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STUDIES ON LIGAND RECOGNITION BY VITELLOGENIN RECEPTORS IN FOLLICLE MEMBRANE PREPARATIONS OF THE GERMAN COCKROACH, *BLATTELLA GERMANICA*

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Abstract—An *in vitro* binding assay was used to analyze the interaction between vitellin and its receptor in follicle membrane preparations of the cockroach, *Blattella germanica*. Binding was found to be optimal at pH 6.8. Specific binding was saturable, and Scatchard analysis yielded a K_d-value of 79 nM and a total receptor concentration of 51 fmol/follicle. Glycopeptides and oligosaccharides prepared from vitellin competed for vitellin binding, whereas yeast mannan did not. Glycopeptides digested with α-mannosidase stimulated vitellin binding. Vitellins of the cockroach species *Simploce capitata* and *Nauphoeta cinerea* competed for binding of *B. germanica* vitellin, whereas vitellin of another cockroach, *Leucophaea maderae*, stimulated binding. Analysis of the oligosaccharide structure of the vitellin in these species revealed differences in the proportions of individual oligosaccharides in the total carbohydrate, however, these differences do not correlate with the ability of the vitellins to compete for binding. The results suggest that the high mannose oligosaccharides of vitellin are necessary but not sufficient for binding to the vitellogenin receptor.

Key Word Index: cockroach, vitellogenin, vitellin, oocytes, receptor-mediated endocytosis, glycopeptide, oligosaccharide

INTRODUCTION

Vitellogenins are the predominant yolk-protein precursors in most egg-laying animals. In insects, they are synthesized in the fat body, secreted into the hemolymph and specifically sequestered by the maturing oocytes, thereafter being termed vitellins. Vitellogenins and vitellins are multimetric phospholipoglycoproteins [for reviews, see Engelmann (1979, 1983), Hagedorn and Kunkel (1979) and Kunkel and Nordin (1985)]. The specificity of vitellogenin uptake has been demonstrated in insects (Kunkel and Pan, 1976; Roth *et al.*, 1976; Ferenz *et al.*, 1981; Lange and Loughton, 1981) and in *Xenopus* (Wallace and Jared, 1976), and it was proposed that this process is mediated by specific receptors (Roth and Porter, 1964; Roth *et al.*, 1976; Opresko *et al.*, 1980). Receptor-mediated endocytosis is a ubiquitous cellular process and involves the binding of the protein molecules to membrane-associated receptors, which are located in so-called coated pits, and internalization of the receptor-ligand complexes by micropinocytosis [for reviews, see Goldstein *et al.* (1979), Anderson and Kaplan (1983) and Wileman *et al.* (1985)]. The first step in vitellogenin uptake, the binding to receptors on the follicle membrane, has been demonstrated in the chicken (Yusko and Roth, 1976; Yusko *et al.*, 1981) and in three insect species (König and Lanzrein, 1985; Osir and Law, 1986; Röhrkasten and Ferenz, 1986). It is not known which part of the vitellogenin molecule is recognized by the receptor but in *Blattella germanica*, studies on vitellin uptake *in vivo* suggested that the oligosaccharide moiety may be necessary for endocytosis (Gochoco *et al.*, 1984). The oligosaccharide structure of vitellin is well-characterized in this insect (Kunkel *et al.*, 1980; Nordin *et al.*, 1984) as well as in *Manduca sexta* (Osir *et al.*, 1986), and consists primarily of $Man_9GlcNAc_2$, which is *N*-glycosidically linked to asparagine (Nordin *et al.*, 1984; Osir *et al.*, 1986). In this report, we analyze the role of the oligosaccharide component in mediating binding of vitellin to follicle membrane preparations of *B. germanica*. We also examine the species specificity of vitellin binding by using the vitellins of three additional cockroach species as competitors and relate their competitive efficiencies to the structure of their oligosaccharide component.

MATERIALS AND METHODS

Animals

Cockroach species *B. germanica*, *Simploce capitata*, *Nauphoeta cinerea*, and *Leucophaea maderae* were obtained from laboratory cultures maintained at 30°C. Cultures of *B. germanica* were grown as described (Kunkel, 1966) providing developmentally synchronous animals for experiments. Oocyte maturation in *B. germanica* was 6–7 days.

Chemicals

[^{14}C]formaldehyde (53 mCi/mmol), Protosol and Liquifluor were obtained from New England Nuclear. Substances used for gel filtration and DEAE-cellulose were purchased from BioRad. Proteinase K (E.C 3.4.21.14) was from Beckman Industries and endo-β-*N*-acetylglucosaminidase

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H (endo H, EC 3.2.1.96) was from Miles Laboratories. Jackbean α-mannosidase (EC 3.2.1.24), phenylmethylsulfonyl fluoride (PMSF), and bovine serum albumin (BSA) were purchased from Sigma Chemical Co. All other chemicals were of analytical grade and purchased from Fisher Scientific Co.

Isolation of vitellin

Vitellin of *B. germanica* was obtained from oocytes 2 days before ovulation and purified by DEAE-cellulose chromatography (Kunkel *et al.*, 1980). The 18S-form of vitellin was prepared from oothecae within 24 h after ovulation and 33S vitellin 3 days after ovulation by salt extraction (Kunkel *et al.*, 1980), and dialyzed against 0.01 M sodium phosphate (pH 6.5)–0.2 M sodium chloride (PBS). Aliquots of 700 μl vol (20 mg protein/ml) were layered onto a 5–30% (w/v) glycerol gradient in PBS (total vol 40 ml) and submitted to ultracentrifugation in an SW27 rotor (Beckman) at 27,000 rpm at 4°C for 18 h. Fractions of 1.5 ml were collected from the bottom using a peristaltic pump and analyzed by measuring the absorbance at 280 nm. Vitellins from *S. capitata*, *N. cinerea* and *L. maderae* were prepared from oothecae within 24 h after ovulation by salt extraction and glycerol gradient ultracentrifugation. Hemolymph proteins from *L. maderae* were obtained as described for *N. cinerea* (König and Lanzrein, 1985). Protein concentrations were determined by absorbance at 280 nm using an extinction coefficient of $E_{280\,nm,\,1\%} = 7.46$ for *B. germanica* and *S. capitata* vitellin (Kunkel *et al.*, 1980), $E_{280\,nm,\,1\%} = 6.25$ for *N. cinerea* vitellin (Buschor and Lanzrein, 1983), and $E_{280\,nm,\,1\%} = 8.3$ for *L. maderae* vitellin (Dejmal and Brookes, 1972). For the hemolymph protein fraction resulting after separation of the vitellogenin an $E_{280\,nm,\,1\%} = 10$ was assumed. All proteins used in the membrane binding assay were previously dialyzed against incubation buffer (IB) that consisted of 15 mM *N*-(Tris(hydroxymethyl)methyl)-2-aminoethanesulfonic acid (TES), pH 6.8, 140 mM sodium chloride, 5 mM potassium chloride, 0.8 mM magnesium sulfate, 0.3 mM calcium chloride and 1 mM PMSF. Molar concentrations were based on a molecular mass of 535 kD for *B. germanica* vitellin (Kunkel, unpublished observation). All protein solutions were made 1 mM in PMSF.

Radiolabeling of proteins

Proteins were labeled by reductive methylation using [^{14}C]formaldehyde (König and Lanzrein, 1985). After stopping the reaction by extensive dialysis against PBS, the labeled proteins were repurified by glycerol gradient ultracentrifugation (5–30% glycerol in PBS, w/v) in an SW41 rotor (12 ml total vol) at 27,000 rpm at 4°C for 18 h. Fractions of 650 μl were collected from the top and the radioactivity was monitored by liquid scintillation counting. Radioactive fractions co-sedimenting with unlabeled 18S vitellin as determined by absorbance at 280 nm were pooled and dialyzed against IB, pH 6.8.

Preparation of glycopeptides and oligosaccharides from vitellin

Glycopeptides were prepared from delipidated vitellin by Proteinase K digestion as described (Kunkel *et al.*, 1980). Digestion of glycopeptides with α-mannosidase was done according to Nordin *et al.* (1984). Oligosaccharides were prepared by enzymatic digestion of vitellin glycopeptides with endo H as described (Kunkel *et al.*, 1980). Endo H oligosaccharides were analyzed by TLC on silica gel plates (Fisher Scientific Co., Cat. No. 6-600C) using butan-1-ol/glacial acetic acid/water (3:3:2, v/v) as solvent with three ascents (Nordin *et al.*, 1984). Mannose concentrations were determined with the phenol–sulfuric acid assay using mannose as a standard (Dubois *et al.*, 1956), and GlcNAc was quantified according to Reissig *et al.* (1955).

Preparation of follicle membranes and membrane binding assay

Membranes were prepared from follicles of *B. germanica* 2 days before ovulation as described (König and Lanzrein, 1985) except that membranes were pelleted at 30,000 *g* at 4°C for 20 min. The membrane preparations used in this study consisted of oocyte plasma membranes, follicular epithelia and basal laminae. The membrane binding assay was done in IB containing 10 mg BSA per ml (IB-BSA) at 4°C as described (König and Lanzrein, 1985). Non-specific binding was determined in the presence of a molar excess of unlabeled 18S vitellin and subtracted from total binding to yield specific binding. Tubes without membranes were incubated and treated in the same way as the experimental tubes, and the radioactivity due to tube binding was subtracted from experimental values. Tube binding was <8% of total binding. Details of the incubation conditions are given with each figure. For liquid scintillation counting, membrane pellets were solubilized in 100 μl distilled water and 600 μl Protosol for 6 h at room temperature, whereafter 3 ml of a toluene/Liquifluor cocktail were added.

RESULTS

To define the characteristics of the vitellogenin receptor in *B. germanica*, follicle membranes were prepared as previously described (König and Lanzrein, 1985), and vitellin was radiolabeled to a high specific activity by reductive methylation. These were then used in binding assays. Vitellin was used instead of vitellogenin because in *B. germanica*, the vitellogenin hemolymph titer does not exceed 2 mg/ml (Kunkel, 1981) and the hemolymph volume is only about 20 μl, rendering collection of large amounts of vitellogenin almost impossible. When vitellin labeled *in vivo* with [^{35}S]methionine was injected into vitellogenic females, more than 90% of the administered dose was taken up by the maturing oocytes (Gochoco *et al.*, 1984). When the vitellin probe labeled by reductive methylation was tested for uptake *in vivo*, 9 h after injection 90% of the injected dose was found in the oocytes (data not shown). The time course of uptake was identical to that of [^{35}S]methionine-labeled vitellin. Thereafter, 18S vitellin labeled by reductive methylation was judged suitable as a probe to study binding to the vitellogenin receptor.

Time course of vitellin binding

To determine the incubation time necessary for vitellin binding to reach steady state, identical aliquots of follicle membrane preparations were incubated with a fixed concentration of [^{14}C]vitellin in IB–BSA at 4°C for various periods of time. Non-specific binding was measured in the presence of a 50-fold molar excess of unlabeled vitellin. This experiment was repeated 4 times with different amounts of follicle membranes (corresponding to 20–50 follicles) and concentrations of [^{14}C]vitellin (15–90 nM). Specific binding of [^{14}C]vitellin to the follicle membrane preparations reached steady state within 1 h (data not shown). Thereafter, the amount of vitellin bound remained constant for at least another hour of incubation. Non-specific binding increased linearly throughout the duration of the experiment and accounted for about 20–30% of total binding at steady state (data not shown). To assure that binding

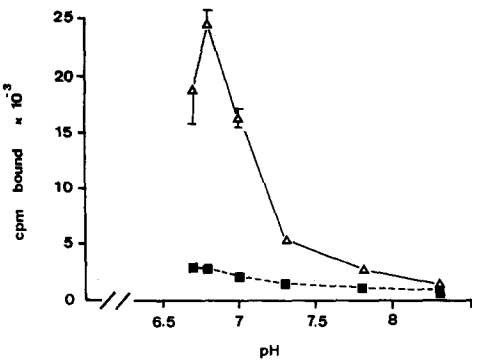

Fig. 1. pH dependence of vitellin binding. Incubation mixtures contained membranes of about 35 follicles and 10.7 μg [^{14}C]vitellin (15,000 cpm/μg) in a total vol of 555 μl IB–BSA (36 nM [^{14}C]vitellin). Non-specific binding was determined in the presence of 2.1 μM unlabeled vitellin. The mixtures were incubated at 4°C for 90 min with shaking. Datum points are means ± SEM of 3 determinations: △, total binding; ■, non-specific binding.

had reached steady state, all subsequent experiments employed a 90 min incubation period.

The influence of pH on vitellin binding

Optimal pH conditions for vitellin binding to follicle membrane preparations of *B. germanica* were studied by adjusting the pH with 15 mM Tris. Membrane preparations were washed five times with IB pH 8 containing 450 mM sodium chloride and 1 mM PMSF to remove yolk proteins adhering to the membranes, and then twice with IB at the chosen pH. The effective pH-value was measured after combining all components of the reaction mixture in a parallel set of samples. A distinctive maximum of specific binding was observed at pH 6.8 (Fig. 1). Non-specific binding was less affected by pH. In a second experiment, membrane preparations were first washed at pH 6.8 and pH-values < 7 were adjusted with 15 mM TES. This experiment gave similar results (data not shown). All subsequent experiments were therefore done at pH 6.8 using TES buffer.

Saturability of vitellin binding and binding parameters at steady state

To estimate the number of vitellin binding sites and the value of the equilibrium dissociation constant, K_d, identical aliquots of a follicle membrane preparation were incubated with increasing concentrations of [^{14}C]vitellin. The specific binding was saturable at a concentration of about 100 nM, while non-specific binding increased linearly (Fig. 2, inset). The data for specific binding were analyzed according to Scatchard (1949) and fitted to a straight line using linear regression analysis. Figure 2 shows the Scatchard plot of a typical experiment. The K_d (79 ± 30 nM; mean ± SEM of 4 independent experiments) was calculated from the slope of the line and the total receptor concentration (51 ± 3 fmol/follicle; mean ± SEM of 4 experiments) from the x-intercept. For these calculations, an M_r of 535,000 for the vitellin was used (Kunkel, unpublished observations. The data obtained from competition experiments using unlabeled vitellin as an inhibitor of [^{14}C]vitellin binding were also plotted according to Scatchard (data not shown). In this case, the amounts of bound and free ligand were calculated from the measured radioactivity and the specific activity, which decreased with increasing concentration of competitor. Non-specific binding, defined as the binding that was observed in the presence of the highest concentration of competitor tested, was subtracted from the measured values of total binding. The results obtained by this method did not significantly differ from those gathered by increasing the amount of labeled ligand. This implies that the unlabeled and the ^{14}C-labeled vitellin bound with equal affinities to the receptor.

Competition for vitellin binding by vitellin glycopeptides and oligosaccharides

To test whether the oligosaccharide moiety of vitellin is involved in the recognition of vitellin by its receptor, glycopeptides and oligosaccharides were prepared from vitellin and their relative potencies for competition of [^{14}C]vitellin binding compared to that of native vitellin. In this experiment, the mannose content in each preparation was used to express the concentration of competitor in μg mannose per ml. The vitellin molecule contains 5% mannose (Kunkel and Pan, 1976), and the mannose content in the glycopeptide and oligosaccharide preparations was measured with the phenol–sulfuric acid assay using mannose as a standard. To inhibit binding of [^{14}C]vitellin (10 μg/ml corresponding to 0.5 μg mannose/ml) by 50%, 2.5 μg mannose/ml in the vitellin preparation was necessary corresponding to an IC_{50} of 93.5 nM (Fig. 3). The glycopeptide fraction, which contains 38% mannose (Kunkel *et al.*, 1980), was a weaker competitor, demanding 15 μg mannose/ml and the oligosaccharide preparation was needed in a concentration of 48 μg mannose/ml to inhibit binding by 50%. Assuming that each glyco-

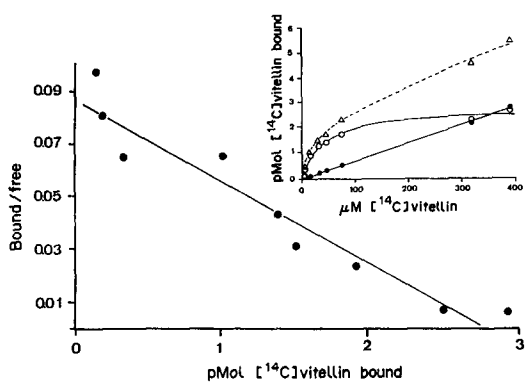

Fig. 2. Scatchard analysis of specific binding of [^{14}C]vitellin to follicle membrane preparations. Membrane preparations of 50 follicles were incubated in 1 ml IB–BSA with increasing concentrations of [^{14}C]vitellin (0.5–400 nM). Non-specific binding was measured in the presence of 30 μM unlabeled vitellin. The line drawn was calculated by linear regression analysis (correlation coefficient $r = -0.94$). *Inset*: saturation isotherm for vitellin binding. Datum points are means of 3 determinations: △, total binding; ○, specific binding; ●, non-specific binding.

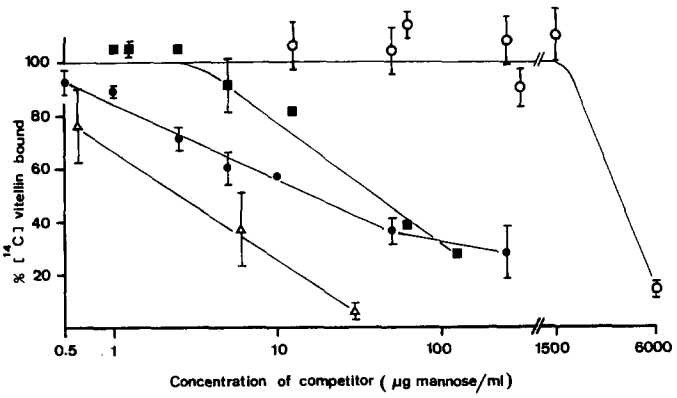

Fig. 3. Competition for [^{14}C]vitellin binding by glycopeptides and oligosaccharides of vitellin. Membrane preparations of 45 follicles were incubated in 780 µl IB–BSA with 10 µg/ml [^{14}C]vitellin (corresponding to 0.5 µg mannose/ml; 19 nM; 64,000 cpm) and 12–600 µg/ml vitellin (△), vitellin glycopeptides (0.5–250 µg mannose/ml; ●), vitellin oligosaccharides (1–125 µg mannose/ml; □), or yeast mannan (12.5–6000 µg/ml; ○). The concentration of a competitor is expressed as its mannose content (µg mannose/ml). 100% binding (1650 cpm) was observed in the absence of competitor. Data from 2 independent experiments with identical membrane, radioligand and competitor concentrations are combined in this figure. Data are means ± SEM of 6 determinations. Error bars are only given where they are larger than the datum points.

peptide molecule contains one oligosaccharide chain with nine mannose units (Nordin *et al.*, 1984) an M_r of 3800 can be calculated. Thus, the IC_{50} of the glycopeptide fraction for competition of vitellin binding was 10 µM. Endo H oligosaccharides of vitellin consist predominantly of $Man_9GlcNAc$, thus an M_r of 1680 was used in calculations yielding an IC_{50} of 29 µM for the oligosaccharides. For comparison, yeast mannan was also tested in this experiment. This substance did not affect binding up to a concentration of 1.5 mg/ml (Fig. 3).

To evaluate the importance of the high mannose oligosaccharide for competition of binding, the glycopeptide fraction of vitellin was enzymatically digested with α-mannosidase, which cleaves α-linked mannose units, yielding a glycopeptide preparation with a Man/GlcNAc ratio of 0.4 as determined colorimetrically (Reissig *et al.*, 1955; Dubois vitellin 1956). Two experiments were done using membrane preparations of 20 follicles and either 22 or 50 nM [^{14}C]vitellin (sp. act. 12,300–14,900 cpm/pmol) from *B. germanica*. Non-specific binding in the presence of a 100-fold excess of unlabeled vitellin was <20% of the total binding. As a competitor the α-mannosidase digested glycopeptide preparation was used in concentrations of 0.3–150 µg mannose-equiv./ml. Concentrations of 0.3 and 1.5 µg mannose/ml had a slight inhibitory effect on total binding of [^{14}C]vitellin to the follicle membranes. Higher concentrations of the glycopeptide, however, stimulated binding (Fig. 4). At the highest concentration tested binding of [^{14}C]vitellin was 2.8-fold the control value, corresponding to 12 fmol [^{14}C]vitellin bound/follicle, whereas the total receptor concentration was 51 fmol/follicle (Fig. 2).

Comparison of 18S and 33S vitellin as competitors for vitellin binding

The vitellin of *B. germanica* oocytes has a sedimentation coefficient of 18S (Storella and Kunkel, 1979; Kunkel *et al.*, 1984). After ovulation, trimerization to a 33S particle occurs (Kunkel *et al.*, 1984). Comparison of the accessibility of the oligosaccharide component of both forms to α-mannosidase and to periodate oxidation suggests that the trimerization produces a conformation in which the oligosaccharide moiety becomes less accessible to α-mannosidase but remains susceptible to periodate oxidation (Gochoco *et al.*, 1984; Kunkel *et al.*, 1984). *In vivo* studies demonstrated that uptake of 33S vitellin by oocytes does not exceed 11% of the administered dose (Gochoco *et al.*, 1984). It was proposed that the trimerization may cover the uptake site by which the follicle recognizes vitellin for endocytosis (Kunkel

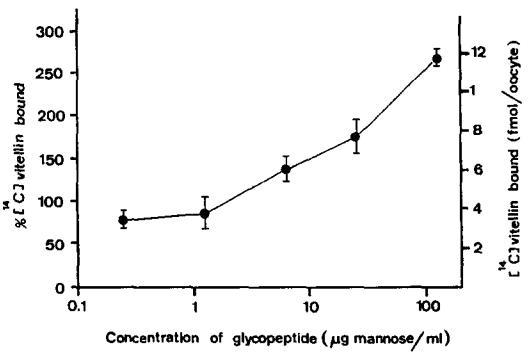

Fig. 4. Stimulation of [^{14}C]vitellin binding by the $ManGlcNAc_2$-peptide from *B. germanica* vitellin. Membrane preparations of 20 follicles from *B. germanica* were incubated in 400 µl IB–BSA with 12.1 µg/ml [^{14}C]vitellin (23 nM; 110,900 cpm) and $ManGlcNAc_2$-peptide (0.3–150 µg mannose/ml). 100% binding (1040 cpm) was observed in the absence of glycopeptide. Data are means ± SEM of 3 determinations and are representative for 2 independent experiments. Non-specific binding in the presence of a 100-fold excess of unlabeled vitellin was 18.5% of the total binding.

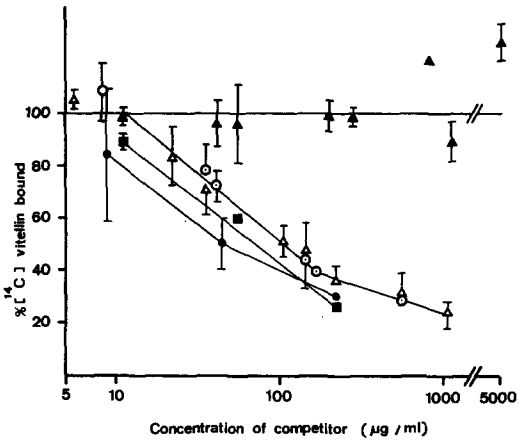

Fig. 5. Competition for binding of [^{14}C]vitellin of *B. germanica* by vitellins of three cockroach species and another hemolymph protein fraction. Membrane preparations of 40 follicles of *B. germanica* were incubated in 750 µl IB–BSA with 13 µg/ml [^{14}C]vitellin (24 nM; 181,000 cpm) and 5.5–1045 µg/ml 18S vitellin (△), 8–550 µg/ml 33S vitellin of *B. germanica* (○), 11–220 µg/ml 18S vitellin of *N. cinerea* (■), 9–220 µg/ml 18S vitellin of *S. capitata* (●), or 11–5000 µg/ml of a hemolymph protein fraction of *L. maderae* not containing vitellogenin (▲). 100% binding (18,200 cpm) was observed in the absence of competitor. Data are means ± SEM of 3 determinations except for the 18S vitellin of *S. capitata* which represent duplicates. Error bars are given only where they are larger than the datum points.

et al., 1984). To test whether the trimerization of vitellin affects receptor recognition of vitellin, the relative potencies of 18S and 33S vitellin for competition of [^{14}C]vitellin binding were compared. In three independent experiments, no difference was found when the concentration of competitors was expressed in µg/ml (Fig. 5). Since the M_r of 33S vitellin is 3 times that of 18S vitellin, on a molar basis 33S vitellin was a 3-fold stronger competitor than 18S vitellin. This suggests that after trimerization, receptor recognition sites of the three vitellin molecules combined in the 33S form are still available for binding. The discrepancy between *in vivo* uptake and membrane binding may be due to the complexity of the *in vivo* system: there, the basal lamina and the matrix in the intercellular spaces of the follicle might hinder easy access of the 33S vitellin with an M_r of >1,500,000 to the oocyte surface. Also, minor changes of the vitellin molecules associated with the trimerization process might render the 33S-form more susceptible for proteolytic enzymes in the hemolymph. To eliminate problems inherent to the *in vivo* system, an *in vitro* system has been developed (Kindle *et al.*, 1988) and is currently being used to study the uptake of different forms of vitellin and of enzymatically modified vitellin by oocytes. In addition, no difference in the competitive potency was observed whether 18S vitellin was obtained from oocytes before ovulation by ion-exchange chromatography or from eggs after ovulation by preparative glycerol gradient ultracentrifugation (data no shown).

Species specificity of vitellin binding

Transplantation of ovaries among different cockroach species revealed that vitellogenin is only sequestered when donor and host are closely related (Bell, 1972). In some cases, ovaries transplanted from one species into female hosts of related species will grow but not vice versa (Bell, 1972). On the level of binding, a similar phenomenon has been demonstrated in the cockroach species *N. cinerea* and *L. maderae* (König and Lanzrein, 1985). Follicle membrane preparations of the former species do not bind [^{14}C]vitellogenin of the closely related species, *L. maderae*, whereas membrane preparations of the latter species bind [^{14}C]vitellogenin of *N. cinerea*. In order to relate a possible species specificity of vitellin binding to structural differences, vitellins of three cockroach species were purified by glycerol gradient ultracentrifugation and used as competitors for binding of [^{14}C]vitellin from *B. germanica* to follicle membrane preparations of this species. To verify the specificity of competition, a hemolymph protein fraction from vitellogenic females of *L. maderae* from which vitellogenin was completely removed by ion-exchange chromatography as confirmed by SDS–PAGE (data not shown) was also used as a competitor in these experiments. The components of this hemolymph protein fraction are not taken up by the follicle and therefore, they should not compete for vitellin binding. Because of the small hemolymph volume of *B. germanica*, the much larger species *L. maderae* was chosen as a source for this protein fraction. Even at concentrations of 5 mg/ml this fraction did not compete for vitellin binding (Fig. 5). The 18S vitellins of *N. cinerea* and *S. capitata* did not significantly differ from 18S or 33S vitellin of *B. germanica* in their relative potencies as competitors for [^{14}C]vitellin binding (Fig. 5). The 33S vitellin of *L.*

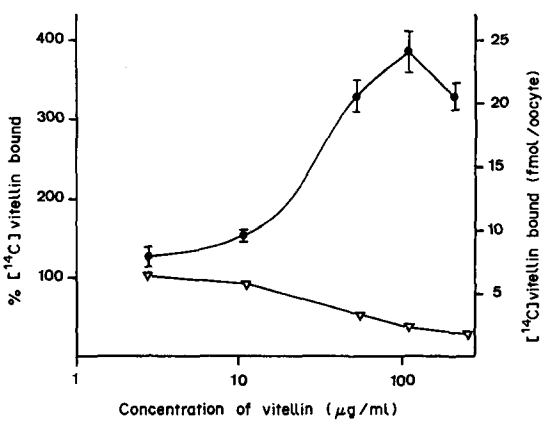

Fig. 6. Stimulation of [^{14}C]vitellin binding by 33S vitellin of *L. maderae*. Membrane preparations of 56 follicles of *B. germanica* were incubated in 930 µl IB–BSA with 5.6 µg/ml [^{14}C]vitellin (10 nM; 82,300 cpm) of *B. germanica* and 2.8–210 µg/ml 33S vitellin of *L. maderae* (●) or 2.8–270 µg/ml 18S vitellin from *B. germanica* (△). 100% binding (2819 cpm) was observed in the absence of competitor. Data are means ± SEM of 3 determinations. Error bars are only given where they are larger than the datum points.

maderae, however, did not act as a competitor but stimulated binding of [^{14}C]vitellin from *B. germanica* linearly up to 3.9-fold the control value (Fig. 6). In this experiment, membrane preparations of 56 follicles from *B. germanica* were incubated with 10.4 nM [^{14}C]vitellin (sp. act. 7865 cpm/pmol) and the control value of [^{14}C]vitellin binding was 2819 cpm (100% value). From these data, it was calculated that the maximal amount [^{14}C]vitellin bound was 25 fmol/follicle, a value that does not surpass the total receptor concentration of 51 ± 4 fmol/follicle as estimated by Scatchard analysis (Fig. 2). A second experiment yielded similar results with a maximal amount of 30 fmol [^{14}C]vitellin bound per follicle (data not shown).

Oligosaccharide structure of the vitellins

A comparative study of the oligosaccharides from vitellins of five insect species has shown that each vitellin contains variably processed high mannose type oligosaccharides and that the vitellins can be arranged into two classes based on the proportion of the individual oligosaccharides present (Nordin *et al.*, 1984). To determine whether a difference between the oligosaccharide structures of the vitellins reflected their distinct competitive efficiencies, the oligosaccharides from purified vitellins of *B. germanica*, *N. cinerea* and *L. maderae* were analyzed by TLC. The oligosaccharides of the three vitellins could be resolved into several components. The vitellins of *B. germanica* and *N. cinerea* showed a unimodal oligosaccharide distribution (Nordin *et al.*, 1984) with $Glc_1Man_9GlcNAc$, $Man_9GlcNAc$ and $Man_8GlcNAc$ as predominant constituants (Fig. 7, lanes 2 and 3). *L. maderae* vitellin oligosaccharides displayed a bimodal distribution because of a higher proportion of small oligosaccharides (Fig. 7, lane 1). Such a bimodal distribution, which reflects greater oligosaccharide processing, is also a characteristic of the oligosaccharides of *S. capitata* and *Blaberus discoidalis* vitellins (Nordin *et al.*, 1984).

DISCUSSION

Vitellogenin enters the oocyte by receptor-mediated endocytosis (Telfer, 1960; Roth and Porter, 1964). The first step, binding of vitellogenin to its membrane-associated receptor has been demonstrated with isolated follicle membrane preparations from chicken (Roth *et al.*, 1976; Yusko and Roth, 1976; Yusko *et al.*, 1981; Woods and Roth, 1984), the cockroach *N. cinerea* (König and Lanzrein, 1985), the locust *Locusta migratoria* (Röhrkasten and Ferenz, 1986) and from the tobacco hornworm *M. sexta* (Osir and Law, 1986).

Number of binding sites and binding properties at steady state

Binding of vitellin to follicle membrane preparations was specific (Fig. 5) and saturable (Fig. 2). The number of binding sites (51 fmol/follicle) and the K_d-value (79 nM) are lower than the values found in *N. cinerea*, where the number of binding sites is 120 fmol/follicle and the K_d-value is 296 nM [recalculated from König and Lanzrein (1985) using an M_r of 500,000 for vitellogenin (Imboden *et al.*, 1987)]. A similar value for K_d was reported for *Locusta migratoria* (Röhrkasten and Ferenz, 1986), whereas in *M. sexta* follicles the K_d-value is about 10-fold lower (Osir and Law, 1986).

A pH dependence of vitellogenin binding to follicle membranes was observed in all species mentioned. In the chicken (Yusko *et al.*, 1981) and in the cockroach *N. cinerea* (König and Lanzrein, 1985), binding reached a minimum at the pH of the blood. It was therefore argued that the microenvironment near the plasma membrane had a pH distinct from that of the blood allowing for an increased specificity of surface binding of vitellogenin (Yusko *et al.*, 1981). In *B. germanica*, the pH-optimum at 6.8 corresponds to the pH-value of the hemolymph. In the locust (Röhrkasten and Ferenz, 1986) and in the tobacco hornworm (Osir and Law, 1986) the reported pH-optima are much broader, spanning 0.6 and 1 pH-unit, respectively, and include the pH-value of the hemolymph.

Role of the vitellin oligosaccharide in binding

Glycopeptides and oligosaccharides prepared from vitellin competed for vitellin binding (Fig. 3), but digestion of glycopeptides with α-mannosidase destroyed its competitive potency; the purified $Man(GlcNAc)_2$-peptide actually stimulated vitellin binding (Fig. 4). The data suggest that although the high mannose oligosaccharides influence ligand binding to the receptor, the oligosaccharide alone is not sufficient for efficient binding as is evidenced by the decreasing competitive potency in the order: native vitellin > glycopeptide > oligosaccharide (Fig. 3), and by a 3-fold decrease in binding affinity following limited trypsin digestion of vitellin (data not shown).

The extent of oligosaccharide processing of the vitellins tested as competitors for binding of *B. germanica* vitellin did not correlate with their competitive potency. Whereas the vitellins of *N. cinerea* and *S. capitata* competed for binding of *B. germanica* vitellin (Fig. 5), that of *L. maderae* did not but actually enhanced binding (Fig. 6) like the $Man(GlcNAc)_2$-peptide. Analysis of the oligosaccharides of the vitellins from *B. germanica*, *N. cinerea* and *L. maderae* (Fig. 7) showed a unimodal oligosaccharide distribution in *B. germanica* and *N. cinerea*, whereas in *L. maderae* vitellin a bimodal distribution with a higher proportion of smaller oligosaccharides was observed as has also been reported for *S. capitata* vitellin (Nordin *et al.*, 1984). It is possible that differences in the total mannose contents of the various vitellins could affect receptor recognition. The vitellin of *L. maderae* is reported to contain a greater proportion of mannose (6.4–7% by wt; Dejmal and Brookes, 1972) than that of *B. germanica* (5% by wt; Kunkel and Pan, 1976) and *N. cinerea* (5.5% by wt; Imboden *et al.*, 1987). Thus, there are at least 4–6 more oligosaccharide units in the *L. maderae* vitellin (if all were of the $Man_9GlcNAc$-type) than in those of the two other species. However, as Fig. 7 shows, oligosaccharides of *L. maderae* vitellin are smaller, on average, than those of *B. germanica* and *N. cinerea*. Thus, the number of extra chains is probably larger than 4–6. In making these calculations the following M_r-values were used: 559,000 for the vitellin of *L. maderae* (Dejmal and Brookes,

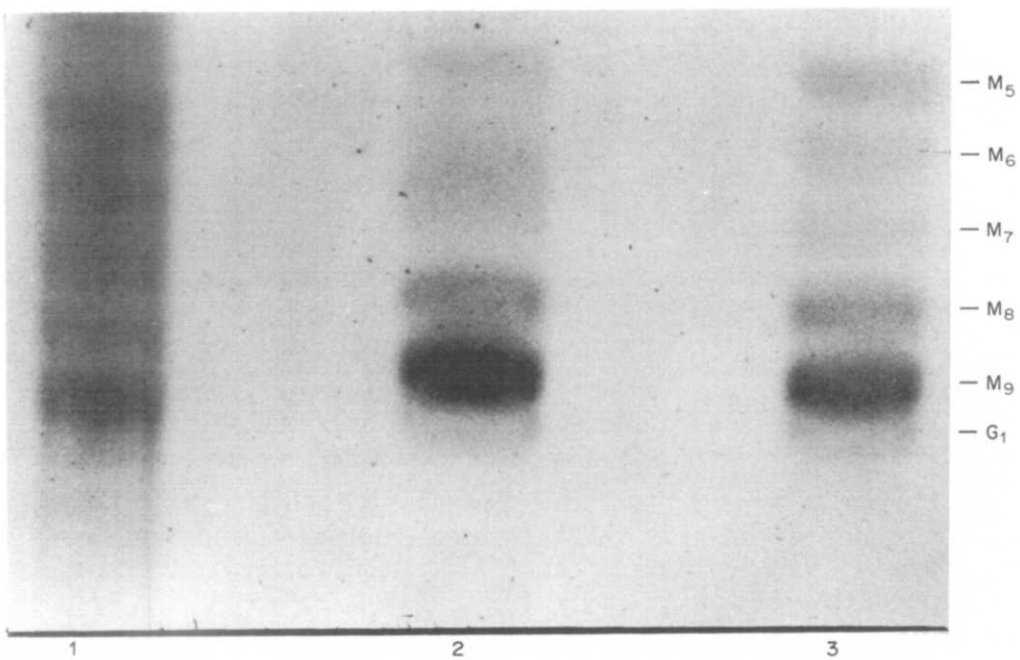

Fig. 7. TLC of vitellin oligosaccharides. Endo H oligosaccharides were prepared from the vitellins of *L. maderae*, *B. germanica* and *N. cinerea* as described (Kunkel *et al.*, 1980). Approx. 20 μg of mannose equivalents of each oligosaccharide preparation were chromatographed on 20 × 20 cm silica gel G plates using a solvent system of butan-1-ol/acetic acid/water (3:3:2, v/v). Carbohydrates were detected by spraying the dried plates with 50% aqueous sulfuric acid and heating them at 110°C for 10 min. Components of the various mixtures include: G_1, $GlcMan_9GlcNAc$; M_9-M_5, $Man_9GlcNAc$ to $Man_5GlcNAc$. Lanes 1–3: endo H oligosaccharides of *L. maderae*, *B. germanica* and *N. cinerea*, respectively.

1972), 535,000 for that of *B. germanica* (Kunkel, unpublished observation) and 500,000 for *N. cinerea* (Imboden *et al.*, 1987). If the receptor recognition sites of vitellogenin include the oligosaccharide moiety, then the larger number of oligosaccharide chains in the *L. maderae* vitellin could prevent a close interaction with the receptor of *B. germanica*, whereas no such steric hindrance would prevent the vitellin of *N. cinerea* from binding to the receptor of *B. germanica*. Conversely, it is also possible that the shorter average chain length of the *L. maderae* vitellin oligosaccharides causes a less avid interaction with the binding site of the receptor in *B. germanica*. Both hypotheses can also explain the fact that follicle membrane preparations of *L. maderae* bind vitellogenin of *N. cinerea* but not vice versa (König and Lanzrein, 1985) although these two species belong to the same subfamily whereas *B. germanica* is more distantly related (Bell, 1972).

The stimulation of binding by the α-mannosidase-treated glycopeptide fraction and *L. maderae* vitellin may be similar to phenomena reported for the interaction of polymannose ligands with receptors. Studies with rabbit alveolar macrophages in culture have shown that their binding to polymannosylated BSA is enhanced in the presence of free mannose (Lee *et al.*, 1986), and that the ability of free mannose to inhibit adhesion of the cells to mannose-derivatized surfaces decreases with incubation time (Largent *et al.*, 1984). Similarly, our data suggest that the $Man(GlcNAc)_2$-peptide and the *L. maderae* vitellin, the latter one probably because of the smaller sizes of its high mannose oligosaccharide residues, interact with unknown components of the follicle membranes thereby causing the stimulatory effect. These observations are consistent with the interpretation of the binding phenomenon as a dynamic process which involves rearrangement or clustering of receptors to a more favorable geometry for multivalent binding by the oligosaccharides. Although the binding studies were done at 4°C, this interpretation is not inconceivable as at this temperature, even uptake of vitellogenin into follicles *in vitro* was observed in *Xenopus laevis* (Wallace *et al.*, 1973), in *Nereis virens* (Rabien and Fischer, personal communication) and in *N. cinerea* (König, unpublished observation).

M. sexta vitellogenin incubated with endo H and subsequently chromatographed over concanavalin A-Sepharose to separate deglycosylated material is still endocytosed by *M. sexta* oocytes *in vitro* (Osir *et al.*, 1986) and competes for binding of radiolabeled vitellogenin to follicle membrane preparations (Osir and Law, 1986). Endo H digestion of *B. germanica* 18S vitellin removed only about 10% of its high mannose oligosaccharides while those of the 33S-form were inaccessible to the glycosidase (Gochoco and Nordin, unpublished) making an evaluation of the binding affinity of enzymatically deglycosylated vitellin in this study impossible.

In the present study, yeast mannan, up to a concentration of 1.5 mg/ml, did not inhibit binding of vitellin to the membranes (Fig. 3). The structures of high mannose oligosaccharides of insect vitellins are identical to those of animal high mannose oligosaccharides (Nordin *et al.*, 1984; Osir *et al.*, 1986), but different from the polymannose chains of yeast mannan (Ballou, 1976). In contrast to vitellin oligosaccharides, which contain exclusively α(1,2)-linked non-reducing termini, mannan consist primarily of non-reducing α(1,3)-linkages. Also, the bulky polymer would probably mask or sterically hinder any interaction between its own high mannose-type core (Ballou, 1976; Byrd *et al.*, 1982) and putative oligosaccharide binding sites on the vitellogenin receptor. The results obtained in the present study are consistent with those of Saunders *et al.* (1985), who investigated uptake by liver cells of uteroferrin, a glycoprotein hydrolase containing high mannose oligosaccharides. They found that, per mole of mannose, yeast mannan was only 5% as effective as the neoglycoprotein mannose–BSA.

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