Isolation, characterization and localization of a lectin within the vitelline membrane of the hen’s egg

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SUMMARY

A lectin with an affinity for certain sulphated polysaccharides, such as fucoidin and dextran sulphate, has been isolated from the vitelline membrane of hens’ eggs and purified to homogeneity as assessed by two-dimensional gel electrophoresis. Polyclonal and monoclonal antibodies have been raised to the lectin and used in indirect immunofluorescence microscopy to localize the agglutinin in the outer layer of the vitelline membrane, where the lectin persists prior to the breakdown of the vitelline membrane. The quantity of lectin extracted from the two layers of the membrane, which have been separated by the method of Bellairs, Harkness & Harkness (1963), correlated well with the results of immunofluorescence microscopy. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis of the two layers of the membrane indicates that each layer has a distinctive polypeptide composition, the outer layer containing in particular lysozyme and avidin.

The evidence obtained in this study indicates that the lectin is not involved in adhesion of the blastoderm to the vitelline membrane; neither is it involved in the expansion of the blastoderm nor in maintaining the strength of the membrane. The possible roles in promoting transport of solutes across the membrane as well as providing bactericidal properties to the egg are discussed.

INTRODUCTION

The vitelline membrane of the hen’s egg is a transparent bag which encloses the yolk. It has two important functions: first, it separates the yolk from the egg white, and apparently acts as a semipermeable barrier permitting certain materials to pass across it, e.g. Callebaut (1983) showed experimentally that tritiated tyrosine

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could pass through the vitelline membrane from labelled albumen into the egg yolk, and there is evidence (New, 1959; Romanoff & Romanoff, 1949) that both water and egg-white proteins normally pass through the membrane in this way. Secondly, the vitelline membrane acts as a substrate for attachment of the early embryo which lies on the surface of the yolk immediately beneath the vitelline membrane. At this stage the embryo is a flat disc attached by its periphery to the inner surface of the vitelline membrane.

The cells at the perimeter of the disc play an important role in helping the embryo to expand in diameter as they migrate beneath the vitelline membrane. The relationship between these edge cells and the vitelline membrane has been investigated experimentally and morphologically by New (1959), Bellairs & New (1962), Bellairs, Boyde & Heaysman (1969), Downie & Pegrum (1971), Downie (1975, 1976) and Chernoff & Overton (1977, 1979). In particular, New (1959) showed that the embryo reacted differently to the outer side of the membrane if it was experimentally attached to it.

Subsequently, it was found by electron microscopy that the vitelline membrane consisted of two major layers (Bellairs et al. 1963). The inner layer is laid down around the yolk while it is still in the ovary, whilst the outer layer is added after ovulation as the yolk passes down the oviduct. The outer layer is surrounded directly by the albumen and is probably impregnated with it. Each layer consists of a meshwork of fibres (Bellairs et al. 1963; Bellairs et al. 1969; Jordanov, Georgiev & Boyadjieva-Mihailova, 1966; Jensen, 1969). Those of the inner layer are about 0.2–0.6 μm in diameter, whilst those of the outer are about 15 nm in diameter. The inner and outer layers have differing amino acid compositions; Bellairs et al. (1963) suggested that the inner layer consisted of a secreted form of a non-collagenous structural connective tissue protein, whilst the outer resembled certain proteins found in egg white. More recently Rutherford & Cook (1981) have detected a haemagglutinating activity associated with the vitelline membrane of the hen's egg. Examination of partially purified extracts of the membrane suggested the presence of a lectin with a specificity for mannan-type structures. We have now purified this lectin to homogeneity as assessed by two-dimensional gel electrophoresis, and from an additional range of oligosaccharides tested find that it has a preferred specificity for sulphated sugars, reminiscent of the lectin with dual fucan/mannan specificity found in teratocarcinoma stem cells (Grabel et al. 1981). Polyclonal and monoclonal antibodies to the purified vitelline membrane lectin have been prepared and we report here the results of immunofluorescence microscopy which indicate that the lectin is concentrated in the outer layers of the membrane. This result is confirmed by haemagglutination assays performed on extracts from the separated layers of the vitelline membrane; in addition we have shown that the polypeptide composition of the separate layers is distinct. The asymmetric distribution of this lectin within the vitelline membrane may contribute to an understanding of the different properties of the inner and outer surfaces of the membrane.
Preparation of the vitelline membrane and its separation into two layers

The yolk was separated from as much albumen as possible and then submerged in Pannett and Compton's saline (Pannett & Compton, 1924). The vitelline membrane was transferred to 0.15 M-NaCl, 5 mM-phosphate (pH 7.2) (PBS) and agitated to remove any adherent yolk.

Samples of vitelline membrane were separated into two layers using the physical separation technique devised by Bellairs et al. (1963). Following separation, samples of the two layers were either immediately processed for electron microscopy, for sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) or stored in batches for up to 2 h in ice-cold PBS until sufficient quantity of separated layers had been obtained for extraction experiments. Normally material from a minimum of six eggs was found to be sufficient for extraction experiments.

Extraction of the vitelline membrane and its separated layers

Washed vitelline membranes and separated layers were homogenized at 4°C, in the same PBS (up to 10 ml) in which they had been stored, using a Polytron PT 10–35 homogenizer (Kinematica GmbH, Luzern, Switzerland) fitted with a PT-10 probe generator operating at No. 5 setting for 10 s, followed by homogenization for 5 min in a close-fitting glass Dounce homogenizer. Routinely the homogenate was centrifuged at 100 000 g for 1 h and the supernatant fluid examined for haemagglutinating activity.

Purification of the lectin activity present in the vitelline membrane

Whole vitelline membranes from 60 eggs were extracted in PBS [0.15 M-NaCl containing 5 mM-phosphate (pH 7.2)] as described above and the 100 000 g supernatant fluid (60 ml) mixed with hydroxyapatite (Sigma Type I; 15 ml of a 25 % suspension in 1 mM-phosphate, pH 6.8 was washed three times in PBS (50 ml) at 450 g for 3 min before use) and shaken at 90 rev. min⁻¹ for 30 min on a gyratory shaker. The mixture was then centrifuged at 450 g for 3 min and the sedimented hydroxyapatite given a single wash in PBS followed by seven washes in 0.15 M-NaCl containing 100 mM-phosphate, pH 7.2 (50 ml) and a single wash in 1.25 M-NaCl made 2 mM with respect to EDTA and containing 20 mM-Tris buffer, pH 7.8 (50 ml). A small quantity of lectin was eluted by a single wash in 1.25 M-NaCl made 2 mM with respect to EDTA and containing 20 mM-Tris buffer, pH 7.8, together with 36 mM-phosphate (50 ml). The bulk (>80 %) of the lectin activity was recovered by a single wash in 1.25 M-NaCl made 20 mM with respect to EDTA and containing 20 mM-Tris buffer, pH 7.8, together with 83 mM-phosphate (50 ml). In this batch elution procedure all the fractions were examined for haemagglutinating activity and for the presence of ovalbumin by single radial immunodiffusion technique (Mancini, Carbonara & Heremans, 1965) using rabbit anti-chicken egg albumin, freed of lipoproteins by resin treatment, (Miles- Yeda Ltd, lot R643; 100 μl) in a 2 % agar (3 ml) with ovalbumin (Sigma, Grade V crystallized preparation essentially salt free, Lot 31F-8061) as a standard.

Haemagglutination assay

Lectin-mediated haemagglutination and the ability of various oligosaccharides to act as inhibitors was determined in microtitre V plates (Linbro/Titertek, Flow Laboratories, Inc) using rabbit erythrocytes (Type I; Mir-Lechaire & Barondes, 1978) as described in detail previously (Cook, Zaglik, Milos & Scott, 1979). One haemagglutination inhibition unit (HAIU) being defined as the minimum concentration of inhibitor required to inhibit completely 4HU. Samples of oligosaccharides were obtained from Sigma with the exception of fucoidin which was purchased from ICN K & K (Plain View, N.Y.). A sample of fucoidin was desulphated by solvolysis (Usow et al. 1971) under conditions which it has been shown do not result in any significant hydrolysis of glycosidic linkages (Grabel et al. 1981). Sulphate content was assayed using the gelatin reagent of Dodgson (1961).
Polyacrylamide gel electrophoresis

Samples of purified lectin were examined by (SDS–PAGE) in 8% acrylamide slab gels with discontinuous buffer (Laemmli, 1970). Quantities of vitelline membrane (both whole and separated layers) and authentic egg proteins were analysed by SDS–PAGE with the discontinuous buffer system on 5–20% acrylamide gradient gels with an appropriate stacking gel. Two types of gradient gels were employed either a 0.7 mm thick gel prepared in a 180 mm x 140 mm x 0.7 mm cassette mounted in GE4 Electrophoresis apparatus (Pharmacia), or a 1.5 mm thick slab gel prepared in a Protean™ 11 Slab cell (Bio-Rad) and mounted in a double slab apparatus. In the latter separations the resolving gels were prepared with ammonium persulphate and sucrose gradients to minimize thermal convection during polymerization.

Gradient gels were subject to electrophoresis for 1h at 70 volt (for 0.7 mm gels) or 78 mA (constant current) (for 1.5 mm gels) before the addition of the samples. Routinely electrophoresis of the samples was at 25 mA (constant current) with the gels maintained at 8°C. Gels were stained with Coomassie brilliant blue R-250 or Silver stain (Oakley, Kirsch & Morris, 1980).

Two-dimensional gel electrophoresis was performed using the method of O'Farrell (1975). Isoelectric focusing was performed in 7.6x0.25 cm rod gels, which were transferred to polyacrylamide slab gels (12.0x7.0x0.3 cm) (Laemmli, 1970) and embedded in 1% agarose as described by O'Farrell (1975). Prestained protein high molecular weight standards (relative molecular mass range 14 300–200 000: BRL lot No. 41101) were cast directly into an isoelectric focusing gel and laid alongside the gel containing the focused sample.

Preparation of polyclonal antibodies to purified vitelline membrane lectin

Type I rabbit erythrocytes (2 ml packed cell volume) were resuspended in a solution (10 ml containing 20 000 HU of the purified lectin) and shaken at 90 rev. min⁻¹ on a gyratory shaker for 30 min at room temperature. The erythrocytes were sedimented at 900g for 5 min and then washed five times in 50 volumes of PBS and finally resuspended in PBS (2 ml) for administration to New Zealand White Rabbits. Initially the animals were given two intraperitoneal injections (2.0 ml) at a one-week interval followed by up to four intramuscular injections (0.5 ml) at weekly intervals.

A model experiment in which the ability of ovalbumin to bind to Type I cells was assessed, consisted of incubating cells (0.5 ml) for 30 min in the presence of ovalbumin (1.0 ml; 0.3 mg ml⁻¹) in 1.25 M-NaCl, 2 mM-EDTA, 20 mM-Tris (pH 7.8) containing 83 mM-phosphate. Following the above washing procedure the ovalbumin content of each wash was assessed by single radial immunodiffusion technique.

Monoclonal antibody production

Monoclonal antibodies were produced following the general procedures of Galfré & Milstein (1981); 10⁸ spleen cells from an immunized Balb/c mouse were fused with 10⁷ mouse myeloma cells (NSO, a non-immunoglobulin secretory derivative, originally isolated from a Balb/c stain and kindly provided by Dr C. Milstein, MRC Laboratory of Molecular Biology, Cambridge). Selection for hybridoma cells was performed using hypoxanthine/aminopterin/thymidine (Miller & Ruddle, 1976). Hybrid clones visibly appeared between 1 and 3 weeks after fusion and the supernatant fluids from well-grown colonies were removed, supplemented with 10 mM-HEPES and 0.1% sodium azide, and stored at 4°C before screening by solid-phase plate assay using ¹²⁵I-rabbit anti-mouse IgG (50 000 c.p.m. well⁻¹). Positive clones were subjected to cloning twice in agarose medium. Ascitic fluid was prepared by intraperitoneal injection of 10⁷ cloned cells into a Balb/c mouse that had been injected 14 days previously with 0.5 ml Pristane (2,6,10,14-tetramethylpentadecane) (Wright, 1980).

Immunodiffusion and immunoelectrophoresis

Ouchterlony immunodiffusion was performed in 1% agar (Noble-Difco) prepared in 0.15 M-phosphate buffer (pH 7.0) containing 1.0% w/v Triton X-100 and 0.04% sodium azide. The gels were maintained in a humid chamber at room temperature and precipitation patterns were
allowed to develop over 48 h. Gels were then washed in PBS containing 0.04% sodium azide over 48 h followed by a 2 h wash in distilled water, dried, fixed with 2% acetic acid and stained with Amido Black 10B (Weeke, 1973).

Rocket immunoelectrophoresis was carried out in an LKB Multiphor apparatus following the procedures described in detail by Weeke (1973). Gels were washed, fixed and stained as above.

Immunoblotting

Electrophoretic blots onto nitrocellulose sheets were prepared as described by Towbin, Staehelin & Gordon (1979). Polyclonal antibody was used at 1/10 dilution and binding detected with either 125I Protein A (10^6 c.p.m. 3 mm^-1 strip) and autoradiography (Fuji X-ray film at -70°C) or by using a commercial ABC kit (Vectastain), in which biotinylated anti-rabbit immunoglobulin was the second antibody and diaminobenzidine tetrahydrochloride the peroxidase substrate.

Staining procedures

Vitelline membrane preparations were fixed for 48 h in absolute ethanol, then rehydrated through a graded series of ethanol to phosphate-buffered saline (0.1 M-phosphate) containing 15% w/v sucrose. They were then embedded in gelatin dissolved in the phosphate buffer. Cryostat sections were examined by indirect immunofluorescence microscopy using both polyclonal and monoclonal antibodies. Sections incubated in a moist atmosphere at 37°C for 30 min with 1/10 dilution of either normal goat serum (when using rabbit polyclonal antibody) or normal rabbit serum (when using mouse monoclonal antibody). On removal of the serum the sections were washed three times with PBS and then incubated with 1/10 dilution of either rabbit anti-lectin serum for 30 min at 37°C or 1/5 dilution of ascites fluid containing mouse anti-lectin antibody overnight at 4°C. Following the removal of the antibodies the sections were washed three times in PBS and incubated for a further 30 min at 37°C with 1/16 dilution of either FITC-conjugated goat anti-rabbit IgG (H+L) affinity-purified antibody (molar ratio F/P = 3-0, Miles-Yeda) or FITC-conjugated rabbit anti-mouse IgG (H+L) antibody (molar ratio F/P = 4-4; Miles-Yeda). Sections were mounted in glycerol containing 1 mM-p-phenylenediamine to prevent fading (Johnson & Nogueira Araujo, 1981) and examined with a Leitz-SM2 Lux Microscope.

RESULTS

Purification of the lectin activity in the vitelline membrane

PBS extracts prepared from the vitelline membrane when examined by SDS-PAGE contain large quantities of a protein of \( M_r \ 4.5 \times 10^4 \), almost certainly ovalbumin (Rutherford & Cook, 1984). In Table 1, where the results of a large-scale purification of the lectin activity in the vitelline membrane are displayed, it may be seen that the quantities (270 mg) of ovalbumin shown to be present in such extracts by the radial immunodiffusion technique compare favourably with the protein level (215 mg) determined chemically with gamma globulin as standard. The haemagglutinating activity present in such extracts is readily absorbed on to hydroxyapatite as is over 90% of the ovalbumin. On washing the hydroxyapatite eight times with 'low' salt (0.15 M-NaCl) 42% of the ovalbumin is recovered with only 2.6% of the haemagglutinating activity. By increasing the salt concentration
to 1.25 M-NaCl and controlling the level of phosphate 93% of the haemagglutinating activity is recovered in the absence of any detectable ovalbumin; it being possible to recover 83% of the lectin activity with up to a 70-fold purification over the starting extract.

In Fig. 1 it may be seen that the active material eluted from hydroxyapatite migrates as a single component of apparent $M_r$ of 62000 in SDS-PAGE. On examination by two-dimensional gel electrophoresis the purified lectin appears to be homogeneous and the subunit of $M_r$, 62000 has a pI of 5.1.

Table 1. *Purification of the lectin activity of the vitelline membrane on hydroxyapatite*

<table>
<thead>
<tr>
<th>Step Description</th>
<th>Haemagglutinating activity</th>
<th>Specific activity (HU mg protein$^{-1}$)</th>
<th>Relative specific activity</th>
<th>Ovalbumin (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitelline membrane extract (100 000 g supernatant fluid)</td>
<td>1229</td>
<td>5769</td>
<td>1</td>
<td>270</td>
</tr>
<tr>
<td>Supernatant fluid after binding to hydroxyapatite</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1st wash of hydroxyapatite (0-15 M-NaCl 5 mM-phosphate pH 7-2)</td>
<td>NA</td>
<td>—</td>
<td>—</td>
<td>2.6</td>
</tr>
<tr>
<td>2nd wash of hydroxyapatite (0-15 M-NaCl 100 mM-phosphate pH 7-2)</td>
<td>32</td>
<td>337</td>
<td>0.06</td>
<td>95</td>
</tr>
<tr>
<td>3rd wash of hydroxyapatite (0-15 M-NaCl 100 mM-phosphate pH 7-2)</td>
<td>NA</td>
<td>—</td>
<td>—</td>
<td>12.5</td>
</tr>
<tr>
<td>4th wash of hydroxyapatite (0-15 M-NaCl 100 mM-phosphate pH 7-2)</td>
<td>NA</td>
<td>—</td>
<td>—</td>
<td>2</td>
</tr>
<tr>
<td>5th wash of hydroxyapatite (0-15 M-NaCl 100 mM-phosphate pH 7-2)</td>
<td>NA</td>
<td>—</td>
<td>—</td>
<td>0.75</td>
</tr>
<tr>
<td>6th wash of hydroxyapatite (0-15 M-NaCl 100 mM-phosphate pH 7-2)</td>
<td>NA</td>
<td>—</td>
<td>—</td>
<td>0.17</td>
</tr>
<tr>
<td>7th wash of hydroxyapatite (0-15 M-NaCl 100 mM-phosphate pH 7-2)</td>
<td>NA</td>
<td>—</td>
<td>—</td>
<td>NA</td>
</tr>
<tr>
<td>8th wash of hydroxyapatite (0-15 M-NaCl 100 mM-phosphate pH 7-2)</td>
<td>NA</td>
<td>—</td>
<td>—</td>
<td>NA</td>
</tr>
<tr>
<td>9th wash of hydroxyapatite (1.25 M-NaCl 2 mM-EDTA 20 mM-Tris pH 7-8)</td>
<td>NA</td>
<td>—</td>
<td>—</td>
<td>NA</td>
</tr>
<tr>
<td>10th wash of hydroxyapatite (1.25 M-NaCl 2 mM-EDTA 20 mM-Tris pH 7-8+36 mM-phosphate)</td>
<td>128</td>
<td>18 286</td>
<td>3</td>
<td>NA</td>
</tr>
<tr>
<td>Elution wash of hydroxyapatite (1.25 M-NaCl 2 mM-EDTA 20 mM-Tris pH 7-8+83 mM-phosphate)</td>
<td>1024</td>
<td>409 600</td>
<td>71</td>
<td>NA</td>
</tr>
</tbody>
</table>

Vitelline membrane extract (60 ml: 100 000 g supernatant fluid) was fractionated on hydroxyapatite as described in detail in the Materials and Methods section; each wash (50 ml) was examined for haemagglutinating activity and protein content. Haemagglutination assays were performed with Type I glutaraldehyde-modified rabbit erythrocytes and protein assay by the Bradford technique. Ovalbumin was determined by radial immunodiffusion assay as described in the text and the quantities refer to total amount of ovalbumin present in each wash. NA = No activity.
Inhibitory activity of various oligosaccharides

A number of charged and neutral saccharides were examined as competitive inhibitors of the lectin activity (Table 2) and of those examined the sulphated polysaccharides dextran sulphate \((M_r \times 5 \times 10^5)\) and fucoidin \((10^5)\) were shown to act as competitive inhibitors of haemagglutinating activity. Heparin and chondroitin sulphate (a sample containing a mixture of isomers was examined), polymers containing both sulphate and carboxyl groups, were without inhibitory activity when tested in concentrations of up to 2.5 mg/assay. Non-sulphated dextrans of different relative molecular masses were ineffective as inhibitors of lectin activity and a sample of desulphated fucoidin (with 69% decrease in sulphate as compared to native fucan) had a specific inhibition activity of only 160 HAIU mg\(^{-1}\).

Preparation of antibodies to the vitelline membrane lectin

A polyclonal rabbit antiserum was raised by re-injecting animals with their own erythrocytes, which had been converted to Type I cells and to which purified lectin had been adsorbed. As shown in Fig. 2A Ouchterlony analysis reveals a single precipitin line, showing a reaction of identity, when crude extracts of whole vitelline membrane and purified lectin are tested against a rabbit polyclonal
antiserum prepared in this manner. No cross reactivity was observed with non-ionic detergent extracts of whole blastoderm or a plasma membrane fraction prepared from a suspension of blastoderm cells (Zalik & Cook, 1976). Importantly no cross reactivity with ovalbumin or ovomucoid (both at 10 mg ml\(^{-1}\)) was observed; indeed as it has been shown in a model experiment that ovalbumin may be recovered quantitatively from Type I cells within two washes, should traces of this glycoprotein be present in the purified lectin beyond the limit of detection, it is unlikely to bind to the cells being used for antibody production. The precipitin pattern remained unaltered when tested against antiserum which had been absorbed with equal volumes of Type I cells, indicating that any antibodies produced to rabbit antigens generated as a result of trypsin treatment or glutaraldehyde fixation in the preparation of the Type I cells, are not responsible for the observed pattern. In addition to ovalbumin and ovomucoid, lysozyme and

<table>
<thead>
<tr>
<th>Substance tested</th>
<th>Specific inhibition activity</th>
<th></th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>HIU mg(^{-1})</td>
<td>HIU (\mu)mol(^{-1})</td>
</tr>
<tr>
<td>Sulphated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dextran sulphate</td>
<td>5(\times)10(^5)</td>
<td>8-3(\times)10(^5) ± 6-2(\times)10(^5)</td>
<td>4-17(\times)10(^8) ± 3-12(\times)10(^8)</td>
</tr>
<tr>
<td>Dextran sulphate</td>
<td>5(\times)10(^3)</td>
<td>NA</td>
<td>—</td>
</tr>
<tr>
<td>Fucoidin</td>
<td>10(^5)</td>
<td>7-4(\times)10(^4) ± 3-8(\times)10(^4)</td>
<td>7-4(\times)10(^6) ± 3-8(\times)10(^6)</td>
</tr>
<tr>
<td>Carboxylated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hyaunonic acid</td>
<td>0-5–80(\times)10(^5)</td>
<td>NA</td>
<td>—</td>
</tr>
<tr>
<td>Colominic acid (Poly-2,8-N-acetyl neuraminic acid)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carboxylated and sulphated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heparin</td>
<td>7–16(\times)10(^3)</td>
<td>NA</td>
<td>—</td>
</tr>
<tr>
<td>Chondroitin sulphate (mixed isomers)</td>
<td>NA</td>
<td>NA</td>
<td>—</td>
</tr>
<tr>
<td>Phosphated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mannose-1-phosphate</td>
<td>260</td>
<td>NA</td>
<td>—</td>
</tr>
<tr>
<td>Mannose-6-phosphate</td>
<td>260</td>
<td>NA</td>
<td>—</td>
</tr>
<tr>
<td>Uncharged</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dextran</td>
<td>2(\times)10(^6)</td>
<td>NA</td>
<td>—</td>
</tr>
<tr>
<td>Dextran</td>
<td>5(\times)10(^5)</td>
<td>NA</td>
<td>—</td>
</tr>
<tr>
<td>Dextran</td>
<td>2-43(\times)10(^5)</td>
<td>NA</td>
<td>—</td>
</tr>
<tr>
<td>Glycogen</td>
<td>2-7–35(\times)10(^5)</td>
<td>NA</td>
<td>—</td>
</tr>
<tr>
<td>Agarose</td>
<td>?</td>
<td>NA</td>
<td>—</td>
</tr>
</tbody>
</table>

The ability of the various oligosaccharides to inhibit the purified lectin (4 HU) was determined as detailed in Materials and Methods. NA = No activity detected. Where inhibitory activity was found the results are given as a mean value ± s.d. The figures in parentheses refer to the number of separate determinations.
Fig. 2. Rabbit polyclonal antibody to the purified lectin. In (A) Ouchterlony gel diffusion analysis was performed with rabbit antiserum (central well: pattern on the left-hand side obtained with unadsorbed serum and on the right-hand side the 0.36 mg protein ml⁻¹) and a solution of purified lectin concentrated with carbowax (p; 1.72 mg protein ml⁻¹) together with solubilized blastoderms (b) and plasma membranes obtained from a suspension of blastoderm cells (m) and ovalbumin (ov) and ovomucoid (o). The precipitin pattern was stained with Amido black 10B. Rocket immunoelectrophoresis (B) was performed on purified lectin concentrated with carbowax (3.32 mg protein ml⁻¹) and then serially diluted (from left to right) with PBS, 3 μl was placed in each well and antiserum (1 ml) was incorporated into agarose (12 ml) running gel. In this case the precipitin pattern was stained with Coomassie brilliant blue R-250. The rabbit anti-serum was shown by immunoblotting (C) to react with the subunit of $M_r$ 62,000. Concentrated lectin solution (1.72 mg protein ml⁻¹) was subjected to electrophoresis in an 8% acrylamide gel before electrophoretic transfer to nitrocellulose paper. Antigen was visualized either by (1) immunoperoxidase staining using rabbit antiserum (1/10 dilution) and the ABC technique (Vectastain kit) in which biotinylated anti-rabbit immunoglobulin was the second antibody and diaminobenzidine tetrahydrochloride the peroxidase substrate, or by (2) autoradiography with $^{125}$I-labelled protein A ($10^6$ c.p.m. 3 mm⁻¹ wide strip). The strip on the right hand side (3) was stained with Coomassie brilliant blue R-250.
avidin (at 10 mg ml\(^{-1}\)) also were found not to give a precipitin line. Identical results were obtained with IgG (12.5 μg), prepared from the rabbit antisera. No precipitin lines were observed with any of the above vitelline membrane or blastoderm extracts or purified egg proteins when pre-immune serum (or IgG) was substituted for immune serum. Rocket immunoelectrophoresis of rabbit antisera against different concentrations of purified lectin (see Fig. 2B) clearly demonstrated that the antiserum was directed against a single antigen and using the immunoblotting technique the antibody is seen to cross react with the lectin subunit of \(M_r\) 62,000 (Fig. 2C). Two monoclonal antibodies were chosen (NL/1 and NL/26), neither of which produced immunoblots. In this work immunostaining was performed with NL/1.

**Indirect immunofluorescence microscopy of the vitelline membrane**

Two separate preparations of vitelline membrane from infertile eggs, a preparation of the vitelline membrane from the edge of a stage-7 embryo (about 28 h of incubation: Hamburger & Hamilton, 1951) and a portion of vitelline membrane taken from above a 72 h embryo were examined as frozen sections in indirect immunofluorescence microscopy with rabbit polyclonal (Fig. 3) and mouse monoclonal antibody (Fig. 4). In all cases there is a clear asymmetric staining of the vitelline membrane, with the outer layer staining intensely. The staining appears to be continuous along the length of sections of the vitelline membrane and in no way localized over the cells at the edge of the blastoderm.

**Separation of the vitelline membrane in to two layers**

Portions of the separated layers were examined in thin transverse sections by electron microscopy (data not shown) and the outer layer was found to consist of a lattice work of fine fibrils with an attached ‘continuous membrane’, exactly as described by Bellairs et al. (1963). The inner layer was seen to be composed of a three-dimensional network of fibres as first described in detail by Bellairs et al. (1963). In no case was there any evidence of cross contamination of the separated layers used in the present study.

The lectin activity in PBS extracts of the outer and inner layers of the vitelline membrane correlates well with the pattern of immunofluorescence staining. As
shown in Table 3 the mean specific activity of the haemagglutinating property of extracts of the outer layer is over five times greater than that from the inner layer; in individual preparations this ratio ranged from 3.8–7.1. As care was taken in
each individual experiment to retain all of the separated layers it was possible to compute the total quantities of lectin activity (in HU) extracted from each layer and $91.0 \pm 6.5\%$ (6) of the lectin was recovered from the outer layer. The specific
Vitelline membrane lectin

Table 3. Specific activity of the lectin in saline extracts of the different layers of the vitelline membrane

<table>
<thead>
<tr>
<th>Layer</th>
<th>Haemagglutinating activity (HU mg protein(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole vitelline membrane</td>
<td>5375 ± 1966 (8)</td>
</tr>
<tr>
<td>Outer layer of the vitelline membrane</td>
<td>13 813 ± 9245 (6)</td>
</tr>
<tr>
<td>Inner layer of the vitelline membrane</td>
<td>2606 ± 1819 (6)</td>
</tr>
</tbody>
</table>

The vitelline membrane and the physically separated layers were extracted with 0-15 M-NaCl 5 mm-phosphate (pH 7-2), centrifuged at 100 000 g for 1 h and the supernatant fluid assayed for protein by the Bradford procedure and for lectin activity by haemagglutination, as described in the Materials and Methods section. Results are quoted as mean ± s.d. and the figures in parentheses represent the number of separate batches of membrane and separated layers examined.

The specific activity of the total lectin extracted from both inner and outer layers in three batches ranged from 4982–5548 HU mg protein\(^{-1}\) and correlates well with the figure of 5375 HU mg protein\(^{-1}\) obtained from extracts of the whole membrane; however, in three other preparations the specific activity ranged from 9760–20 191 HU mg protein\(^{-1}\) and may reflect variations in individual batches of eggs. As in the latter case the percentage recovery of activity in the outer layer ranged from 80–97 % it is possible that on drying the vitelline membranes on to glass, a necessary step in the separation process, quantities of non-lectin protein are retained on the glass surface.

SDS-PAGE of the polypeptides of the vitelline membrane

The range of polypeptides present in the vitelline membrane are displayed in Fig. 5 together with a number of authentic egg proteins for comparison. It will be seen that the staining pattern of the polypeptides present in the membrane is substantially the same whether Coomassie brilliant blue or silver reagent was used. In the case of the outer layer the polypeptides with apparent molecular weight...
Fig. 5. SDS–PAGE of whole vitelline membrane (W, 142 µg protein), outer (O, 85 µg protein) and inner (I, 87 µg protein) layers and authentic egg proteins on gradient (5–20%) acrylamide gels. The gel on the left (A) has been stained with Coomassie brilliant blue R-250 and the one on the right (B) with silver stain. In gel (A) the following authentic egg proteins (Sigma: 10 µg each) have been examined, ovalbumin (track 1), lysozyme (track 2), avidin (track 3), vitellin (track 4), conalbumin and avidin (track 6). Molecular weight markers (carbonic anhydrase $M_r$ 30 K, ovalbumin $M_r$ 43 K, bovine serum albumin $M_r$ 68 K, phosphorylase B $M_r$ 94 K, $\beta$-galactosidase $M_r$ 116 K and myosin $M_r$ 200 K) were placed in track 5. Note the presence of lysozyme and avidin in the outer layer and their much reduced presence in the inner layer. In gel (B) a mixture of protein molecular weight standards (Bethesda Research Laboratories) was used as detailed in Materials and Methods.
close to $43 \times 10^3$ are stained more intensely with silver reagent. From a comparison with the standard egg proteins the presence of lysozyme and avidin in the vitelline membrane is clearly indicated; these latter two proteins appear to be concentrated in the outer layer of the membrane, the inner layer being largely devoid of these polypeptides.

No attempt has been made to assign any of the bands shown on these gels to the lectin. When saline extracts (20 µg protein) of the vitelline membrane have been examined by polyacrylamide gel electrophoresis followed by staining with Coomassie brilliant blue the lectin has appeared as a faintly staining band relative to ovalbumin, the major polypeptide present. The material examined in the gels in Fig. 5 represents the total amounts of protein available in the layers of the vitelline membrane and not just saline soluble protein. Even though four times the quantity of protein has been examined in these gels (Fig. 5), as compared to the experiments with the saline extracts, the amount of lectin present is likely to be below the limit of detection and in this respect it may be noted that the ovalbumin band, present in the whole membrane and outer layer, is relatively faint as compared to the other polypeptide species present.

**DISCUSSION**

Lectins are carbohydrate-binding proteins of non-immune origin, originally identified in extracts of plant material as haemagglutinins. Over the last decade there has been a considerable increase in the interest shown by biologists in these materials, especially as they are now being identified in association with animal cells (Barondes, 1981). The lectins can be conveniently subdivided into two classes on the basis of whether they are soluble or integrated into membranes (Barondes, 1984). Integral membrane lectins are involved in the carbohydrate recognition of glycoproteins for receptor-mediated pinocytosis (Harford & Ashwell, 1982) and are possibly involved in intercellular recognition (Weigel et al. 1979). Soluble lectins, which do not require detergents for solubilization, it has been suggested (Barondes, 1984) may give rise to properties of the developing extracellular matrix that regulate cell migration and adhesion. In addition this latter class may also function intracellularly, possibly in the process of glycoconjugate secretion and in some instances Barondes (1984) suggests their role may not be to hold glycoconjugates together but to keep them apart.

The lectin obtained from the vitelline membrane may be classified as a soluble lectin for two reasons, first it has been isolated in the absence of detergent and secondly the haemagglutination titre obtained is identical irrespective of whether the extract has been centrifuged at 450 g for 10 min or 100,000 g for 1 h. This latter result suggests the absence of particle associated lectin as has been found in the gastrulating chick embryo (Zalik, Milos & Ledsham, 1983).

We have obtained evidence that a high degree of structural specificity is required by the polyanions that act as inhibitors of the lectin and that the inhibition obtained is not the result of non-specific electrostatic interaction. This evidence is
based upon the inhibition data obtained with isolated polysaccharides. We conclude from our inhibition experiments with polymers carrying only one type of anionic group that it is the chemical nature of the group which is important. Carboxylated polymers, such as hyaluronic acid, are ineffective as inhibitors, and fucoidin on desulphation by solvolysis (Usov et al. 1971) rapidly loses its inhibitory activity. A similar lectin has been found in murine lymphocytes which recognizes sulphated polysaccharides and which is inhibited by dextran sulphate and fucoidin (Parish, Rylatt & Snowden, 1984). The murine lectin differs from the vitelline membrane lectin, however, in that it is inhibited by dextran sulphate irrespective of its molecular weight. In our earlier studies (Rutherford & Cook, 1984) a number of glycoproteins possessing ‘high mannose’ oligosaccharide groups were found to have modest inhibitory activity (yeast mannan, 7.5; thyroglobulin, 13.4; invertase, 96.0 HAIU mol\(^{-1}\) \times 10\(^{-3}\)). An important finding was the fact that ovalbumin, though containing high mannose structures, was not an inhibitor of the agglutinin, suggesting that the vitelline membrane lectin is designed to be effective even in the presence of egg white. Another of the interesting features of our present results is the dual fucan/mannan specificity, as most lectins characterized to date have a preferred specificity for a particular saccharide residue. However, a lectin with the same dual fucan/mannan specificity has also been found as a soluble haemagglutinin from teratocarcinoma stem cells (Grabel et al. 1981).

We do not as yet understand the role of the lectin in the physiology of the egg or in the differentiation of the embryo, but several possibilities will be considered here.

1) Adhesion. It has been suggested (Barondes, 1984) that soluble lectins may be important in cell adhesion. Certainly there are indications that this lectin may be able to provoke strong adhesions of cells to substrates. Thus, New (1959) showed that if the embryo was explanted to the outer surface of the vitelline membrane it adhered extremely tightly and the migration of its edge cells was inhibited. It seems possible therefore that embryonic cells will adhere more tightly to the outer than to the inner surface of the vitelline membrane because the lectin is present on the outer surface but not on the inner. Under normal conditions however, the embryonic cells never come into contact with the outer surface. We conclude therefore that this lectin cannot play a direct role in cell adhesion under normal circumstances.

2) Expansion of the blastoderm. In order to migrate cells must adhere to a substrate (though not so firmly that they are incapable of moving at all as in New’s experiments described above). One possibility therefore is that some as yet undetected product of this lectin may pass through the vitelline membrane and coat the inner surface thus aiding adhesion and expansion. An experimental analysis however in which embryos in culture (New, 1955) were treated with fucoidin (50 and 200 \(\mu\)g doses were tested) to counteract the lectin had no significant effect on the rate of blastoderm expansion, experimental and control embryos expanding at the same rate. We therefore conclude that the lectin plays no role in blastoderm expansion.
3) **Strength of the membrane.** It has been shown by transmission electron microscopy that morphological changes begin to take place in the inner layer of the vitelline membrane after about 48 h of incubation (Jensen, 1969) prior to the breakdown of the membrane at about 80 h. No changes could be detected by Jensen (1969) in the outer layer during this period. It is of interest therefore that we too in this present work have been unable to show any changes in the outer layer during this period, no differences in immunostaining being detected. We therefore conclude that the lectin plays no important role in maintaining the strength of the vitelline membrane.

4) **Transport across the vitelline membrane.** There is extensive evidence that water and water-soluble proteins can pass across the vitelline membrane (Romanoff & Romanoff, 1960; New, 1959; Callebaut, 1982; Hassell & Klein, 1971; Jordanov et al. 1966). Under normal conditions these materials pass from the albumen into the yolk, though it is possible to reverse the direction if an intact yolk is immersed in a 10–20 % solution of sodium chloride (Jordanov et al. 1966). The ability of the vitelline membrane to act as a dialysing membrane probably depends not only on the biochemical composition of the yolk and the albumen, but also on its own structure. It seems possible that the presence of the lectin in the outer layer may be an important factor. Experiments to test this possibility are in progress.

5) **Bactericidal properties.** The albumen of the hen's egg possesses strong bactericidal and fungicidal properties (Romanoff & Romanoff, 1949) which protect the yolk and embryo from infections. It seems possible that the lectin may play a similar role as it interacts with yeast mannan and it may be relevant that lysozyme is also located in the outer layer of the vitelline membrane together with avidin. Romanoff & Romanoff (1949) pointed out that lysozyme is the lytic principle in egg albumen and suggested that avidin probably aids in inhibiting bacterial growth by depriving micro-organisms of biotin. Experiments to test the bactericidal properties of the lectin are in progress.

Owing to the ready availability of the hen's egg and the production of the antibodies described here it should be possible to investigate some of these biological properties further.

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