannopyranoside</td>
<td>194</td>
<td>48–54</td>
<td>262</td>
<td>2.59 × 10^4</td>
</tr>
<tr>
<td>Methyl-α-d-glucopyranoside</td>
<td>194</td>
<td>50–70</td>
<td>305</td>
<td>3.02 × 10^4</td>
</tr>
<tr>
<td>d-Mannose</td>
<td>180</td>
<td>99–112</td>
<td>583</td>
<td>5.76 × 10^3</td>
</tr>
<tr>
<td>Sucrose</td>
<td>342</td>
<td>424–583</td>
<td>1453</td>
<td>1.44 × 10^6</td>
</tr>
<tr>
<td>α-Glucose</td>
<td>180</td>
<td>237–276</td>
<td>1421</td>
<td>1.41 × 10^6</td>
</tr>
</tbody>
</table>

* The P-2 glycopeptide fraction has a hexose content of 38 ± 3%. The mol. wt is calculated assuming that each glycopeptide contains one oligosaccharide chain with eleven mannose units.

endo H resulted in the release of reducing sugars. The liberated saccharide fraction was reduced with tritium labelled NaBH₄. The products were chromatographed on a Bio-Gel P-6 column and both neutral hexose and radioactivity monitored (Fig. 3). Only a single radioactive component, which eluted with the neutral hexose, was observed.

To determine the percentage of the oligosaccharide chains cleaved by endo H, a sample of AG 50 glycopeptide fraction was digested with enzyme for 36 hr and directly applied to a Bio-Gel P-6 column and monitored for carbohydrate and protein (Fig. 4). The peptide material separated completely from all the carbohydrate which eluted as a single peak proving that the oligosaccharide chains are quantitatively cleaved from the peptides. Because of the well-characterized specificity of endo H it is clear that the oligosaccharide is N-glycosidically bound to the protein exclusively through a diacetyl chitobiosyl linkage to asparagine (Tarentino and Maley, 1974; Kornfeld and Kornfeld, 1976; Tarentino and Maley, 1976).

Total separation of intact glycopeptide from tritiated endo H oligosaccharide alcohol by gel filtration on P-6 was also observed (data not shown). The fact that the oligosaccharide alcohol fraction elutes as a single peak suggests that all chains are the
same size. Therefore, the number of hexose units in a
tritiated specimen was estimated using several
oligosaccharide standards by Bio-Gel P-6 column
chromatography in 0.1 M Tris buffer, pH 8.0. The
oligosaccharide alcohol again eluted as a single
component and on the basis of the elution positions
of the standards employed is judged to contain a total
of twelve monosaccharides (Fig. 5). Colourimetric and
GLC analyses of the sugar composition of vitellin
oligosaccharides yield on average Man:GlcNAc ratio
of approximately five suggesting that two GlcNAc and
ten or eleven Man units are present on each complete
oligosaccharide chain. Based on the subunit weights
of Blattella Vt (100 and 50 K) and the percentage of
carbohydrate (4-5%) in native vitellin, it was
calculated that there are three or four oligosaccharide
chains per 100 K subunit.

Phosphorus localization

The phosphorus contents of the vitellin, delipidated
vitellin, the AG 50 glycopeptide fraction and the endo
H oligosaccharide were measured and the results
are tabulated in Table 3. During preparation of the
glycoprotein for phosphorus analysis, it was
exhaustively delipidified. Phospholipids are reported
to comprise a portion of the total lipid vitellogenin,
(Chino et al., 1977). Phosphate removed during
delipification is due presumably to phospholipid
noncovalently bonded to the protein. Use of two
different ratios of methanol to chloroform provided a
product with the same phosphate content, suggesting
that the delipification is complete.

Since preparation of both the glycopeptide and the
oligosaccharide involved the use of enzymes,
proteinase K and endo H were tested for possible
contaminating phosphatase activity. None was
detected. The percentage of phosphorus is enriched
approximately ten-fold in the purified glycopeptide
fraction relative to that of the protein while the serine
content is increased by only about 43% above that of
vitellin itself (Table 1). This suggests that a higher
proportion of serine residues proximal to the
oligosaccharide chains are phosphorylated compared
to those more distant. While phosphorus is definitely
associated with protein, the results show conclusively
that the oligosaccharide itself contains none.

Subunit location of oligosaccharide

Localization of the majority of protein-bound
carbohydrate on the 100 K subunit of vitellin was
noted by specific carbohydrate staining (Mc Manus,
1946) of subunits after SDS polyacrylamide gel
electrophoresis (data not shown). Because of the

Table 3. Phosphate content of Blattella germanica vitellin
and its various fractions

<table>
<thead>
<tr>
<th>Component</th>
<th>% Phosphorus</th>
<th>% Mannose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitellin</td>
<td>0.60</td>
<td>—</td>
</tr>
<tr>
<td>Delipidated vitellin*</td>
<td>0.30</td>
<td>4.5</td>
</tr>
<tr>
<td>Delipidated vitellin†</td>
<td>0.28</td>
<td>4.5</td>
</tr>
<tr>
<td>AG-50 glycopeptide</td>
<td>3.00</td>
<td>38</td>
</tr>
<tr>
<td>Endo H oligosaccharide</td>
<td>0‡</td>
<td>89§</td>
</tr>
</tbody>
</table>

All values are based on dry weight of material and are
calculated from the average of duplicate colourimeter
readings. One sample of each component was analysed. The
native vitellin (glycolipoprotein) was delipidified (Kunkel and
Pan, 1976) by two methods:

* Delipidified by extraction with CHCl₃:CH₂OH (2:1); and
† Delipidified by extraction with CHCl₃:CH₂OH (1:2).
‡ Less than 0.2 mole of phosphorous per mole of
oligosaccharide.
§ Calculated on the basis of an oligosaccharide
composition of eleven Man and two GlcNAc residues.
difficulty of obtaining labelled vitellin of a sufficiently high specific activity for quantitative analysis, vitellogenin subunits labelled with \[^3H\]-d-mannose for 3 hr \textit{in vivo} were examined following purification. SDS gel electrophoresis was used to determine the ratio of labelled carbohydrate in each subunit. Figure 6 illustrates the \[^3H\]-profile of the newly synthesized vitellogenin.

The label is found in two major peaks. The most extensively labelled components (150 K) corresponds to a serum precursor of the 100 and 50 K subunits of mature vitellogenin (Fig. 6). The second large \[^3H\]-labelled peak is the 100 K subunit of the mature vitellogenin corresponding to the major subunit of vitellin and contains 93\% of the mannose found in the combined 50 and 100 K subunits. The other subunit (50 K) contains only 6--7\% of the label. Identical results were obtained when 2-[\(^3H\)]-mannose used to label the carbohydrate moiety precluding incorporation of label into protein. The peak at slice number 33 is similar in molecular weight to a cleavage product of the 100 K subunit obtained in high yield when native vitellin is treated with trypsin (data not shown) and may represent the result of degradation during isolation or an alternative cleavage product of the 150 K precursor.

Fig. 6. Subunit localization of oligosaccharide in vitellogenin. Vitellogenin was labelled \textit{in vivo} with \[^3H\]-D-mannose and accumulated for 3 hr in the serum of ovariectomized females. Each was then bled as described in experimental procedures and vitellogenin purified (Künkel and Pan, 1976). A measured aliquot of known radioactivity was subjected to SDS acrylamide gel electrophoresis (5\% gels). A 150 K vitellogenin precursor, never observed in newly ovulated eggs, as well as the 100 K and 50 K subunits of vitellogenin contain radioactive mannose.

Fig. 7. Size comparison of vitellogenin (Vg) and vitellin (Vt) oligosaccharides. Samples of vitellogenin, labelled \textit{in vivo} with 2-[\(^3H\)]-mannose (76,000 cpm) and unlabelled vitellin (700 mg mannose) were combined and converted into a glycopeptide mixture as described in Materials and Methods. The glycopeptides were incubated with endo H and the oligosaccharides chromatographed on a 1.6 cm i.d. \(\times\) 100 cm Bio-Gel P-6 column at 22°C. The flow rate was 12 ml/hr and 0.87 ml aliquots were collected. Fractions were monitored for their contents of \[^3H\] by liquid scintillation spectrometry and for mannose by the phenol sulphuric acid assay. \(V_o\), void volume; \(V_e\), elution position of mannose.
Size comparison of oligosaccharides

The profiles in Fig. 7 show coincident elution of vitellogenin and vitellin oligosaccharides. Therefore, as judged by this criterion it appears that no detectable change occurs in the size of the carbohydrate chains between the time they are secreted by the fat body and immediately after their uptake by the egg.

DISCUSSION

The experiments with Con A argue for multiple Con A-reactive groups on the surface of two serum proteins of B. germanica. Both vitellogenin and S-I can serve as models for Con A reactivity with insect serum glycoproteins. Both proteins precipitate with Con A in agarose gel. Both vitellin and vitellogenin of B. germanica react with Con A, a result consistent with chemical studies described above and showing similar saccharide substituents are present on each. By virtue of its precipitin reaction in immuno-electrophoresis, the nonvitellogenic S-I from B. germanica contains multiple combining sites for Con A. This leads to the question of whether the carbohydrate of vitellogenin is qualitatively different from that on the other serum proteins. Analysis of the carbohydrate specificity of Con A has been used to identify certain reactive sugar groups and structures of saccharides on the cell membranes (Goldshtein et al., 1965, 1967; Goldstein et al., 1974).

Precipitation inhibition results show α-D-mannosides to be the strongest vitellin-Con A precipitation inhibitor. On a carbohydrate weight basis the strongest is vitellin glycopeptide followed in order by S. cerevisae mannan, ovalbumin and methyl-α-D-mannopyranoside. The vitellin glycopeptide competes very well with the vitellin for access to Con A. This is especially noteworthy since the native vitellin is estimated to have sixteen to twenty of these chains in its structure. This may suggest that not all of the oligosaccharide chains are equally available to react with Con A. Since the binding of Con A to vitellin is reversible, and does not involve disruption of the structure of the reacting molecule, at least some of the manno-oligosaccharide chains are probably located on the exterior surface of the native vitellin molecule. This location would allow direct oligosaccharide interaction with Con A and perhaps with various recognition marker receptors (see below).

The results of the chemical experiments described above indicate that vitellin carbohydrate resembles a 'high mannose' type of oligosaccharide of uniform size (ten to eleven Man residues plus two of GlcNAc) attached via asparagine moieties exclusively to the 100 K subunits (Kornfeld and Kornfeld, 1976). These findings are of interest in view of the fact that the carbohydrate structures of various insect species and the carbohydrate moieties on the molecule. It is also of interest that since the oligosaccharide is not an immunodeterminant of B. germanica vitellin, the loss of cross reactivity that occurs through evolution between cockroach species vitellins is not due to changes in oligosaccharide structure.

Since endo H digestion clearly gives quantitative removal of the oligosaccharide chains from vitellin glycopeptides (Fig. 4) and they are of a single size (Fig. 5) it is presumed that the 50 K subunit is also substituted with the same sized saccharide. However, the small percentage of [14C]-mannose label incorporated in this subunit suggests that they are incompletely glycosylated (i.e. less than one chain per subunit).

The average Man:GlcNAc ratios obtained by colourimetric and gas chromatographic analyses (11:2 and 9:2 respectively) are in reasonable agreement. The elution volume of the endo H oligosaccharide alcohol from the P-6 column (Fig. 5) is consistent with it containing either ten or eleven mannose units; some uncertainty exists regarding the effect of GlcNAc reduction and branching characteristics on the elution position of such an oligosaccharide alcohol, relative to those of unreduced standards. Therefore, a single number cannot be assigned using the available data. The well established substrate specificity of endo H permits assignment of a chitobiosyl-asparagine linkage region to this saccharide (Tarentino and Maley, 1974; 1976) and since this type of oligosaccharide generally contains mannoside joined α(1→4) to the distal GlcNAc (Kornfeld and Kornfeld, 1976) such an assignment for this particular linkage can be reasonably presumed. It is likely that all other mannosyl units on the vitellin oligosaccharide are linked in the α configuration (Kornfeld and Kornfeld, 1976).

Susceptibility of all of the detectable carbohydrate in vitellin glycopeptides to endo H suggests that no O-glycosidic linkages from mannose to protein such as those present in yeast mannan (Ballo, 1976) exist in vitellin.

Phosphomannosyl groups present on the carbohydrate portion of certain lysosomal hydrolases are reported to function in their recognition by cells in vitro (Hughes, 1977; Distler et al., 1979) therefore it was considered important to ascertain whether the oligosaccharide of vitellin is similarly substituted. Analysis of both the endo H and proteinase K for possible contamination by phosphatase indicates that they do not contain any such associated activity which is detectable by the assay used. Therefore, the absence of phosphatase on the vitellin oligosaccharide can not be attributed to removal by phosphatase during its preparation. The physiological 'life' of vitellogenin–vitellin involves a complex series of known events including synthesis in the fat body, secretion, circulation in the haemolymph and finally specific endocytic uptake, aggregation and degradation in the egg. Processing (maturation) probably also occurs (Warren et al., 1979) although there is no evidence for this in the B. germanica system. It is probably that some of these events involve
REFERENCES


