

CONCAVALIN A REACTIVITY AND CARBOHYDRATE STRUCTURE OF *BLATTELLA GERMANICA* VITELLIN*

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

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

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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 asparagine 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 glucosaminidase 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

* By convention, the term vitellogenin is used to define the yolk protein prior to its uptake by the developing oöcyte. The eggbound species is referred to as vitellin.

Abbreviations used: Con A, Concanavalin A; S-I, serum protein I; TEAE cellulose, triethylamino ethyl cellulose; endo 'H', endo β -N-acetyl glucosaminidase H; GlcNAc, N-acetyl glucosamine; GalNAc, N-acetyl galactosamine; ManNAc, N-acetylmanosamine; PBS, phosphate buffered saline, 0.1 M sodium phosphate, 0.15 M NaCl, pH = 7.2; PMSF, Phenylmethylsulphonyl fluoride.

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-[1-³H9N]-mannose 13.2 Ci/mmmole, and D-[2-³H]-mannose, 1 Ci/mmmole, 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) BioGel 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 from Mallinckrodt. 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 230 nm absorbance, or by the procedure of LOWRY *et al.* (1951). However, 280 nm absorbance was used for purified vitellogenin or vitellin ($E_{280, 1\%}^{1\text{cm}} = 7.46$). 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-I 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 in NaCl and chromatographed at 4°C on a 2.5 cm i.d. \times 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. \times 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 immunoelectrophoresis 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 $100 \times (A - B)/A$, 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 delipidated (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. \times 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(H⁺) column (2.2 cm i.d. \times 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 μ l of conc. HCl:propionic acid (1:1 v/v). The acid mixture 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 N_2 and 2 M HCl for 6–8 hr at 100°C gave quantitative release of hexosamine and mannose with no measurable destruction of either sugar. Samples of AG 50 glycopeptide (8–10 mg) were acid hydrolyzed as described above. After cooling, the sample volume was reduced to 1.0 ml and 0.2 ml of saturated sodium bicarbonate added. *N*-Acetylation was performed using 0.3 ml of 2% (v/v) acetic anhydride in water at 22°C for 5 min. Excess bicarbonate was neutralized with acetic acid and the reaction mixture was deionized on an 0.5 cm i.d. \times 8 cm column containing equal portions of Dowex 50-X2(H^+) (top) and Dowex 1-X10(OH^-) (bottom) and the eluate concentrated. A portion of this solution was used for colorimetric estimation of neutral sugar and GlcNAc. Sodium borohydride (15 mg) was added to the remaining portion and reduction was carried out at 65°C for 15 min. Acetic acid was used to neutralize the excess $NaBH_4$ and the mixture was deionized on a column (0.5 cm i.d. \times 8 cm) of IR-120(H^+). Boric acid was then removed by co-distillation five times with methanol. The residue was dried under a stream of nitrogen. To the dry sample were added 1.5 ml of acetic anhydride and 15 mg sodium acetate. *O*-acetylation was carried out at 105°C for 3 hr. The sample was then treated with ice cold 20% (w/v) $KHCO_3$ and after 60 min at 20°C the solution was extracted three times with 15 ml portions of chloroform and backwashed with two 10 ml portions of water. The combined chloroform extracts were dried with anhydrous sodium sulphate and the solution evaporated to a small volume. Alditol acetates were analyzed by gas-liquid chromatography on a 3 mm i.d. \times 100 cm glass column packed with 3% ECNNS-M. Separations were carried out isothermally at either 200° (neutral sugars) or 220°C (amino sugars) with an injection and detector temperature at 245° and 250°C respectively. Identification and quantitation of alditol acetates was accomplished by co-chromatography with authentic standards.

Vitellin oligosaccharide preparation

The oligosaccharide units of vitellin were prepared by enzymatic digestion of glycopeptides with endo-H, 7.5×10^{-4} units/ μ l of enzyme solution (TARENTINO and MALEY, 1974). Five mg of AG-50 glycopeptide were dissolved in 500 μ l of 0.2 M citrate buffer, pH 5.5, and 5 μ l of endo-H were added to the solution. Digestion was carried out at 37°C under toluene vapour for 36 hr and then stopped by boiling the reaction mixture for five minutes in a water bath. After adding 500 μ l of water to the cooled solution it was chromatographed on a Bio-Gel P-6 column (1.6 cm i.d. \times 100 cm) at 22°C with a flow rate of 20 ml/hr. The carbohydrate-containing fractions were pooled and evaporated to a small volume by rotary evaporation.

[3H]-Sodium borohydride reduction of endo H oligosaccharides

The terminal reducing sugar of certain oligosaccharide preparations was radioactively labelled using tritiated borohydride. Reduction was performed on 0.5–1.0 mg samples of neutral hexose in a reaction volume of 1.6 ml. After addition of 100 μ l of tritium labelled $NaBH_4$ (1 mCi) the reaction proceeded for 1 hr at room temperature and then

20 mg non-labelled $NaBH_4$ were added and incubation continued at 40°C for 2 hr. The borohydride was neutralized by lowering the pH to 5.5 with acetic acid and the sample concentrated to dryness under reduced pressure. It was redissolved in 1.0 ml of water, and deionized on an IR-120 (H^+) column (0.5 cm i.d. \times 8 cm). The eluant was co-distilled five times with methanol, redissolved in 1.0 ml of water and chromatographed in a 0.5 cm i.d. \times 10 cm Dowex 1 (OH^-) 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 R_f 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 μ g neutral hexose) and vitellogenin labelled *in vivo* with [3H] in the mannosyl residues (76,000 cpm) were mixed and delipidated. 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. \times 100 cm column of 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 proteinase 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). Proteinase K was adjusted to a concentration of 5.0 mg/ml in buffer. Enzyme solution (0.2 ml) was added to 50 μ l substrate and incubated at 22°C for 2 hr. The reaction mixture was then made alkaline with 3.0 ml or 0.2 M Na_2CO_3 and the absorbance read at 405 nm. Potential contaminating phosphatase activity in the endo H preparation was tested using 2 μ l of enzyme in 250 μ l of citrate buffer, pH 5.5 with 50 μ l of substrate. The reaction mixture was incubated for 30 hr at 37°C under toluene vapour. An aliquot (50 μ l) was made alkaline with 5.0 ml of 0.2 M Na_2CO_3 and absorbance read at 405 nm.

RESULTS

Preparation of vitellin glycopeptides

Vitellin digested with proteinase 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 V_1) indicating the proteinase 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.

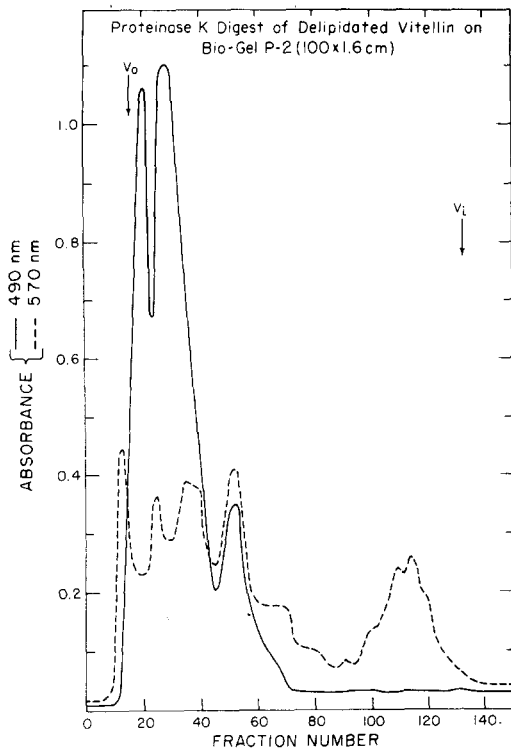


Fig. 1. Elution profile of vitellin glycopeptides on Bio-Gel P-2 (minus 400 mesh). Delipidated Vt (approx. 100 mg, 4.5% neutral hexose) was digested exhaustively with proteinase K at 55°C for 36 hr in water and the products chromatographed at 50°C on a 100 × 1.6 cm i.d. column of Bio-Gel P-2 (minus 400 mesh) with water as eluant. Solid line carbohydrate; dashed line, ninhydrin positive material. Tubes 12–65 were pooled. V_0 , void volume; V_p , 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

Table 1. Compositions of vitellin and the AG 50 glycopeptide fraction

Amino acid	Mole %	
	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		
Colourimetric	ND	5.7, 5.5, 5.0 (5.4)*
Gas-liquid chromatography	ND	4.4, 4.5, (4.5)*

ND = not determined.

* Average.

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 μ moles) and vitellin (0.3 μ moles). 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 moieties on vitellin are not major immunodeterminants.

Protein-oligosaccharide linkage

Digestion of the AG 50 glycopeptide fraction with

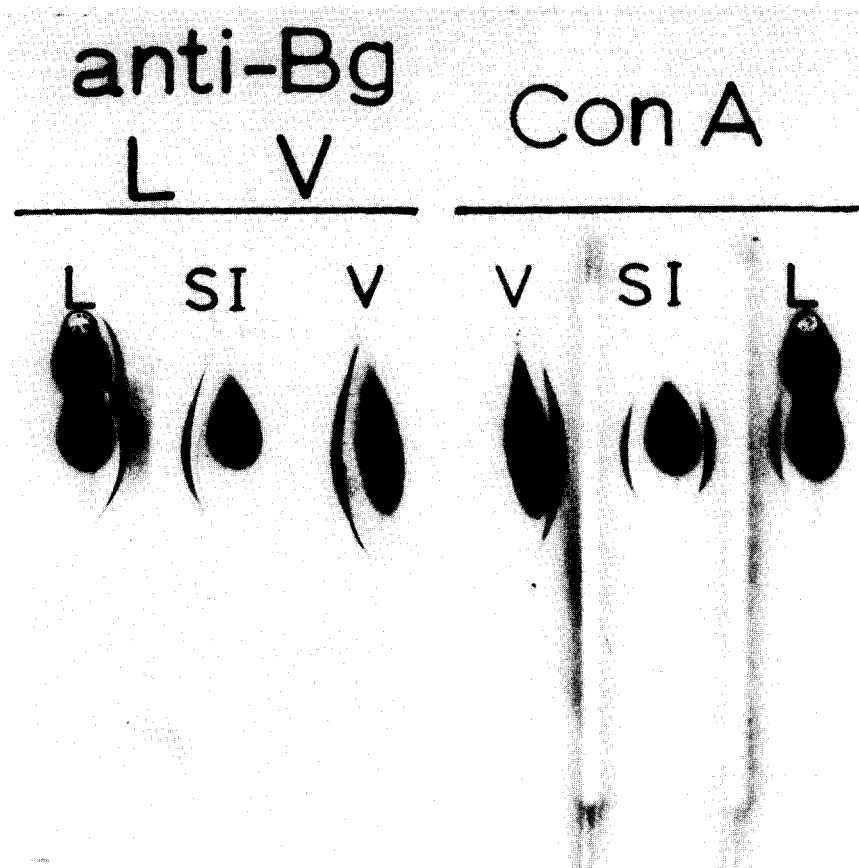


Fig. 2. Immunoelectrophoretic demonstration of Con A reactivity of cockroach serum proteins. Electrophoresis was carried out at pH 8.6 in agarose as described previously (KUNKEL and LAWLER, 1974). Aliquots of larval serum (L), purified serum protein I (SI) and vitellogenin (V) were placed in sample wells. 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

Inhibitor	Mol. wt	50% inhibition		Moles inhibitor/ mole vitellin at 50% inhibition
		g/l	95% CI titre in mMoles	
Vitellin P2 glycopeptide*	4700	0.25	0.00031	0.3
Ovalbumin	43,000	4.5	0.0030	3.1
<i>S. cerevisiae</i> mannan	28,000	1.17-1.35	0.045	44.3
Trehalose	343	55-81	195	1.93×10^5
Methyl- α D-mannopyranoside	194	48-54	262	2.59×10^5
Methyl- α D-glucopyranoside	194	50-70	305	3.02×10^5
D-Mannose	180	99-112	583	5.76×10^5
Sucrose	342	424-583	1453	1.44×10^6
D-Glucose	180	237-276	1421	1.41×10^6

* The P-2 glycopeptide fraction has a hexose content of $38 \pm 3\%$. 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 sugar. The liberated saccharide fraction was reduced with tritium labelled NaBH_4 . 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; KORNFIELD and KORNFIELD, 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

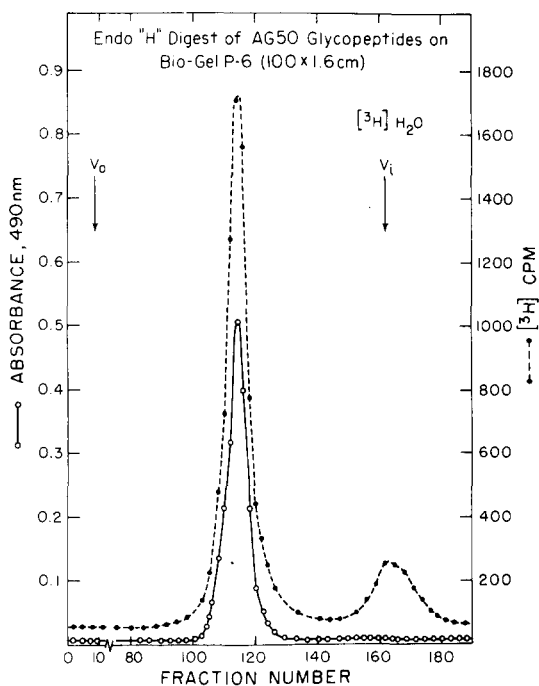


Fig. 3. Bio-Gel P-6 elution profile of $[^3\text{H}]$ -reduced endo H oligosaccharide alcohol. Five mg of vitellin AG 50 glycopeptide fraction were digested with endo H as described under experimental procedures. The reaction was desalted and reduced with tritium labelled KBH_4 . The radioactive products were chromatographed at 22°C and $700 \mu\text{l}$ fractions were collected using water as eluant. Solid line, carbohydrate; dashed line, radioactivity. Tritiated water elutes with the included volume of the column (V_i , fraction 163).

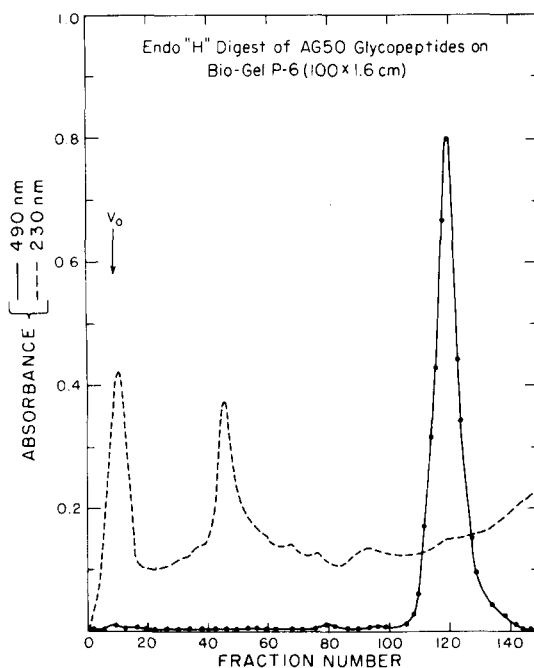


Fig. 4. Bio-Gel P-6 (minus 400 mesh) elution profile of endo H digestion mixture of AG 50 glycopeptide fraction. Glycopeptide (5 mg) was digested with endo H as described under Experimental Procedures. The reaction mixture was applied to the column and chromatographed at 22°C . Fractions ($700 \mu\text{l}$) were collected using water as eluant. Solid line, carbohydrate; dashed line, peptide material.

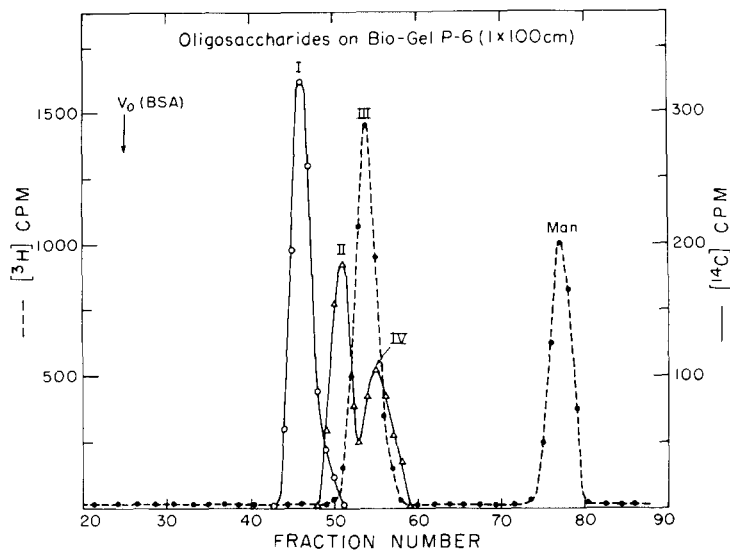


Fig. 5. Sizing of vitellin [^3H]-oligosaccharide alcohol on Bio-Gel P-6. Tritium labelled oligosaccharide alcohol prepared by endo H digestion and tritiated KBH_4 reduction of glycopeptide was chromatographed on a Bio-Gel P-6 (minus 400 mesh) column at room temperature with other oligosaccharide standards. Fractions (1.1 μl) were collected using 0.1 M Tris buffer (pH 8.0) as eluant. I, $(\text{Glc})_3(\text{Man})_9(\text{GlcNAc})_2$; II, $(\text{Glc})_3(\text{Man})_9\text{GlcNAc}$; III, endo H [^3H] oligosaccharide alcohol; IV, $(\text{Man})_9(\text{GlcNAc})_2$. The elution positions of bovine serum albumin, (V_0) and mannose (Man) are indicated.

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 delipidated. Phospholipids are reported to comprise a portion of the total lipid vitellogenin (CHINO *et al.*, 1977). Phosphate removed during delipidation 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 delipidation 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

Component	% Phosphorus	% Mannose
Vitellin	0.60	—
Delipidated vitellin*	0.30	4.5
Delipidated vitellin†	0.28	4.5
AG-50 glycopeptide	3.00	38
Endo H oligosaccharide	0‡	89§

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 delipidated (KUNKEL and PAN, 1976) by two methods:

* Delipidated by extraction with $\text{CHCl}_3 : \text{CH}_3\text{OH}$ (2 : 1); and
 † Delipidated by extraction with $\text{CHCl}_3 : \text{CH}_3\text{OH}$ (1 : 2). Phosphate removed during delipidation presumably occurs as phospholipid noncovalently bonded to the protein.

‡ Less than 0.2 mole of phosphorus per mole of oligosaccharide.

§ Calculated on the basis of an oligosaccharide composition of eleven Man and two GlcNAc residues.

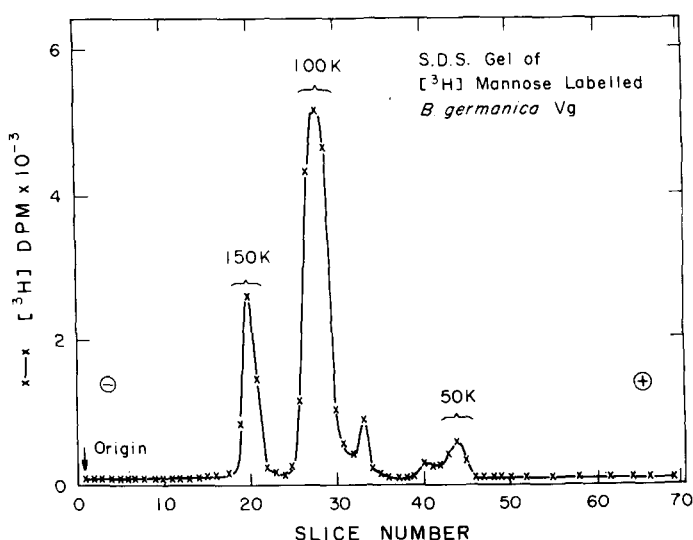


Fig. 6. Subunit localization of oligosaccharide in vitellogenin. Vitellogenin was labelled *in vivo* with $[^3\text{H}]$ -D-mannose and accumulated for 3 hr in the serum of ovariectomized females. Each was then bled as described in experimental procedures and vitellogenin purified (KUNKEL and PAN, 1976). 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.

difficulty of obtaining labelled vitellin of a sufficiently high specific activity for quantitative analysis, vitellogenin subunits labelled with $[^3\text{H}]$ -mannose for 3 hr *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 $[^3\text{H}]$ -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 $[^3\text{H}]$ -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- $[^3\text{H}]$ -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 products 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.

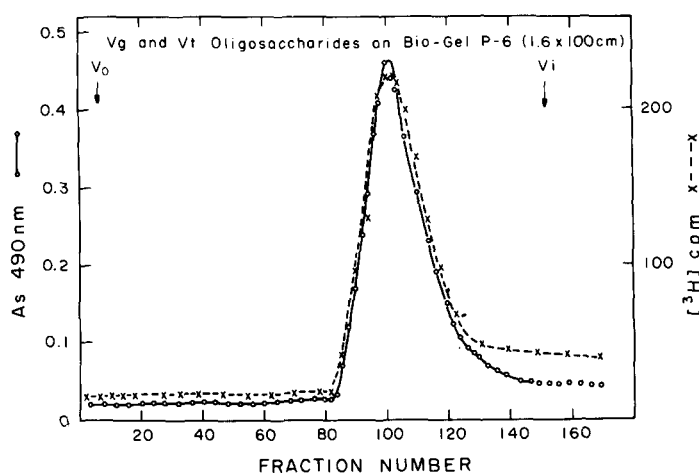


Fig. 7. Size comparison of vitellogenin (Vg) and vitellin (Vt) oligosaccharides. Samples of vitellogenin, labelled *in vivo* with 2- $[^3\text{H}]$ -mannose (76,000 cpm) and unlabelled vitellin (700 μg mannose) were combined and converted into a glycopeptide mixture as described in Materials and Methods. 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 $[^3\text{H}]$ by liquid scintillation spectrometry and for mannose by the phenol sulphuric acid assay. V_0 , void volume; V_i , elution position of mannose.

Size comparison of oligosaccharides

The profiles in Fig. 7 show co-incident 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 immunoelectrophoresis, 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 (GOLDSTEIN *et al.*, 1965a,b; SO and GOLDSTEIN, 1967; GOLDSTEIN *et al.*, 1974).

Precipitation inhibition results show α -D mannoses to be the strongest vitellin-Con A precipitation inhibitor. On a carbohydrate weight basis the strongest is vitellin glycopeptide followed in order by *S. cerevisiae* 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 asparaginyl moieties exclusively to the 100 K subunits (KORNFELD and KORNFELD, 1976). These findings are of interest in view of the fact that the vitellins of two other insect species, *Locusta migratoria* (YAMASAKI, 1974; CHEN *et al.*, 1978) and *Manduca sexta* (MUNDALL and LAW, 1979), also contain mannose and glucosamine as the only carbohydrates. In addition, the vitellin of the latter species bears all of the carbohydrate on the heavier of its two-sized subunit classes (MUNDALL and LAW, 1979). One can speculate that all vitellins probably contain a similar

type of oligosaccharide and this generalized conservation of composition, location (and structure?) among diverse species of Insecta suggests a possible function for the saccharide 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 [³H]-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 mannose joined $\beta(1\rightarrow4)$ 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 (BALLOU, 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 assays used. Therefore, the absence of phosphate 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 endocytotic 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

recognition-marker processes. While any role played by the oligosaccharide of the vitellogenin and vitellin in these is unknown, this segment of the molecule has potential for participating in these functions.

Figure 7 reveals that there is no significant difference in the size of the oligosaccharide of vitellogenin and vitellin as measured by gel filtration. A difference of one hexose unit in size between the two oligosaccharides would result in a shift in the peaks of about six to seven fractions. However, vitellin was always purified from eggs taken within 24 hr of egg case extrusion. Any structural changes which might occur after this time remain to be evaluated by analyses of the glycoprotein prepared from older embryos.

The oligosaccharide portion could protect the molecule from degradation during secretion and circulation in the haemolymph or from premature degradation in the egg. [During the first four days after ovulation vitellin in the egg is not proteolytically degraded (STORELLA and KUNKEL, 1979).] It may serve to prevent aggregation during the passage of vitellogenin through cell membranes as does the carbohydrate portion of vesicular stomatitis virus glycoprotein during virion assembly (GIBSON *et al.*, 1979).

The mannosyl residues may play some role in endocytosis at least partially explaining the species specificity known to occur in vitellogenin uptake (KUNKEL and PAN, 1976). This suggestion must be qualified by the knowledge that many insect vitellogenins may contain very similar oligosaccharides and subtle structural features in the protein portion may have to be invoked to explain the known recognition specificity of vitellogenin by oocytes. However, the variety of structural changes in yeast mannans accomplished by simple gene changes (BALLOU, 1976) provide a possible model for recognition site(s) on vitellogenin and vitellin. The quantitatively different carbohydrate structure of the nonvitellogenic serum proteins, as shown by different haemagglutination inhibition titres, could account for their observed low rate of incorporation into oocytes.

The degradation of vitellin commences rather abruptly between day four and day five after ovulation of the egg. The possibility exists that the oligosaccharide could function to attenuate degradation during this period. A sharp difference in susceptibility to proteolysis between glycosylated and nonglycosylated invertase after uptake by rat egg yolk sacs has recently been reported (BROWN *et al.*, 1979). Finally, it is possible that the saccharide serves somehow to direct vitellin to the vitellophages where its catabolism occurs (TANAKA, 1976). Experiments aimed at gaining further insight into the possible function of the vitellin carbohydrate in these events are in progress.

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REFERENCES

- ANDERSON E. (1970) Two types of coated vesicles in oocyte development. *J. Microscop. (France)* **8**, 721–738.
- BALLOU C. E. (1976) Structure and biosynthesis of the mannan component of the yeast cell envelope. *Adv. Microbiol. Physiol.* **14**, 93–158.
- BARTLETT G. R. (1959) Phosphorus assay in column chromatography. *J. biol. Chem.* **234**, 466–468.
- BROWN J. A., SEGAL H. L., MALEY F., TRIMBLE R. B. and CHU F. (1979) Effect of deglycosylation of yeast invertase on its uptake and digestion in rat yolk sacs. *J. biol. Chem.* **254**, 3689–3691.
- CHEN T. T., STRAHLENDORF P. W. and WYATT G. R. (1978) Vitellin and vitellogenin from locusts (*Locusta migratoria*): properties and post translational modification in the fat body. *J. biol. Chem.* **253**, 5325–5331.
- CHINO H., YAMAGATA M. and SATA S. (1977) Further characterization of lepidopteran vitellogenin from haemolymph and mature eggs. *Insect Biochem.* **7**, 125–131.
- DISTLER J., HIEBER V., SAHAGIAN G., SCHICKEL R. and JOURDIAN G. W. (1979) Identification of mannose 6-phosphate in glycoproteins that inhibit the assimilation of β -galactosidase by fibroblasts. *Proc. Natn. Acad. Sci. U.S.A.* **76**, 4235–4239.
- DUBOIS M., GILLIES K. A., HAMILTON J. D., REBERS P. A. and SMITH F. (1956) Colorimetric method for determination of sugars and related substances. *Analyt. Chem.* **28**, 350–356.
- GELLISON G., WAJC F., COEHN E., EMMERICH H., APPLEBAUN S. W. and FLOSSDORF J. (1976) Purification and properties of oocyte vitellin from the migratory locust. *J. comp. Physiol. B*, **108**, 287–301.
- GIBSON R., SCHLESINGER S. and KORNFELD S. (1979) The nonglycosylated glycoprotein of vesicular stomatitis virus is temperature-sensitive and undergoes intracellular aggregation at elevated temperatures. *J. biol. Chem.* **254**, 3600–3607.
- GOLDSTEIN I. J., HELLERMAN C. E. and MERRICK J. M. (1965a) Protein carbohydrate interaction I. The interaction of polysaccharides with concanavalin A. *Biochem. biophys. Acta* **97**, 68–76.
- GOLDSTEIN I. J., HELLERMAN C. E. and MERRICK J. M. (1965b) Protein carbohydrate interaction II. Inhibition studies on the interaction of Concanavalin A with polysaccharides. *Biochemistry* **4**, 876–883.
- GOLDSTEIN I. J., REICHERT C. M. and MISAKI A. (1974) Interaction of Concanavalin A with model substrates. *Ann. N.Y. Acad. Sci.* **234**, 283–296.
- HAGEDORN H. H. and KUNKEL J. G. (1979) Vitellogenin and vitellin in insects. *A. Rev. Ent.* **24**, 475–505.
- HOLLANDER V. P. (1971) Acid phosphatases. In *The Enzymes*, 3rd Ed., Vol. 4. Academic Press, New York.
- HUGHES C. (1977) Recognition of lysosomal enzymes. *Nature, New Biol* **269**, 288–290.
- KORNFELD R. and KORNFELD S. (1976) Comparative aspects of glycoprotein structure. *A. Rev. Biochem.* **45**, 217–237.
- KUNKEL J. G. (1966) Development and the availability of food in the *Blattella germanica* (L.). *J. Insect Physiol.* **12**, 227–235.
- KUNKEL J. G., ETHIER D. B. and NORDIN J. H. (1978) Carbohydrate structure and immunological properties of *Blattella vitellin*. *Fedn Proc.* **37**, 948.
- KUNKEL J. G. and LAWLER D. M. (1974) Larval-specific serum protein in the order Dictyoptera—immunologic characterization in larval *Blattella germanica* and cross-reaction throughout the order. *Comp. Biochem. Physiol.* **47**, 697–710.
- KUNKEL J. G. and PAN M. L. (1976) Selectivity of yolk protein uptake: Comparison of vitellogenins of two insects. *J. Insect Physiol.* **22**, 809–818.
- LOWRY O. H., ROSEBROUGH N., FARR A. and RANDALL R. (1951) Protein measurement with Folin Phenol reagent. *J. biol. Chem.* **193**, 265–275.

- McMANUS F. A. (1946) Histological demonstration of mucin after periodic acid. *Nature, Lond.* **158**, 202.
- MUNDALL E. C. and LAW J. R. (1979) Physical and chemical characterization of vitellogenin from the hemolymph and eggs of the tobacco hornworm. *Comp. Biochem. Physiol.* **63B**, 459-468.
- PARK J. T. and JOHNSON M. J. (1949) A submicrodetermination of glucose. *J. biol. Chem.* **181**, 149-151.
- REISSIG J. L., STROMINGER J. L. and LENOIR L. F. (1955) A modified colorimetric method for the estimation of *N*-acetylamino sugars. *J. biol. Chem.* **217**, 959-966.
- ROSS M. and COCHRAN D. G. (1975) The German cockroach, *Blattella germanica*. In *Handbook of Genetics* (Ed. by KING R. C.), Vol. 3, pp. 35-62. Plenum Press, New York.
- ROTH T. F. and PORTER K. R. (1964) Yolk protein uptake in the oöcyte of the mosquito *Aedes aegypti* L. *J. Cell Biol.* **20**, 313-320.
- ROTH T. F., CUTTING J. A. and ATLAS S. B. (1976) Protein transport: a selective membrane mechanism. *J. supramol. Struct.* **4**, 527-548.
- SHARON N. (1966) Polysaccharides. *A. Rev. Biochem.* **35**, 485-520.
- SO L. L. and GOLDSTEIN I. J. (1967) Protein-carbohydrate interaction IV. Application of the quantitative precipitation method of polysaccharide-Concanavalin A interaction. *J. biol. Chem.* **242**, 1617-1622.
- SWEET D. P., SHAPIRO R. H. and ALBERSHEIM P. (1975) Quantitative analysis by various G.L.C. response-factor theories for partially methylated and partially ethylated alditol acetates. *Carbohydr. Res.* **40**.
- STORELLA J. B. and KUNKEL J. G. (1979) Processing and utilization of vitellin in the cockroach egg. *Am. Zool.* **19**, 999.
- TANAKA A. (1976) Stages in the embryonic development of the German cockroach, *Blattella germanica* Linee (Blattaria, Blattellidae). *Kontyû* **44**, 512-525.
- TARENTINO A. L. and MALEY F. (1974) Purification and properties of Endo- β -*N*-acetylglucosaminidase from *Streptomyces griseus*. *J. biol. Chem.* **249**, 811-817.
- TARENTINO A. L. and MALEY F. (1976) Purification and properties of an Endo- β -*N*-acetylglucosaminidase from hen oviduct. *J. biol. Chem.* **251**, 6537-6543.
- WARREN T. G., BRENNAN M. D. and MAHOWALD A. P. (1979) Two processing steps in maturation of vitellogenin polypeptide in *Drosophila melanogaster*. *Proc. Natn. Acad. Sci. U.S.A.* **76**, 2848-2852.
- WEBER K., PRINGLE J. R. and OSBORN M. (1972) Measurement of molecular weights by electrophoresis on SDS-acrylamide gels. *Meth. Enzym.* **26**, 3-27.
- YAMASAKI K. (1974) Yolk protein from eggs of *Locusta migratoria* and glycopeptides from the protein. *Insect Biochem.* **4**, 411-422.